DOPAMINE RECEPTOR EXPRESSION AND FUNCTION IN THE NORMAL AND PATHOLOGICAL HYPOTHALAMUS-PITUITARY-ADRENAL AXIS

Rosario Pivonello

Dopamine receptor expression and function in the normal and pathological hypothalamus-pituitary-adrenal axis

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Thesis Erasmus University Rotterdam

Subjects headings: dopamine, dopamine receptors, hypothalamus-pituitary-adrenal axis

Front Cover:	Prenatal ultrasound scans of Désirée and Christian Pivonello, inexhaustible source of vitality for their father during the entire period of the achievement of this thesis. The scan were performed by Dr. Carlo Alviggi, Dr. Francesco Savarese, and Dr. Ferdinando Sannino, Naples, Italy. The picture and the design of the cover was performed by Luciano De Venezia and Luca Daniele, Avellino, Italy, on the basis of a personal idea.
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Printing:	HAVEKA BV, Alblasserdam, The Netherlands

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EXPRESSIE EN FUNCTIE VAN DOPAMINE RECEPTOREN IN DE NORMALE EN PATHOLOGISCHE HYPOTHALAMUS-HYPOFYSE-BIJNIER AS

THESIS

to obtain the degree of Doctor
from the Erasmus University Rotterdam
by command of the
rector magnificus

Prof. dr. S.W.J. Lamberts

and in accordance with the decision of the Doctorate Board.
The public defence shall be held on
Wednesday, 9 November 2005 at 15.45 hrs
by

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born in Naples, Italy

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Diego Ferone

The research project described in this thesis has been carried out at the Department of Internal Medicine of the Erasmus University of Rotterdam (The Netherlands) and the Department of Molecular and Clinical Endocrinology and Oncology of "Federico II" University of Naples (Italy). The realization of this thesis has been supported by Pfizer.

A Désirée e Christian
la ragione della mia vita

"Erano solo una promessa di vita
e già diventavano tutta la mia vita"
RP

To Désirée e Christian
The reason of my life

"They were just a promise of life
and were already becoming all my life"
RP

IF you can keep your head when all about you
Are losing theirs and blaming it on you,
If you can trust yourself when all men doubt you,
But make allowance for their doubting too;
If you can wait and not be tired by waiting,
Or being lied about, don't deal in lies,
Or being hated, don't give way to hating,
And yet don't look too good, nor talk too wise:

If you can dream - and not make dreams your master;
If you can think - and not make thoughts your aim;
If you can meet with Triumph and Disaster
And treat those two impostors just the same;
If you can bear to hear the truth you've spoken
Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to, broken,
And stoop and build 'em up with worn-out tools:

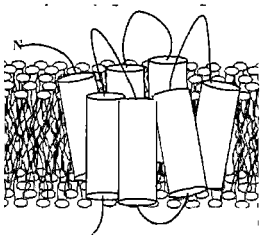
If you can make one heap of all your winnings
And risk it on one turn of pitch-and-toss,
And lose, and start again at your beginnings
And never breathe a word about your loss;
If you can force your heart and nerve and sinew
To serve your turn long after they are gone,
And so hold on when there is nothing in you
Except the Will which says to them: 'Hold on!'

If you can talk with crowds and keep your virtue,
' Or walk with Kings - nor lose the common touch,
if neither foes nor loving friends can hurt you,
If all men count with you, but none too much;
If you can fill the unforgiving minute
With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
And - which is more - you'll be a Man, my son!

Rudyard Kipling

CONTENT

I



INTRODUCTION

DOPAMINE RECEPTOR EXPRESSION AND FUNCTION AND THE HYPOTHALAMUS-PITUITARY-ADRENAL AXIS: THE STATE OF THE ART

1. DOPAMINE RECEPTORS

Structural characteristics

Dopamine is the predominant catecholamine neurotransmitter in the human central nervous system, where it controls a variety of functions including cognition, emotion, locomotor activity, food intake and endocrine regulation. Dopamine also plays multiple roles in the periphery as a modulator of cardiovascular and renal function, gastrointestinal motility and the endocrine system (1). Dopamine exerts its functions *via* the binding with dopamine receptors (1). Dopamine receptors belong to the family of seven transmembrane domain G protein-coupled receptors and include five different receptor subtypes, named D_1-D_5. The members of dopamine receptor family are encoded by genes localized on different chromosome loci, displaying a considerable homology in their protein structure and function. The analysis of dopamine receptor structure and function suggests the existence of two different groups of receptors: D_1-like, including D_1 and D_5 receptors, associated to a stimulatory function, and D_2-like, including D_2, D_3 and D_4 receptors, associated to an inhibitory function. The D_1 and D_5 receptors are encoded by intronless genes and share an 80% homology in their transmembrane domains. The D_2 receptor shares a 75% homology with the D_3 and a 53% homology with the D_4 transmembrane domains and all three receptor subtypes are encoded by genes, which are interrupted by introns. The D_2 receptor exists in two main variants, called D_{2long} and D_{2short}, generated by an alternative splicing of an 87 base pairs exon. These two D_2 receptor isoforms differ for the presence or absence of a stretch of 29 amino acids in the third cytoplasmic loop in their protein structure. Splicing variants of the D_3 receptor encoding nonfunctional proteins have been also identified. The analysis of the D_4 receptor reveals the existence of polymorphic variations within the coding sequence, being a 48 base pairs sequence existent as a direct repeat sequence ($D_{4.1}$), fourfold ($D_{4.4}$), sevenfold ($D_{4.7}$) or eleven fold ($D_{4.11}$) repeat sequence. Therefore, the D_4 receptor isoforms differ for the length of the third cytoplasmic loop and have one, four, seven or eleven times the same insert of a stretch of 19 amino acids in their protein structure. The D_5 receptor has two related pseudogenes, which share a 95% homology with the gene and encode for truncated non functional forms of the receptor (1). The molecular characteristics of human dopamine receptor family are summarized in **Table 1.** A schematic representation of the human dopamine receptor is shown in **Fig. 1.**

Table 1: Molecular characteristics of human dopamine receptors

	D_1-like		D_2-like			
			D_2			
	D_1	D_5	D_{2short}	D_{2long}	D_3	D_4
Amino acids	446	467	414	443	400	387-515
Amino acids in the third cytoplasmic loop	57	50	134	163	120	101-261
Introns	0	0	5	6	5	3
Chromosomal localization	5q35	4p15-16	11q22-23		3q13	11p15

Data derived from ref. 1

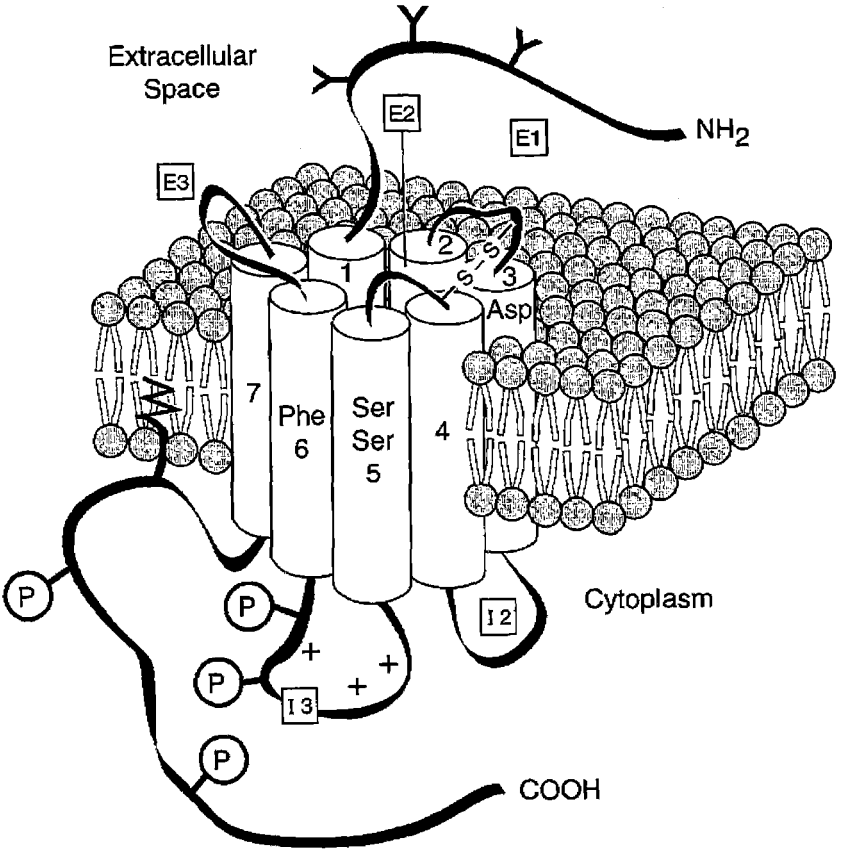


Figure 1: Schematic structure of the dopamine receptor. The figure represents the structural features of D_1-like receptors. D_2-like receptors are characterized by a shorter COOH-terminal tail and by a bigger third intracellular loop. Residues involved in dopamine binding are highlighted in transmembrane domains. Potential phosphorylation sites are represented on third intracellular loop and on COOH terminal tail. Potential glycosylation sites are represented on NH_3 terminal tail. E1-E3, extracellular loops, I1-I3, intracellular loop, 1-7, transmembrane domains.

Pharmacological characteristics

The pharmacological profile of dopamine receptors displays a difference between D_1-like and D_2-like receptors, mainly characterized by a variable binding affinity of certain dopamine agonists and antagonists (1). Dopamine binds all five receptors although among the D_1-like receptors it binds the D_1 receptor with lower affinity than D_5 receptors, and among the D_2-like receptors it binds the D_2 receptor with lower affinity than D_3 and D_4 receptors. Beyond dopamine, different agonists or antagonists preferentially or exclusively bind D_1-like or D_2-like receptors. For instance, among the dopamine agonists, bromocriptine preferentially binds D_2-like receptors but is able to bind D_1-like receptors as well whereas among the dopamine antagonists, sulpiride binds exclusively D_2-like receptors. It is noteworthy that D_1 and D_5 receptors cannot be clearly differentiated pharmacologically, since no dopamine agonist or antagonist exclusively or preferentially bind D_1 or D_5 receptors and they generally display similar binding affinities for both receptor subtypes. Conversely, the availability of dopamine agonists and/or antagonists, which exclusively or preferentially bind the different D_2, D_3 or D_4 receptors make it possible to clearly differentiate them pharmacologically. No compound is able to clearly discriminate between D_{2long} and D_{2short}, although a marginal difference in the affinities of the two D_2 receptor isoforms has been described for the dopamine antagonist sulpiride (1). Among the dopamine agonists, cabergoline is able, like bromocriptine, to bind both D_1 and D_2 receptors, although with higher affinities for both receptors and higher selectivity for D_2 receptors (2). Moreover, cabergoline has recently been demonstrated to possess a higher affinity than bromocriptine not only for both D_2 receptor isoforms, but also for D_3 and D_4 receptors (3,4). No study has ever compared the binding affinity of bromocriptine and cabergoline for the D_5 receptor. The different pharmacological characteristics of bromocriptine and cabergoline definitely demonstrated that cabergoline is more potent compared to bromocriptine in the binding and activation of the D_2-like receptors. The pharmacological profile of the D_2-like dopamine receptors in relation to dopamine and the dopamine agonists bromocriptine and cabergoline is shown in **Table 2.**

Functional characteristics

Dopamine receptors mediate dopamine and dopamine agonist effects *via* a number of different mechanisms of signal transduction (1). Among these, the most important one is the modulation of adenylyl cyclase activity resulting in either stimulation or inhibition of cyclic AMP (cAMP) accumulation. This mechanism is

Table 2: Pharmacological profile of human dopamine receptors

	D_2				D_3		D_4	
	D_{2short}		D_{2long}					
	E_{max} (%)	IC_{50} (nM)	E_{max} (%)	IC_{50} (nM)	E_{max} (%)	IC_{50} (nM)	E_{max} (%)	IC_{50} (nM)
Dopamine	100	350	100	320	100	11	100	100
Bromocriptine	41	4.5	28	3.9	68	4.2	0	>1000
Cabergoline	102	0.53	75	0.41	86	0.78	49	81

The efficacy and potency of the different dopaminergic compounds are expressed as E_{max} and IC_{50}, respectively. E_{max} is represented as the percentage of the maximal efficacious (E_{max}= 100%) concentration of dopamine. Data derived from ref. 3

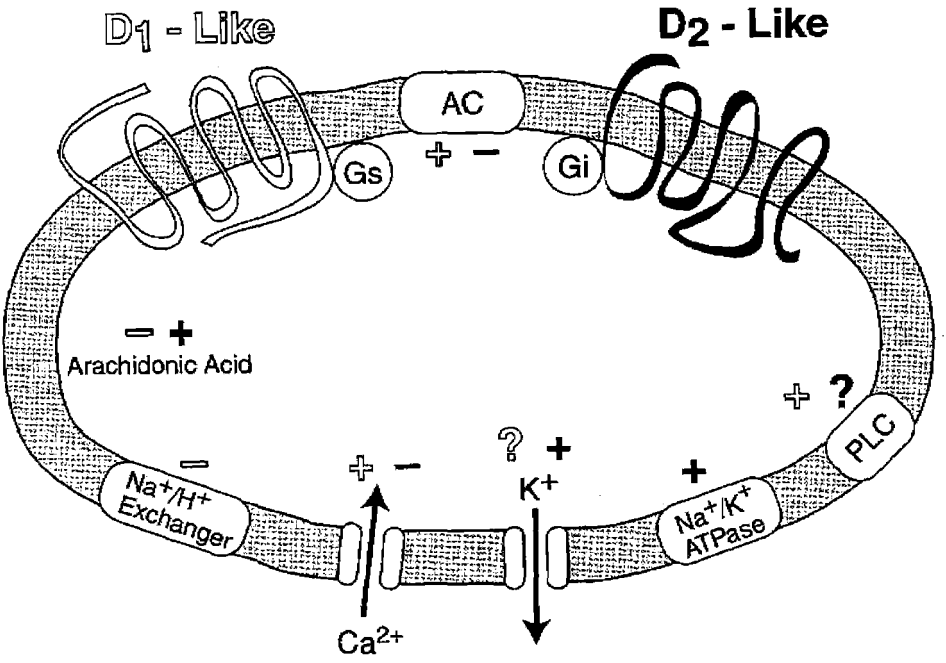


Figure 2. Schematic representation of signal transduction mechanisms associated to the dopamine receptor. AC, adenylil cyclase; PLC, phospholipase C. Open symbols, D_1-like effects, closed symbols, D_2-like effects

mediated by the activation of different G proteins, mainly $G_{s\alpha}$ for the stimulation and $G_{i\alpha}$ for the inhibition of adenylyl cyclase. D_1-like receptors generally stimulate, whereas D_2-like receptors inhibit adenylyl cyclase activity and cAMP accumulation (1). Dopamine receptors are also able to activate different mechanisms of signal transduction, including the modulation of the activity of phospholipase C or the release of arachidonic acid, as well as the activity of the calcium and potassium channels. Moreover, dopamine receptors seem also to modulate the activity of Na/H exchangers and the Na-K ATPase (1). It is noteworthy that dopamine receptors activate signal transduction mechanisms involved not only in the regulation of hormone synthesis and secretion but also in the control of cell growth and differentiation (1). A schematic representation of the different signal transduction mechanisms mediated by the dopamine receptors is shown in **Fig. 2.**

