Cortisol-secreting adrenocortical tumours in dogs; considering the related aspects dopamine and somatostatin receptors and Gs alpha.

C.G. Spandauw

Research internship Master Veterinary Medicine
’11 – ‘12
Faculty of Veterinary Medicine, Utrecht

Supervisors:
S. Galac, M. Kool, J. Mol, H.S. Kooistra

ABBREVIATIONS

3β-HSD	3β-hydroxysteroid dehydrogenase
AC	Adenylyl cyclase
ACTH	Adrenocorticotropic hormone
CNS	Central nervous system
CRH	Corticotrophin releasing hormone
CPA	Cortisol-secreting adenoma
D2	Dopamine receptor type 2
D2l	Dopamine receptor type 2 long isoform
D2s	Dopamine receptor type 2 short isoform
ERK	extracellular signal-regulated kinase
GDP	Guanine diphosphate
Gi	inhibitory G-protein
GPCR	G-protein-coupled receptor
GNAS	Guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide
GRKs	G-protein-coupled receptor-kinases
Gs	stimulatory G-protein
Gsα	stimulatory G-protein alpha (α) subunit
GTP	Guanine triphosphate
HPA axis	Hypothalamic-pituitary-adrenal axis
IHC	Immunohistochemistry
MAPK	Mitogen-activated protein kinase
NET	Neuro-endocrine tumour
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
PI	Phosphatidylinositol
PTP	Phosphotyrosine phosphatase
qPCR	quantitative polymerase chain reaction

SNP	Single nucleotide polymorphism
SSTR	Somatostatin receptor
Rb	Retinoblastoma tumour suppressor protein

INDEX

GENERAL INTRODUCTION

Imagine a small West Highland White Terrier walking into your examining room. She has a very thin coat with the pink skin showing all through, a tail which you could almost compare with one of a rat, a huge round belly compared to the rest of her body, of which the skinny legs are very obvious. She is anxiously wandering around and will eventually pee in a corner, to which the owner will react with: “she just pied outside before we got in”. Things that the owners mention during the anamnesis is that the dog is very tired, does not want to walk the normal round anymore, is constantly searching for water and food and will even eat non eatable things. After the execution of some tests, it is proven that this dog has Cushing’s syndrome, or also called hypercortisolemia.

Cushing’s syndrome is a frequently seen endocrine problem in dogs.[1] It has been estimated by some epidemiological studies that one or two on every 1000 dogs suffers from this syndrome every year.[2] The syndrome is also known in men, but the incidence is much lower, estimated around 1.2 till 2.4 patients on every million individuals per year.[3, 4]

Spontaneous Cushing’s syndrome or hypercortisolism is characterized by a chronic overproduction of cortisol by the adrenal glands. In physiological conditions, the production of cortisol is under the influence of ACTH (adrenocorticotropic hormone) and regulated by a feedback mechanism. From the hypothalamus CRH (corticotrophin releasing hormone) and vasopressin are released, which stimulate the release of ACTH. ACTH is released in the bloodstream from the adenopituitary, and stimulates the synthesis and release of cortisol. Cortisol, which is the ‘end’ product of this chain, inhibits the release of ACTH and CRH. With this feedback loop, the cortisol concentration remains within physiological limits.

Looking at this regulatory mechanism of cortisol secretion, it can be understood that Cushing’s syndrome can find its origin when there is a disregulation somewhere in the feedback loop. In the majority of cases (80-85%), hypercortisolism results from overproduction of ACTH by a tumour of the pituitary gland. This ACTH-dependent hypercortisolism is known as Cushing’s disease. In the remaining cases (15-20%), hypercortisolemia is due to an ACTH-independent or autonomic production of cortisol by an adrenocortical tumour.[5] Apart from these two forms of hypercortisolism, ectopic ACTH-secretion and food-dependent hypercortisolism have been described as well, but remain an exception.[1]

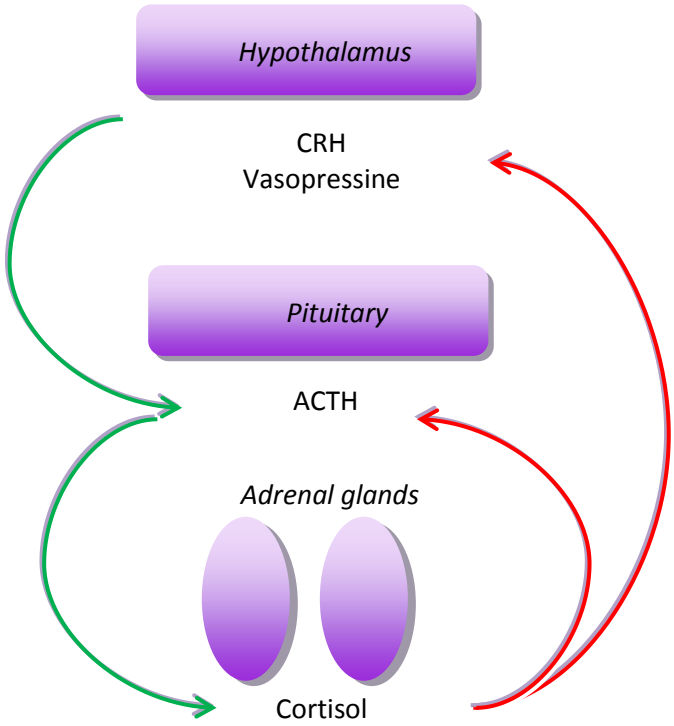


Figure 1. Hypothalamic-pituitary-adrenal axis (HPA axis). Corticotrophin releasing hormone (CRH) and vasopressin from the hypothalamus stimulate the pituitary to release ACTH. ACTH reaches the adrenal glands via the bloodstream, where it stimulates the synthesis and release of cortisol. Cortisol has an inhibitory effect on the pituitary and hypothalamus, by which ACTH release will decrease. This negative feedback will keep the cortisol levels within physiological limits. ***Green arrow; stimulation, Red arrow; inhibition.***

Normally cortisol is of importance for a lot of processes in the body. It is involved in gluconeogenesis, fat-, protein-, and carbohydrate-metabolism, and has a protective function against stress. If cortisol is present in excessive amounts, all these processes are disturbed, which will lead to the clinical signs mentioned earlier.[6] The clinical presentation of dogs with hypercortisolemia is comparable to that of men. Figure 2 gives an overview of the clinical signs in dogs and men with Cushing's syndrome. When you study this figure it comes across quite clear, that the clinical signs are highly comparable. Keeping in mind the higher incidence of Cushing's syndrome in dogs compared to men, the dog could serve as an animal model to study hypercortisolism in men.[7]

Clinical sign	Human	Canine
Fatigue	+	+
Weight gain	+	+
Truncal obesity	+	+
Muscle atrophy	+	+
Osteoporosis	+	–
Lordosis	–	+
Hypertension	+	+
Polyuria polydipsia	–	+
Thinning of skin	+	+
Easy bruising	+	–
Hirsutism	+	–
Alopecia	+/–	+

Figure 2. Overview of clinical signs of Humans and Canines with Cushing's syndrome.[1]

Nowadays, different kinds of treatment for hypercortisolism are possible, depending on the origin of the disease. The most effective treatment is surgery: (1) adrenalectomy in case of a unilateral adrenocortical tumour, provided there are no metastases and no ingrowth into the adjacent blood vessels or (2) hypophysectomy in case of a pituitary tumour of which the size allows a complete removal. However, surgery is extremely difficult and can be performed only in specialized institutions by an experienced surgeon. An alternative to surgery is medical treatment with the adrenocorticolytic drug mitotane (o,p`-DDD) or adrenocorticostatic trilostane. Mitotane has potential toxic effects in both humans and animals, therefore the owners need to be given careful instructions on how to use the drug and how to recognize toxic effects. Due to its side effects, mitotane should not be used in a household with small children and/or pregnant women. Also, hormone substitution is needed, because of the induction of hypoadrenocorticism due to the destroying effects on the zona glomerulosa and zona fasciculata. Recently, mitotane has been replaced widely by trilostane, a competitive inhibitor of 3β-HSD (3β-hydroxysteroid dehydrogenase), an enzyme active in the steroid pathway by which cortisol is synthesized. Trilostane effectively decreases cortisol synthesis and leads to the improvement of clinical signs, however, it has no effect on the growth of AT and/or pituitary tumour tissue.[1]

Because each of these medical treatments have obvious downsides and none of them ensures a total and permanent cure, there is need for new medical treatment options.[8] Recently there is a lot of interest among (neuro)endocrine tumour research for the somatostatin and dopamine receptors. These receptors are confirmed to have inhibitory effects on hormone secretion, for example growth hormone secretion of the pituitary, and apart from that, there seems to be an antiproliferative and pro-apoptotic effect as well. Regarding Cushing's syndrome, some research has been done on pituitary tumours, but much less on adrenal gland tumours and none on canine adrenal gland tumours. Therefore, in this research project we aimed to survey some subjects regarding these receptors. The dopamine receptors will be covered in **Chapter 1**. In the next chapter, **Chapter 2**, the somatostatin receptors will be discussed. The last chapter, **Chapter 3**, will tackle a different subject regarding cortisol secreting ATs, namely one of the possible causes of the tumour development. In this chapter, possible mutations in Gs alpha (Gsα), a stimulatory protein involved in the cascade of hormone syntheses/secretion, will be discussed.

CHAPTER 1

DOPAMINE 2 SHORT AND LONG ISOFORM RATIOS

ABSTRACT

Background: Dopamine is a catecholamine, which plays an important role in the CNS as a neurotransmitter, but also in the periphery where it has multiple functions, including modulation of hormone secretion. Its receptor exists in 5 different subtypes, of which subtype 2 (D2) exists in two isoforms, a short (D2s) and long (D2l) one. These two isoforms are thought to have different effects on hormone secretion; the short isoform is thought to be a more potent inhibitor. The D2 receptor has been found in several neuroendocrine tumours, but has not been looked at in cortisol-secreting AT in dogs.

Aim: In this study the expression of the two isoforms is examined in canine cortisol-secreting adrenocortical tumours. It is expected that carcinomas will show expression of the long isoform only, while both isoforms will be present in adenomas.

Materials and methods: The mRNA expression of both isoforms was studied by means of quantitative RT-PCR of 36 adrenocortical tumours and 15 normal adrenal glands.

Results: The results of the short isoform were excluded, as well as the results of one of the two qPCR plates of the long isoform, due to unreliable results. Statistical analysis of the last plate did not show any significant results, but the stability of the reference genes was questionable. The overall protein expression of D2l was very low.

Conclusion: Due to unreliable results, no conclusion can be drawn. But based on the low expression and on other research, the dopamine receptor D2 probably does not have potential in the development of new therapeutic options for cortisol-secreting adrenocortical tumours.

Dopamine is a catecholamine, which plays an important role in the central nervous system (CNS) as a neurotransmitter, but also in the periphery where it has multiple functions, including the modulation of hormone secretion. The effects of dopamine in the central nervous system were first noticed in 1972. These effects are obtained via the binding of dopamine to one of its five different receptors (D1 – D5). The same receptors have been found outside the CNS. In 1978, based on the biological and pharmacological effects it was suggested that there are two different groups of dopamine receptors. This classification is based on the coupling of the receptor to adenylyl cyclase (AC), where the D1-like dopamine receptors (including D1 and D5) are positively coupled to AC and the D2-like dopamine receptors (including D2, D3, and D4) are not. Another difference between the two dopamine receptor groups can be found in their genomic organization. The D1-like receptors do not have introns in their coding region and the D2-like receptors have different amounts of introns. Because of these introns, two splice variants of the D2 receptor are generated by alternative splicing of a 87 basepare exon, resulting in a short (D2s) and long (D2l) variant of the D2 receptor.[9]

Characteristics of the D2-like receptors

The dopamine receptors are part of the seven transmembrane domain G protein-coupled receptor family (GPCR). The D2-like receptors share a considerable amount of similarity amongst each other regarding the transmembrane domains. All D2-like receptors have the same amount of amino acids in the NH_2-terminal, but the number of *N*-glycosylation sites differs. The D2-like receptors have a distinctly shorter COOH terminal when compared to the D1-like receptors. The final characteristic of the D2-like receptor, which is also biologically important, is the existence of a long third intracellular loop. This long loop interacts with inhibiting G proteins (G_i) which inhibit cyclic AMP (cAMP) production [10], while a short loop interacts with stimulatory G proteins (G_s). The D1-like receptors have a short loop shown in Figure 3, due to which they have an inhibitory effect. [9]

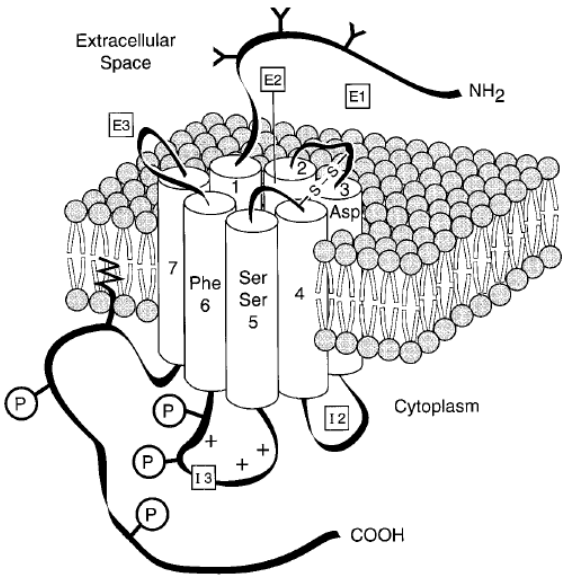


Figure 3. Representation of the dopamine receptor with the structural features of the D_1-like receptor. D_2-like receptors are characterized by a bigger 3rd intracellular loop and a shorter COOH-terminal tail. Residues involved in dopamine binding are highlighted in the transmembrane domains. Potential phosphorylation sites are represented on the 3rd intracellular loop (I3) and on the COOH terminus. Potential glycosylation sites are represented on the NH_2 terminal. E1-E3, extracellular loops; 1–7, transmembrane domains; I2-I3, intracellular loops. [9]

Signal transduction in dopamine receptors

Dopamine receptors use three distinct signal transduction routes to mediate their actions. Adenylyl cyclase (AC), which has been mentioned as a differentiation mark between the D1-like and the D2-like receptors, is the most important signal transduction mechanism for Dopamine receptors. AC activity is inhibited by D2 receptors, leading to the inhibition of cAMP production.[9, 10] This results in inactivation of the protein kinase A (PKA), which regulates the function of membrane channels, the synthesis of different cytoplasmic and nuclear proteins, and the (de)sensitization of several G protein coupled receptors.[10, 11]

The second signal transduction route used by Dopamine receptors, employs calcium as a second messenger. Due to stimulation of phosphatidylinositol (PI) hydrolysis by activated D2 receptors, the mobilization of intracellular calcium stores is induced and the intracellular calcium concentration is increased. The coupling to this pathway has only been shown in a few cell lines, including Ltk^- fibroblasts and CCL1.3 cells. An opposite effect can also be seen, in which Dopamine receptor signaling inhibits inward calcium currents. This can either be due to the activation of potassium currents, which leads to a change in the membrane potential, or to direct inhibition of calcium channels by G proteins.[9]

A third possible mechanism for signal transduction is the modulation of potassium (K^+) currents. The outward current is increased by D2-like receptor signaling, resulting in hyperpolarization.[9] The effect of this hyperpolarization has not been established outside the CNS.