Tissue Distribution and Role

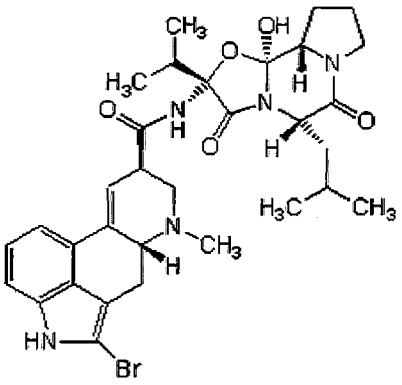
Dopamine receptors are mainly and widely distributed in the central nervous system (1). In particular, the dopaminergic neurons in the substantia nigra, tegmental area and hypothalamus, give origin to three main pathways, the nigrostriatal, the mesolimbocortical and the tuberoinfundibular pathways. Dopamine receptors are, therefore, mainly localized in the striatum, the limbic system, the brain cortex and in the infundibulum. However, the presence of dopamine receptors has been demonstrated in most areas of the central nervous system, where they mediate the effect of dopamine on cognition, emotion, regulation of food intake, locomotor activity and the endocrine system (1). Dopamine receptors are also expressed in the pituitary gland, where they mediate the effect of dopamine in the regulation of hormone synthesis and secretion (1). Finally, dopamine receptors are widely distributed in the periphery, mainly at the level of the cardiovascular system, kidney, and adrenal gland, beyond the peripheral nervous system. In the cardiovascular system, dopamine is known to induce vasodilation and decrease of cardiac contractility. In the kidney, dopamine induces an increase of the renal filtration rate and a decrease of salt reabsorption, as well as a stimulation of renin secretion activating the renin-angiotensin-aldosterone system, and inhibition of vasopressin action. The presence of dopamine receptors in the adrenal gland suggests a role of these receptors in the regulation of adrenal hormone synthesis or secretion (1).

2 DOPAMINE AGONISTS

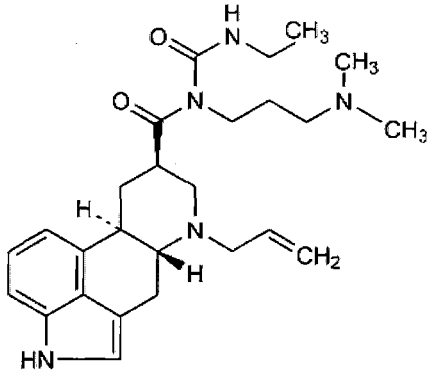
General characteristics

Dopamine agonists are a category of compounds, which are able to bind dopamine receptors thereby mimicking dopamine actions. The currently available dopamine agonists which were previously used, or are presently used, or which are potentially useful in the clinical practice may be divided into two different groups on the basis of their derivation: *ergot* and *non-ergot* derivatives (5). The *ergot* derivative dopamine agonists mainly include bromocriptine, pergolide, lisuride, and cabergoline. Conversely, quinagolide is the most well known *non-ergot* derivatives dopamine agonists. The *ergot* derivative compounds have attracted a remarkable interest for the wide spectrum of pharmacological actions including central, neurohormoral and peripheral effects mediated by catecholamine, serotonin and dopamine receptors. Indeed, their wide range of biological activities may be explained by assuming that *ergot* derivative compounds interact with more than one receptor site, that the population of receptor sites to which they bind varies from organ to organ, and that affinity for receptor sites and intrinsic activity vary from compound to compound. Considering the biological activities of *ergot* derivative compounds, it is not surprising that the drugs which have been developed do not form a single pharmacological or therapeutic entity, since they have different indications but also display a number of side effects. Indeed, the *ergot* derivatives find an application in the treatment of a variety of clinical conditions, including migraine, post-partum hemorrhage, orthostatic hypotension, senile cerebral insufficiency, and Parkinson's disease (2,5). In addition, the evidence that these compounds may act at the level of the hypothalamus-pituitary axis to inhibit PRL secretion and PRL-secreting cell growth results in a wide application in the treatment of hyperprolactinemic syndromes, frequently due to PRL-secreting pituitary tumors. The most widely used ergot derived dopamine agonist in the treatment of PRL-secreting pituitary tumors has been bromocriptine. In the past, pergolide and lisuride were also used, though their employment in this disease was not successful. More recently, cabergoline is becoming the most relevant dopamine agonist in the treatment of PRL-secreting pituitary tumors on the basis of its specific characteristics, which make it a more potent, tolerated and, therefore, effective drug compared with bromocriptine (2,5). The chemical structure of the two most important dopamine agonists bromocriptine and cabergoline are shown in **Fig. 3**. The *non-ergot*-derivatives compounds are represented by the octahydrobenzyl(g)-quinolines, among which quinagolide is the most active compound. In different studies on experimental animals, quinolines

were demonstrated to have a higher affinity for D_1-like and D_2-like receptors and a higher selectivity for D_2-like receptors compared with the *ergot* derived dopamine agonist bromocriptine (5,6). In humans, quinagolide was confirmed to have a potent action in inhibiting physiological and pathological PRL secretion without significant adverse effects (5,7). However, the demonstration of an equal or even higher potency and tolerability of the *ergot* derive4d dopamine agonist cabergoline compared to quinagolide and, in particular, a better compliance related to the long-lasting activity and the "once or twice a week" administration of cabergoline compared to the short-lasting activity and the "once a day" administration of quinagolide, have resulted in the use of cabergoline as primary drug of choice in the treatment of PRL-secreting pituitary tumors.



Bromocriptine



Cabergoline

Figure 3: Molecular structure of the dopamine agonists bromocriptine and cabergoline

Cabergoline

Cabergoline is currently the most widely used dopamine agonist included in the pharmacotherapy of PRL-secreting pituitary tumor (8). Cabergoline has the most potent and long-lasting activity in controlling PRL-secretion both *in vitro* and *in vivo* in different animal models. In the last decade, several studies in humans have demonstrated the efficacy of cabergoline in resolving the hyperprolactinemic syndrome in the majority of patients, accompanied by a potent anti-tumoral effect on PRL-secreting pituitary tumors with an excellent tolerability (9,10). In line with

previous observations on antitumoral effects of different dopamine agonists, cabergoline was demonstrated to induce a reduction in cell volume, subsequent to the early inhibition of secretory mechanisms and late inhibition of PRL gene transcription and protein synthesis as well as perivascular fibrosis and cell necrosis (11). In various animal models, cabergoline markedly reduced plasma PRL levels *in vivo* after single or multiple doses; the PRL-lowering effects appeared 2-8 hrs after administration and lasted for more than 72 hrs (12). In addition, a single dose of cabergoline (0.6 mg/Kg) in rats significantly inhibited the plasma PRL levels for 6 days, whereas the continued oral administration of cabergoline significantly reduced plasma PRL levels as well as weight of the pituitary during 15 to 60 days of treatment (13). In human healthy volunteers, a single dose of cabergoline (0.2 to 0.6 mg) produced a dose-dependent inhibition of PRL secretion, 0.2 mg being the minimal effective dose, while maximal PRL suppression occurred within 5 hrs after administration at all doses; at a dose lower than 0.3 mg, cabergoline exerted a significant PRL inhibition for approximately 4 days and at doses of 0.4-0.6 mg for at least 7 days (14). In healthy men, cabergoline at the doses of 0.5, 1.0, and 1.5 mg produced a complete PRL suppression, which occurred earlier and persisted longer with the two higher doses (15). In healthy women with regular menses, cabergoline at the dose of 0.4-0.6 mg induced maximal inhibition of PRL secretion of 43-76%. After the low 0.4 mg dose, PRL levels returned to baseline within 24 hrs, while the PRL inhibitory effects persisted until the fifth day after the administration of 0.6 mg (16). The absolute oral bioavailability of cabergoline has not been established: studies using radiolabeled cabergoline showed that up to 72% of the dose administered orally is eliminated in the feces within 10 days. In particular, after 24 hrs, less than 10% of the total administered radioactivity was excreted in the urine and none was present in the feces, whereas after 240 hrs, the overall radioactivity excreted was 10-20% in urine and 55-72% in feces. This suggests that cabergoline is absorbed from the gastrointestinal tract but is extensively metabolized, possibly with enterohepatic recycling. Indeed, less than 15% of the radioactivity excreted in the urine was due to unchanged drug (17,18). Finally, cabergoline was reported to have a lower prevalence and severity of side effects, compared to the other dopamine agonists, probably due to the higher affinity and selectivity for D_2 receptors, permitting to administer the compound at a lower dose for reaching the same therapeutic effect. In addition, side effects occur early, generally at the beginning of treatment, and tend to disappear spontaneously with treatment continuation. It is likely that side effects associated with dopamine agonist administration are due to changes in drug concentrations in the blood (19). In this respect cabergoline

represents an advantage as its long half-life results in a relatively flat plasma drug concentration. The most common adverse event in patients with hyperprolactinemia is nausea, associated or not with vomiting (~35%), followed by headache (~30%), and dizziness or vertigo (~25%) (19). Diarrhea, drowsiness, somnolence, paresthesia and dyspnea are less commonly reported and the prevalence of cabergoline treatment withdrawal due to side effects is reported in less than 3% of patients (20). Hypotension is reported in approximately 50% of women with hyperprolactinemia during cabergoline treatment, although it is usually asymptomatic (21). A wide spectrum of therapeutic applications is foreseen for cabergoline, besides its well known use in the field of hyperprolactinemic syndromes and inhibition of lactation Parkinson's disease. In particular, cabergoline has been suggested to be useful also in non PRL-secreting pituitary tumors (8).

3. DOPAMINE RECEPTORS IN THE NORMAL PITUITARY GLAND

Morphology of the pituitary gland

The human pituitary gland is anatomically composed of three different parts: two major lobes and an intermediate zone or area, also called *"pars intermedia"*. The two pituitary lobes have a completely different histology, since the anterior lobe or *"pars distalis"* has an epithelial structure whereas the posterior lobe or *"pars nervosa"* has a neural structure. The lobes contain different cell populations which are the source of multiple hormones and which are differently regulated, as hormone secretion is modulated *via* the vascular system for the anterior lobe and *via* the nervous system for the posterior lobe (22-24). A scheme of the structure and histology of the normal pituitary gland is shown in **Fig. 4**. The intermediate zone is a peculiar structure of the pituitary gland, which shows different characteristics among mammals: it is well defined in rats and poorly defined in humans (25). In humans, it is better defined in fetal life, but undergoes a progressive involution to a group of colloid-filled cysts that mingle with the neural lobe in the adult life (25). These observations most likely explain why the majority of studies on the intermediate zone of the pituitary gland have been performed in rats rather than in humans. The anterior lobe contains different endocrine cell populations, including somatotrophs, which release GH, lactotrophs, which release PRL, corticotrophs, which release ACTH, thyrotrophs, which release TSH and gonadotrophs, which release FSH and LH (22-24). The posterior lobe is formed by groups of neural cells producing adiuretin and oxytocin (22-24). Conversely, the intermediate zone contains one major endocrine cell population, the melanotroph cells, a subgroup of corticotroph cells which processes the precursor molecule proopiomelanocortin (POMC) and mainly releases MSH. These cells usually stain for MSH and in the majority also for ACTH (26). The melanotroph cells differ from the corticotroph cells of the anterior lobe, which also process POMC but almost exclusively release ACTH. These cells generally stain for ACTH, and in a minority also for MSH (27-29). A scheme of POMC cleavage to produce ACTH and/or MSH is shown in **Fig. 5**. An unusual feature of the intermediate zone is the relative lack of vascular supply, which remains at the periphery of the cell bodies, whereas nerve fibers arising from the hypothalamic cell bodies, pass through the neural lobe and form synaptic connections onto the endocrine cells (30,31). The most important innervations reaching the intermediate zone are represented by dopaminergic nerves, which seem to be involved in the effect of dopamine on growth and function of melanotroph cells (32).

Expression of dopamine receptors

Dopamine receptors have been clearly demonstrated in the anterior lobe of the pituitary gland. In rats, the D_2 receptor is expressed mainly in lactotrophs (33), but it was found also in non-lactotroph cells, mainly somatotrophs and thyrotrophs (34,35), as well as in gonadotroph cells (36). Moreover, D_2 receptor expression in corticotroph cells has been suggested by the demonstration of its expression in AtT20 cells, a mouse pituitary corticotroph cell line (37,38). Similarly, in humans, the D_2 receptor is also expressed mainly in lactotroph cells (39), although it was found in more than 75% of cells of the anterior pituitary gland, indicating its expression not only in lactotroph, but also in non-lactotroph cell populations (40). Dopamine receptors, and particularly D_2 receptors, have been clearly demonstrated in the intermediate zone of the pituitary gland as well. Indeed, several studies on experimental animals have demonstrated the presence of D_2 receptor in melanotroph cells (41,42). These data suggests the possible D_2 receptor expression in melanotroph cells in humans as well. With respect to D_2 receptor isoforms, several studies on experimental animals have demonstrated that both D_{2long} and D_{2short} receptor isoforms are expressed in lactotroph and melanotroph cells, although D_{2long} is predominantly expressed compared to the D_{2short} isoform (43). Finally, it is noteworthy that the D_2 receptor is not the only dopamine receptor expressed in the pituitary gland. Indeed, the D_4 receptor, in particular its $D_{4.4}$ variant, is expressed in the pituitary gland as well, although its role in the physiology of the gland is not known (44).

The currently available information does not allow to draw definitive conclusions on the expression of dopamine receptors in the corticotroph cells of the normal human pituitary gland. The first important aim of this thesis is the evaluation of the expression of dopamine receptors, in particular D_2 receptors, in the corticotroph cell population, and the comparison between D_2 receptor expression in the corticotroph cells of the anterior lobe and the melanotroph cells of the intermediate zone of the normal human pituitary gland. This issue is addressed in chapter II of this thesis.

Function of dopamine receptors

The role of dopamine receptors, in particular the D_2 receptor, in the pituitary gland is to mediate the regulatory effects of hypothalamic dopamine on the different pituitary cell populations. In the pituitary gland, multiple transduction mechanisms are activated by the D_2 receptor. In addition to the inhibition of adenylyl cyclase,

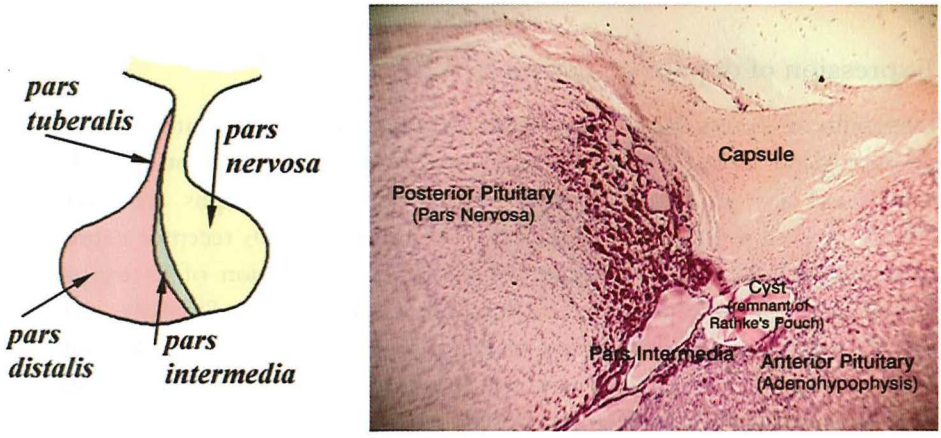


Figure 4: Schematic anatomical structure (left) and exemplary histological structure (right) of the normal pituitary gland

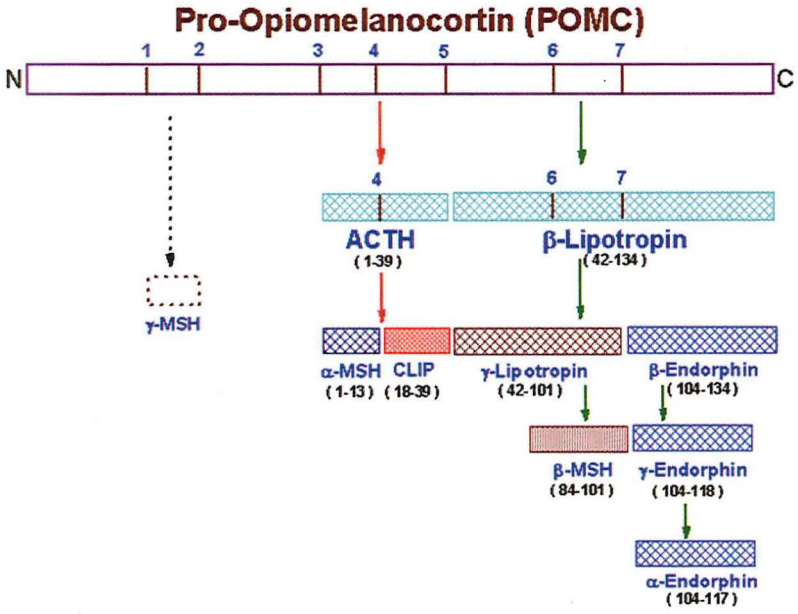


Figure 5: Scheme of the POMC cleavage and formation of ACTH and MSH

pituitary D_2 receptors inhibit phosphatidylinositol metabolism, activate potassium channels and inhibit calcium currents. All these effects are mediated by G proteins, mainly $G_{s\alpha}$ and $G_{i\alpha}$. In rats and humans, the major role of the pituitary D_2 receptor is the inhibitory control of PRL synthesis and secretion, as well as the growth of lactotroph cells (39). Moreover, it has been demonstrated in rats and hypothesized in humans that the D_2 receptor is also involved in the inhibitory control of the synthesis and/or secretion of MSH and growth of the melanotroph cells (45,46). The finding that the expression of the Pit 1 transcription factor, which is involved in pituitary hormone gene expression, is inhibited by activation of D_2 receptors in transfected cell lines supports the existence of a dopaminergic control on pituitary hormone gene expression (47). In addition, a series of *in vitro* and/or *in vivo* studies in experimental animals and/or in humans suggests a possible modulatory role of dopamine on GH, TSH, and FSH/LH release from the somatotroph, thyrotroph and gonadotroph cells, respectively (48).

4. DOPAMINE RECEPTORS IN THE NORMAL ADRENAL GLAND

Morphology of the adrenal gland

The human adrenal gland is anatomically composed of two different structures: the cortex and the medulla. The adrenal cortex is responsible for the release of mineralcorticoids, glucocorticoids and sex steroids, whereas the medulla is responsible for the secretion of catecholamines. The adrenal cortex is mainly regulated via the vascular system by the kidney and the pituitary, whereas the medulla is mainly regulated via the nervous system through different innervations, among which the dopaminergic innervation has a pivotal role. Histologically, the adrenal cortex is characterized by three different zones, the external "*zona glomerulosa*", which is responsible for the secretion of the mineralcorticoids, mainly aldosterone, the intermediate "*zona fasciculata*", which is responsible for the secretion of glucocorticoids, mainly cortisol, and the internal "*zona reticularis*", which is responsible for the secretion of sex steroids (49). A scheme of the structure and histology of the normal adrenal gland is shown in **Fig 6.**

Expression of dopamine receptor

Dopamine receptors are expressed in the adrenal cortex. In experimental animals, as well as in humans, receptor ligand binding studies have demonstrated the presence of specific and saturable binding sites for different radiolabeled dopaminergic compounds such as spiperone, which bind both D_1-like and D_2-like receptors, and sulpiride, which selectively binds D_2-like receptors (50). These studies demonstrated that both D_1-like and D_2-like receptors are expressed in the adrenal cortex. In addition, the expression of D_1-like and D_2-like receptors in the adrenal medulla has been clearly demonstrated to mediate the dopaminergic regulation of catecholamine secretion (51). A recent study has evaluated the expression of D_2-like receptor subtypes in human normal adrenal glands, demonstrating that D_2 and D_4 receptors are expressed in all three zones of the adrenal cortex and in the adrenal medulla, being localized mainly in the zona glomerulosa and reticularis and at a lesser extent in the zona fasciculata of the adrenal cortex (52).

The currently available information does not allow to draw definitive conclusions on the expression of dopamine receptors in the normal human adrenal gland. An important aim of this thesis is the evaluation of the expression of dopamine receptor subtypes, including the D_2 receptor isoforms, as

well as the distribution of D_2 receptors in the different areas of the cortex and the medulla of the human normal adrenal gland. This issue is addressed in chapter IV of this thesis.

Function of dopamine receptors

Dopamine receptor expression in the adrenal cortex has been initially hypothesized after showing a role of dopamine in the control of aldosterone secretion in *in vivo* studies in both experimental animals and humans (53). The administration of the D_2 antagonist metoclopramide to both rats and humans increased plasma aldosterone levels without influencing any stimulator of aldosterone release. This effect was blocked by the intravenous injection of dopamine (54-56). However, the administration of dopamine or the dopamine agonist bromocriptine did not modify plasma aldosterone levels (57). These observations suggested that aldosterone production was under maximum tonic dopaminergic inhibition. Subsequent studies demonstrated that the sodium balance status is crucial for the effects of dopamine or dopamine agonists on aldosterone secretion. Indeed, dopamine and D_2 agonists were shown to inhibit angiotensin-stimulated and upright posture-induced increased aldosterone secretion in sodium depleted, but not in sodium repleted normal subjects (58,59). Moreover, *in vitro* studies with isolated adrenal glomerulosa cells demonstrated that the activation of D_2 receptors resulted in a remarkable inhibition of angiotensin II-induced aldosterone secretion, whereas it did not influence basal and ACTH-induced aldosterone secretion (60). These studies suggested the expression of D_2 or D_2-like receptors in the cells of the zona glomerulosa of the adrenal cortex and a selective functional interaction between dopamine and angiotensin II in the regulation of aldosterone secretion. While no definitive data are available on the role of dopamine and dopamine receptors in the adrenal cortex, their role in controlling catecholamine secretion in the adrenal medulla is well documented (61).

The currently available information does not allow to draw definitive conclusions on the functional role of dopamine and dopamine receptors in the regulation of cortisol and/or sex steroids secretion by the adrenal gland. An important aim of this thesis is to evaluate the in vitro effect of the dopamine agonists bromocriptine and cabergoline on the production of aldosterone, cortisol and the androgen androstenedione by cultured human adrenal cortical cells. This issue is addressed in chapter IV of this thesis.

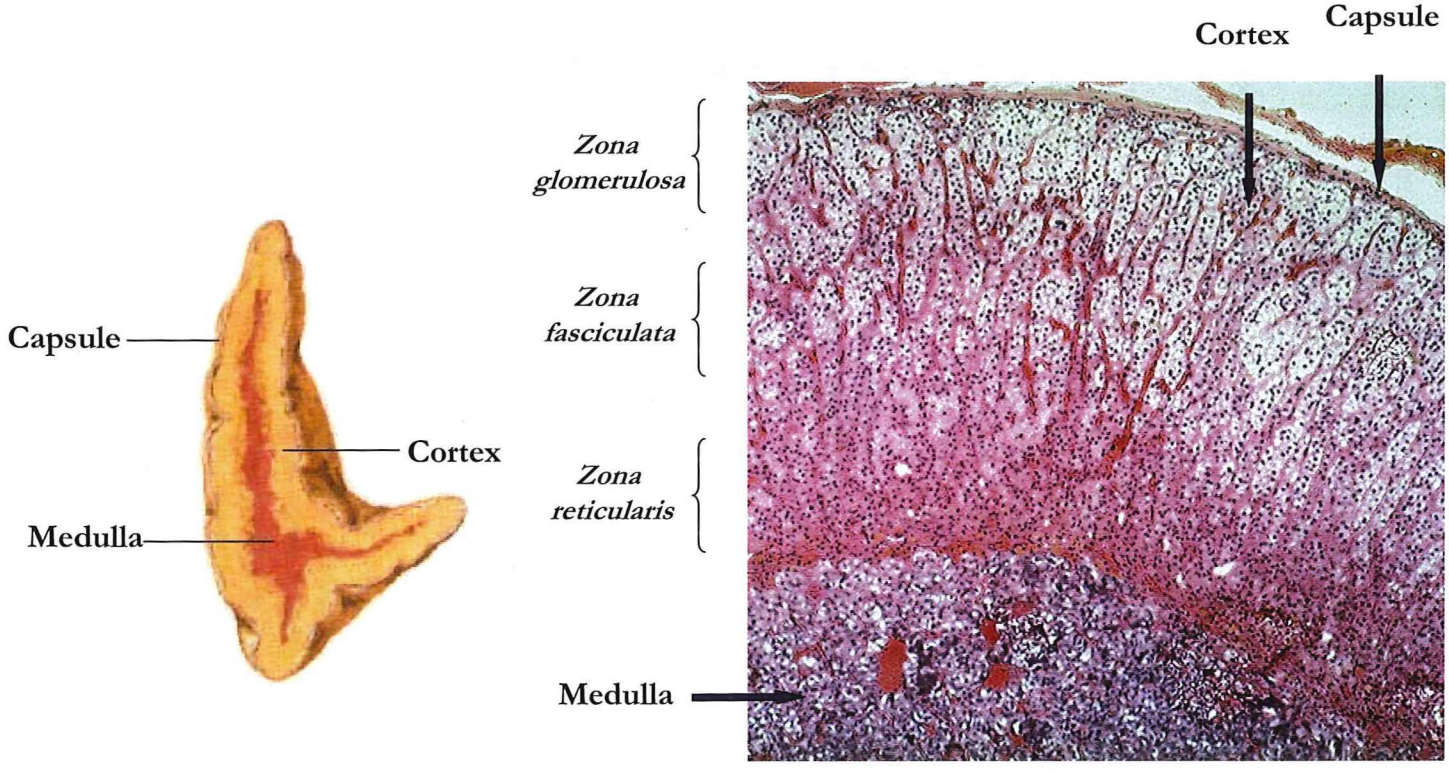


Figure 6: Schematic anatomical structure (left) and exemplary histological structure of the normal adrenal gland

5. DOPAMINE RECEPTORS IN THE PATHOLOGICAL PITUITARY AND ADRENAL GLANDS

Dopamine receptors and pituitary disease

A significant number of studies have evaluated D_2 receptor expression in human pituitary tumors. D_2 receptor expression has been clearly demonstrated in PRL-secreting pituitary tumors, where the presence of a considerable number of receptors explains the good therapeutic effect of dopamine agonists, inducing inhibition of PRL secretion and tumor shrinkage (5,62). Actually, the presence of a functional D_2 receptor inhibiting PRL secretion not only in PRL-secreting tumors but also in the normal anterior pituitary gland, leads to a major therapeutic application in the treatment of hyperprolactinemia either due to functional hypothalamus-pituitary defects or to a pituitary tumor. D_2 receptor agonists are the most effective pharmacological tools to normalize plasma PRL levels in all clinical conditions associated to hyperprolactinemia. D_2 receptors have been demonstrated in non PRL-secreting pituitary tumors as well. Indeed, D_2 receptors have been found in GH-secreting (63,64) as well as in clinically non functioning (65-67) pituitary tumors, where they also seem to be a pre-requisite for the effectiveness of treatment with dopamine agonists. Moreover, D_2 receptors have been demonstrated in TSH- and FSH/LH-secreting pituitary tumors, although their role in the treatment with dopamine agonists has not been definitely investigated. In these tumors, however, the effectiveness of dopamine agonists seems to be less pronounced compared to PRL-secreting pituitary tumors, probably because of a lower density of D_2 receptors or a different pattern of dopamine receptor subtypes and/or D_2 receptor isoforms. On the other hand, the dopamine agonist cabergoline has been recently found to overcome the relative resistance of GH-secreting (68) and clinically non functioning pituitary tumors (69) to the previously used dopamine agonists, such as bromocriptine and quinagolide, thereby opening a new way to a possible medical treatment of these tumors. Finally, a variable and heterogeneous expression of D_2 receptors has recently been demonstrated both by in situ hybridization (ISH) and immunohistochemistry (IHC) in nearly 90% of all types of pituitary tumors and particularly in nearly 70% of silent or functioning ACTH-secreting pituitary tumors (70). Taken together, these observations suggest a widespread expression of D_2 receptors in human pituitary tumors forming molecular targets for medical treatment with dopamine agonists.

While dopamine receptor expression has been extensively studied in most types of pituitary tumors, there is very limited information on the expression and function of dopamine receptors, in particular D_2 receptors, in human corticotroph pituitary tumors. An important aim of this thesis is to evaluate the D_2 receptor expression, as well as the in vitro effect of the dopamine agonists bromocriptine and cabergoline on ACTH secretion, and the in vivo effect of cabergoline on ACTH and cortisol secretion in corticotrophin pituitary tumors. This issue is addressed in chapter III of this thesis.

Dopamine receptors and adrenal disease

Dopamine receptors have been unequivocally demonstrated in pheochromocytomas, deriving from the medulla of the adrenal gland (51). Moreover, a recent study evaluating the expression of D_2-like receptors in pheochromocytomas and aldosterone-secreting adenomas, demonstrated that D_2 and D_4 receptors are the only D_2-like receptors expressed in these two types of adrenal tumors (52).