Other pathways which can be influenced by the D2 receptor are the release of arachidonic acid (AA), the Na^+/H^+ exchanger, and the Na^+-K^+-ATPase.[9]

Apart from these signal transduction pathways, the D2-like receptors are also involved in mitogenesis and cell differentiation. The D2 receptor has been shown to have either mitogenic effects, or cell growth inhibiting effects, depending on the cell line. Also, the D2 receptor might promote some cell differentiation aspects.[9]

Receptor regulation

The regulatory mechanisms behind dopamine receptor presence and activity are not completely elucidated yet. However, some rather general G protein coupled receptor mechanisms, including desensitization, might hold true for D1- and D2-like receptors as well. Desensitization indicates the decrease of receptor responsiveness upon ligand binding, after prolonged stimulation. Also, phosphorylation of the receptor occurs, followed by binding of arrestin-like proteins. This induces the uncoupling of the receptor from its G protein

followed by internalization of the receptor in an endosome via clathrin-coated pits. Finally, dephosphorylation and recycling or degradation via lysosomes occurs.[10] The D2 receptor forms an exception to the above mentioned mechanism, because desensitization does not occur solely on agonist binding. D2 receptor activation variably results in functional desensitization or sensitization and up- or down-regulation. This depends on the concentration and type of agonist or antagonist, the receptor isoform, and the cell system.[12] D2 receptor phosphorylation, desensitization, and internalization is mediated by PKC.[13]

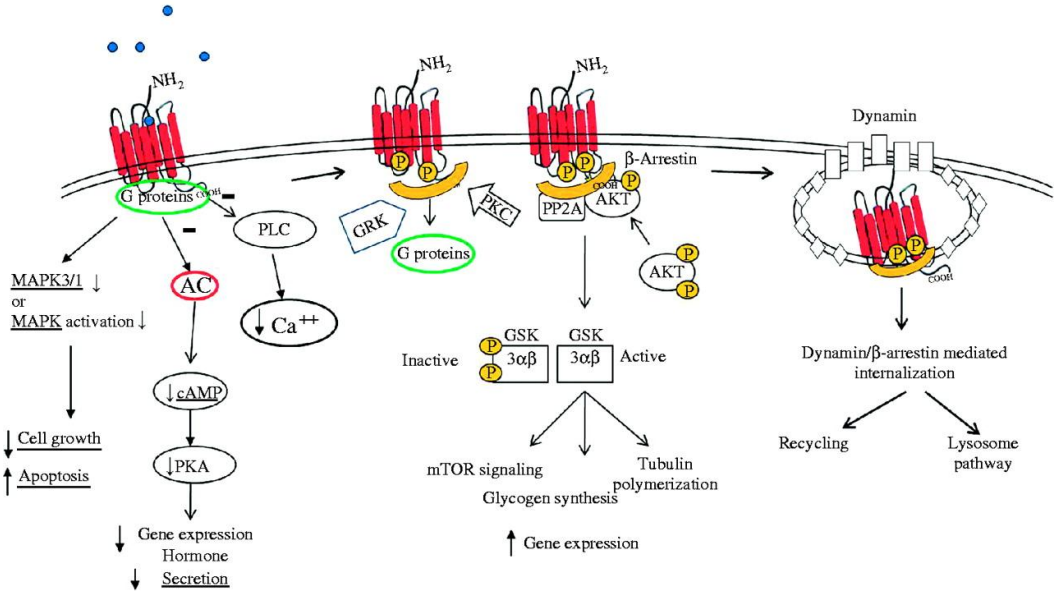


Figure 4. Representation of signaling and trafficking of the D2 receptor.[69]

Short and long dopamine receptor D2 isoforms

As mentioned before, the D2 receptor exists in two isoforms. The alternative splicing of 87 basepairs results in a protein that is 29 amino acids shorter. These amino acids are located in the third intracellular loop. Because this loop is coupled with the inhibitory G protein and involved in signal transmission[10], its alternative splicing could have functional implications. While both variants inhibit AC, the affinity for this inhibition differs in favor of the short variant, which has a stronger inhibiting effect. There is also a different effect on the inhibition of calcium and potassium currents.[11] Apart from these differences, there also seems to be a difference in the modulation of the transmembrane signaling induced by the long or short variant by protein kinase C (PKC), in which the mediated response of the short variant is selectively inhibited. A pathway through which only the short isoform of the D2 receptors transmits its signal, is the stimulation of phospholipase D via specific G proteins and a specific PKC isoform.[10] Both isoforms influence the regulation of cell growth, differentiation, and apoptosis by activating the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways.[10, 14] The specific inhibiting G protein

coupling, through which the different isoforms signal, is dependent on the tissue and the isoform.[15, 16] There are no specific compounds that could discriminate between the two isoforms[9], but the dopamine antagonist sulpiride has a very small difference in affinity.[10]

The short isoform of the D2 receptor has been associated with a more potent dopaminergic effect, implicated by a stronger inhibitory effect shown in studies of pituitary tumours.[17, 18] This difference in inhibitory effect is probably due to the insertion in the third cytoplasmic loop of the D2 long isoform, which affects the overall structure of the loop.[15] Studies in cortisol-secreting adrenal gland tumours of humans have already shown a change in expression of both isoforms in adenomas compared to carcinomas.[19] However, in this study no decrease in cortisol levels was seen after administering the dopamine agonist cabergoline.

The third intracellular loop of the D2 receptor is also involved in the internalization and desensitization of the receptor. Specific internalization pathways used by either the long or short variant [20], activate the MAPK pathway. For the short D2 isoform this includes clathrin-mediated endocytosis and dynamin-dependent internalization, but for the long D2 isoform the internalization is probably independent of dynamin.[10, 14]

Expression pattern

In the adrenal gland, the mainly expressed dopamine receptor is the D2 receptor. Since this receptor has two isoforms, probably with different effects, it is of interest to explore if these isoforms have different expression ratios in canine adrenal gland tumours. It has already been proven that the D2 receptor is present in human cortisol-secreting adenomas and carcinomas. The D2 long isoform was found to be the only isoform present in carcinomas, while both isoforms were present in adenomas.[19]

Aim

The aim of is study is to examine the expression of the short and long isoform of the D2 receptor in cortisol-secreting adrenocortical tumours in relation to normal adrenal glands. Based on the studies in humans, it is expected that in carcinomas the long isoform of D2 receptor will solely be found, while both isoforms will be found in adenomas.

Patient material

The tissue for this study was collected from 36 dogs with ACTH-independent hypercortisolism due to adrenocortical tumours. Among these adrenocortical tumours there were 26 carcinomas and 12 adenomas, based on criteria proposed by Labelle *et al.* 2004.[21] The adrenocortical tissue was obtained by adrenalectomy, performed at the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine of Utrecht University between 2001 and 2010. Permission to use the tissues for further research prospects was given by the owners. The dogs' ages at the time of surgery ranged from 2 to 13 years. The dogs were from different breeds, mixed breed dogs were the most common (6). Of the dogs 18 were female (12 neutered) and 18 were male (8 neutered).

Fifteen adrenal glands (whole tissue explants) of healthy laboratory beagle dogs served as control tissues. The dogs were euthanized due to reasons not related to this study. Their use has been approved by the Ethical Committee on Animal Experimentation. The adrenal glands were collected within approximately ten minutes after euthanasia.

After adrenalectomy, a part of the tumour tissue was snap-frozen in liquid nitrogen and stored at -70°C until its use for qPCR analysis. At the histopathological examination performed by the pathologist, the tumours were classified into either adenoma or carcinoma based on the criteria by Labelle *et al.* (2004).[21]

RNA isolation

Total RNA was isolated from normal and tumorous adrenal glands of dogs and treated with DNase, using the RNeasy mini kit (Qiagen, the Netherlands) according to the manufacturer's protocol.

cDNA synthesis

cDNA of the isolated RNA was synthesized by reverse transcription (RT). RT was performed in a 80 µl reaction using 2000 ng total RNA, 16 µl iScript Reaction mix and 4 µl iScript Reverse Transcriptase (iScript cDNA Synthesis kit, BioRad, Veenendaal). The mixture was incubated for 5 minutes at 25 °C, 30 minutes at 42 °C followed by 5 minutes at 85 °C.

Minus RT controls were prepared with 500 ng of the same RNA under the same conditions, but without the addition of reverse transcriptase.

(q)PCR primers

(q)PCR primers were designed using the PerlPrimer software and Primer Select software according to the parameters outlined in the BioRad i-cycler manual. The specificity of each primer pair was confirmed by gel electrophoresis, sequencing of its product, and/or also in qPCR by checking the meltcurve for one peek.

Primer		Sequence	Size of product	GenBank accession no.	Annealing temperature (At)
D2	Fw	5' TGCCCACTGCTCTTCGGACGCAAC 3'	D2s: 319 bp (502 – 820)	AF293963.1	61.0 °C
	Rv	5' GGTGGCTGGGTGGTATGGGACTGT 3'	D2l: 406 bp (502 – 907)		
D2s	Fw	5' CACTCAAGGAGGCTGC 3'	180 bp	AF293964.1	64.0 °C
	Rv	5' TTCTTCTCTGGTTTGGCG 3'	(715 – 806)		
D2l	Fw	5' CCACTCAAGGGCAACTG 3'	86 bp	AF293963.1	60.0 °C
	Rv	5' CCTGTTCACTGGGAA 3'	(714 – 800)		

Table 1. Overview of used primers. With D2 primers showing the presence of two D2 isoforms and D2s and D2l primers for the qPCR of the short and long D2 receptor, respectively.

The products of the PCR were separated on a 1% Agarose gel and were run at 100V to show the presence of the two isoforms in the different adrenal tissues. A PCR reaction will result in 2 different bands on gel with this specific primer pair, because the product of the short isoform will be 319 basepairs in length, while the product of the long isoform will be 495 basepairs in length.

PCR

The PCR is performed with a 20 µl reaction, which contains 1 µl of cDNA and 19 µl of the reaction mix. The reaction mix was composed according to Table 3. The PCR amplification program used is shown in Table 2.

To be able to determine the long and short isoform ratios in normal adrenocortical tissue, adenomas and carcinomas, a quantitative PCR (qPCR) was performed.

Temperature	Time	
98 °C	30“	
98 °C	10”	35x
At	10”	
72 °C	10”	
72 °C	5’	
20 °C	∞	

Table 2. PCR program. At; annealing temperature according to Table 1.

Compound	Volume 1 PCR reaction
5x Phusion buffer HF	4
10 mM dNTP	0.4
10 mM Forward primer	1
10 mM Reverse primer	1
Milli Q	12.4
Phusion enzyme	0.2
Total	19

Table 3. Composition PCR reaction mix.

qPCR

A qPCR for the two specific isoforms was performed after the PCR previously discussed. To be able to quantify the short and long isoforms separately, new primers were composed (Table 1). The forward primer for the short isoform is located at the junction of exon 4 to 6, where compared to the long isoform exon 5 is deleted. The forward primer for the long isoform is located at the junction of exon 4 to 5, and the reverse primer is located at the end of exon 5. In this way it is secured, that the primers will differentiate between the two isoforms. RPS19, GUSB, RPS5, and SRPR, were used as the non-regulated reference genes for normalization of target gene expression.

qPCR was performed using the Bio-Rad detection system (Bio-Rad Laboratories Ltd.) with SYBR green fluorophore. Reactions were performed in duplicate with a volume of 25 µl each, acquired by combining 54 µl of qPCR Master Mix, composed according to Table 5, with 2 µl cDNA. qPCR reactions for each primer set were optimized by performing reactions under a gradient of annealing temperatures, using four serial 10x dilutions per primer pair on pooled cDNA from ten random tissue samples. The protocol used is depicted in Table 4: denaturation (95°C), amplification cycle repeated 45 times (95°C for 10 sec, 30 sec at the primer specific annealing temperature (Table 1) and 30 sec at 72°C.) For the primers of the short isoform, two instead of one gradient reaction had to be performed in order to establish the annealing temperature, with the first gradient between 50-60 ˚C and the second between 60-70 ˚C. A melting curve analysis was performed following every run to ensure a single amplified product for each reaction. The reference standard dilution series was repeated on every plate. Duplicate negative controls were run with every experimental plate to assess the specificity and to identify any potential contamination.

Compound	Volume 1 qPCR reaction (96 wells)
SYBR Green	1250
10 nM Fw primer	113
10 nM Rv primer	113
MilliQ	1250
Total	2726

Table 5. Composition of Master Mix for qPCR reactions, values in µl.

Temperature	Time	
95 °C	3’	
95 °C	10”	45x
At (or gradient)	30”	
72 °C	30”	24x

Table 4. qPCR program;
At Annealing temperature

Statistical analysis

Statistical analysis was performed with Rest 2008 according to the manufacturers’ protocol and GeNorm software was used for analysing reference gene stability [22].

As the qPCR product of the D2 short showed several bands after gel electerophoresis, the qPCR results of the D2 short had to been excluded from further analyses. Strangely the melt curve of the qPCR products had one, rather broad peek, which would indicate that there is only one product with rather high Ct values starting at 32.

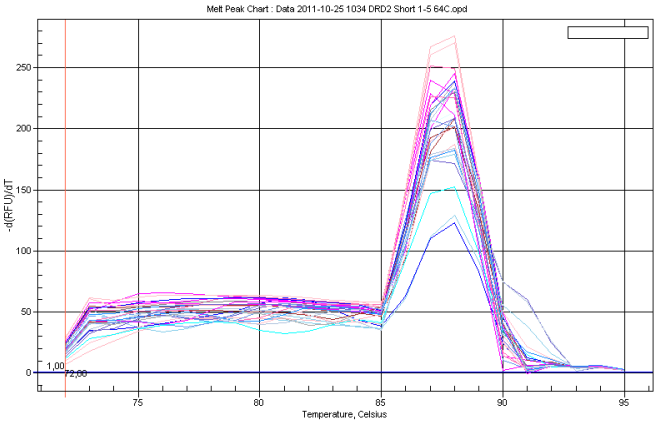


Figure 5. Melt curve of the D2 short qPCR products, showing one rather broad peek.

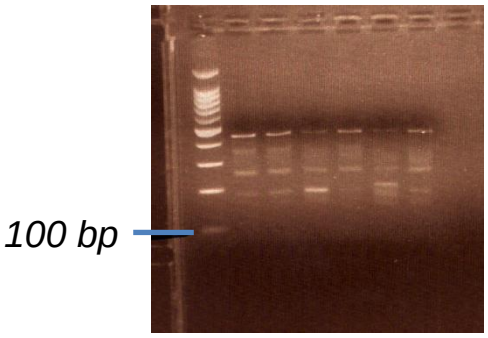
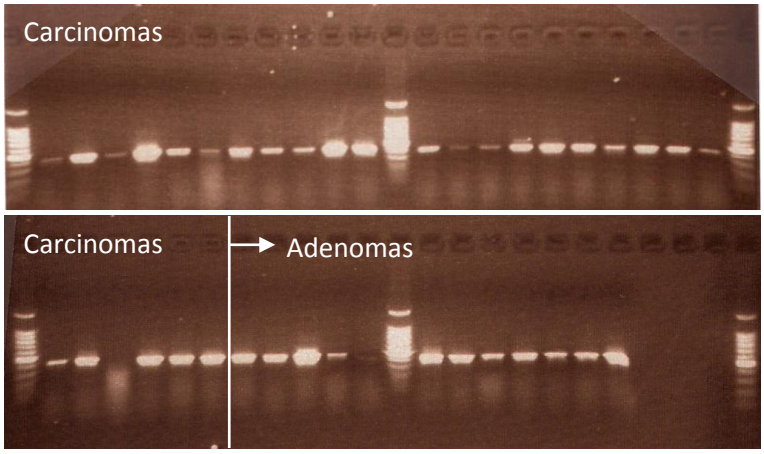


Figure 6. Gel electrophoresis result of qPCR product of D2 short, showing several bands.

Figure 7. Gel electerophoresis of D2 long and short isoform in adrenocortical adenomas and carcinomas. In all the samples, mainly one band is shown of 400bp, which corresponds with the length of de PCR product of D2 long. There is no band visible around 300bp, which would correspond to the length of the D2 short product.



The qPCR products of the D2 long were checked by sequencing the product. This confirmed the sequence of D2 long, which allowed the qPCR results to be further analyzed. The efficiency of the standard curve of one of the two plates was very low, 45% instead of 90-110%, and therefore this plate could not be used for further analysis. The finally obtained relative expressions based on one 96 wells qPCR plate were plotted in

Figure 8. There were no significant differences between the adenomas, carcinomas, and/or normal adrenal glands. When carcinomas are compared to normal adrenal glands, a relative expression of 0.231 with a p-value of 0.146 is seen. Adenomas compared to normal adrenal glands results in a relative expression of 0.055 with a p-value of 0.086. Comparing the adenomas and carcinomas together with the normal adrenal glands results in a relative expression of 0.136 and a p-value of 0.11. At last the adenomas and carcinomas were compared which results in a relative expression of 0.238 with a p-value of 0.334.

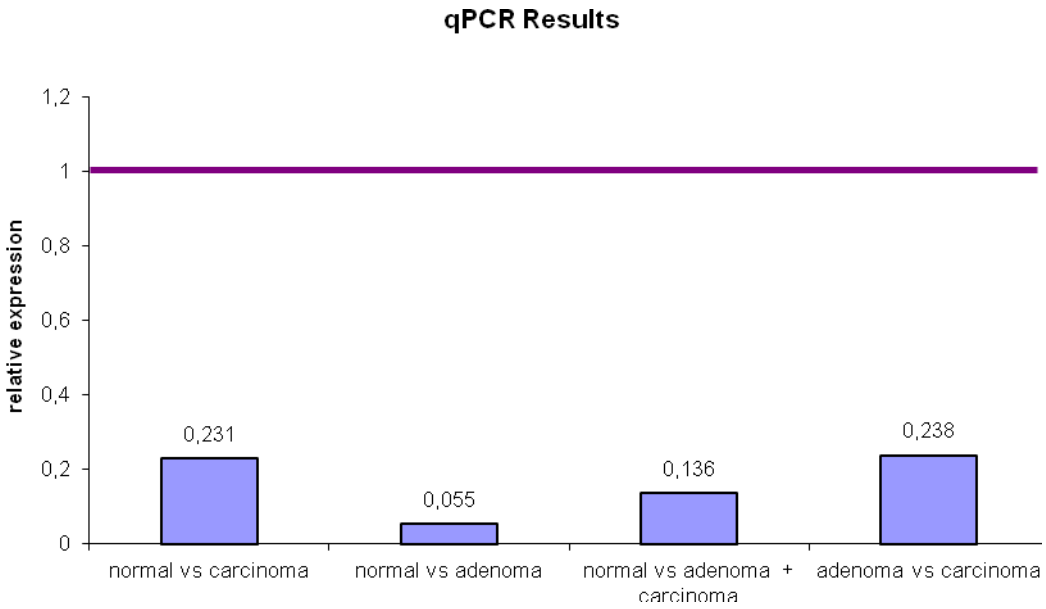


Figure 8. Relative expression of the Dopamine receptor type 2 long isoform (D2 long) in adrenal gland carcinomas, adenomas, and both compared to normal adrenal glands.

DISCUSSION

The dopamine receptor type 2 did not show any significant differences in expression between adenomas, carcinomas and/or normal adrenal glands. Due to a bad standard line in one of the two D2 long qPCR plates, which unfortunately could not be redone, the results are based on only one plate with thirteen carcinomas, seven adenomas and eight normal adrenal gland samples.

The reference genes did not show good results either. In GeNorm, two out of four reference genes, SRPR and RPS19, had a M-value of above the cut-off value of 1.5, with values of 1.552 and 2.506 respectively. This means that the expression of these reference genes in the three groups, adenomas, carcinomas, and normal adrenal gland tissues was not sufficiently stable. The same was seen when calculation was performed in rest 2008. No down-regulation was noticed in the expression of the mRNA of D2 long when normalized with the reference genes, but a down-regulation was seen if there was no normalization with the reference genes. This indicates that one or more reference genes differ in expression in the three groups, the same way as D2 long, neutralizing the possible down-regulation of D2 long. Other reference genes should be used to calculate if D2 long is down-regulated in tumorous adrenal gland tissue.

Whether the results of the qPCR of the long and short isoforms of D2 are useful for concluding if the receptor expression is down-regulated or not, is questionable. Cortisol producing adrenocortical tumours consist of only zona fasciculata cells. These cells have the lowest amount of D2 receptors.[19] Therefore if normal adrenal gland tissue is compared with tumorous adrenal gland tissue by qPCR, the differences could also be due to the fact, that we did not compare the tissue equals. Immunohistochemistry would be needed to sort this out. Furthermore, with immunohistochemistry the expression of the protein is examined, while with qPCR no data about the protein are obtained. Unfortunately, de Bruin *et al*. (2008) have already tried to immunohistochemically stain the D2 receptor in the pituitary of the dog, but were unable to get immunopositivity due to the unavailability of canine-specific antibodies.[23]

The results of the D2 short remain questionable. It is very unlikely to see one peek in a melt-curve and for the same samples, several bands on a gel. This would indicate that the different products denature at the same temperature, which would be almost impossible, because they probably do not have the same amount of hydrogen bonds. These bonds will resolve and at a certain temperature all DNA will become single stranded, which is shown in the melt-curve. Since there is only one rather broad peek, this means that all DNA in the sample becomes single stranded at about the same temperature. However, as demonstrated in Figure 7 the expression of the D2 short is practically absent in the adenomas and

carcinomas, which implicates that there will be no role for the short isoform in medical treatment of ACTH-independent hypercortisolism.

What could be the future of the D2 receptor regarding ACTH-independent hypercortisolism? In humans, the dopamine agonist cabergoline has not been able to inhibit cortisol levels in cortisol-secreting adenomas and carcinomas.[19] Cabergoline binds to the D1-like and D2-like receptors, but binds more strongly to D2-like receptors.[10] There is no data about ACTH release being regulated by dopamine. The only hormone that has been shown to be inhibited by the dopamine agonist bromocriptine, is prolactin.[24] The dopamine agonist bromocriptine also binds D2-like receptors preferentially. Sulpiride, a dopamine antagonist, binds exclusively to D2-like receptors.[10] The two D2 receptor isoforms are thought to be relevant in signaling pathways related to proliferation and cell death in pituitary cells.[25] A study of Pivonello *et al.* showed an association between D2 receptor expression and tumour shrinkage after one year of treatment with cabergoline.[17] Therefore, more than in medical management of cortisol release, the dopamine agonist might play a role in suppressing the tumour growth. The effects on adrenocortical cells needs further examination.

According to Wu *et al*. (2001), the effect of dopamine in the adrenal gland is limited to the zona glomerulosa. Noradrenergic endings in the cortex of the adrenal gland are suggested to release and take up dopamine. These local noradrenergic endings are limited to the zona glomerulosa and therefore would indicate that the modulation of steroid biosynthesis and/or secretion by dopamine is limited to the ZG.[26] This corresponds with the fact that the expression of the D2 receptor is mainly seen in the zona glomerulosa and expression is rather low to absent in the zona fasciculata.[19] Furthermore, dopamine antagonists are unable to induce a rise in cortisol levels.[26] This could also indicate that cortisol is not under permanent inhibition by dopamine, unlike aldosterone [19]. It would be expected that if dopamine would have an effect on cortisol levels, treatment with dopamine agonists would result in inhibition of plasma cortisol levels.

The main focus of research regarding dopamine is focused towards its effects in the CNS, because dopamine has a lot of pre- and postsynaptic effects in different areas of the brain. At this point, there are no data suggesting that dopamine agonists or antagonists would be implicated in the treatment of ACTH-independent hypercortisolism. Their expression is very low in the zona fasciculata, there have been no effects of agonists or antagonists on plasma cortisol levels in the study of Pivonello *et al.*[19], and a low expression of the dopamine receptor has been shown in adrenocortical tumours in this study.

CHAPTER 2

SOMATOSTATIN RECEPTOR LOCALIZATION

ABSTRACT

Background: Somatostatin receptors are recently of interest as targets for new drug therapy in neuroendocrine tumours, because of their inhibiting effects on hormone secretion. Their expression has been studied on several tumours and also on cortisol-secreting adrenocortical tumours, but not in dogs.

Aim: To determine the protein expression and localization of several somatostatin receptor subtypes in canine cortisol-secreting adrenocortical tumours. It is expected that the expression pattern varies individually.

Material and Methods: Immunohistochemical staining of SSTR1, SSTR2, and SSTR3 in several adenomas and carcinomas, compared with a normal adrenal gland.

Results: The immunohistochemical staining of SSTR3 was excluded due to unreliable results. The staining patterns of SSTR1 and SSTR2 were very variable compared to the normal adrenal gland.

Conclusion: It could be that somatostatin analogues will be of use in the future as personalized therapy.

INTRODUCTION

The somatostatin receptors (SSTR) are of interest for developing new therapy for (neuro-) endocrine tumours, because of their inhibitory effect on hormone secretion.[27] These receptors have been shown to be present in several (neuro-) endocrine tumours as well as in the pituitary gland of humans and dogs. Research on canine pituitary tumours showed different expression ratios of the somatostatin receptor subtypes compared to humans.[7] Canine tumours mainly express SSTR 2 and have a low expression of SSTR 5, while the opposite is the case in human tumours. Regarding the adrenal glands, data are lacking. There are few reports about SSTR expression in human [28-32] and rat [33] adrenal glands. The reports about adrenal gland tumours in humans were not very supportive of future use of somatostatin analogue therapy, but recognized some potential for somatostatin analogues in diagnostics or personalized therapy in the future. However, regarding the differences between human and dog in SSTR subtype expression in the pituitary, it is important to investigate if the same pattern holds true for the adrenal gland. It is possible that the SSTR subtype expression rate in dogs may be of use for new drug therapy.

Somatostatin and the somatostatin receptor

Somatostatin is a neuropeptide which was originally identified by Krulich in 1968.[34] After isolation and characterization by Brazeau in 1973[35] it is nowadays still a protein which gains a lot of interest from researchers all over the world. Somastatin was originally characterized as a hypothalamic peptide, while nowadays its presence has been detected almost everywhere in the body. Is has a widespread effect on multiple (neuro)endocrine systems by functioning as a neurohormone, neurotransmitter, and autocrine or paracrine hormone.[8, 36] Somatostatin is produced by specific cells, which are widely distributed throughout the body and are also present in the adrenal gland.[37] Due to the short circulation half-life of 1.5-3 minutes[36] as a result of inactivation by peptidases[38], it is thought that the cells that produce somatostatin are located close to the target cells.[36] When somatostatin binds to its receptor it regulates both endocrine and exocrine hormone secretion, with primarily an inhibitory effect.[37, 39] Somatostatin has no effect on ACTH secretion in healthy human individuals, but it does inhibit ACTH secretion in diseases with an elevated ACTH level like Addison's disease and ACTH-producing tumours. In normal adrenals, somatostatin has an inhibitory effect on aldosterone and catecholamine secretion. A biphasic effect of somatostatin on glucocorticoid secretion, in which with low doses have stimulatory effects, while large doses are inhibiting, has been described.[38]

Somatostatin exists in two physiologically active variants, containing either 14 or 28 amino acids which are called somatostatin-14 and somatostatin-28 respectively. SST binds

with different affinities to the six different somatostatin receptor subtypes (sstr 1, sstr 2A and sstr 2B, sstr 3, sstr 4, and sstr 5), which are encoded by five genes.[37] The subtypes have a sequence homology of about 39-57% with a specific highly conserved sequence motif, while the variation is mainly located at the amino- and carboxy-terminal segments.[38] The seven transmembrane G-protein coupled somatostatin receptors are part of the G-protein-coupled receptor (GPCR) superfamily, which indicates that their actions are mediated by G proteins.[37] While the receptors normally consist of seven transmembrane regions, the SSTR 5 also has two truncated isoforms with four and five transmembrane regions respectively. These splice variants have been shown in human pituitary tumours to be variably expressed.[39]

The somatostatin receptors have been found to appear in high density in neuroendocrine tumours.[40] Based on the fact that they can regulate hormone secretion in an inhibitory way, these receptors are of interest in developing new treatments for hypersecretion of hormones due to a tumour.[37] Apart from the influence on hormone secretion, the SSTRs also have antiproliferative and pro-apoptotic effects upon stimulation[28], which is also of interest in tumour therapy. The exact mechanisms behind these effects will be explained next.

Effects of receptor activation

The inhibition of hormone secretion is due to the effect of somatostatin binding to its receptor. Several second messenger systems are modulated by the G-coupled proteins (mainly G_i, an inhibiting G protein) linked to the receptors, which become activated after binding takes place. The signaling pathways which are influenced by all SSTRs are: inhibition of adenylyl cyclase, activation of phosphotyrosine phosphatase (PTP), and mitogen-activated protein kinase (MAPK) modulation. Other pathways which are modulated by one or more of the receptors are: K^+ and Ca^{2+} ion channels, Na^+/H^+ antiporter, guanylate cyclase, phospholipase C, phospholipase A2, and serine and threonine phosphatase.[38]

Coupling of the SSTR to K^+ channels, induces a hyperpolarization of the membrane [41] by a K^+ influx upon the opening of K^+ channels. This hyperpolarization prevents the voltage-sensitive Ca^{2+} channels to open and depolarization-induced Ca^{2+} influx to appear, and therefore the intracellular concentration of Ca^{2+} will decline. There are also some direct and other indirect effects on these channels which result in lower Ca^{2+} concentrations.[38] The low intracellular Ca^{2+} concentration results in a decrease in hormone excretion.[42] This inhibitory pathway has been described in the pituitary, and whether the same holds true for the adrenal gland has not yet been proven. Apart from the effect on Ca^{2+}, all SSTR subtypes have inhibitory effects on adenylate cyclase and cAMP production after ligand binding. The low cAMP and Ca^{2+} concentrations are known to block regulated secretion, which explains the effect of somatostatin on hormone secretion.[41] There also seems to be a rather direct

inhibitory effect on exocytosis, mediated through activation of the protein phosphatase calcineurin.[36, 38]

SSTRs can influence several pathways of which some are acted upon by all receptor subtypes, while several receptor subtypes can be expressed in a single cell. It is unknown whether this pattern of expression is functional, for instance by the formation of homodimers and heterodimers which could alter functional properties.[38]

The mechanisms behind the antiproliferative and pro-apoptotic effects in tumours are not totally clear yet.[25, 42] The effects can be divided into direct and indirect effects. The indirect effects are due to SSTRs present on non-tumour cells, which secrete hormones and growth factors that stimulate tumour growth, e.g. by angiogenesis, and modulate immune cell function. By binding of SST to its receptor on these cells, these effects will be inhibited. The direct effects are due to SSTRs present on the tumour cells. Examples of direct effects are the stimulation of PTP activity and the inhibition of MAPK.[38] The activation of PTPases induces dephosphorylation of the growth factor receptor, which inhibits this receptor and is thereby responsible for the anti-proliferative effect. Apoptosis is induced by SSTR3, and also slightly by SSTR 2[43] via activation and dephosphorylation of p53, a tumour suppressor protein, and Bax, an pro-apoptotic protein.[38, 41] The other four SSTR subtypes only induce G_i cell cycle arrest, which is associated with induction of p21 and retinoblastoma tumour suppressor protein (Rb).[38]

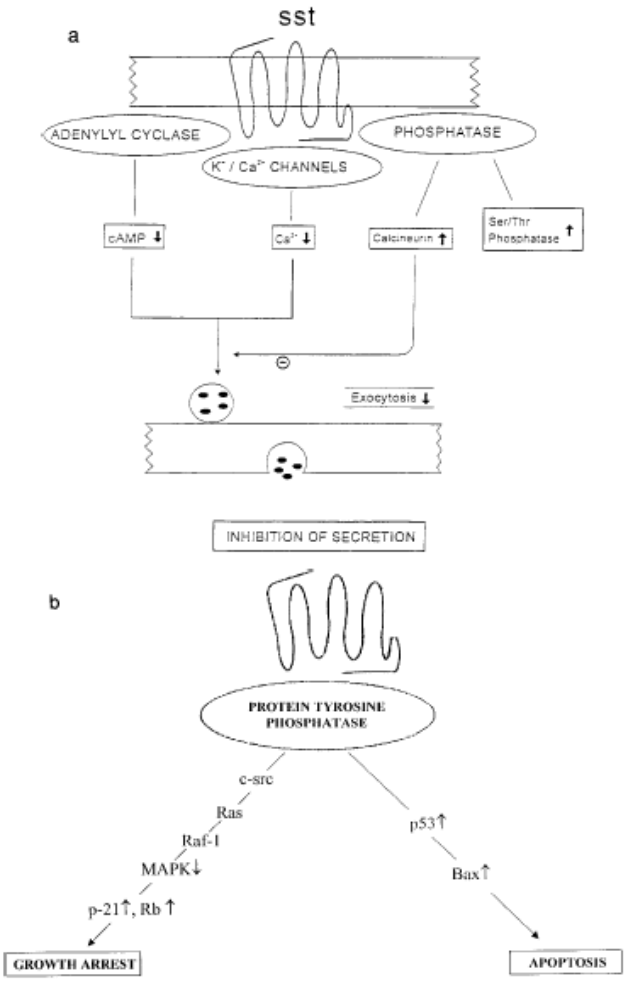


Figure 9. SSTR signaling pathway inducing inhibition of secretion, apoptosis, and growth arrest.[38]

Internalisation and desensitisation

Despite the fact that the effect of somatostatin after activation of the SSTR is in many ways inhibitory, this effect wears off with continued exposure.[38] This is due to the ability of the receptors to regulate their own presence and responsiveness by uncoupling the G proteins from the receptor, which induces desensitization, and also by internalization and

degradation. Crucial for the internalization and desensitization is the phosphorylation of cytoplasmic loops of the receptor.[38] SSTR 2, 3 and 5 seem to be internalized at a higher rate after agonist binding than SSTR 4, whereas SSTR 1 is not internalized at all.[38, 44] This internalization pattern seems to species related, because in the rat, only SSTR 1 can be internalized.[37] After internalization, SSTR 2 seems to be recycled to the cell surface efficiently, while SSTR 3 stays clustered in large intracellular vesicles.[44]

The internalization pathway is composed of the following steps. First, the SSTR will be phosphorylated upon agonist binding, which will terminate the signal. This phosphorylation is executed by G-protein-coupled receptor-kinases (GRKs). Subsequently, the coupling between the G-proteins and the receptor will be interrupted by arrestins. Following the phosphorylation, β-arrestins will form a stable complex with the receptor and will be internalized together via a dynamin/clathrin-dependent pathway. The recycling rate is regulated by the β-arrestins in the endosome compartment. Because the binding properties of β-arrestins differ among the receptor subtypes, differences in recycling rates between the subtypes occur. The binding properties seem to be related to expression of specific phosphate acceptor sites at the C-terminal tail. Due to differences between species in the presence of the acceptor sites, species related internalization patterns could be explained.[37, 44, 45] After internalization, the receptors can be dephosphorylated and recycled or ubiquitiniated and degraded.[37, 45] The discussed pathways are visualized in Figure 10.