While dopamine receptor expression has been extensively studied in medullary adrenal tumors, there is very limited information on the expression of dopamine receptors in cortical adrenal tumors. An important aim of this thesis is to evaluate dopamine receptor subtype and D_2 receptor isoform expression in the different types of benign and malignant adrenal tumors, including aldosterone-secreting, cortisol-secreting and the androgen-secreting tumors as well as the pheochromocytomas. This issue is addressed in chapter IV of this thesis

6. THE CUSHING'S SYNDROME

Cushing's syndrome (CS) is the most important disease associated with the hypothalamus-pituitary-adrenal axis. CS is a rare disease due to chronic exaggerated endogenous glucocorticoid production: it is commonly caused by an ACTH-secreting pituitary adenoma, accounting for around 80% of cases, and rarely by a cortisol-producing adrenal tumor, accounting for 15%, or to an ACTH-secreting neuroendocrine tumor of the lung, thymus or gastroenteropancreatic system, accounting for 5% of the cases. The ACTH-dependent CS of pituitary origin is called Cushing's disease (CD), whereas the ACTH-dependent CS of non-pituitary origin is called ectopic CS or ectopic ACTH syndrome (EAS) (71). A scheme of a normal and pathological hypothalamus-pituitary-adrenal axis is shown in **Fig. 7**. CS is a severe disease associated with an increased morbidity and mortality for cardiovascular diseases, and with several systemic complications, among which the most common are hypertension and impairment of glucose tolerance that definitely contribute to the increased cardiovascular risk. (72). The diagnosis of CS and the differential diagnosis between the different forms of CS, in particular the ACTH-dependent forms, is difficult because of their similar clinical and biochemical picture, and the limitations of the imaging techniques, which are often not able to visualize the pituitary and the ectopic tumors because of their very small size. The diagnosis is suggested by the results of a combination of dynamic tests, among which the dexamethasone suppression test and the CRH test are most commonly used in clinical practice. The differential diagnosis of the ACTH-dependent forms of CS often requires the bilateral inferior petrosal sinus sampling (BIPSS), which allows to evaluate the presence or absence of an ACTH gradient between the blood deriving from the pituitary and the peripheral blood, suggesting CD or EAS, respectively (73). The treatment of CS is mainly based on surgery. However, the surgical removal of the pituitary or ectopic tumors is affected by a consistent number of failures. The alternative treatments, including bilateral adrenalectomy and radiotherapy, are associated with several complications. The medical therapy currently plays only a minor role in the treatment of CS since no drug has ever been demonstrated to have a significant effectiveness allowing its routine use in clinical practice. Adrenal blocking drugs, which act at the adrenal level, are commonly used before or after the standard treatments to achieve normalization of glucocorticoid levels before the definitive cure of the disease. Among drugs, which may act directly at the pituitary or the ectopic tumor, dopamine agonists, in particular bromocriptine, showed a certain effectiveness in normalizing cortisol levels in a sub-group of

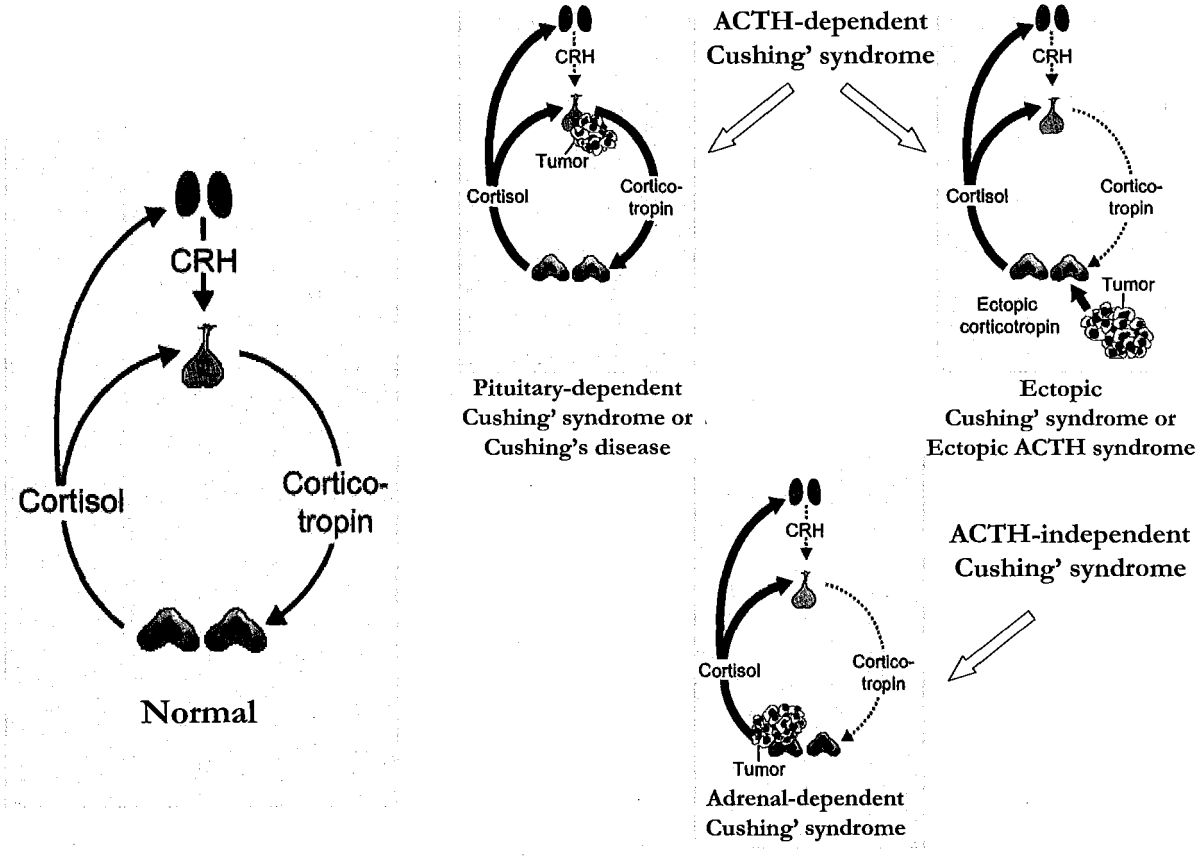


Figure 7: Schematic representation of the normal and pathological hypothalamus-pituitary-adrenal axis. The presence of an ACTH-secreting pituitary tumor induce Cushing's disease, the presence of an ACTH-secreting extra-pituitary tumor induce the ectopic ACTH syndrome and the presence of a cortisol-secreting adrenal tumor induce the adrenal-dependent Cushing's syndrome

patients with CD, whereas somatostatin analogues, in particular octreotide, have shown a certain effectiveness in normalizing cortisol levels in a subgroup of patients with EAS (74). The most potent dopamine agonist cabergoline has never been used in the treatment of CD. Moreover, the expression of dopamine receptors in ACTH-producing ectopic tumors, representing a prerequisite for the effectiveness of dopamine agonists, as well as for the effectiveness of cabergoline in the treatment of patients with EAS, has not been investigated so far.

The main aim of the thesis is to evaluate the effectiveness of cabergoline treatment in the ACTH-dependent forms of CS, namely CD and EAS, and to correlate the effectiveness of cabergoline treatment to the expression of dopamine receptors, in particular D_2 receptors, in the pituitary or ectopic tumors causing the diseases. This issues have been addressed in chapter III and chapter V of this thesis.

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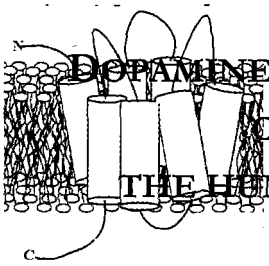

DOPAMINE D_2 RECEPTOR EXPRESSION IN THE CORTICOTROPH CELLS OF THE HUMAN NORMAL PITUITARY GLAND

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Submitted for publication

Abstract

The dopamine D_2 receptor is the main dopamine receptor expressed in the pituitary gland. In humans, it is commonly accepted that a functional D_2 receptor is mainly expressed in lactotroph cells of the anterior lobe and in melanotroph cells of the intermediate zone of the normal pituitary gland, where it mediates the tonic inhibitory control of hypothalamic dopamine on prolactin (PRL) and melanocyte-stimulating hormone (MSH) secretion, respectively. Although indirect evidence suggests D_2 receptor expression in non PRL and non MSH-secreting cells, including ACTH-secreting cells, this has never unequivocally been demonstrated. The aim of the current study was to evaluate dopamine D_2 receptor expression in the corticotroph cell populations by immunohistochemistry and to compare the D_2 receptor expression in the different corticotroph cell populations of the anterior lobe and intermediate zone of the human normal pituitary gland. Human normal pituitary glands obtained from routine autopsies were used for the study. In all cases, histology together with immunostaining for ACTH, MSH, PRL, and neurofilament as neural marker, were performed and compared to the immunostaining for D_2 receptor. D_2 receptor immunostaining was performed using a polyclonal and a monoclonal antibody. The results of the current study demonstrate that the D_2 receptor is heterogeneously expressed in the majority of the cell populations of the anterior lobe and relatively homogeneously expressed in the intermediate zone of the normal pituitary gland. The intermediate zone of the pituitary gland is recognized by the presence of the colloid-filled cysts and nerve fibers. The D_2 receptor was clearly expressed in the corticotroph cells of the anterior lobe, although usually with a weak intensity, as well as in the melanotroph cells of the intermediate zone, where the intensity of the expression seems to be higher than that registered in corticotroph cells of the anterior lobe, but still lower than that registered in lactotroph cells of the anterior lobe of the pituitary gland. The D_2 receptor was diffusely expressed in the posterior lobe of the pituitary gland as well, and intensely expressed in basophilic cells, which were part of "basophilic invasion of the neurohypophysis". The pattern of the immunostaining was comparable using both types of D_2 receptor antibodies. In conclusion, the results of this study demonstrate for the first time that the D_2 dopamine receptor is expressed in the majority of the cell populations of the human normal pituitary gland, and particularly, in the different corticotroph cell populations present in the anterior lobe, the intermediate zone or in the "basophilic invasion" of the neurohypophysis. These data support the findings of D_2 receptor expression in the majority of

corticotroph pituitary tumors and suggest that tumors derived from the intermediate zone and/or the basophilic invasion of the neurohypophysis might be those with the higher expression of functional D_2 receptors and, consequently, the most sensitive to dopaminergic drugs.

Introduction

Dopamine is the predominant catecholamine neurotransmitter in the human central nervous system and plays multiple roles in the periphery. It has a pivotal role in the hypothalamus-pituitary system, which represents an important connection between the central nervous system and the peripheral endocrine system (1). The various actions of dopamine are mediated *via* five specific receptors (D_1-D_5), which can be subdivided in two different receptor families on the basis of their biochemical and pharmacological characteristics: the D_1-like family, including D_1 and D_5, and the D_2-like family, including D_2, D_3 and D_4 receptors (2).

The dopamine D_2 receptor represents the main dopamine receptor expressed in the pituitary gland: it is expressed both in the anterior and the intermediate lobe of the pituitary gland, where it mediates the tonic inhibitory control of hypothalamic dopamine on prolactin (PRL) and melanocyte-stimulating hormone (MSH) secretion, respectively (3-6). Although these data have been mostly derived from animal studies, it is commonly accepted that a functional D_2 receptor is mainly expressed in the lactotroph cell population, belonging to the anterior lobe and the melanotroph cell populations, a peculiar group of corticotroph cell population forming the *pars intermedia* or intermediate zone of human normal pituitary gland. On the other hand, the D_2 receptor has recently been demonstrated to be expressed in more than 75% of the cells of human normal pituitary cells (7), indicating that it is not only expressed in lactotroph and melanotroph cells, which represent not more than 30% of the entire pituitary cell population (5). However, no study has systematically evaluated the co-localization of D_2 receptor with the different pituitary hormones in human normal pituitary gland. A variable and heterogeneous expression of D_2 receptors has been demonstrated either by *in situ* hybridization (ISH) or immunohistochemistry (IHC) in nearly 90% of all types of pituitary tumors, including PRL-, GH- and ACTH-secreting as well as clinically non functioning pituitary tumors (8). In addition, it has been recently demonstrated using different techniques that the D_2 receptor is expressed in around 75% of cases of ACTH-secreting pituitary tumors, in which it mediates an *in vitro* as well as *in vivo* inhibitory effect by dopaminergic drugs on ACTH secretion (9). However, until now D_2 receptor expression in normal corticotroph cells has never been unequivocally demonstrated.

The aim of the current study was to evaluate dopamine D_2 receptor expression in the corticotroph cell populations by IHC and to compare the D_2 receptor

expression in the different corticotroph cell populations of the anterior lobe and intermediate zone of the human normal pituitary gland.

Materials and Methods

Samples: Normal pituitary glands were obtained from routine autopsies of 7 different subjects. The samples were fixed in 10% paraformaldehyde overnight and subsequently embedded in paraffin for the immunohistochemistry study. The samples were selected on the basis of the recognition of all three areas of the pituitary gland, namely the anterior epithelial lobe, the posterior neural lobe and the intermediate zone, in the tissue sections. Histology of the normal pituitary tissues was performed on sequential sections in order to confirm the normality of the pituitary gland, and the presence of a clear-cut intermediate zone by the evidence of colloid-filled cysts between the anterior and posterior lobe of the glands.

Study protocol: The study protocol included: 1) the evaluation of D_2 receptor expression; 2) the evaluation of ACTH, MSH and PRL expression, in order to individuate the corticotrophs and to distinguish them from lactotroph and melanotroph cells and 3) the evaluation of the presence of nerve fibers, in order to clearly design the limitation between the anterior and posterior lobe and to characterize the intermediate zone of the normal pituitary gland and 4) the evaluation of the histology. The evaluation of hormone content, presence of nerve fibers and D_2 receptor expression in the tissue samples was performed by IHC. The IHC for D_2 receptor, hormone and nerve markers was performed on sequential sections, in order to visualize and compare the same cell clusters in all the tissue sections.