If the exposure to the SSTR agonist is prolonged up to 22 hours, the expression of several SSTR subtypes is upregulated at the membrane. However, the mechanism has not been elucidated yet.[38]

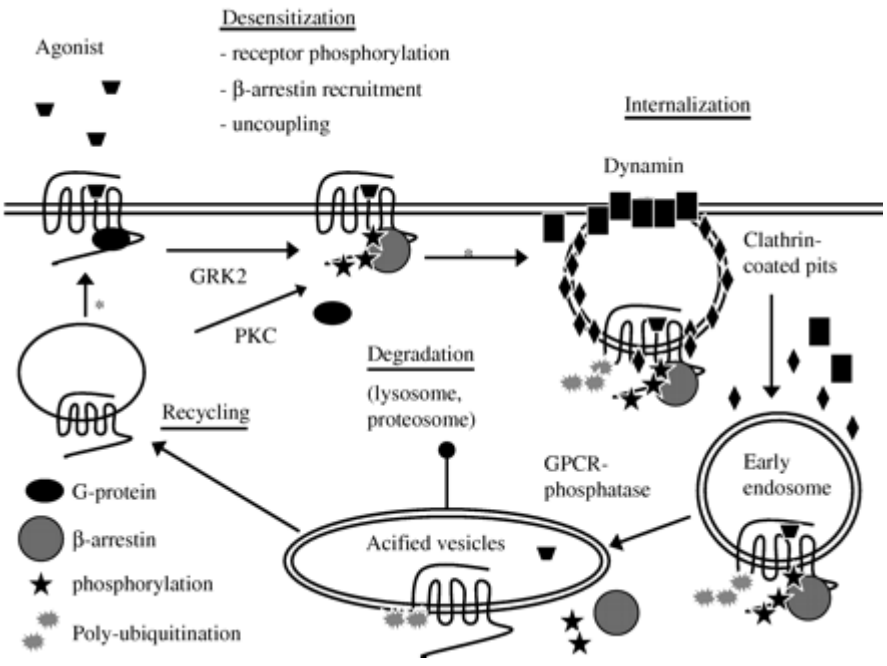


Figure 10. Intracellular components involved in GPCR desensitization, internalization and intracellular trafficking, after agonist binding.[37]

Receptor subtype expression in humans and dogs

The somatostatin receptors are expressed in a lot of different organs and tissues (Table 6). The SSTR subtype which is mainly expressed in normal tissue and human tumours is SSTR 2.[40, 41, 45] While the main SSTR expressed in human corticotroph adenomas is SSTR 5, only low values of this receptor are expressed in canine corticotroph adenomas.[8, 23] In dogs, SSTR 2 is mainly expressed[23] and the glucocorticoid-induced down regulation of this receptor which is thought to occur in men[8], is not seen in canine corticotroph adenomas. There was even an increase in SSTR 2 expression upon treatment with dexamethasone.[23] In contrast to the above, Jacobs *et al.* state that SSTR 2 analogues could be an ideal therapy because of the lack of down-regulation.[44]

So far, there are only a few studies on the expression of somatostatin receptors in the adrenal gland. Of the study of Pisarek *et al*. (2008), the most striking finding is that immunohistochemistry of all SSTR subtypes demonstrated a varied expression pattern, which was specific in each case, or rather very individual.[29]

Location	SSTR 1	SSTR 2
Brain	Yes (divergent)	Yes (divergent)
Pituitary	Yes (low)	Yes (high)
Pancreas	Yes (β cells)	Yes (α cells)
Stomach	Yes	Yes
Liver	Yes	No
Kidneys	Yes	Yes
Intestines	Yes	Yes
Adrenals	Yes (modest expression)	Yes (high)
Immune cells	No ?	Yes (activated cells)
Tumours (insulinoma)	Yes	Yes (high)

Table 6. Distribution of somatostatin receptor subtype 1 and 2 based on mRNA expression studies in humans and rats, with some differences in expression elucidated between brackets. (Based on Patel; 1999)[38]

The use of somatostatin analogues in human medicine is widespread compared to the use in veterinary medicine. It is used for clinical treatment of various tumour types and for diagnostic purposes. By studying the SSTR expression in cortisol-secreting adrenocortical tumours, the potential for use of somatostatin analogues will become clear. Based upon the few studies which have been done in men[31, 32], the results could lead to individual linked expression patterns upon which individual therapy could be based. Despite the low CT values measured with qPCR, it is still of importance to look for protein expression with immunohistochemistry (IHC). With this technique the localization of the receptor subtypes

and a rough estimation of the protein expression can be determined. With the previously performed qPCR, there is only information about the amount of mRNA. Pisarek and coworkers showed that the levels of mRNA and the corresponding protein expression were unrelated.[28] Also, the eventual therapy is based on protein expression and not mRNA expression. Another reason why IHC has to be performed is that different tissues have been used for the mRNA measurements. Measurements with the qPCR method were performed on normal adrenal gland tissue and on tumorous tissue. As mentioned in the former chapter, this indicates that no tissue equals were compared due to the high amount of zona fasciculata cells in a cortisol-secreting adrenocortical tumour compared to a normal adrenal gland. Therefore the expression of several SSTR protein subtypes in canine cortisol-secreting adrenocortical tumours needs to be investigated by immunohistochemistry in this study.

Aim

The aim of this part of the research project is to determine the protein expression and localization of several somatostatin receptors in canine cortisol-secreting adrenocortical tumours compared to normal adrenal glands. It is expected that the expression pattern varies individually.

Patient material

The adrenocortical tumours (ATs) used in this study were obtained from 19 dogs (7 adenomas and 12 carcinoma's, with a total of 9 slides per receptor subtype) with ACTH-independent hypercortisolism that underwent adrenalectomy at the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine of Utrecht University between 2001 and 2010. Permission was obtained from the dogs' owners to use the ATs for this study. The dogs' ages at the time of surgery, ranged from 6 to 13 year. The dogs were of different breeds. Of the dogs 7 were male (2 neutered) and 12 female (9 neutered).

Based on the clinical history and physical examination, there was a suspicion of hypercortisolism, which was confirmed using laboratory tests as mentioned in 'Clinical Endocrinology of Dogs and Cats'[6] and Gilor *et al*.[46]

Adrenal glands (whole tissue explants) of healthy laboratory beagle dogs served as control tissue. The dogs were euthanized in other experiments, unrelated to the present study, that had been approved by the Ethical Committee Animal Experimentation of the Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. The adrenal glands were collected within approximately ten minutes after euthanasia. The same accounts for the other control tissues.

After adrenalectomy, a part of the tumour tissue was fixed in formaldehyde for histopathological examination and IHC. At the histopathological examination performed by the pathologist, the classification of adenoma or carcinoma was performed based on the criteria described by Labelle *et al.*[21]

Immunohistochemistry

Before the adrenocortical tumour tissue could be stained, optimization of the immunohistochemical protocol was needed. This has been performed on tissues in which a high expression of the specific SSTR subtype was expected. Expression of SSTR subtype 1 was expected in colon[47], insulinomas (unpublished qPCR data, [38]) and adrenal glands. Expression of SSTR subtype 2 was expected in pancreas and insulinomas.[38, 48, 49] Expression of SSTR subtype 3 was expected in insulinomas (unpublished qPCR data,[38]), colon[47], and normal adrenal glands.

After the protocol was optimized the adrenocortical tumours were stained. Based on the qPCR results of the mRNA expression of the receptor subtypes 1, 2 and 3, the 9 samples with the lowest Ct value were used for the immunohistochemical staining. These 9 samples were equally divided amongst adenomas and carcinomas.

	SSTR 1		SSTR 2		SSTR 3	
	Nr	Ct value	Nr	Ct value	Nr	Ct value
Adenomas	III	28.9361	III	25.2634	I	29.4638
	VI	28.2606	VI	25.1356	VIII	31.9711
	XI	28.0143	VIII	23.1418	IX	29.1779
	XII	28.9391	XI	24.8172		
Carcinomas					6	31.4431
	10	27.1918	10	24.5560	10	28.5950
	11	28.4922	21	26.1308	14	29.6255
	23	27.83078	24	25.5596	16	30.1969
	27	28.3898	25	25.1283	23	31.6164
	29	29.0723	28	25.0514	24	30.9724

Table 7. Overview of used tissues, based on their lowest Ct values. Roman numbers correspond to the adenomas, the others the carcinomas.

The immunohistochemistry protocol was performed on 4-µm sections of paraffin embedded tumours. Deparaffinization of the slices was performed twice with xylene, after which rehydration with a graded series of ethanol took place. For antigen retrieval, the sections were incubated for 10 minutes at 98 °C in preheated sodium citrate with a pH of 6 (SSTR 1) or in Tris-EDTA with a pH of 9 (SSTR 2 and 3). Slides were cooled for 20 minutes at room temperature and washed with TBS, after which endogenous peroxidase activity was blocked with 0.35% H_2O_2 in TBS for 15 minutes. Aspecific binding sites were blocked with 10% Normal Goat Serum (NGS) and 1% BSA in TBS for 30 minutes. Incubation with the primary antibody, rabbit anti human, was performed overnight at 4 °C with a working dilution of 1:1500 (SSTR 1), 1:1000 (SSTR 2), and 1:3000 (SSTR 3), in 1% BSA in TBS. The primary antibodies were based on the human SSTR with which the SSTRs of the dog have a 100% query coverage. The primary antibody against SSTR 1 attaches to residues 380-391 of the human SSTR 1 -RNGTCTSRITTL-, against SSTR 2A residues 355-369 of the human SSTR 2A -TQRTLLNGDLQTSI-, and against residues 385-393 of the SSTR 3 -ERPPSRVA- (Thermo Scientific; PA3108, PA3109, and PA3110 respectively), of which the amino acid sequences are located at the same position as the dog, which was checked by blasting the human sequence against the dogs' sequence in NCBI. The secondary antibody was a commercially available anti-rabbit HRP conjugated secondary antibody (EnVision Dako K4003) with which the slides were incubated for 30 minutes. Antibody detection was performed by using DAB substrate kit for peroxidase (Vector SK-4100) for 5 minutes. Counterstaining was performed using heamatoxylin, followed by dehydration in a graded series of ethanol, incubation in xylene twice and covering the slides with VectaMount mounting medium (Vector H-5000)

and a coverslip. TBS 0.05M was used for all washing steps. For negative controls, 1% BSA in TBS was used instead of the primary antibody.

Substance	Amount for 11 slides in µL
10% BSA	220
NGS	220
0.05M TBS	1760

Substance	Amount for 10 slides in µL
Antibody	1.33; 2; 0.66
10% BSA	200
0.05M TBS	1800

Table 8 Composition of Blocking buffer (left) and first antibody mixture (right). ***BSA; Bovine serum albumin, NGS; Normal goat serum, TBS; Tris buffered saline.*** **Antibody concentrations for respectively SSTR 1; SSTR 2; SSTR 3**

Evaluation

The evaluation of the stained slides, with regard to the localisation and intensity of the staining was performed by a pathologist.

RESULTS

Results of SSTR 3 were excluded, because the antibody did not have a 100% query coverage with the amino acid sequence in the dog, due to a failure in blasting the sequences of SSTR 3. As a result, the properly sized band expected for SSTR 3 did not show on the western blot. The results of the staining of SSTR 1 and 2 were criticized by the pathologist. The staining was commented not to be ideal and the method should be optimized thoroughly again before the slides can be properly evaluated.

Despite these comments, photo's haven been taken from several slides and will be shown for the SSTR 1 and SSTR 2 subtype staining separately.

SSTR 1

Material

Nine slides of tumorous tissue were used. Four of those were from adenomas and 5 from carcinomas. Normal colon was used as a positive control tissue (Figure 11 A), and shows that the staining was successful. A normal adrenal gland was stained as well.

There was no general staining pattern that could be identified in the different tumours. In Figure 11 staining patterns in some slides are shown.

Normal adrenal gland

The normal adrenal gland showed different staining intensities depending on the localization. There were several tumours that did not show the same staining pattern in the zona glomerulosa as the normal adrenal did.

Adenomas

A variable staining pattern was seen in the adenomas. Some randomly stained cells throughout the slide were seen in one adenoma, another adenoma showed staining of some nuclei.

Carcinomas

Some carcinomas showed staining of single cells or groups of cells which were seemingly randomly distributed. One slide showed a dark staining throughout all the layers of the adrenal gland.

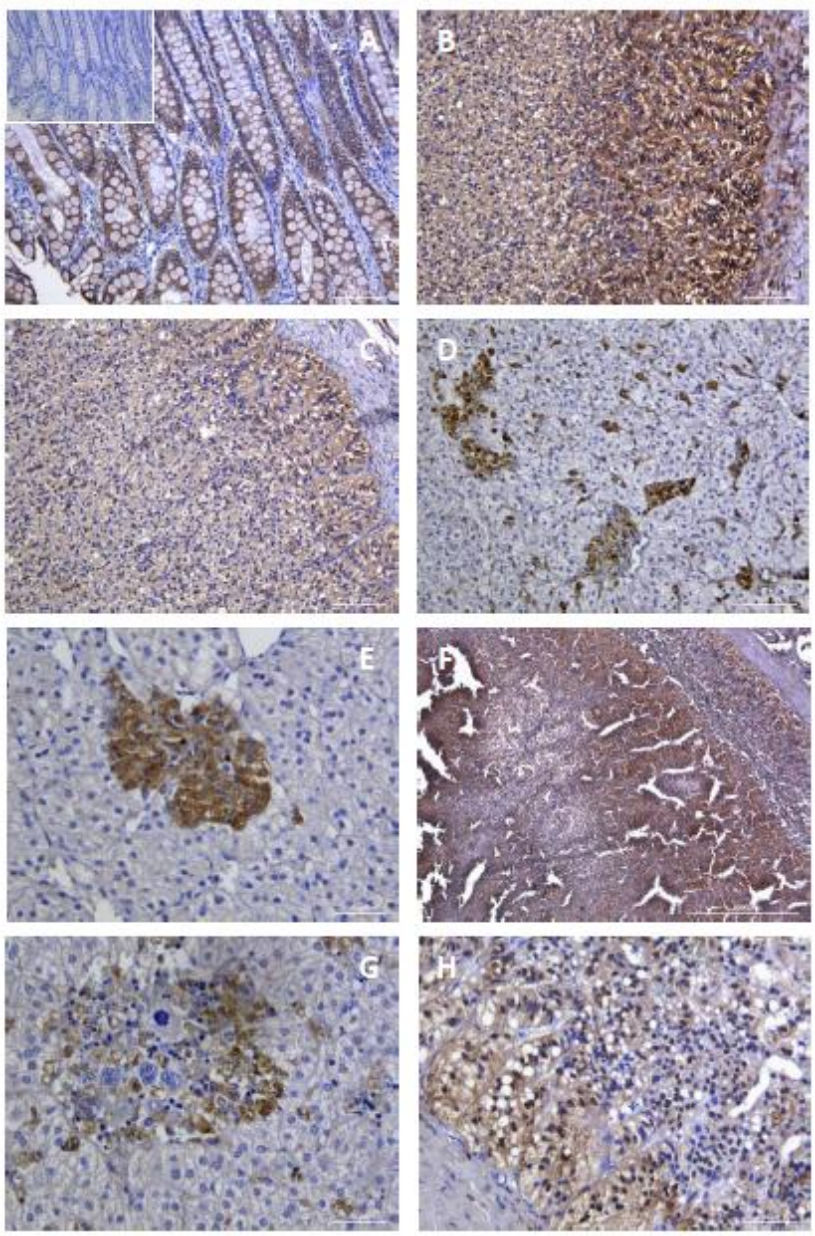


Figure 11. Staining of the SSTR 1 in the positive control tissue, colon (A), with the negative control without the secondary antibody inserted in the corner. Normal adrenal gland shows different staining intensities at different sites (B + C). Some carcinomas show some seemingly random positive cells or groups of cells (D + E), or a dark staining throughout all the layers (F) which is more comparable with the normal adrenal gland (B). An adenoma also shows some randomly stained cells which are seen together with giant cells and premature immune cells (G). Another adenoma shows staining of the nuclei (H).

SSTR 2

Material

Nine slides of tumorous tissue were used in total. Four of those were derived from adenomas and 5 from carcinomas. Normal pancreas served as a control tissue (Figure 12 A), which shows positive staining of the islets. A normal adrenal gland was stained as well.

Normal adrenal gland

The normal adrenal gland does not showed any staining at all.

Adenomas

The adenomas show an inconsistent staining pattern. Two adenomas showed randomly stained cells or groups of cells throughout the slides, whereas other adenomas showed an overall staining throughout the layers, including the zona glomerulosa, which is very different from the absence of staining in the normal adrenal gland.

Carcinomas

The carcinomas also showed different staining patterns, of which one showed membranous staining in one part of the tumour and yet another showed an intense staining pattern throughout the slide and throughout the layers of the tumorous adrenal gland.

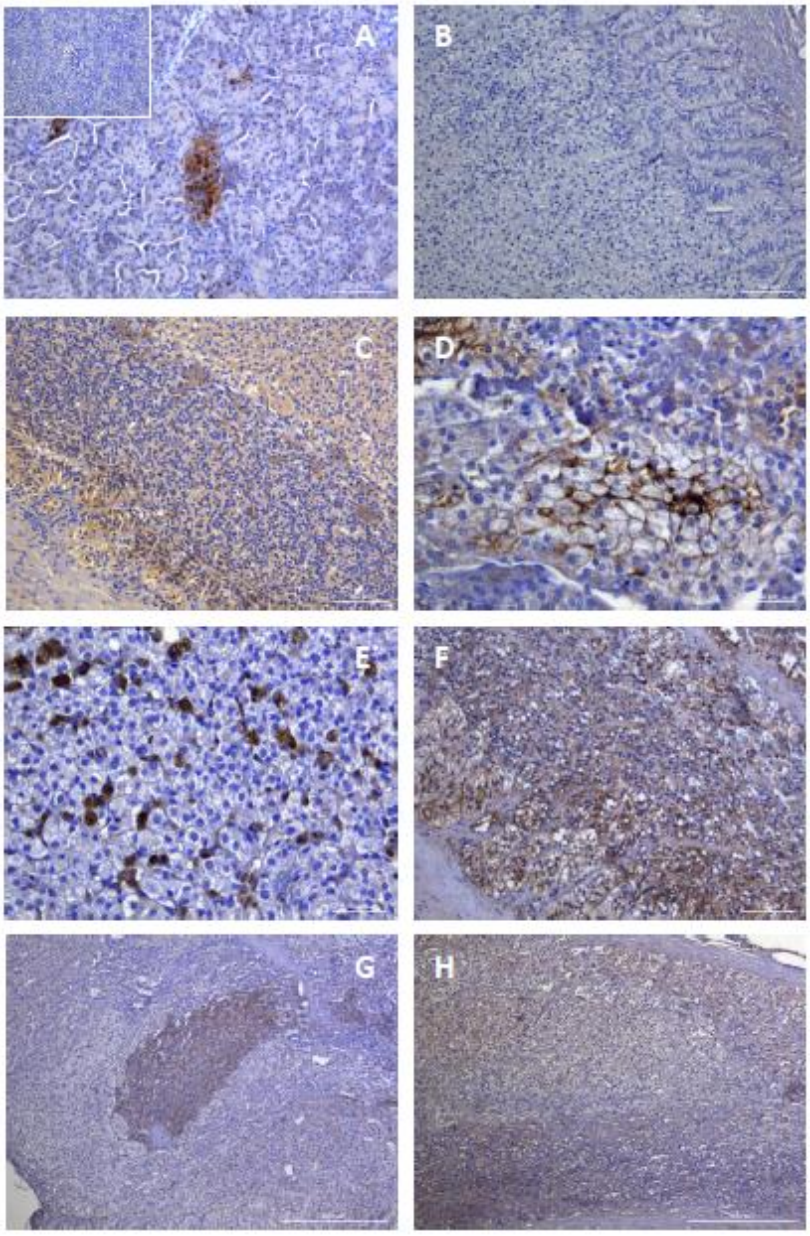


Figure 12. Staining of the SSTR 2 in the positive control tissue, pancreas (A), with the negative control without the secondary antibody inserted in the corner. Normal adrenal gland shows no staining (B). The carcinomas show different staining intensities, in which one is overall quite intensively stained (C), especially compared to the normal adrenal, and the other shows some membranous staining in a small part (D). The adenomas also show an inconsistent staining pattern, of which one shows some randomly stained cells (E), some others show an overall staining throughout the layers (F + H) even with nuclear staining (F), or a group of cells appears to be stained (G).

DISCUSSION

This study is performed with the eventual goal of providing evidence of the use of somatostatin analogues in the treatment of cortisol-secreting adrenocortical tumours. Because of the short half-life of somatostatin, this naturally occurring peptide would not be applicable in drug therapy. Therefore, different somatosatin analogues have been developed for clinical use. There are four analogues, called Octreotide, Lanreotide, Vapreotide, and Seglitide. All analogues have a low affinity for SSTR subtypes 1 and 4 in humans, but a normal to high affinity for subtypes 2, 3, and 5. Octreotide and Lanreotide are available for clinical use.[37-39] Newly synthesized analogues, like SOM 230 and KE 108, could also be considered when choosing analogue therapy. But the decision for which analogue to use, should be made for each individual separately.[29]

If analogue therapy is used, the effectiveness should be monitored, because of the potential up- or downregulation of the receptors. For example, SSTR 2 is downregulated with high glucocorticoid levels.[8] If these levels are lowered during therapy, this could influence the expression of SSTR 2 and thereby the effectiveness of the treatment. Also, there is the effect upon prolonged activation on the expression of the receptor. But the desensitization-, degradation-, and recycling rates seem to be species specific. There is some information on this in humans and rats[33], but for dogs it is yet unknown which receptors will be degraded and which will be recycled. The receptor subtypes that will be down-regulated upon prolonged activation will be less favorable for analogue therapy, while more interest will be gained for the receptor subtypes which will be up-regulated. Due to the wide range of tissues in which the SSTR is expressed and its multiple physiological actions, therapy with somatostatin analogues could be problematic. When the expression of SSTRs is low in adrenal glands, compared to other tissues, a lot of adverse effects could be seen.

There are already promising results regarding the somatostatin receptors and Cushing's syndrome caused by a pituitary tumour. Pro-apoptotic effects on pituitary tumours have been implicated[42], *in vitro* studies showed that ACTH secretion could be suppressed using pasireotide, which targets SSTR 2 and 5. Results with octreotide, a SSTR2 preferring somatostatin analogue were less promising [50]. This might be due to the fact that the STTR 2 promoter is under direct control of glucocorticoids, while STTR 5 is not, inducing downregulation of the receptor during to hypercortisolism.[39, 51] Clinical trials in humans with pasireotide also showed a reduction in free cortisol[50], while in dogs octreotide showed the strongest ACTH-suppressive effects[23], due to the different expression pattern of receptors compared to humans.

In human adrenal glands and adrenal gland tumours the expression of somatostatin receptors is very variable.[31] But because of the differences in the expression patterns between humans and dogs regarding their pituitaries it could be that the expression in the

dog is more favorable for drug therapy. Despite the fact that the expression of the receptors is very low (SSTR 3 and 5), or even down regulated (SSTR 1) in cortisol-secreting adenomas and carcinomas (unpublished qPCR data), the possibility of positive immunohistochemical staining could not be excluded. In a study of Pisarek *et al*. it was shown that in samples with low mRNA concentration the receptor protein was mainly localized at the membrane, whereas in samples with high mRNA concentrations the receptor protein was mainly localized in the cytoplasm.[28] The same results were also found in a study of Reubi *et al.*[52] Therefor the low qPCR values for the different SSTR receptors in adrenal gland tumours could still reveal a high membrane bound expression with immunohistochemistry. Research on SSTR expression is complicated by the fact that even in tumours of the same type like carcinomas, the variation in SSTR type expression can be quite considerable.[53] In the present study, the tumours were classified as adenomas or carcinomas based on the criteria of Labelle *et al.*[21] But according to the pathological reports, a lot of the tumours were not classified in one of the two groups with a 100% certainty. This could interfere with the results of the study. It would be better to use only the tumours which are without a doubt categorized as adenoma or carcinoma for further research, in order to get more reliable results when the two groups need to be compared. The tumours which cannot clearly be classified could be studied as well, but statistical analysis could only be performed regarding the differences between normal adrenal glands and tumours in general.

According to the pathologist, the method used for the IHC in this research project was not fully optimized. This conclusion was drawn because the staining looked hazy instead of showing specific stained dots. Also in some slides the plasma in the blood vessels was stained. Furthermore, the inspection of the positive control tissue showed unequal staining in different parts of the slide. Comparison of the zona glomerulosa of a normal adrenal gland with tumorous adrenal gland showed that the staining of the tumour was not the same as in the normal adrenal gland. It was hypothesized, that since the zona glomerulosa is not altered when comparing normal and tumorous adrenals, the staining of this zone should be comparable. But according to Unger *et al*., only 3 out of 8 normal adrenal glands showed staining in the zona glomerulosa. This variable expression pattern also holds true for the other zona's of normal adrenal glands and for tumour cells of cortisol-secreting adenomas (CPAs).[31] Therefore it is difficult to relate expression patterns of tumorous tissue to only one normal adrenal gland. A control group of several normal adrenal glands should be considered in future immunohistochemical research to be able to make a better comparison.

To draw a valid conclusion from the IHC results, the staining of the control tissues will have to be fully optimized, to make sure the staining is correct and reliable and no aspecific binding is present. After optimization, the tumours can be stained again. If after optimization the staining pattern would turn out to be comparable to the present results, then it would be impossible to formulate a general medical therapy for adrenal gland tumours based on STTR expression. There will be no SSTR subtype predominantly expressed in all tumours,

therefore a future possibility could be to determine receptor subtype concentrations for each individual tumour and adapt the therapy to the results.[29] Further research is needed to determine if the expression patterns are that variable that individual examination would be needed. Also, the biological effects of intracellular receptors need to be cleared.[29] If the intracellular staining is due to receptor internalization, then the receptor and G protein are probably uncoupled, which would lead to no further biological effects upon ligand binding.

For research purposes, it is necessary to look at the SSTR expression profile of many canine adrenal gland tumours. The antibodies currently available are mainly produced against human SSTR's. In a paper of Schmid *et al*.[27] it has been studied which antibody could be used best in a specific immunohistochemistry method for routine identification of the SSTR expression profile in human patients. This profile seems to change regarding the aggressiveness of the tumour.[27] It is a long road before this could be developed in dogs, but it shows the difference of the expression of this receptor and the importance of submitting the tumorous tissues to specific groups. At this moment there are no commercially available and well-characterized antibodies against somatostatin receptors in dogs. The amino acid sequence of dog and human somatostatin receptors is more or less similar, depending on the receptor subtype, but this is no guarantee for a successful and specific staining. Because of the differences in amino acid sequences in some receptors, especially SSTR subtype 5, it might be necessary to produce specific antibodies against the receptors in dogs.

The expression pattern of SSTR's can be influenced by for example a specific mutation. Toboada and coworkers demonstrated a relation between mRNA expression of the somatostatin receptors and an activating mutation in the G-protein α-subunit gene (GNAS) in somatotropinomas. It appeared that tumours positive for the mutation had higher mRNA levels of SSTR1 and SSTR2, and lower levels of SSTR3.[54] Whether the high prevalence of the gsp oncogene in somatotropinomas can also be found in adrenal gland tumours of dogs will be discussed in the next chapter, wherein a screening of the adrenal glands in this study for mutations in the Gsα gene is described.

If in the end, somatostatin analogue therapy will not be applicable for adrenal gland tumours, it may still be of interest in the diagnostics of these tumours. In humans but also in veterinary medicine [49] octreoscan is used for diagnostic imaging of tumours. This technique is based on a high expression of SSTR 2 in the tumour. Indium or technetium labeled analogues, called 111 In-pentetreotide (111 In-DTPA) octreotide and 99mTc-EDDA/HYNIC-Tate octreotide respectively, will be administered. The receptors can then be visualized using scintigraphy. The localization and size of the tumours can thus be determined *in vivo*, but as already said, this depends on the expression level of SSTR 2.[29]

Conclusively, there still is a lot of work to be done on cortisol-secreting adrenocortical tumours with respect to somatostatin receptor expression. Although the results of qPCR and IHC on adrenal glands were not promising for the potential of somatostatin analogue

therapy, a clinical trial could be the last option to determine with more certainty if this therapy will probably have or not have an additional therapeutic value at this time, but no cortisol inhibiting effect has been notified so far in an *in vivo* study in rats regarding the adrenal gland.[55] Besides, adrenal gland tumours are often aggressive tumours. This means that for a medical therapy such as somatostatin analogue treatment to be successful, it would need to prohibit further cell growth and metastasis of the tumours. Otherwise it would still be necessary to surgically remove the tumour.

CHAPTER 3

GS ALPHA

ABSTRACT

Background: After binding of ACTH to the G-protein coupled ACTH receptor in adrenocortical cells, cortisol is synthesized and secreted. This effect is mediated by a G-protein subunit, Gsα. Mutations in this subunit could lead to constitutive activation, which has been shown in other endocrine tumours.

Aim: To identify a possible molecular basis of ACTH-independent hypercortisolism in dogs in the Gsα protein subunit. It is expected that the incidence of the gsp oncogene in cortisol-secreting adrenocortical tumours of dogs is low.

Materials and Methods: A total of 44 tumours were sequenced, consisting of 14 adrenocortical adenomas and 30 carcinomas.

Results: 14 tumours showed an amino acid changing mutation, of which 11 tumours showed a mutation which corresponds with the mutations found in humans leading to constitutive activation.

Conclusion: The mutations corresponding to constitutive activation could explain the ACTH-independent cortisol secretion in about 25-35% of the ACTH-independent hypercotisolism and explain the origin of some of the adrenocortical tumours due to the stimulating effect of the mutation on proliferation.

Cortisol production and secretion is regulated by ACTH, and mediated by the ACTH receptor, which is a G-protein coupled receptor (GPCR).[56] The G-proteins (guanine nucleotide binding proteins) coupled to the receptor are in fact composed of three different subunits and are therefore heterotrimers which are together cytoplasmatic membrane-associated. The G-protein links the extracellular signal, ACTH, to the intracellular second messenger enzyme systems, which will ultimately result in a specific response, the cortisol production and secretion. This is mediated by the α subunit of the G-protein.[57]

G-protein subunits

The three different subunits of which the G-protein is composed, are the α-, β-, and γ-subunit. The α-subunit exists in several distinct subtypes which can be either stimulatory or inhibitory. The stimulatory α-subunit (Gsα) can stimulate adenylate cyclase when it is bound to GTP, whereas in the inactive state it is bound to GDP. GDP is exchanged for GTP upon binding of ACTH with the G-protein coupled receptor, which induces the activation of the α-subunit and subsequently dissociation from the βγ-subunits.[57]

After the activation of the α subunit, adenylyl cyclase is activated, which subsequently converts ATP to cAMP. cAMP levels will elevate and result in the activation of PKA, which phosphorylates several targets with the catalytic subunits. This will result among other effects in the transcription of genes via CREB binding to the cAMP response element CRE.[58, 59] In the case of ACTH stimulation, this will result in production of cortisol. The stimulation of adenylate cyclase and the resulting cAMP generation will be terminated by the hydrolysis of the bound GTP to GDP by the intrinsic GTPase of the α subunit.[57]

If in the cascade a protein is mutated, this could lead to an increased or decreased gene transcription. In this research the focus is on the first protein subunit after receptor stimulation, namely the Gsα.

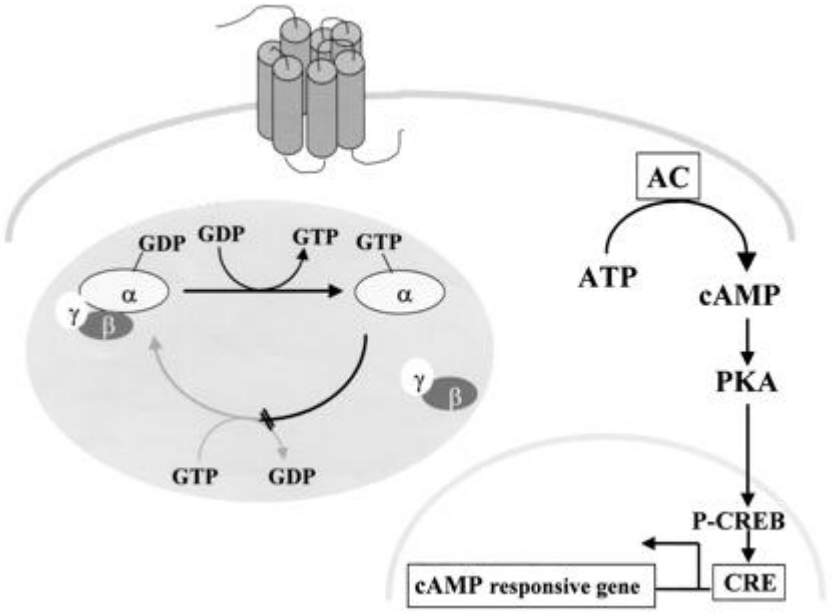


Figure 13. Schematic representation of the cascade after ligand binding at the GPCR. The replacement of GDP with GTP on the α subunit is facilitated by the receptor after a conformational changes due to ligand binding. This causes the dissociation form this subunit and the activation of adenylyl cyclase (AC). AC catalyses the conversion of ATP into cAMP of which the levels will rise. PKA is subsequently activated and will activate the transcription factor CREB by phosphorylation (P-CREB). P-CREB will bind to the cAMP response element (CRE) and start the transcription of cAMP responsive genes. Figure adjusted from Lania 2003.[59]

Gain of function mutation

In the endocrinology, a hypofunction of endocrine organs like hypocorticism can be a result of loss of function of the G-protein coupled to the receptors. Contrary, gain of function mutations have been related to hyperfunction of endocrine organs. It could for example lead to increased sensitivity of the receptor to its usual agonist. In endocrinology, this effect would mostly lead to a decrease in concentration of the agonist, via negative feedback.[60] A gain of function mutation which would induce constitutive activation, indicating activation in absence of the ligand[60], would be able to cause ACTH-independent hypercortisolism in the adrenal gland. In fact, in humans endocrine gland dysfunction is the main observed consequence of a mutation in the GPCR.[61]

Gsα mutations

In humans, mutations of Gsα that occur early in development can induce the so called McCune-Albright syndrome, a disease which affects the skin, bone marrow, and endocrine glands. More focal effects of somatic mutations in Gsα are observed if the mutation occurs later in life, resulting in for example a pituitary tumour.[61] In several endocrine tumours mutations in Gsα have been documented. In humans mutations leading to the replacement of the wild type amino acid Arginine and Glutamine in codon 201 and/or 227, respectively,

have been detected in pituitary tumours[62], the McCune-Albright syndrome[63], thyroid adenomas[63], and adrenal gland tumours[56, 64]. In the Gsα of cat thyroid adenomas two mutations have also been found at base 128 and 207 which were stated to correspond with the situation in humans after amino acid translation.[65]

The mentioned mutations appear to cause inhibition of the intrinsic GTPase activity of Gsα, which is necessary to 'turn off' Gsα. The mutation therefore results in constitutive activation.[62] This constitutive activation of Gsα induces also a constitutive activation of adenylyl cyclase which results in an increase in cAMP by activation of its synthesis[64] which in many endocrine cells leads to increased hormone secretion but also to cell proliferation.[61] This is due to the fact that cAMP is an intracellular second messenger increased upon binding of several trophic hormones and also involved in the stimulation of growth.[62]

Other activating and inactivating mutations have also been indentified in humans[66], but no study has looked at mutations in Gsα of the dog.