Immunohistochemistry: The IHC study was performed on tissue samples according to previous reports (9,10). D_2 receptor expression was evaluated by using two different antibodies: a rabbit anti-human polyclonal antibody (Chemicon International, Temecula, CA, USA) and a mouse anti-human monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, the formalin-fixed and paraffin-embedded tissue specimens were cut as 5 μm thick sections. The sections were deparaffinized, dehydrated, exposed to microwave heating (in citric acid buffer) at 100°C for 15 min, rinsed in tap water followed by phosphate buffer solution (PBS), and subsequently incubated for 15 min in normal goat serum (1:10

dilution in PBS + 5% bovine serum albumin, BSA). The sections were then incubated overnight at 4°C with the primary polyclonal antibody at a dilution of 1:500, or for 1 hour at room temperature with the primary monoclonal antibody at a dilution of 1:100, according to the manufacturer's recommendations. A standard streptavidin-biotinylated-peroxidase complex (ABC kit, Biogenix, San Ramon, CA, USA) was used to visualize the bound antibodies. Negative controls included: *a*) omission of the primary antibody for both the monoclonal and polyclonal antibodies; and *b*) preabsorption of the antibodies with the immunizing receptor peptide (at a concentration of 100 nM), for the polyclonal antibody. The immunostaining for D_2 receptor and the negative controls were performed on sequential sections of the tissue samples. The immunostaining for ACTH (Neomarkers, Duiven, The Netherlands), alpha-MSH (Chemicon International, Temecula, CA, USA), PRL (Dako, Glostrup, Denmark), as well as neurofilaments (Monosan, Uden, The Netherlands) was performed using specific antibodies at dilutions of 1:500, 1:500, 1:3000 and 1:300, respectively. Histological evaluation was performed on hematoxylin-eosin stained sequential sections. Positive and negative controls for D_2 receptor IHC included a dopamine agonist sensitive and resistant PRL-secreting pituitary tumor, respectively. The sensitivity of the D_2 receptor antibodies had been previously tested and the optimal antibody concentration was chosen to obtain the maximal specific signal with the minimal non-specific background The specificity of the polyclonal D_2 receptor antibody has been demonstrated previously by immunoblotting performed on glycoproteins isolated from a pituitary and an adrenal tumor (9,10).

Results

Fig. 1-5 represent exemplary histology (A) and IHC (B-F) of human normal pituitary tissues, where D_2 receptor is visualized using the monoclonal antibody. In particular, **Fig. 1-2** show the entire pituitary gland, including the anterior lobe, the intermediate zone and the posterior lobe, whereas **Fig. 3-4** focused on the intermediate zone, **Fig. 5** on the anterior lobe and **Fig. 6** on the posterior lobe of the pituitary gland. **Fig. 7** represents exemplary histology (A) and IHC (B-F) of human normal pituitary tissues, where D_2 receptor is visualized using the polyclonal antibody.

Histology: A completely normal histology without evidence of pathological features was confirmed in 5 of 7 samples (**Fig. 1A**) whereas an infiltration of

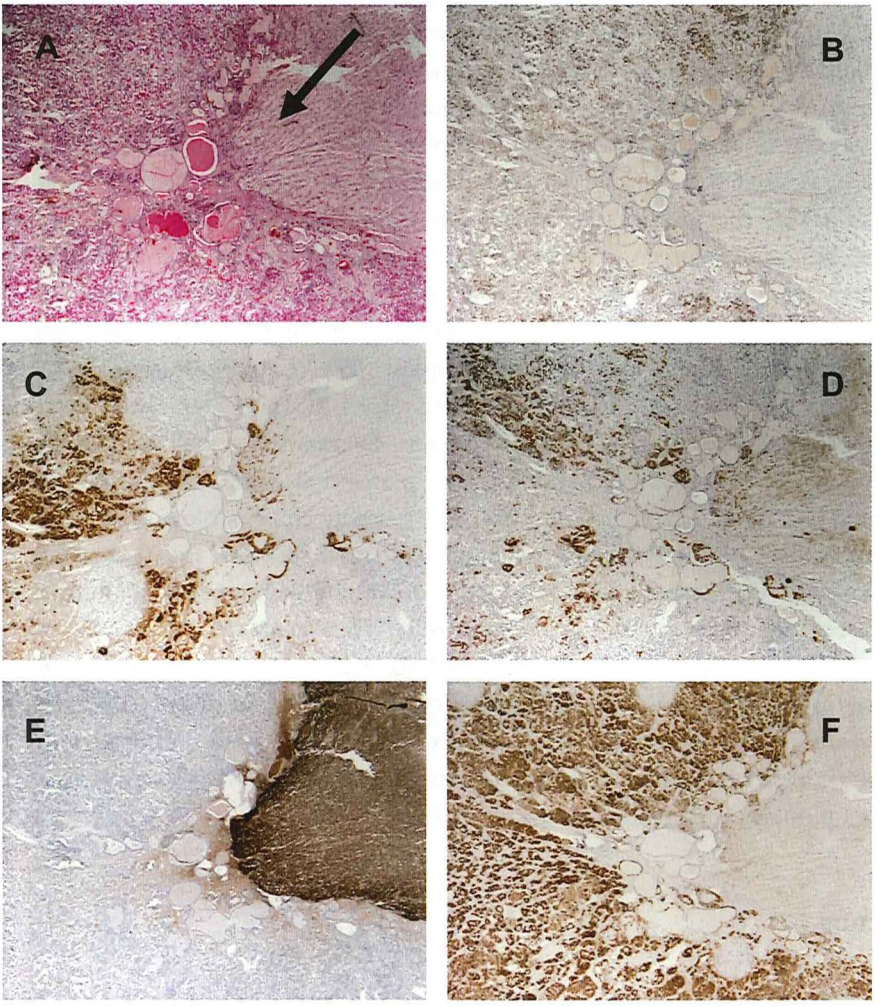

FIGURE 1: Example of IHC experiment. Histology (A), PRL (B), ACTH (C), MSH (D), Neurofilaments (E), and D_2 receptor (F) in a normal human pituitary gland. The picture gives an overview of the entire gland, including the anterior lobe (left), the intermediate zone (middle) and the posterior lobe (right). The intermediate zone is clearly recognized by the presence of the colloid-filled cysts (arrow) between the epithelial anterior lobe and the neural posterior lobe. The D_2 receptor immunostaining is performed by a monoclonal antibody. The picture shows a widespread expression for D_2 receptor throughout all three areas of the pituitary gland. Magnification is 20X.

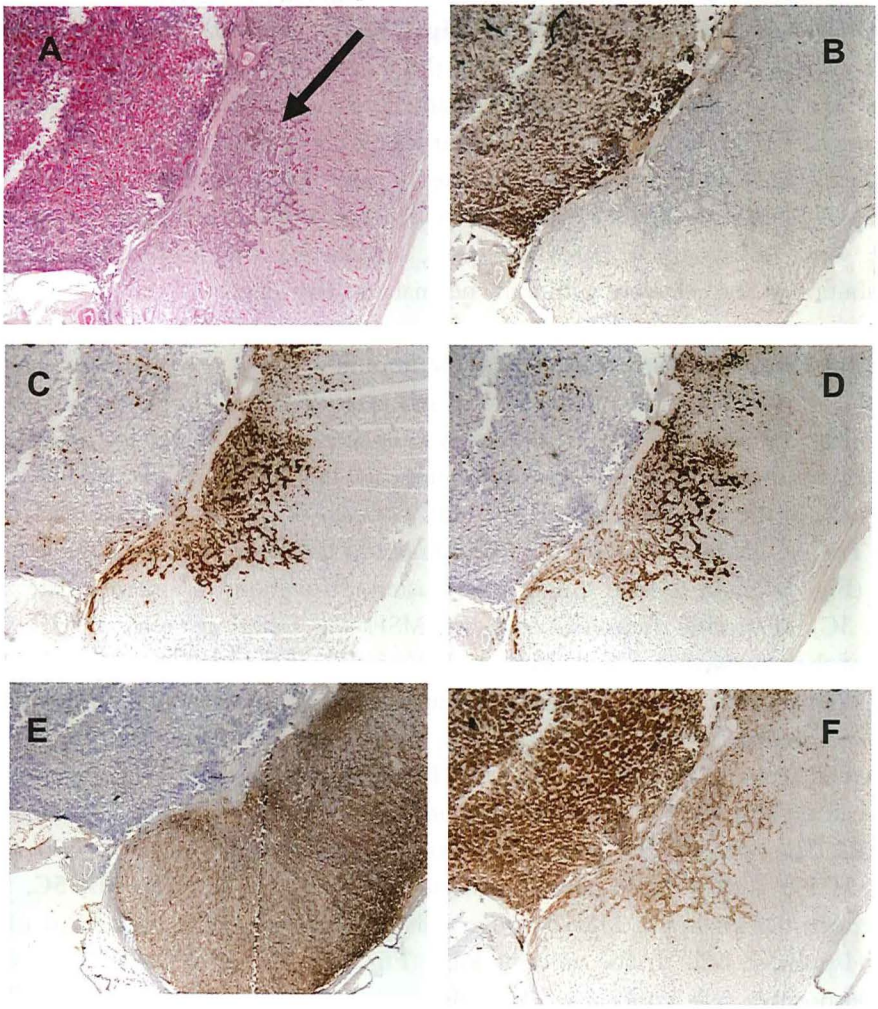

FIGURE 2: Example of IHC experiment. Histology (A), PRL (B), ACTH (C), MSH (D), Neurofilaments (E), and D_2 receptor (F) in a peculiar case of a normal human pituitary gland, containing a "basophilic invasion of the neurohypophysis" (arrow). The picture gives an overview of the entire gland, including the anterior lobe (left), the intermediate zone (middle) and the posterior lobe (right). The intermediate zone is partially replaced by the groups of the basophic cell cluster invading the posterior lobe. The D_2 receptor immunostaining is performed by a monoclonal antibody. The picture shows a widespread expression for D_2 receptor throughout all three areas of the pituitary gland. The arrow indicated the basophilic invasion of the neurohypophysis. Magnification is 20X.

basophilic cells within the neural lobe (**Fig. 2A**), the so-called "basophilic invasion of neurohypophysis", was found in 2 of the 7 cases of normal pituitary gland. All three areas of the pituitary gland, namely the epithelial anterior lobe, the intermediate zone and the neural posterior lobe were consistently represented in all samples. The epithelial cells of the anterior lobe were represented by 60-70% acidophilic cells and 30-40% basophilic cells, whereas the cells of the intermediate zone were nearly 100% basophilic cells, confirming the known physiological distribution of the cell types within the normal pituitary gland.

IHC for Pituitary Hormones: A specific immunostaining for PRL was found in 30-40% of the cells of the anterior lobe and in scattered cells of the intermediate zone (**Fig. 1B and 2B**). The PRL-positive cells were spread, scattered or clustered, throughout the entire lobe. A specific immunostaining for ACTH was found in 20-30% cells of the anterior lobe and in 70-90% of cells of the intermediate zone (**Fig. 1C**). The ACTH-positive cells were distributed in different clusters in the anterior lobe (**Fig. 5C**) and formed one or two clusters throughout the intermediate zone (**Fig. 3C**). A specific immunostaining for MSH was found in nearly 100% of the cells of the intermediate zone and in 20-30%of cells of the anterior lobe (**Fig. 1D**). The MSH-positive cells were distributed in clusters both in the anterior lobe (**Fig. 5D**) and the intermediate zone (**Fig. 3D**). At the comparison and matching of the sequential sections stained for PRL, ACTH and MSH, scattered PRL-positive cells co-localized with ACTH and/or MSH-positive cells in the intermediate zone (**Fig. 3B, 3C, 3D**), whereas the great amount of PRL-positive cells did not co-localized with ACTH and/or MSH-positive cells in the anterior lobe (**Fig. 5B, 5C, 5D**). Moreover, the totality of MSH-positive cells co-localized with a minor part of the ACTH-positive cells in the anterior lobe (**Fig. 5C, 5D**) whereas the totality of ACTH-positive cells co-localized with the great part of the MSH-positive cells of the intermediate zone (**Fig. 3C, 3D**). In the cases with the basophilic invasion of the neurohypophysis, a strong positivity for both ACTH and MSH was found in the cells invading the neural lobe of the pituitary gland (**Fig. 2C, 2D, 4C, 4D**). A comparable pattern of immunostaining for all the three different pituitary hormones was found in all 7 cases of normal pituitary glands.

IHC for nerve fibers: A specific immunostaining for neurofilaments was found both in the neural lobe and in the intermediate zone of the pituitary glands (**Fig, 1E, 2E**). The immunostaining displayed a punctuate pattern; it was strong and homogeneous throughout the entire neural lobe as well as the intermediate zone,

where it is localized around the clusters of ACTH and/or MSH-positive cells both in case of absence and presence of the basophilic invasion of neurohypophysis (**Fig. 3E, 4E**), whereas it was virtually absent in the anterior lobe of the pituitary glands (**Fig. 5E**). A comparable pattern of immunostaining for nerve fibers was found in all 7 cases of the pituitary glands.

IHC for D_2 receptor: A specific immunostaining for D_2 receptor was found in the great majority of the cells belonging to the anterior lobe, the intermediate zone, and in a consistent number of cells belonging to the posterior lobe of the pituitary gland, using either the polyclonal or the monoclonal antibody (**Fig. 1F, 2F, 7F**). Independently on the antibody, the immunostaining was heterogeneous, ranging from negative to strongly positive in the anterior lobe, and relatively homogeneous in the intermediate zone and posterior lobe, where it is generally weakly or moderately positive. At the comparison of the D_2 receptor with hormone immunostaining, both PRL-positive cells, mainly localized in the anterior lobe, and purely MSH-positive cells, mainly localized in the intermediate zone, were strongly positive for D_2 receptor (**Fig. 1, 2 and 7**). Conversely, a heterogeneous pattern of immunostaining for D_2 receptors was found in purely ACTH and ACTH/MSH-positive cells: those located in the anterior lobe displayed a relatively weak immunostaining (**Fig. 5F**), whereas those located in the intermediate zone displayed a weak or moderate immunostaining (**Fig. 3F**). Moreover, a strong immunostaining for D_2 receptor was found in the cells belonging to the basophilic invasion of the neurohypophisis (**Fig. 4F**). Both polyclonal and monoclonal antibodies demonstrated a comparable pattern of immunostaining for the D_2 receptor, among the different cases of normal pituitary glands.

Discussion

The human pituitary gland is anatomically composed of three different parts: two major lobes and an intermediate area or zone, also called "*pars intermedia*". The two pituitary lobes display a completely different histology, since the anterior lobe has an epithelial structure whereas the posterior lobe has a neural structure. The lobes contain different cell populations, which are the source of multiple hormones, and are differentially regulated, since hormone secretion is modulated *via* the vascular system in the anterior lobe and via the nervous system in the posterior lobe (11-13). The intermediate zone is a peculiar structure of the pituitary gland, which displays

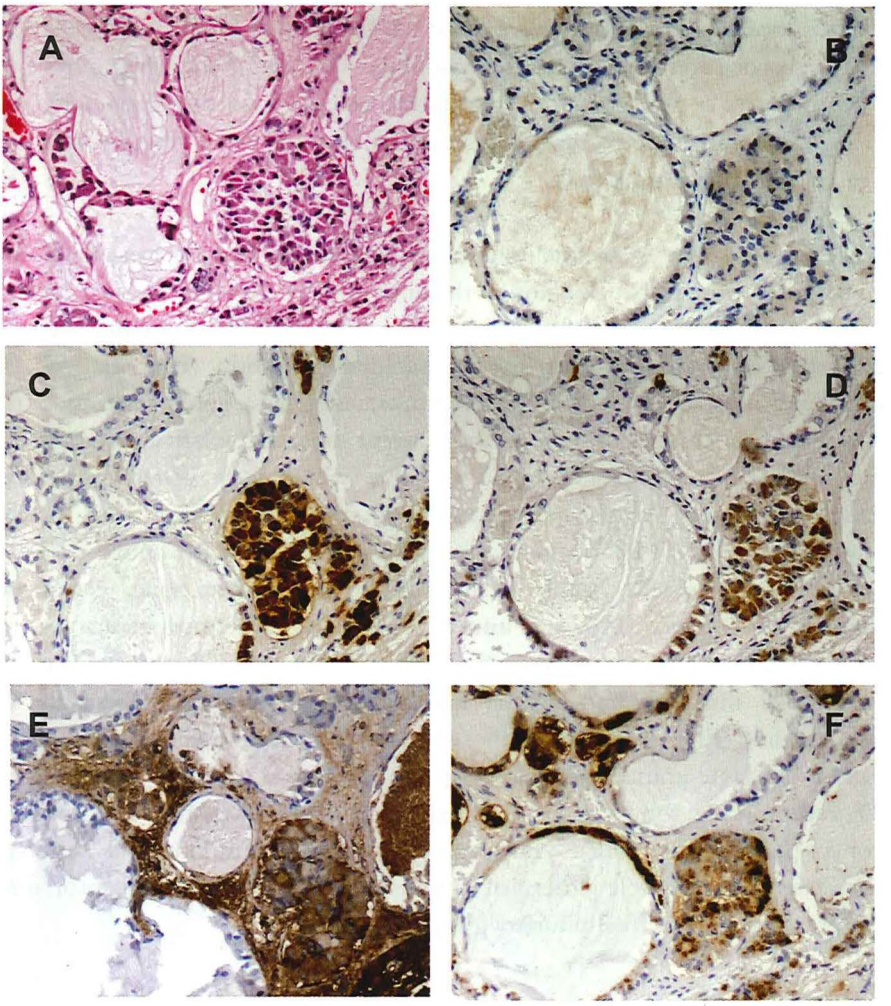

FIGURE 3: Example of IHC experiment. Histology (A), PRL (B), ACTH (C), MSH (D), Neurofilaments (E), and D_2 receptor (F) in a normal human pituitary gland. The picture focuses on the intermediate zone, clearly recognized by the presence of the colloid-filled cysts between the epithelial anterior lobe and the neural posterior lobe. The D_2 receptor immunostaining is performed by a monoclonal antibody. The picture shows a relatively diffuse expression of D_2 receptor in the melanotroph cells, where, however, cells with different intensity of D_2 expression can be recognized. Magnification is 200X

different characteristics among mammals: it is well defined in rats and poorly defined in humans (11-14). In humans it is better defined during fetal life, but undergoes a progressive involution to a group of colloid-filled cysts that mingle with the neural lobe in the adult life (14). These observations most likely explain why the majority of studies on this structure have been performed in rats rather than in humans. The intermediate zone contains one major endocrine cell population, the melanotroph cells, a subgroup of corticotroph cells which processes the precursor compound proopiomelanocortin (POMC) and mainly releases MSH: these cells usually stain for MSH and/or ACTH (15). The melanotroph cells differ from the corticotroph cells of the anterior lobe, which also process POMC but almost exclusively release ACTH: these cells generally stain for ACTH, but may also stain for MSH (16-18). An unusual feature of the intermediate zone is the relative lack of vascular supply, which remains at the periphery of the cell bodies, whereas innervations arise from the hypothalamic cell bodies, passes through the neural lobe and forms synaptic connections onto the endocrine cells (14,19,20). The most important innervations reaching the intermediate zone are represented by dopaminergic nerves (21,22). This evidence, together with the demonstration of dopamine D_2 receptor expression and a dopamine effect on growth and function in melanotroph cells supported the concept of a pivotal role of dopamine in the control of the intermediate zone of the pituitary gland by the hypothalamic dopaminergic nervous pathway (2,4,6,23,25).

Dopamine receptors have been unequivocally demonstrated in the anterior lobe of the pituitary gland. In rats, the D_2 receptor is expressed mainly in lactotroph cells, where it mediates the modulation of synthesis and secretion of PRL by hypothalamic dopamine (2,3,26), but it was found also in non-lactotroph cells, mainly somatotrophs and thyrotrophs, where dopamine seems to regulate GH and TSH secretion (27,28), and gonadotrophs, where dopamine has been proposed to have a regulatory role as well (26). Moreover, D_2 receptor expression in corticotroph cells has been suggested by the demonstration of its expression in AtT20 cells, a mouse pituitary corticotroph cell line (29,30). Similarly, in humans, the D_2 receptor is also expressed mainly in lactotroph cells, where it is involved in the modulation of PRL synthesis and secretion by hypothalamic dopamine (3). Moreover, it was found in more than 75% of cells, indicating its expression not only in lactotroph, but also in non-lactotroph cell populations of the pituitary gland (7). Most studies so far have evaluated D_2 receptor expression in human pituitary tumors. D_2 receptor expression has been clearly demonstrated in PRL-secreting pituitary tumors, where the presence of a considerable amount of the receptor explains the good therapeutic

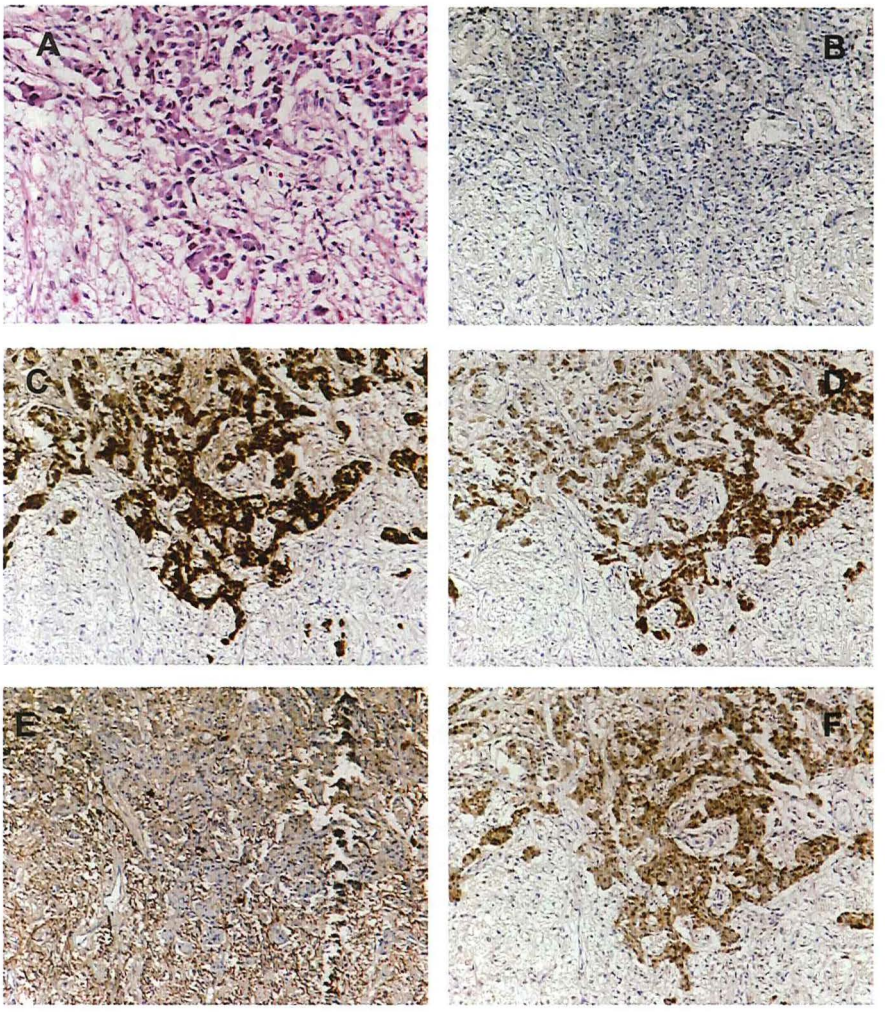

FIGURE 4: Example of IHC experiment. Histology (A), PRL (B), ACTH (C), MSH (D), Neurofilaments (E), and D_2 receptor (F) in a peculiar case of a normal human pituitary gland. The picture focuses on the intermediate area between the anterior and the posterior lobe containing the basophilic invasion of the neurohypophysis. The D_2 receptor immunostaining is performed by a monoclonal antibody. The picture shows a strong homogeneous and diffuse expression of D_2 receptor in the corticotroph cell population invading the neurohypophysis. Magnification is 200X.

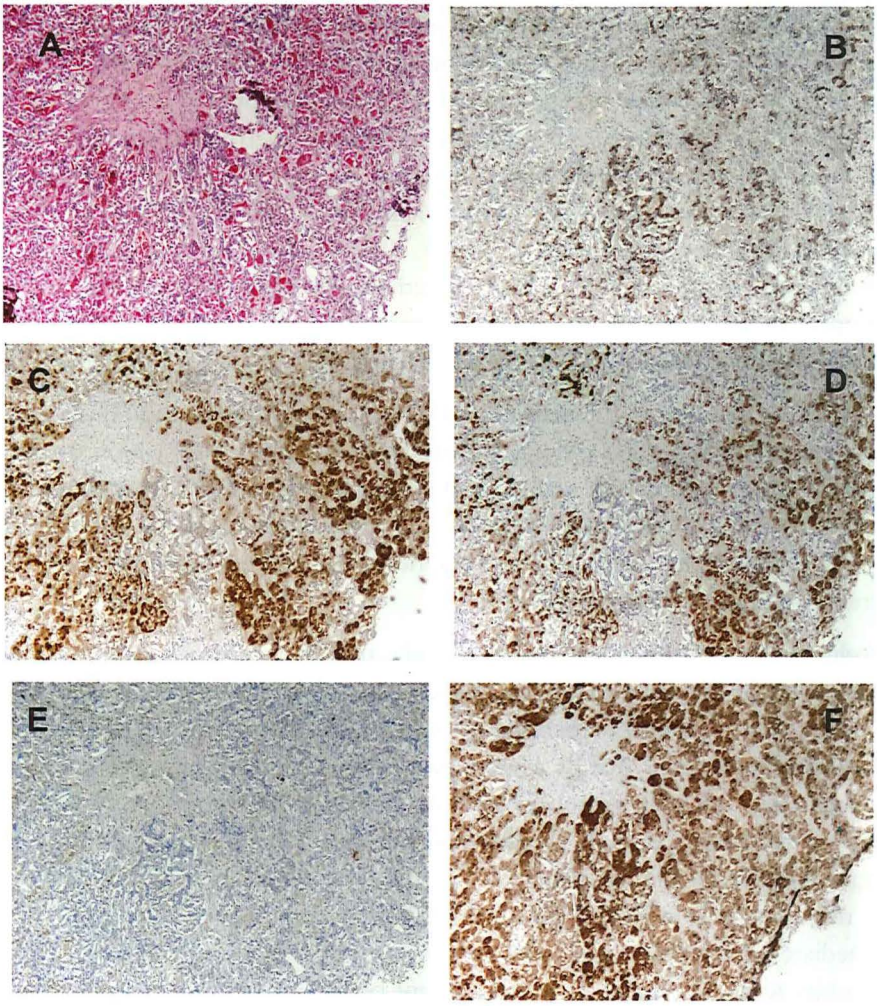

FIGURE 5: Example of IHC experiment. Histology (A), PRL (B), ACTH (C), MSH (D), Neurofilaments (E), and D_2 receptor (F) in a normal human pituitary gland. The picture focuses on a part of the anterior lobe, with significant presence of corticotroph cells. The D_2 receptor immunostaining is performed by a monoclonal antibody. The picture shows a weak and relatively homogeneous expression of D_2 receptor in the corticotroph cells, where, however, different cells with different intensity of D_2 expression can be recognized. Magnification is 100X.

effect of dopamine agonists, inducing inhibition of PRL secretion and tumor shrinkage (31,32). In non PRL-secreting pituitary tumors, like GH-secreting (33,34), clinically non functioning (35), and ACTH-secreting pituitary tumors (9), D_2 receptors also are a prerequisite for the effectiveness of treatment with dopamine agonists. In addition, a variable and heterogeneous expression of D_2 receptors has been recently demonstrated both by ISH and IHC in nearly 90% of all types of pituitary tumors and particularly in nearly 70% of silent or functioning ACTH-secreting pituitary tumors (8). Taken together, all these observations suggest a widespread expression and a predominant role of the D_2 receptor in different cell populations of the anterior lobe of the pituitary gland.

The current study evaluated whether the D_2 receptor is expressed in non-lactotroph and non-melanotroph cells, and particularly, in corticotroph cells within the human normal pituitary gland, as well as whether the two main corticotroph cell populations, the melanotrophs located in the intermediate zone on the one hand, and the corticotrophs located in the anterior lobe on the other hand, display a differential pattern of D_2 expression.

According to the results of the current study, the D_2 receptor is expressed in the great majority of the cell populations of the anterior lobe, as well as in the totality of the cell clusters forming the intermediate zone of the human normal pituitary gland. These data demonstrate that 1) a great part of the cell population of the anterior lobe, lactotroph as well as non-lactotroph, expresses D_2 receptors, although the amount of receptor is extremely variable within the different cell populations, being strongly positive mainly in lactotroph cells, and moderately or weakly positive or even negative in non-lactotroph cells; 2) the melanotroph cell clusters of the intermediate zone clearly express D_2 receptors, although, even in this area different cell groups seem to express different amount of the receptor, despite the fact that they are represented by a relatively homogeneous type of cells.