Aim

The aim of this study is to investigate if there is a molecular basis for ACTH-independent hypercortisolism in dogs in the Gsα protein subunit. It is expected that the incidence of the gsp oncogene in cortisol-secreting adrenocortical tumours of dogs is low, like the prevalence in adrenal gland tumours in humans.

Patient material

Adrenocortical tumour tissue of 14 random patients has been used to be able to detect mutations with an incidence of about 10%. The material was of 14 patients mentioned in Chapter 1. All the used tumours were characterized as carcinomas. Tissue of two normal adrenal glands as mentioned in Chapter 1, were used for comparison.

Primers

Primer		Sequence	Location
F352	Fw	CCATGGGCTGCCTCGGAAACA	Bp 352
F777	Fw	TCCCTCCTGAGTTCTATGAG	Bp 777
F1226	Fw	AACAAGCAAGACCTGCTC	Bp 1226
R1708	Rv	TTAAGCAAGCGGAAGGGAAGAAA	Bp 1708
R1504	Rv	CTGAATGATGTCACGGCA	Bp 1504
R951	Rv	CAGCGAAGCAGATCCTG	Bp 951
R845	Rv	CTCATAGCAGGCACGCACTCC	Bp 845

Table 9. Primer overview for sequencing Gsα.

Primers were designed with Perlprimer and Primer Select on the Gsα sequence of the dog, NCBI genbank association number NM_001003263.1.

PCR

The PCR reaction was performed using cDNA obtained via the method described in Chapter 1. Per reaction, 1 µl of cDNA was added to the PCR reaction mix (Table 10) and amplified with the program shown in Table 11.

Compound	Volume 1 PCR reaction
5x Phusion buffer HF	4
10 mM dNTP	0.4
10 mM Forward primer	1
10 mM Reverse primer	1
Milli Q	12.4
Phusion enzyme	0.2
Total	19

Table 10. PCR reaction mix composition.

Temperature	Time	
98 °C	30“	
98 °C	10”	35x
56 °C	10”	
72 °C	10”	
75 °C	5’	
20 °C	∞	

Table 11. PCR program. “; seconds, ‘; minutes.

Gel electrophoresis

With a 1 % agarose gel, the PCR products were separated and made visible by adding the products mixed with 6x loading buffer to the gel and comparing the PCR product bands with the bands of the 1kB ladder after running with 100V, until the bands were individually visible.

Sequencing

For sequencing, the PCR products were diluted ten times and amplified with the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Composition for each amplification reaction, called tercycle reaction, is shown in Table 12. The tercycle programme is shown in Table 13. Amplification products were filtrated using Sephadex G-50 Superfine (Amersham, Buckinghamshire, United Kingdom) and sequenced using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were compared with the NCBI database using BLAST (Basic Local Alignment Search Tool).

Compound	Volume (µl) 1 Tercycle reaction
5x sequence buffer	2
3 mM primer	1
BDT	1
MilliQ	3
PCR product (10x diluted)	3
Total	10

Table 12. Tercycle reaction mix.

Temperature	Time	
96 °C	30”	
55 °C	15”	25 x
65 °C	1’	
20 °C	∞	

Table 13. Tercycle programme. “; seconds, ‘; minutes.

Analysis

The resulting sequences were viewed with refseq and compared with the Gs alpha sequence from NCBI with number NM_001003263.1.

ADDITIONAL

In continuation of the described random check of the fourteen (14) samples, all the available tumours were sequenced. This included a total of 44 tumours of which were fourteen (14) adenomas and thirty (30) carcinomas. A PCR was performed on all of them, with the same method as discussed and the sequence reaction was performed using only the F777 and R1504 primers.

First the analysis of the sequences from the randomly checked tumours showed peaks of two different nucleotides at basepair 954, 956, and 1034. At basepair 954 the normal nucleotide according to NCBI is a C, while two sequences of carcinomas showed the presence of a T and C. At basepair 956 the normal nucleotide according to NCBI is a T, while in two samples, this T is replaced by a C both with a forward and reverse primer. At basepair 1034 the normal nucleotide that should be present according to NCBI is a G, but in one sample there was also a T shown with three primers.

The height of the peaks was half of the height of the peaks at the same position in other samples showing only one nucleotide, indicating a heterozygous mutation.

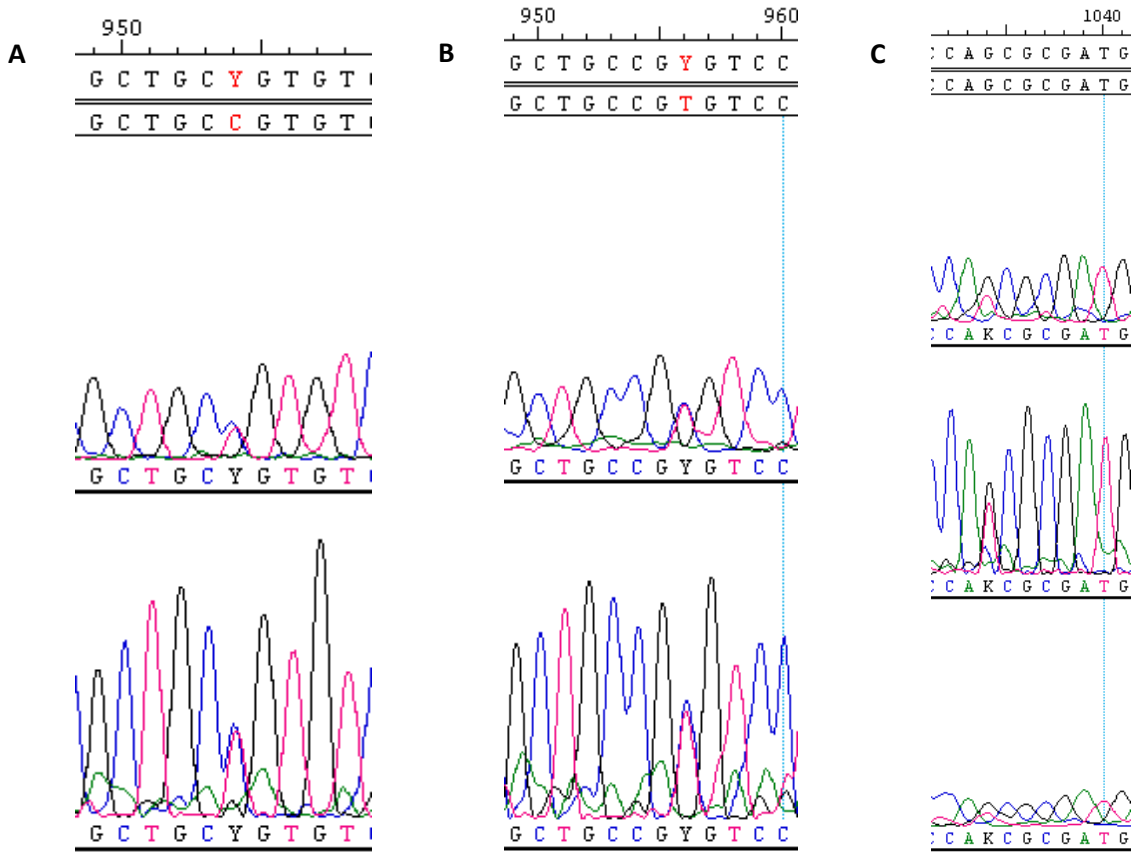


Figure 14. Sequence results of Gs alpha. A; At basepair 954 two nucleotides, C & T, are shown with two different primers. B; At basepair 956 two nucleotides, C & T, are shown with two different primers. C; At basepair 1034 two nucleotides, G & T, are shown with three different primers.

Two of these possible mutations also resulted in amino acid replacements. The basepair 954 mutation leads to a Cysteine (Cys) instead of an Arginine (Arg) and the basepair 1034 mutations leads to an Histidine instead of an Glutamine (Gln). The mutation at basepair 956 does not result in an amino acid change, both sequences will lead to an Arginine.

Apart from the three possible mutations, there was also a region between basepair 566 till 610 which gave in all samples, including the normal adrenal glands, a very chaotic result (Figure 15). It was not possible to get a nice sequence in this area, which would only show one possible nucleotide at each spot. Finally it turned out to be a splice variant, with exon 2 (bp 566 till bp 610) being alternatively spliced. When the reference sequence of Gsα was substracted from the sequences of the samples, it showed the sequence of exon 3 after exon 1 with the forward primers (green arrows) and the sequence of exon 1 before exon 3 with the reverse primers (red arrows).

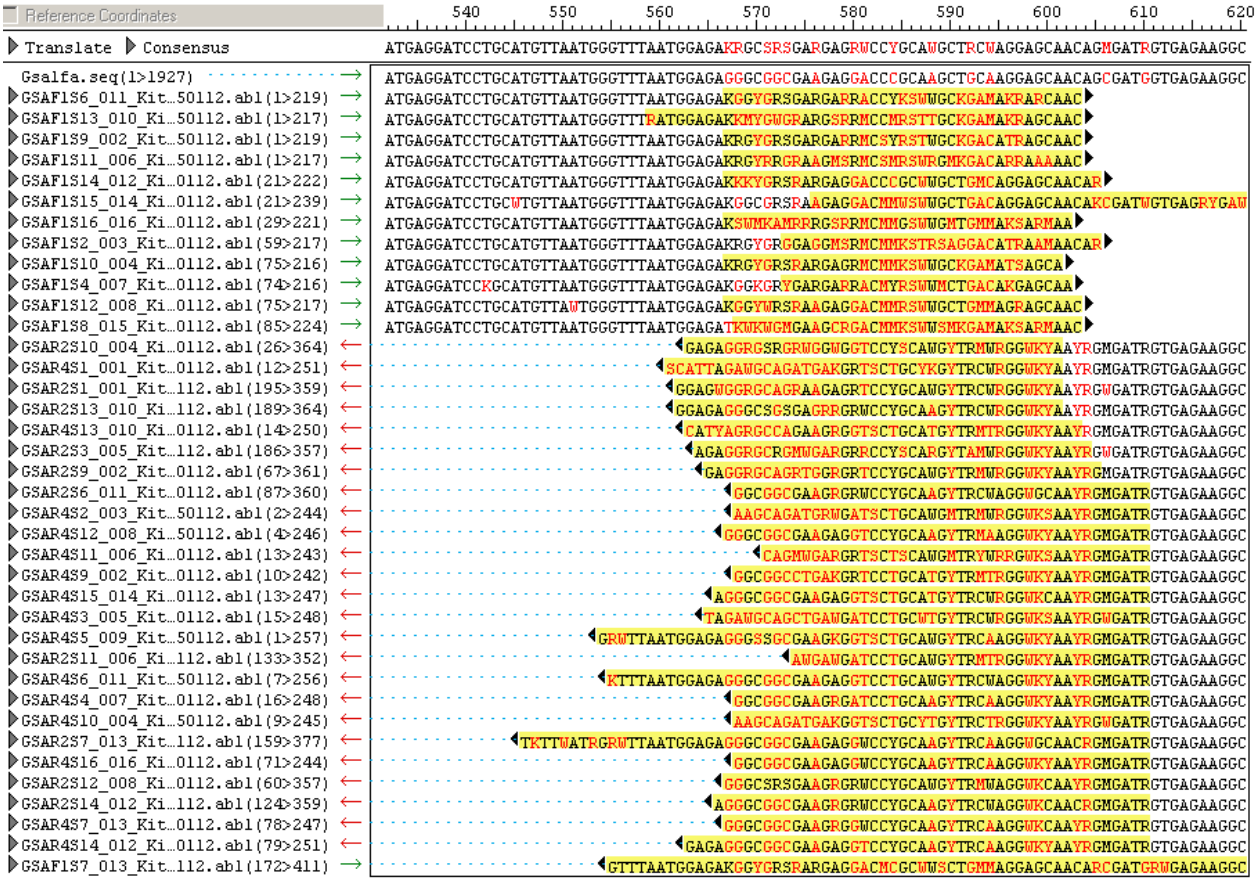


Figure 15. Gsα sequence result of different samples, showing chaotic results between basepair 566 and 610 with a lot of inconclusive nucleotide results.

The analysis of all the adrenal gland tumours showed the same mutations as found with the random check, with one additional mutation at basepair 955. An overview of all the found mutations can be seen in Table 14.

Tumour No	Mutation site	Nucleotide	Amino Acid
A1	954	Y	Arg → Cys
A2	958	R	Val → Met
A7	955	R	Arg → His
	956	Y	-
A8	956	Y	-
A13	961	Y	Leu → Pro
C2	954	Y	Arg → Cys
	956	Y	-
C3	1034	K	Gln → His
C5	954	Y	Arg → Cys
C6	954	Y	Arg → Cys
	956	Y	-
C12	956	C	-
C14	954	M	Arg → Ser
	956	Y	-
C16	956	Y	-
C20	954	Y	Arg → Cys
C21	955	K	Arg → Leu
C22	956	C	-
C23	956	Y	-
C24	955	R	Arg → His
C26	1035	R	Ser → Gly
C28	955	R	Arg → His

Table 14. Overview of found mutations in the Gs alpha mRNA of adrenal gland adenomas (A) and carcinomas (C). Arg; Arginine, Cys; Cysteïne, Val; Valine, Met; Methionine, His; Histidine, Leu; Leucine, Pro; Proline, Ser; Serine, Gly; Glycine.

The summation of the found mutations results in a total of fourteen (14) amino acid replacing mutations. Of these mutations six (6) were at basepair 954, resulting in Cysteïne five (5) times and one time in a Serine. Mutations at basepair 955 were also seen, this resulted in the same amino acid to be replaced as the mutations of basepair 954. Four (4) mutations were present at basepair 955, which resulted in Histidine three (3) times and one time in a Leucine. A mutation at basepair 958 was also found resulting in a Methionine and a mutation at basepair 961 resulting in a Proline. The mutation at basepair 1034 was found once, resulting in a Histidine, even though the mutation was located at the last basepair of the triplet. One mutation was found at basepair 1035, which resulted in a Glycine.

The silent mutation at basepair 956 was found in a total of nine tumours, of which two were adenomas and seven were carcinomas.

In total four of the thirteen adenomas showed an amino acid changing mutation, of which two are known to give continuous stimulation. This corresponds with an incidence of 12.5%.

Ten carcinomas showed a mutation in a total of thirty carcinomas investigated. Nine of these tumours showed a mutation known to result in constitutive stimulation, which is an incidence of almost 35%.

DISCUSSION

Seven different heterogeneous occurring sequences have been identified, which differ from the reference sequence given in NCBI. One of these mutations does not result in an amino acid replacement and therefore does not influence the protein expression in any way and is probably a single nucleotide polymorphism (SNP). Of three other mutations, at basepair 958, 961, and 1035, it is unknown what their amino acid changing effect will be on the function of the Gsα protein. The three mutations found at basepair 954, 955, and 1034 are known to result in constitutive activation of adenylyl cyclase in human pituitary tumours[59]. This constitutive activation could result from the inhibition of the intrinsic GTPase or increase the dissociating rate of GDP from its inactive form, whereby the binding of GTP is accelerated resulting in the active state of the α subunit[62]. Such a mutation could therefore cause the hypercortisolism, as cortisol synthesis and secretion are mediated by Gsα activation[64]. With DNA from other tissue of the same individuals, it should be tested if the found deviations are tumour related. But the fact that the mutations at basepair 954, 955, and 1034 correspond with the mutations found in humans at codon 201 and 227 and in cats[65] indicates that the mutations, which have been found in some tissue samples of cortisol producing adrenocortical tumours, will probably be tumour related.

Mutations in Gsα are also reported to be able to lead to cellular proliferation and nodule formation. Mutations in Gsα in adrenocortical neoplasms in men are far less common than Gsα mutations in pituitary or thyroid tumours[56, 64], whereas the incidence in GH-secreting adenomas is around 40% [62]. This indicates that there are several different mutations which could induce tumour formation in adrenal glands, because mutations in Gsα are not seen in 100% percent of the tumours.[64] But if it should be confirmed that the mutations in this research are tumour related, a little piece of the puzzle regarding the cause of developing cortisol-secreting adrenocortical tumours in dogs has possibly been solved. A research of Bimpaki *et al*. (2009) also indicates that there is far more to be studied, even if causative mutations are known. In that paper cAMP signalling was studied in adrenal glands of patients with ACTH-independent hypercortisolism in the absence of known mutations, including Gsα. They showed that even if the now known mutations are excluded, there is still a functional abnormality in the cAMP signalling.[67] Further research is needed to clarify this phenomenon after all the causative mutations have been identified.

An additional cause for increased hormone secretion, could be enhanced receptor expression, in this case ACTH receptors. It has been found that in ACTH-secreting adenomas of the pituitary the CRH receptors genes were overexpressed.[59] Whether an enhanced ACTH receptor expression would result in a higher cortisol secretion is doubtful, because of the negative feedback of cortisol towards the pituitary which induces an inhibition in ACTH secretion resulting in very low ACTH concentration.

If the mutations are proven to be tumour related, the expression of Gsα in tumours with and without the mutations should be compared. Due to increased protein degradation of the mutated Gsα, it is expected that the expression of the protein in tumours carrying a mutation will be lowered. It is thought that due to the activated free state of the mutated protein it becomes very susceptible to degradation.[59]

The splice variant of exon 3, which has been shown in every tissue sample, is a well-known splice variant.[66] But in an article of Thiele *et al*. (2007) it is discussed that the long and short variant of the Gsα subunit have a different affinity for GDP, which is lower in the long variant and subsequently the receptor will more frequently be stabilized in the active state. The long variant may therefore be necessary for the constitutive activation.[57] Further research is needed to determine if these minimally underpinned speculations hold true in the cortisol-secreting adrenal gland tumours.

OVERALL CONCLUSION

The expectation regarding the dopamine receptor as stated in the first chapter could not be proven due to the inability to measure the expression of the short variant. But considering the two splice variants of the dopamine receptors and the dopamine receptors in the adrenal gland in general, it can be concluded that they probably will not play a considerable role in potential medical management of cortisol-secreting adrenocortical tumours. The prevalence of the dopamine type 2 receptor has been shown to be very low in this study. Besides, based on other studies, the expression is mainly in the zona glomerulosa and dopamine analogues were unable to inhibit or lower cortisol secretion. Therefore these receptors should be excluded from further research regarding ACTH-independent hypercortisolism.

The somatostatin receptor showed an individually varying expression pattern as was expected. But it could still be a potential target for the development of new drug therapy. In this study the individual expression shows occasional increased receptor expression when compared to the normal adrenal gland. More research is needed to visualize the expression of the receptors at a large scale and study if the analogues have cortisol lowering effects *in vivo*, after which steps can be taken to the development of individual therapy.

Finally the Gsα mutations have an important share in explaining the origin of the cortisol-secreting adrenocortical tumour. Although this mutation does not explain a large portion of adrenocortical tumours in humans and we expected this to be the same in dogs, it seems to be otherwise. It is important to make sure that these mutations are able to cause hypercortisolism without ACTH stimulation. But overall this discovery is a very interesting lead to build new therapy upon, specifically adjusted for tumours carrying Gsα mutations. This could be worthwhile because of the high incidence of the mutation.

FUTURE PROSPECTIVES

Somatostatin analogues are already used in human medicine. Octreotide and lanreotide preferentially bind SSTR 2 and have been shown to reduce neuroendocrine tumour related symptoms, and frequently cause tumour stabilization or even a reduction in tumour mass.[68, 69] Another therapeutic used in humans is radiolabeled somatostatin-analogs in case of radionuclide therapy. This therapy is discussed to be used in patients with sst-positive neuroendocrine tumours which are advanced and/or metastasized.[70] One step ahead of therapy, in diagnostics, somatostatin analogues can also be used. For example, the sst-analogues labeled with indium can be used for visualizing the tumour by scintigraphy. This has been proven to be useful *in vivo* to determine the tumour localization and size.[29] The effectiveness is of course dependent on the receptor expression pattern, but this might be an interesting subject for further development. A potential complication is that internalized receptors might be visualized as well, but their biological effect has not been elucidated yet.[29]

In the future it is important to focus on the use of somatostatin in diagnostics and eventually therapy. Before these steps can be taken, the receptor protein expression pattern of all the somatostatin receptor subtypes in dogs has to be established more firmly. If specific receptor subtypes are highly expressed in several tumours, a trial can be done, using labeled somatostatin analogues to determine tumour size and localization. Confirmation of the receptor expression can be obtained after adrenalectomy. Tumour directed radiotherapy or just administering somatostatin anologues as future therapy could be the final step of using somatostatin receptors for medicinal treatment in cortisol-secreting adrenocortical tumours.

Something like hetero oligomers, which bind somatostatin and dopamine receptors at the same time, are also indicated as future perspectives. According to Boschetti *et al.* (2010) SSTRs and DRs work synergistically when coexpressed, via membrane interaction or dimerization. Chimeric molecules have been synthesized which may draw the receptors together, leading to a higher potency of chimeric drugs.[71] But this is probably not a field of interest in cortisol-secreting adrenocortical tumours, because of their low dopamine receptor expression. Besides, there are no clinical studies which report the effect in neuroendocrine tumours of somatostatin/dopamine combined therapy[69].

A study which can integrate the somatostatin receptor expression and the Gsα mutations, is a study which groups tumours with and without the Gsα mutations together and compares the receptor expression patterns of the two groups. An article of Taboada *et al.* (2011) showed that human somatotropinomas with the Gsα mutation showed higher expression of SSTR 1 and SSTR 2, and lower expression of SSTR 3.[54] It is interesting to see

whether some pattern can be shown in cortisol-secreting adrenal glands in dogs as well. If this can be shown, it could be used in adjusting potential somatostatin receptor based therapy upon tumours with or without the Gsα mutation causing constitutive activation. Apart from adjusting potential somatostatin receptor based therapy, it could be a possibility to look more into the physiologic effects of the Gsα mutations on cell level to mark specific targets which could be a basis for new drug therapy. Gsα is a generally used protein in many cells in the cascade from GPCR to the DNA or to other intended effects. This could complicate the development of drug therapy based upon the presence of the Gsα mutation without affecting other cell types in which physiological actions are mediated by Gsα, but is also an interesting puzzle to solve.

Apart from the Gsα mutations, it is of interest to look for other causative mutations in the cortisol-secreting adrenocortical tumours. To finally obtain an overview of causative mutations, onto which specific medication might be developed, as is done in human intestinal cancers. Other proteins in the cascade form GPCR till the transcription of DNA should be evaluated for mutations, but also the TP53 gene and steroidogenic factor 1 (SF1) should be evaluated. A mutation in the TP53 gene caused a high incidence of adrenocortical carcinomas in children in southern Brazil and SF1 is important in adrenal gland development, but is also overexpressed in many cases of childhood and adult onset adrenocortical tumours in humans. More genes of interest for the evaluation for causative mutations can be found in a review of Fassnacht *et al.* (2011)[72]

Overall, there are a lot of interesting subjects for further research which all can contribute to the ability to develop better therapy for cortisol-secreting adrenocortical tumours in dogs.

REFERENCES

1. Galac, S. Recent developments in canine Cushing's syndrome. (2010).

2. Willeberg P, P. W. Epidemiological aspects of clinical hyperadrenocorticism in dogs (canine Cushing's syndrome)
. **18**, 717-724 (1982).

3. Lindholm, J. *et al*. Incidence and late prognosis of cushing's syndrome: a population-based study. *J. Clin. Endocrinol. Metab.* **86**, 117-123 (2001).

4. Etxabe, J. & Vazquez, J. A. Morbidity and mortality in Cushing's disease: an epidemiological approach. *Clin. Endocrinol. (Oxf)* **40**, 479-484 (1994).

5. Kooistra, H. S., Galac, S., Buijtels, J. J. & Meij, B. P. Endocrine diseases in animals. *Horm. Res.* **71 Suppl 1**, 144-147 (2009).

6. Rijnberk, A. & Kooistra, H. S. in *Clinical endocrinology of dogs and cats : an illustrated text* (Schlütersche, Hannover, 2010).

7. de Bruin, C. *et al*. Cushing's disease in dogs and humans. *Horm. Res.* **71 Suppl 1**, 140-143 (2009).

8. Hofland, L. J. Somatostatin and somatostatin receptors in Cushing's disease. *Mol. Cell. Endocrinol.* **286**, 199-205 (2008).

9. Missale, C., Nash, S. R., Robinson, S. W., Jaber, M. & Caron, M. G. Dopamine receptors: from structure to function. *Physiol. Rev.* **78**, 189-225 (1998).

10. Pivonello, R. *et al*. Novel insights in dopamine receptor physiology. *Eur. J. Endocrinol.* **156 Suppl 1**, S13-21 (2007).

11. Vallone, D., Picetti, R. & Borrelli, E. Structure and function of dopamine receptors. *Neurosci. Biobehav. Rev.* **24**, 125-132 (2000).

12. Sibley, D. R. & Neve, K. A. in *The dopamine receptors* (eds Neve, K. A. & Neve, R. L.) 383-424 (Humana, Totowa, N.J., 1997).

13. Namkung, Y. & Sibley, D. R. Protein kinase C mediates phosphorylation, desensitization, and trafficking of the D2 dopamine receptor. *J. Biol. Chem.* **279**, 49533-49541 (2004).

14. Kim, S. J., Kim, M. Y., Lee, E. J., Ahn, Y. S. & Baik, J. H. Distinct regulation of internalization and mitogen-activated protein kinase activation by two isoforms of the dopamine D2 receptor. *Mol. Endocrinol.* **18**, 640-652 (2004).

15. Kendall, R. T. & Senogles, S. E. Isoform-specific uncoupling of the D2 dopamine receptors subtypes. *Neuropharmacology* **60**, 336-342 (2011).

16. Senogles, S. E., Heimert, T. L., Odife, E. R. & Quasney, M. W. A region of the third intracellular loop of the short form of the D2 dopamine receptor dictates Gi coupling specificity. *J. Biol. Chem.* **279**, 1601-1606 (2004).

17. Pivonello, R. *et al*. Dopamine receptor expression and function in clinically nonfunctioning pituitary tumors: comparison with the effectiveness of cabergoline treatment. *J. Clin. Endocrinol. Metab.* **89**, 1674-1683 (2004).

18. Pivonello, R. *et al*. Dopamine receptor expression and function in corticotroph pituitary tumors. *J. Clin. Endocrinol. Metab.* **89**, 2452-2462 (2004).

19. Pivonello, R. *et al*. Dopamine receptor expression and function in human normal adrenal gland and adrenal tumors. *J. Clin. Endocrinol. Metab.* **89**, 4493-4502 (2004).

20. Perreault, M. L., Verma, V., O'Dowd, B. F. & George, S. R. in *The Dopamine Receptors* (ed Neve, K. A.) 193-218 (Humana Press, New York, 2010).

21. Labelle, P., Kyles, A. E., Farver, T. B. & De Cock, H. E. Indicators of malignancy of canine adrenocortical tumors: histopathology and proliferation index. *Vet. Pathol.* **41**, 490-497 (2004).

22. Vandesompele, J. *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034 (2002).

23. Bruin, C. d. *et al*. Expression and functional analysis of dopamine receptor subtype 2 and somatostatin receptor subtypes in canine cushing's disease. *Endocrinology* **149**, 4357-4366 (2008).

24. de Bruin, C., Feelders, R. A., Lamberts, S. W. & Hofland, L. J. Somatostatin and dopamine receptors as targets for medical treatment of Cushing's Syndrome. *Rev. Endocr Metab. Disord.* **10**, 91-102 (2009).

25. Ferone, D. *et al*. The clinical-molecular interface of somatostatin, dopamine and their receptors in pituitary pathophysiology. *J. Mol. Endocrinol.* **42**, 361-370 (2009).

26. Wu, K. D. *et al*. Expression and localization of human dopamine D2 and D4 receptor mRNA in the adrenal gland, aldosterone-producing adenoma, and pheochromocytoma. *J. Clin. Endocrinol. Metab.* **86**, 4460-4467 (2001).

27. Schmid, H. A. *et al*. Monoclonal Antibodies against the Human Somatostatin Receptor Subtypes 1-5: Development and Immunohistochemical Application in Neuroendocrine Tumors. *Neuroendocrinology* (2011).

28. Pisarek, H. *et al*. Differential expression of somatostatin receptor subtype-related genes and proteins in non-functioning and functioning adrenal cortex adenomas. *Mol. Med. Report* **4**, 963-969 (2011).

29. Pisarek, H., Stepien, T., Kubiak, R. & Pawlikowski, M. Somatostatin receptors in human adrenal gland tumors--immunohistochemical study. *Folia Histochem. Cytobiol.* **46**, 345-351 (2008).

30. Unger, N. *et al*. Immunohistochemical determination of somatostatin receptor subtypes 1, 2A, 3, 4, and 5 in various adrenal tumors. *Endocr. Res.* **30**, 931-934 (2004).

31. Unger, N. *et al*. Immunohistochemical localization of somatostatin receptor subtypes in benign and malignant adrenal tumours. *Clin. Endocrinol. (Oxf)* **68**, 850-857 (2008).

32. Ueberberg, B. *et al*. Differential expression of the human somatostatin receptor subtypes sst1 to sst5 in various adrenal tumors and normal adrenal gland. *Horm. Metab. Res.* **37**, 722-728 (2005).

33. Pisarek, H. & Pawlikowski, M. Immunohistochemical localization of the somatostatin receptor subtype 2A in the rat adrenal gland. *Folia Histochem. Cytobiol.* **40**, 27-30 (2002).

34. Krulich, L., Dhariwal, A. P. & McCann, S. M. Stimulatory and inhibitory effects of purified hypothalamic extracts on growth hormone release from rat pituitary in vitro. *Endocrinology* **83**, 783-790 (1968).

35. Brazeau, P. *et al*. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* **179**, 77-79 (1973).

36. Barnett, P. Somatostatin and somatostatin receptor physiology. *Endocrine* **20**, 255-264 (2003).

37. Tulipano, G. & Schulz, S. Novel insights in somatostatin receptor physiology. *Eur. J. Endocrinol.* **156 Suppl 1**, S3-11 (2007).

38. Patel, Y. C. Somatostatin and its receptor family. *Front. Neuroendocrinol.* **20**, 157-198 (1999).

39. Hofland, L. J., Feelders, R. A., de Herder, W. W. & Lamberts, S. W. Pituitary tumours: the sst/D2 receptors as molecular targets. *Mol. Cell. Endocrinol.* **326**, 89-98 (2010).

40. Reubi, J. C. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr. Rev.* **24**, 389-427 (2003).

41. Bronstein-Sitton, N. Somatostatin and the Somatostatin Receptors: Versatile Regulators of Biological Activity. *Pathways* **2**, 25-26, 27 (2006).

42. Ben-Shlomo, A. & Melmed, S. Pituitary somatostatin receptor signaling. *Trends Endocrinol. Metab.* **21**, 123-133 (2010).

43. Grimberg, A. Somatostatin and cancer: applying endocrinology to oncology. *Cancer. Biol. Ther.* **3**, 731-733 (2004).

44. Jacobs, S. & Schulz, S. Intracellular trafficking of somatostatin receptors. *Mol. Cell. Endocrinol.* **286**, 58-62 (2008).

45. Ghosh, M. & Schonbrunn, A. Differential temporal and spatial regulation of somatostatin receptor phosphorylation and dephosphorylation. *J. Biol. Chem.* **286**, 13561-13573 (2011).

46. Gilor, C. & Graves, T. K. Interpretation of Laboratory Tests for Canine Cushing's Syndrome. *Topics in Companion Animal Medicine* **26**, 98-108 (2011).

47. Corleto, V. D. Somatostatin and the gastrointestinal tract. *Curr. Opin. Endocrinol. Diabetes Obes.* **17**, 63-68 (2010).

48. Robben, J. H. *et al*. In vitro and in vivo detection of functional somatostatin receptors in canine insulinomas. *J. Nucl. Med.* **38**, 1036-1042 (1997).

49. Robben, J., Claude Reubi, J., Pollak, Y. & Voorhout, G. Biodistribution of [111In-DTPA-D-Phe1]-octreotide in dogs: uptake in the stomach and intestines but not in the spleen points towards interspecies differences. *Nucl. Med. Biol.* **30**, 225-232 (2003).

50. Hofland, L. J., Lamberts, S. W. & Feelders, R. A. Role of somatostatin receptors in normal and tumoral pituitary corticotropic cells. *Neuroendocrinology* **92 Suppl 1**, 11-16 (2010).

51. de Bruin, C. *et al*. Differential regulation of human dopamine D2 and somatostatin receptor subtype expression by glucocorticoids in vitro. *J. Mol. Endocrinol.* **42**, 47-56 (2009).

52. Reubi, J. C., Waser, B., Liu, Q., Laissue, J. A. & Schonbrunn, A. Subcellular distribution of somatostatin sst2A receptors in human tumors of the nervous and neuroendocrine systems: membranous versus intracellular location. *J. Clin. Endocrinol. Metab.* **85**, 3882-3891 (2000).

53. Pisarek, H., Pawlikowski, M., Kunert-Radek, J., Kubiak, R. & Winczyk, K. SSTR1 and SSTR5 subtypes are the dominant forms of somatostatin receptor in neuroendocrine tumors. *Folia Histochem. Cytobiol.* **48**, 142-147 (2010).