Beyond the widespread expression of the D_2 receptor throughout the gland, an important conclusion of this study is that the corticotroph cell populations of the normal human pituitary gland do express D_2 receptors. This suggests that dopamine may play a regulatory role in the function of corticotroph cells, although the lower amount of D_2 receptor expressed in corticotrophs, compared with lactotrophs, suggests that in physiological conditions dopamine plays a minor role in the control of the former compared with the latter cell population. The second conclusion is that the D_2 receptor is expressed in higher amount in melanotroph cells of the intermediate zone than in the corticotroph cells of the anterior lobe, suggesting a

more relevant physiological role of the receptor in the former than in the latter cell population. The third conclusion of this study is that the melanotroph cells of the intermediate zone display a variable expression of D_2 receptors. The observation of a group of cells expressing a lower, and another group of cells expressing a higher amount of D_2 receptor, is suggestive for the existence of two or more cell populations of melanotrophs with different features, behavior, regulation and role, and possibly dopamine control, in the intermediate zone of the human normal pituitary gland. The physiological role of dopamine in the control of corticotroph cell populations of the pituitary gland has been extensively investigated in rats, but much less in humans. Dopamine is assumed to inhibit hormone secretion from the melanotroph cells in the human and rat pituitary gland, although this role is likely to be more relevant in rats where the intermediate zone is much better defined, compared with humans where the intermediate zone is only a rudimental structure. Nonetheless, the role of dopamine in inhibiting hormone secretion from melanotroph cells, at least in a specific period of life or in specific physiological conditions in humans, is supported by the increase in MSH and endorphin levels after blockade of dopamine control demonstrated in infancy and pregnancy (36,37). Conversely, the physiological role of dopamine on corticotroph cells of the anterior lobe has been poorly investigated both in rats and humans. It is known in rats and suggested in humans that the regulation of POMC synthesis is differentially controlled in the melanotroph cells of the intermediate zone and the corticotroph cells of the anterior lobe. In fact, although in both cell types POMC synthesis is ensured by the hypothalamic corticotrophin releasing factor, the negative control upon POMC expression is regulated by hypothalamic dopamine in melanotrophs and by peripheral glucocorticoids in corticotrophs (38,39). However, dopamine agonists have been reported to enhance-, and dopamine antagonists to blunt the release of ACTH or ACTH-related compounds from anterior lobe corticotroph cells in rats (40,41) whereas controversial data were reported in humans (42,43). On the other hand, dopaminergic drugs have been reported to inhibit ACTH secretion from tumoral corticotroph cells in experimental studies in humans (44,45).

The potential role of dopamine in the control of corticotroph cells has been reported in the clinical trials with dopamine agonists in patients with Cushing's disease, bearing a corticotroph pituitary tumor. Previously, bromocriptine was found to inhibit ACTH and cortisol secretion in a minority of patients after short-term and even less after long-term treatment (46,47). Recently, cabergoline was demonstrated to give better results than bromocriptine in the treatment of Cushing's disease (9). On the other hand, D_2 receptors have been recently reported to be expressed in as

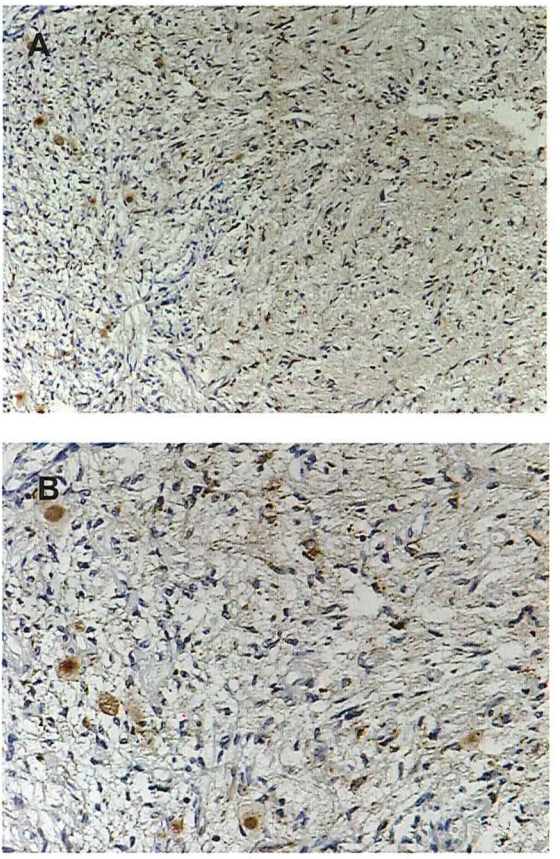

FIGURE 6: Example of IHC experiment. D_2 receptor in a case of autoptic normal human pituitary gland. The picture focuses on the posterior lobe. The D_2 receptor immunostaining is performed by a monoclonal antibody. The picture shows a homogeneous and scattered expression of D_2 receptor in the neural cells and fibers of the lobe. Magnification is 100X (A) and 200X (B).

many as 75% of corticotroph pituitary tumors (9). These observations suggest that the D_2 receptors or functional D_2 receptors are expressed only in a group of corticotroph pituitary tumors. It was hypothesized that corticotroph tumors expressing D_2 receptor and/or responsive to dopamine agonists, were derived from

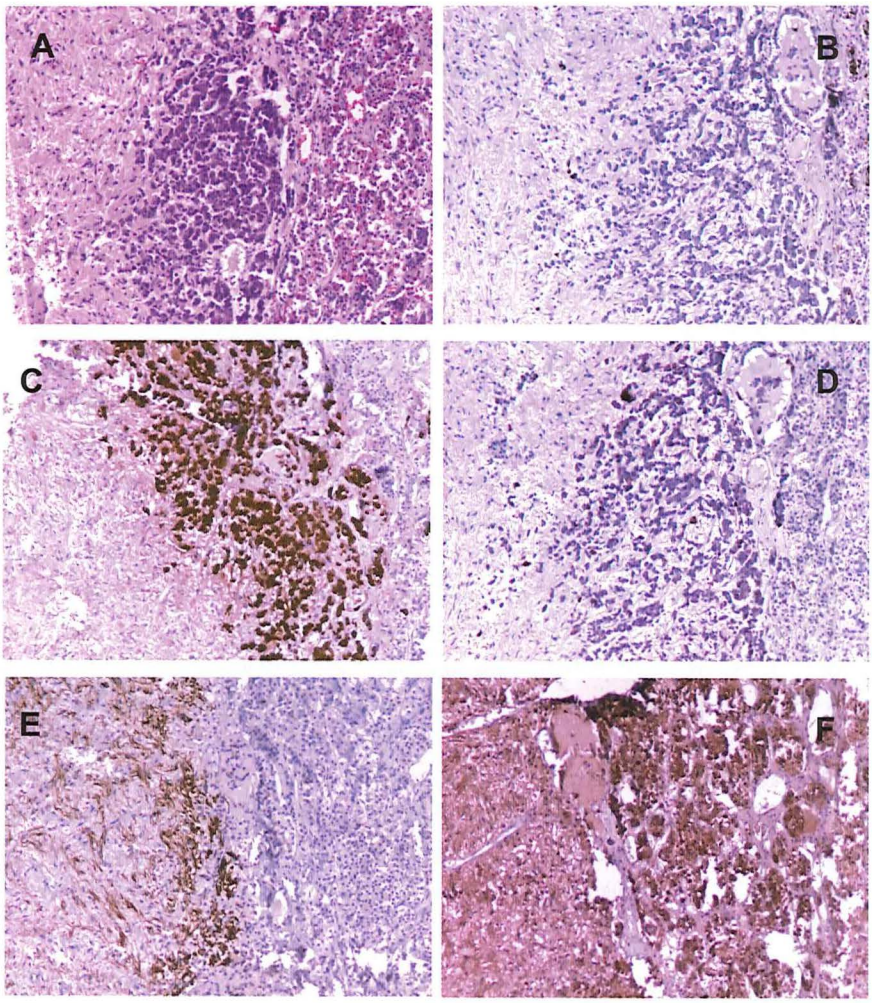

FIGURE 7: Example of IHC experiment. Histology (A), PRL (B), ACTH (C), MSH (D), Neurofilaments (E) and D_2 receptor (F) in a normal human pituitary gland. The picture gives an overview of the entire gland, including the anterior lobe (right), the intermediate zone (middle) and the posterior lobe (left). The D_2 receptor immunostaining is performed by a polyclonal antibody. The picture shows a widespread expression for D_2 receptor throughout all three areas of the pituitary gland. Magnification is 100X.

the intermediate zone of the pituitary gland, where a functional D_2 receptor is known to be significantly expressed, and that dopamine depletion could play a role in the development of such tumors (48,49). This hypothesis seems to be supported by animal models like D_2 receptor knock-out mice, which develop Cushing's disease associated with a hyperplasia of the intermediate lobe of the pituitary gland, where melanotroph cells loose their cell identity and start to produce and release not only MSH but also ACTH (50). On the other hand, in humans, despite the description of cases of patients with Cushing's disease bearing a corticotroph tumor strongly staining for MSH and responsive to treatment with dopamine agonists (51), the finding of MSH staining in the majority of corticotroph tumors makes MSH not an accurate marker of tumor origin from the intermediate zone of the pituitary gland (52). Alternatively, the responsiveness of corticotroph tumors to dopaminergic drugs can be dependent on the expression of a significant amount of functional D_2 receptors on tumoral cells independently of the corticotroph tumor originating from the anterior lobe or intermediate zone. The results of the current study may support both hypotheses, although the higher amount of D_2 receptor on melanotroph cells of the intermediate zone compared with corticotroph cells of the anterior lobe suggests that the most responsive tumors may derive from the intermediate zone, whereas corticotroph tumors less responsive or unresponsive to dopaminergic drugs may derive from the anterior lobe of the pituitary gland. Moreover, it has to be taken into account that dopamine D_4 receptors also have been demonstrated in the rat and human normal pituitary gland (53,54), as well as in human corticotroph pituitary tumors (10). Therefore, the possible influence of the D_4 receptors on the responsiveness of the tumors to the dopaminergic drugs cannot be completely ruled out.

Finally, the results of the current study demonstrate that D_2 receptors are diffusely expressed in the posterior lobe, being localized in the body and/or terminal axons of neurons forming the neural portion of the pituitary gland. These data confirmed the previous reports describing the presence of D_2 receptors in the posterior lobe of the pituitary gland in animal models and the hypothesis that dopamine may also play a role in the regulation of the neurohypophyseal hormone secretion or in the neural pathways reaching and controlling the adenohypophyisis (55,56). A fascinating finding of the current study is the high levels of D_2 receptor expression in the cells, which are part of a basophilic invasion of the neurohypophysis. This represent an enigmatic phenomenon, commonly detected in human normal pituitary glands (57). It consists of an extension of ACTH/MSH-immunoreactive cells from the intermediate zone into the posterior lobe and it seems to occur as early as fetal life,

increasing with age in intensity and frequency (57). It has been observed that these corticotroph cells invading the neurohypophysis show the presence of occasional cellular atypia and mitotic activity, suggesting a role in the development of tumors located in the posterior lobe of the pituitary gland (57). Moreover, it has been recently found that the corticotrophs in this invasion are phenotypically different from those located in the anterior or even intermediate zone of the human normal pituitary gland and that they are actively proliferating, suggesting that they may derive from the migration of corticotrophs of the intermediate zone into the posterior lobe and/or from the proliferation of at least a cell cluster of the corticotrophs of the intermediate zone, which might induce the development of tumors of the posterior lobe of the pituitary gland (58). The results of the current study confirmed that 1) the basophilic invasion of neurohypophysis is a relatively common phenomenon in human normal pituitary gland, as it was found in around 30% of cases; 2) it is formed by ACTH- and MSH-immunoreactive cells, probably belonging to the melanotrophs of the intermediate zone; and 3) it is formed by cells displaying a significant immunostaining for the D_2 receptor, comparable to that of some groups of normal melanotroph cells. These observations suggest that a peculiar cluster of melanotrophs with an increased neoplastic potential may be responsible of this phenomenon and might also be the origin of the corticotroph pituitary tumors, which are the most responsive to dopaminergic drugs.

In conclusion, this is the first study clearly demonstrating that the dopamine D_2 receptor is significantly expressed in the majority of cell populations of all three parts of the normal pituitary gland, and particularly in the different populations of corticotroph cells in humans. These data strongly suggest that dopamine plays a pivotal role in the control of the complete pituitary gland, including the corticotroph cell populations of both the anterior lobe and the intermediate zone of the gland. This finding further supports the observations of functional D_2 receptor expression in corticotroph pituitary tumors.

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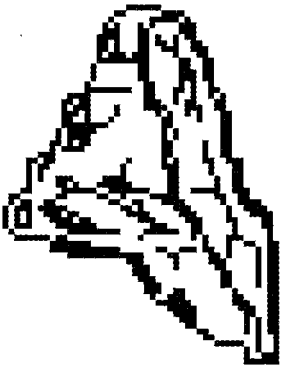
III

DOPAMINE RECEPTOR EXPRESSION AND DOPAMINE AGONIST EFFECTIVENESS IN CORTICOTROPH PITUITARY TUMORS



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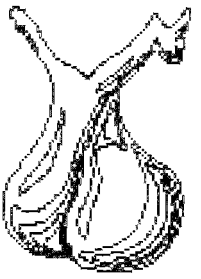

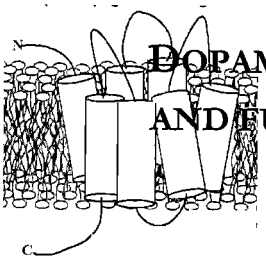

DOPAMINE RECEPTOR EXPRESSION AND FUNCTION IN CORTICOTROPH PITUITARY TUMORS

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Journal of Clinical Endocrinology and Metabolism 2004;89:2452-2462

ABSTRACT

The role of dopamine agonists treatment in corticotroph pituitary tumors is controversial. The aim of this study was to evaluate D_2 receptor expression in 20 corticotroph pituitary tumors and to correlate it to the *in vitro* effect of dopamine agonists on ACTH secretion and the *in vivo* effect of short-term cabergoline treatment on cortisol secretion. D_2 expression was evaluated by receptor-ligand binding, immunohistochemistry, and RT-PCR. A 50% or more decrease in daily urinary cortisol levels was considered as significant clinical response. At receptor-ligand binding, a specific binding of ^{125}I-epidepride was found in 80% of cases. At immunohistochemistry, a specific D_2 immunostaining was found in 75% of cases. D_2 expression was found in 83.3% of cases (D_{2long} in 40%, D_{2short} in 20% and both in 40%) by RT-PCR. Significant *in vitro* inhibition of ACTH secretion was found in 100% of D_2 positive cases and not in 100% of D_2 negative cases by either bromocriptine or cabergoline. A significant *in vivo* inhibition of cortisol secretion after 3-month cabergoline treatment was found in 60%, although a normalization of cortisol secretion was found in 40% of cases. All cabergoline responsive cases were associated with D_2 expression, whereas all noncabergoline-responsive cases but one were not associated with D_2 expression. In conclusion, functional D_2 receptors were expressed in around 80% of corticotroph pituitary tumors. The effectiveness of cabergoline in normalizing cortisol secretion in 40% of cases supports its therapeutical use in the management of Cushing's disease.