54. Taboada, G. F. *et al*. Impact of gsp oncogene on the mRNA content for somatostatin and dopamine receptors in human somatotropinomas. *Neuroendocrinology* **93**, 40-47 (2011).

55. Rebuffat, P., Robba, C., Mazzocchi, G. & Nussdorfer, G. G. Inhibitory effect of somatostatin on the growth and steroidogenic capacity of rat adrenal zona glomerulosa. *J. Steroid Biochem.* **21**, 387-390 (1984).

56. Dall'Asta, C. *et al*. Assessing the presence of abnormal regulation of cortisol secretion by membrane hormone receptors: in vivo and in vitro studies in patients with functioning and non-functioning adrenal adenoma. *Horm. Metab. Res.* **36**, 578-583 (2004).

57. Thiele, S. *et al*. A disruptive mutation in exon 3 of the GNAS gene with albright hereditary osteodystrophy, normocalcemic pseudohypoparathyroidism, and selective long transcript variant Gsalpha-L deficiency. *J. Clin. Endocrinol. Metab.* **92**, 1764-1768 (2007).

58. Vezzosi, D., Bertherat, J. & Groussin, L. Pathogenesis of benign adrenocortical tumors. *Best Practice & Research Clinical Endocrinology & Metabolism* **24**, 893-905 (2010).

59. Lania, A., Mantovani, G. & Spada, A. Genetics of pituitary tumors: Focus on G-protein mutations. *Exp. Biol. Med. (Maywood)* **228**, 1004-1017 (2003).

60. Vassart, G. & Costagliola, S. G protein-coupled receptors: mutations and endocrine diseases. *Nat. Rev. Endocrinol.* **7**, 362-372 (2011).

61. Spiegel, A. in *Insights into receptor function and new drug development targets* (eds Conn, M., Kordon, C. & Christen, Y.) 139-150 (Springer-Verlag, Berlin, 2006).

62. Landis, C. A. *et al*. GTPase inhibiting mutations activate the alpha chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. *Nature* **340**, 692-696 (1989).

63. Mantovani, G. *et al*. Parental origin of Gsalpha mutations in the McCune-Albright syndrome and in isolated endocrine tumors. *J. Clin. Endocrinol. Metab.* **89**, 3007-3009 (2004).

64. Kobayashi, H. *et al*. Mutation analysis of Gsalpha, adrenocorticotropin receptor and p53 genes in Japanese patients with adrenocortical neoplasms: including a case of Gsalpha mutation. *Endocr. J.* **47**, 461-466 (2000).

65. Peeters, M. E., Timmermans-Sprang, E. P. & Mol, J. A. Feline thyroid adenomas are in part associated with mutations in the G(s alpha) gene and not with polymorphisms found in the thyrotropin receptor. *Thyroid* **12**, 571-575 (2002).

66. Aldred, M. A. & Trembath, R. C. Activating and inactivating mutations in the human GNAS1 gene. *Hum. Mutat.* **16**, 183-189 (2000).

67. Bimpaki, E. I., Nesterova, M. & Stratakis, C. A. Abnormalities of cAMP signaling are present in adrenocortical lesions associated with ACTH-independent Cushing syndrome despite the absence of mutations in known genes. *Eur. J. Endocrinol.* **161**, 153-161 (2009).

68. Grozinsky-Glasberg, S., Grossman, A. B. & Korbonits, M. The role of somatostatin analogues in the treatment of neuroendocrine tumours. *Mol. Cell. Endocrinol.* **286**, 238-250 (2008).

69. Gatto, F. & Hofland, L. J. The role of somatostatin and dopamine D2 receptors in endocrine tumors. *Endocr. Relat. Cancer* **18**, R233-51 (2011).

70. Hofland, L. J. & Lamberts, S. W. The pathophysiological consequences of somatostatin receptor internalization and resistance. *Endocr. Rev.* **24**, 28-47 (2003).

71. Boschetti, M. *et al*. Role of dopamine receptors in normal and tumoral pituitary corticotropic cells and adrenal cells. *Neuroendocrinology* **92 Suppl 1**, 17-22 (2010).

72. Fassnacht, M., Libe, R., Kroiss, M., Allolio, B. & Medscape. Adrenocortical carcinoma: a clinician's update. *Nat. Rev. Endocrinol.* **7**, 323-335 (2011).

APPENDICES

APPENDIX 1

LOCALISATION QPCR PRIMERS SHORT AND LONG ISOFORM DRD2

DRD2 Short

```
   1 atggatccac tgaacctgtc ctggtacgat gatgatctgg agagccagaa ctggagccgg
  61 cccttcaacg ggtccgaagg aaagcccggc aagccccact acaactacta cgccatgctg
 121 cttaccctgc tcatcttcat catcgtcttc ggcaatgtgc tggtgtgcat ggccgtgtcc
 181 cgcgagaagg cgctgcagac caccaccaac tacctgattg tcagccttgc cgtggccgac
 241 ctcctggtgg ccacgctcgt catgccctgg gttgtctacc tggaggtggt aggtgagtgg
 301 aaattcagca ggattcactg tgacatcttt gtcactctgg acgtcatgat gtgcacggca
 361 agcatcctga acctgtgtgc catcagcatt gacaggtaca cagctgtggc catgcccatg
 421 ctctacaaca cccgctacag ctccaagcgc cgagtcactg tcatgatcgc catcgtctgg
 481 gtcctgtcct tcaccatctc ctgcccactg ctcttcggac tcaacaacac agaccagaac
 541 gagtgcataa tcgccaaccc cgcattcgtg gtgtactcct ccatcgtctc cttctacgtg
 601 cccttcatcg tcaccctgct ggtctacatc aagatctaca tcgtcctccg caggcgccgc
 661 aagcgggtca acacgaagcg tagcagccgg gctttcaggg ccaacctgaa ggccccactc
 721 aaggaggctg cccgccgagc ccaggaactg gagatggaga tgctgtccag caccagcccc
 781 cccgagagga cccggtacag tcccatacca cccagccacc accagctgac cctccccgac
 841 ccgtcccacc acggcctcca cagcactgcc gacagccccg ccaaaccaga gaagaatggg
 901 catgccaaag accaccccaa gattgccaag atctttgaga tccagtccat gcccaatggc
 961 aaaacccgga cctctctcaa gaccatgagc cgcaggaagc tctcccagca gaaggagaag
1021 aaagccactc agatgctcgc cattgttctc ggcgtgttca tcatctgctg gctgcccttc
1081 ttcatcaccc acatcctgaa catacactgt gagtgcaaca tcccgcccgt cctgtacagc
1141 gccttcacgt ggctgggcta tgtcaacagc gccgtgaacc ccatcatcta cacgaccttc
1201 aacattgagt tccgcaaggc cttcctgaag atcctgcact gctga
```

Inscription

Exon boundary

Primer

DRD2 Long

```
   1 atggatccac tgaacctgtc ctggtacgat gatgatctgg agagccagaa ctggagccgg
  61 cccttcaacg ggtccgaagg aaagcccggc aagccccact acaactacta cgccatgctg
 121 cttaccctgc tcatcttcat catcgtcttc ggcaatgtgc tggtgtgcat ggccgtgtcc
 181 cgcgagaagg cgctgcagac caccaccaac tacctgattg tcagccttgc tgtggccgac
 241 ctcctggtgg ccacgctcgt catgccctgg gttgtctacc tggaggtggt aggtgagtgg
 301 aaattcagca ggattcactg tgacatcttt gtcactctgg acgtcatgat gtgcacggca
 361 agcatcctga acctgtgtgc catcagcatt gacaggtaca cagctgtggc catgcccatg
 421 ctctacaaca cccgctacag ctccaagcgc cgagtcactg tcatgatcgc catcgtctgg
 481 gtcctgtcct tcaccatctc ctgcccactg ctcttcggac tcaacaacac agaccagaac
 541 gagtgcataa tcgccaaccc cgcattcgtg gtgtactcct ccatcgtctc cttctacgtg
 601 cccttcatcg tcaccctgct ggtctacatc aagatctaca tcgtcctccg caggcgccgc
 661 aagcgggtca acacggagcg tagcagccgg gctttcaggg ccaacctgaa ggccccactc
 721 aagggcaact gcactcaccc tgaggacatg aaactctgca ccgttatcat gaagtccaat
 781 gggagtttcc cagtgaacag gcggagagtg gaggctgccc gccgagccca ggaactggag
 841 atggagatgc tgtccagcac cagccccccc gagaggaccc ggtacagtcc cataccaccc
 901 agccaccacc agctgaccct ccccgacccg tcccaccacg gcctccacag cactgccgac
 961 agccccgcca aaccagagaa gaatgggcat gccaaagacc accccaagat tgccaagatc
1021 tttgagatcc agtccatgcc caatggcaaa acccggacct ctctcaagac catgagccgc
1081 aggaagctct cccagcagaa ggagaagaaa gccactcaga tgctcgccat tgttctcggc
1141 gtgttcatca tctgctggct gcccttcttc atcacccaca tcctgaacat acactgtgag
1201 tgcaacatcc cgcccgtcct gtacagcgcc ttcacgtggc tgggctatgt caacagcgcc
1261 gtgaacccca tcatctacac gaccttcaac attgagttcc gcaaggcctt cctgaagatc
1321 ctgcactgct ga
```

Inscription

Exon boundary

Primer

APPENDIX 2

RAW QPCR DATA

Mean Ct values of the duplo qPCR measurement of the reference genes SRPR, GUSB, RPS 19, and RPS 5 together with the mean Ct values of DRD2 long are shown in next table. Values missing in the standard line are left out to make the best standard line possible.

	SRPR	GUSB	RPS 19	RSP 5	DRD2
Standard line	18,01	18,38	11,89	11,88	24,46
	20,05		14,19	14,16	26,17
	21,88	21,56	15,69	15,70	27,56
	23,84	23,56	17,85	17,29	29,70
	25,86		19,72	19,07	32,69
	28,50	27,67	22,26	21,85	33,86
	30,10	29,12	23,87	23,44	
Carcinoma	19,84	20,86	7,45	12,89	32,97
	21,19	22,78	15,76	15,33	28,00
	18,08	19,41	13,60	13,22	24,09
	21,16	22,17	14,37	14,08	32,34
	25,49	27,21	19,18	16,22	29,19
	20,14	21,23	13,62	14,07	29,32
	18,71	20,59	13,67	13,84	30,29
	19,96	19,22	14,72	13,08	24,94
	18,68	18,64	11,13	11,23	28,45
	21,36	21,32	15,46	15,63	30,40
	20,51	21,74	14,07	14,38	29,73
	22,38	23,06	15,32	16,60	26,78
Adenoma	20,75	21,80	15,73	16,29	21,89
	21,91	22,05	13,96	14,69	32,02
	20,41	21,79	13,80	13,89	40,38
	19,72	19,76	14,22	13,80	29,39
	19,11	19,77	12,20	13,46	32,15
	19,83	19,99	13,12	13,33	30,94
	22,10	22,40	14,76	15,62	29,26
Normal adrenals	21,23	21,75	13,37	14,62	28,95
	20,46	20,89	13,03	13,71	26,26
	21,01	21,31	13,06	13,88	26,52
	18,74	20,24	12,01	12,78	25,17
	18,50	19,40	11,03	11,20	22,41
	19,68	20,75	13,09	13,25	27,57
	19,90	20,79	13,40	13,71	23,99

APPENDIX 3

IMMUNOHISTOCHEMISTRY PROTOCOL

Preheat antigen retrieval solutions
2x 5min xylene
3 min ethanol 96%
3 min ehtanol 80%
2 min ethanol 70%
2 min ehtanol 60%
2x 5min TBS

Antigen retrieval 10 min at 98 °C, or at 37 °C for pepsin
20 min cooling down at RT
2x 5 min TBS
15 min in 0.35% H_2O_2 in TBS
2x 5 min TBS

Dry slides and take off as much liquid as possible
Draw circles round section with ImmEdgde pen
Blocking in 10% NGS, 1% BSA in TBS with around 200 µL per slide (cover complete slide)
30 min incubation at RT

Remove Blocking solution, add diluted 1st antibody
Store slides in a container with a high humidity grade
Overnight incubation at 4 °C

Next day
2x 5 min TBS
Dry slides and take off as much liquid as possible
Exactly 5 min incubation of each slide with about 200 µL DAB solution at RT
Put all the slides in milliQ
Dry slides and take of as much liquid as possible

Cover the sections with some drops of hematoxylin for counterstaining for a few seconds
Drain hematoxilin from slide
10 min flushing of slides with tap water

2 min ethanol 60%
2 min ethanol 70%
3 min ethanol 80%
3 min ethanol 96%
3 min ethanol 96%
3 min ethanol 100%:xylene (1:1)
2x 5 min xylene
Dry slides and take of as much liquid as possible
Mount a cover slip with mounting medium

Dry slides before microscopic evaluation

Antigen retrieval: 10mM Sodium Citrate pH6, TrisEDTA pH9, pepsin stock 0.4%
1st antibody: SSTR 1, SSTR2A, SSTR 3 (Thermo Scientific), raised in rabbit, diluted in 1% BSA in TBS
2nd antibody: Envision K4003 (anti rabbit)
DAB: DAS substrate kit for peroxidase, Vector SK-4100
Hematoxylin QS: Vector H3404
Mounting medium: Vecta Mount, Vector H-5000

APPENDIX 4

GS ALPHA SEQUENCE WITH PRIMER LOCALISATION

```
   1 tccggcaata agagcggcgg cggcggcacg cgggcagcag ctccccgcag ctcctgctct
  61 ggtccgcctc ggcccggcgg cggccatcag ccccctcgg cctcggctcg aggggcgggg
 121 agcttcgcgc tcccctcggt acgaccgcca acctcccttc cccgcgcgcc ccgcgcccgc
 181 agtccgcccc gcgcgctcct ccccgaggag cgagcccgcg ccaggcccgc ccgcccggcg
 241 ctgcccggcc ctcccggccc tcccggccct cccggcccgc gtgaggccgc ccgcgcccgg
 301 ccccgccgcc gccgccgcag cccggccgcg ccccgcgccc cgccgccgcc gccatgggct
 361 gcctcggaaa cagtaagacc gaggaccagc gcaacgagga gaaggcgcag cgcgaggcca
 421 acaaaaagat cgagaagcag ctgcagaagg acaagcaggt ctaccgggcc acgcaccgcc
 481 tgctgctgct gggtgccgga gaatctggta aaagcaccat tgtgaagcag atgaggatcc
 541 tgcatgttaa tgggtttaat ggagaggcg gcgaagagga cccgcaagct gcaaggagca
 601 acagcgatgg tgagaaggca accaaagttc aggacatcaa aaacaacctg aaagaggcga
 661 tcgaaaccat cgtggccgcc atgagcaacc tggtgccccc cgtggagctg gccaaccctg
 721 agaaccagtt cagagtggac tacattctga gcgtgatgaa cgtgcctgac tttgacttcc
 781 ctcctgagtt ctatgagcat gccaaggctc tgtgggagga tgaaggagtg cgtgcctgct
 841 atgagcgctc caacgagtac cagctgattg actgcgccca gtacttcctg gacaagattg
 901 atgtcatcaa gcaggctgac tacgtgccca gtgatcagga tctgcttcgc tgccgtgtcc
 961 tgacttctgg aatctttgag accaagttcc aggtggacaa agtcaacttc cacatgtttg
1021 acgtgggtgg ccagcgcgat gaacgccgca aatggatcca atgcttcaat gatgtgactg
1081 ccatcatctt cgtggtggcc agcagcagct acaacatggt cattcgggag gacaaccaga
1141 ccaaccgcct gcaggaggct ctgaacctct tcaagagcat ctggaacaac agatggctgc
1201 gcaccatctc tgtgattctg ttcctcaaca agcaagacct gctcgctgag aaagtccttg
1261 ctggaaaatc gaagattgag gactactttc cagaatttgc tcgctacact actcctgagg
1321 atgctactcc cgagcccgga gaggacccac gcgtgacccg ggccaagtac ttcatccgcg
1381 acgaatttct gagaatcagc actgctagtg gagatgggcg ccactactgc tacccccact
1441 tcacttgcgc tgtggacacc gagaacattc gccgtgtgtt caacgactgc cgtgacatca
1501 ttcagcgcat gcaccttcgt cagtatgagc tgctctaaga agggaacacc caaatttaat
1561 taaagcctta agcacaatta attaagagtg aaacgtaatt gtacacgcag ttgatcaccc
1621 accatagggc atgattaaca acgcaacctt tccttttctc cccaagtgat tttgcgaaac
1681 ccccttttct tcccttccgc ttgcttaaat attccaaatt tagaaggctt aaggcggcct
1741 acagagaaaa agaaaaggaa aaaggccaca aaagttccct gtctctttca gtaattaaaa
1801 taacagcagc aaacagagat aatgaaataa aagaaataaa tgaaataaaa gaaataaatg
1861 aaataaatga aataaatatt gtcttgtgca gcattaaaaa aatcaaaata aaaattaaat
1921 gtgagca
```

Insciption

- *Exon boundary*
- *Start codon*
- *Forward primer*
- *Reverse primer*
- *Splice variant*