Introduction

Dopamine is the predominant catecholamine neurotransmitter in the human central nervous system, where it controls a variety of functions including cognition, emotion, locomotor activity and regulation of the endocrine system (1). Dopamine also plays multiple roles in the periphery as a modulator of cardiovascular and renal function, gastrointestinal motility, and hormone synthesis and secretion (1). The various actions of dopamine are mediated by five specific receptors (D_1-D_5), which can be subdivided into two different receptor families on the basis of their biochemical and pharmacological characteristics: D_1-like, including D_1 and D_5, and D_2-like, including D_2, D_3, and D_4 receptor (1). The different dopamine receptor (DR) subtypes have a different distribution and play different roles in the various organs and tissues (1). D_2 receptor is expressed in the anterior and intermediate lobes of the pituitary gland (2,3), where it mediates the tonic inhibitory control of hypothalamic dopamine on prolactin (PRL) and melanocyte-stimulating hormone (MSH) secretion, respectively (4-6). The presence of a functional D_2 receptor on tumoral PRL-secreting cells (7-10) led to a major therapeutic application in the treatment of PRL-secreting pituitary tumors. Indeed, medical therapy with dopamine agonists represents the first choice treatment of these type of pituitary tumors, being effective in suppressing PRL secretion and inducing tumor shrinkage (11-13). Moreover, among the different dopamine agonists, the more recently developed drug cabergoline has been demonstrated to be more effective than the most widely used drug bromocriptine in the treatment of PRL-secreting pituitary tumors (14,15). Treatment with bromocriptine has been also investigated in ACTH-secreting or corticotroph pituitary tumors, although with controversial results (12,16). However, no study has ever evaluated DR expression and the effect of cabergoline treatment in controlling the ACTH and cortisol hypersecretion associated to corticotroph pituitary tumors.

The current study was designed with a twofold purpose: 1) to evaluate DR expression in corticotroph pituitary tumors by different techniques, namely receptor-ligand binding (R-LB), immunohistochemistry (IHC) and RT-PCR; and 2) to correlate DR expression to the *in vitro* effect of dopamine agonists on ACTH secretion in cultured tumoral corticotroph pituitary cells and to the *in vivo* effect of cabergoline treatment on ACTH and cortisol secretion in patients with Cushing's disease (CD), characterized by excessive endogenous ACTH and cortisol secretion induced by a corticotroph pituitary tumor (17).

Patients and Methods

Patients: Twenty patients (16 women and 4 men, 25-60 years of age) with a diagnosis of CD entered the study after their informed consent had been obtained. Ten patients were admitted to the Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands (Group 1), and 10 were admitted to the Department of Molecular and Clinical Endocrinology and Oncology, "Federico II" University, Naples, Italy (Group 2) over a period of two years. The patients of *Group 1* were retrospectively selected on the basis of the availability of a tumor specimen collected and frozen at the time of neurosurgery; the patients of *Group 2* were selected on the basis of the presence of persistent or recurrent disease after neurosurgery for the inclusion in a prospective study. The diagnosis of CD was based on: 1) increase in daily urinary cortisol excretion with inappropriately high plasma ACTH concentrations; 2) increase in basal serum cortisol concentrations with lack of the physiological circadian rhythm; 3) failure of urinary and serum cortisol suppression after low dose, but a decrease in urinary cortisol greater than 50% after high dose oral dexamethasone suppression test or a decrease in serum cortisol of at least 7 μg/dl in the 7-hours continuous intravenous dexamethasone suppression test (18-20). The diagnosis of CD was supported by the evidence of a pituitary tumor at magnetic resonance imaging of pituitary gland or at the bilateral inferior petrosal sinus sampling (20,21). Indeed, five of the 20 patients had pituitary macroadenoma, 10 had microadenoma, and the remaining five patients had normal pituitaries; the pituitary source of ACTH hypersecretion in these five patients was confirmed by bilateral inferior petrosal sinus sampling. All patients were subjected to neurosurgical operation by the transsphenoidal approach for the removal of the pituitary tumor. The histological and immunohistochemical study of the tumor removed by neurosurgery documented a corticotroph pituitary lesion (adenoma or hyperplasia) in all cases, definitely confirming the diagnosis of CD in the totality of patients. After neurosurgery, clinical, hormonal and radiological remission of CD was documented in 12 patients whereas disease persisted in the remaining eight patients; moreover, a disease recurrence occurred in six of the 12 remitted patients of the study 1-3 years after neurosurgery. The patients' profile IS shown in **Table 1**.

Samples: Pituitary tumor specimens were obtained at the time of tumor excision by neurosurgery. Samples of these specimens were taken fresh directly at the operation. They were fixed in 10% paraformaldehyde overnight and embedded in paraffin for the IHC study and/or quickly frozen on dry ice and stored in a freezer at -80°C for

Table 1: Patients' clinical profile at the diagnosis of Cushing's disease

Patient (sex/age)	Plasma ACTH (pg/ml)	0800 Serum Cortisol (µg/dL)	1600 Serum Cortisol (µg/dL)	Daily Urinary Cortisol (µg/day)	Serum prolactin (µg/L)	DMX test results*	Radiological findings	Surgical findings	Histological findings	Treatment outcome
Group 1										
1. m/60	90.5	17.7	14.2	352.3	4.3	14.0	no tumor	diffuse enlargement	basophilic hyperplasia	disease persistence
2. f/55	101.6	18.9	20.8	362.4	4.4	16.0	microadenoma	microadenoma	basophilic adenoma	cure
3. f/60	247.5	24.3	25.4	622.9	6.8	14.1	macroadenoma	macroadenoma	chromofobe adenoma	disease persistence
4. f/33	70.4	25.3	18.9	707.9	4.9	19.8	microadenoma	microadenoma	chromofobe adenoma	cure
5. f/33	16.0	25.1	15.3	483.9	4.8	12.4	microadenoma	microadenoma	basophilic adenoma	cure
6. m/27	55.1	26.4	26.3	1481.0	6.6	13.9	microadenoma	microadenoma	basophilic adenoma	cure
7. f/56	120.4	14.5	16.2	413.6	5.9	22.9	macroadenoma	macroadenoma	basophilic adenoma	cure
8. f/35	30.1	31.6	29.1	849.9	5.8	19.6	no tumor	diffuse enlargement	basophilic hyperplasia	disease persistence
9. f/38	49.0	17.4	11.6	458.4	0.2	8.0	microadenoma	microadenoma	basophilic adenoma	cure
10. f/27	49.8	30.7	32.5	1044.2	9.3	20.0	no tumor	microadenoma	basophilic adenoma	disease persistence
Group 2										
11. f/49	70.0	20.2	20.0	563.0	22.4	124	microadenoma	microadenoma	basophilic adenoma	cure/disease recurrence
12. f/48	12.6	18.9	19.8	157.9	7.1	88.9	macroadenoma	macroadenoma	chromofobe adenoma	cure/disease recurrence
13. f/25	53.0	35.4	28.9	717.7	13.7	444.9	microadenoma	microadenoma	basophilic adenoma	disease persistence
14. f/35	54.9	24.1	25.5	400.0	9.2	233.5	microadenoma	microadenoma	acidophilic adenoma	disease persistence
15. m/31	11.0	32.1	20.1	314.5	7.2	99.9	no tumor	microadenoma	basophilic adenoma	disease persistence
16. f/42	82.0	26.8	10.7	254.0	30.1	72.8	no tumor	diffuse enlargement	basophilic hyperplasia	disease persistence
17. m/45	58.9	39.8	27.9	555.9	6.7	245.9	microadenoma	microadenoma	basophilic adenoma	cure/disease recurrence
18. f/43	107.0	31.9	30.0	431.9	38.9	213.9	macroadenoma	macroadenoma	chromofobe adenoma	cure/disease recurrence
19. f/47	89.5	36.0	32.7	316.7	13.0	134.6	microadenoma	microadenoma	basophilic adenoma	cure/disease recurrence
20. f/26	56.9	36.5	34.5	800.0	15.0	560.9	macroadenoma	macroadenoma	basophilic adenoma	cure/disease recurrence

*Dexamethasone test= *group 1*: Change in serum cortisol between and after high dose iv DMX (µg/dL); *group 2*: urinary cortisol levels after high dose oral DXM (µg/day)

R-LB and/or RT-PCR study. In selected cases, a sample was also used for the establishment of a pituitary tumor primary culture. The study included only samples in which the tumoral tissue represented at least 90% of the section at the histological evaluation, to exclude the influence of normal pituitary tissue on the results of the studies.

Study design: The study protocol was diversified in the two different groups of patients. In Group 1, D_2 receptor expression was evaluated by R-LB and IHC studies. DR subtypes expression was evaluated by RT-PCR in five of 10 cases. In Group 2, D_2 receptor expression evaluated by IHC was correlated to the *in vitro* effect of dopamine agonists (bromocriptine and cabergoline) on ACTH secretion and to the *in vivo* effect of 3-month cabergoline treatment on cortisol secretion. DR subtypes expression was evaluated by RT-PCR in seven of 10 cases and also correlated to the results of the *in vitro* and *in vivo* functional studies. The protocol was in accordance with the Helsinki Doctrine on Human Experimentation, and it was approved by the local Ethical Committees.

Receptor-ligand binding: The R-LB study was performed on tissue samples according to a previous report (22). The frozen tissue samples were cut in 10-μm thick sections. These sections were mounted onto pre-cleaned gelatine-coated microscope glass slides and stored in a freezer at -80°C for at least 3 days before the experiment, in order to improve the adhesion of the tissue to the slide. The D_2 analog ^{125}I-epidepride (Radiopharmaka, Seibersdorf, Austria) was used as radioligand. The sections were pre-incubated at room temperature for 10 min in binding buffer [50 mM Tris-HCl (pH 7.7), 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ $2H_2O$, 1 mM $MgCl_2$ $6H_2O$, 0.1% ascorbic acid]. Thereafter, they were incubated for 60 min at room temperature in binding buffer with ^{125}I-epidepride. Non-specific binding was determined in a sequential section in the presence of excess unlabeled cabergoline (1 μM). A sample was considered positive for ^{125}I-epidepride binding when the signal was displaced by more than 50%. The incubated sections were washed twice for 5 min each time in binding buffer. After a short wash in distilled water to remove salts, the sections were air dried and exposed to Biomax film (Eastman Kodak, Rochester, NY, USA) or HyperFilm-^{3}H (Amersham, Houten, The Netherlands) for 7-15 and 30-60 days respectively in X-ray cassettes. Histological evaluation was performed on hematoxylin-eosin stained sequential cryostat sections. In addition, a positive control, represented by a sample of rat brain cut at the level of the basal ganglia and negative controls, represented by rat tissues known to have

no expression of DRs, were used in any experiment. The binding signals obtained were analyzed densitometrically using a computer-assisted image processing system and were quantified by calculating the ratios between the regions of interest delineated on the total (T) and non-specific (NS) binding sections. Using the T/NS ratios, the amount of binding in every section was graded as negative (-), for T/NS ranging from 0-1.9, moderately positive (+), for T/NS ranging from 2-3, and strongly positive (++) for T/NS greater than 3.

Immunohistochemistry: The IHC study was performed on tissue samples according to a previous report (23). The formalin-fixed and paraffin-embedded tissue samples were cut in 5-μm-thick sections. These sections were deparaffinized, dehydrated, exposed to microwave heating in citric acid buffer at 100°C for 15 min, rinsed in tap water followed by phosphate buffer solution (PBS) and subsequently incubated for 15 min in normal goat serum (1:10 dilution in PBS + 5% bovine serum albumin, BSA). The sections were then incubated overnight at 4°C with a rabbit anti-human D_2 receptor polyclonal antibody (Chemicon International, Temecula, CA, USA) in a dilution of 1:500. A standard streptavidin-biotinylated-alkaline phosphatase or -peroxidase complex (ABC kit, Biogenix, San Ramon, CA) was used to visualize the bound antibodies. Negative controls for the IHC included: *a*) omission of the primary antibody; and *b*) preabsorption of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 nM), both performed in sequential sections. Immunostaining for ACTH and PRL as well as GH, TSH, FSH and LH was also performed on sequential sections using specific antibodies at the standard dilution. Histology evaluation was performed on hematoxylin-eosin stained sequential sections. Positive and negative controls were represented by D_2 receptor immunostaining on sections of dopamine agonist-sensitive and -resistant, PRL-secreting pituitary tumors, respectively and were carried out in the same experiments of the corticotroph pituitary tumors. The specificity of the D_2 receptor antibody was tested by immunoblotting using a corticotroph pituitary tumor sample.

Immunoblotting: The immunoblotting was performed on tissue samples according to a previous report (24). Membranes were extracted from a frozen tissue sample. The tissue sample was suspended in an ice-cold Tris-buffer (10 mM Tris-HCl pH 7.6, 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml soybean-trypsin-inhibitor, 50 μg/ml bacitracin), homogenized with a Polytron homogenizer at 900 rpm for 10 strokes, and then ultracentrifuged

for 1 h at 4°C at 100000 x g. Membrane pellet was solubilized in a lysis buffer (20 mM HEPES, pH=7.4, 5 mM EDTA, 3 mM EGTA, 150 mM NaCl, 4 mg/ml dodecyl-B-D-maltoside) for 1h at 4°C and then ultracentrifuged at 100000 x g for 1 h at 4°C. Glycosylated proteins were purified from membrane pellet obtained after high-speed centrifugation, by wheat germ agglutinin (WGA) affinity chromatography: the pellet was re-suspended in lysis buffer and cycled twice over a 0.5-mL WGA (Vector Laboratories Inc., Burlingame, CA, USA) column equilibrated with lysis buffer. The column was washed and eluted with lysis buffer containing 3 mM N,N',N"triacetyl-chitotriose (Sigma Chemical Co., St. Louis, MO, USA). The protein-containing fractions was determined with the Bradford assay standardized with BSA, pooled and stored at -80 °C. Starting material and WGA-purified membrane proteins were denatured and fractionated under reducing conditions on 12.5% SDS-PAGE, then transferred electrophoretically to Hybond C-extra nitrocellulose membranes (Amersham Life Science, Oakville, Canada). After transfer, non-specific binding sites were blocked by Tris-buffered saline-Tween (TBS-T) containing 5% non-fat dried milk. After five washes with TBS-T, membranes were incubated for 16 h at 4°C with a 1:500 dilution of rabbit anti-human D_2 receptor polyclonal antibody (Chemicon International, Temecula, CA, USA) in TBS-T containing 1% BSA. Membranes were washed five times with TBS-T, and then incubated for 1 h at 22°C with 1:1000 dilution of horseradish peroxidase-linked anti-rabbit IgG (Amersham Life Science, Oakville, Canada) and again washed. The specificity of the antibody was confirmed by pre-incubating the antibody with the respective immunizing receptor peptide (at a concentration of 100 nM). Immunoreactive bands were detected by chemiluminescence detection system (ECL western blot analysis system, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The immunoreactive bands were visualized by autoradiography after 0.5-min exposure to Biomax film (Eastman Kodak Company, Rochester, NY, USA). As the antibody recognize both the native and the denatured forms of D_2 receptor, bands of 110, 68, and 47 KDa may be visualized by western blot. The expected band with the procedure used in our lab was the 68-kDa denatured form.

RT-PCR: Messenger RNA isolation and cDNA synthesis were carried out according to a previous report (22). Messenger RNA was isolated using Dynabeads oligo(deoxythymidine)$_{25}$ [oligo(dT)$_{25}$; Dynal AS, Oslo, Norway] from a frozen tissue sample. The cells were lysed for 2 min in an ice-cold Tris-buffer (100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT and 5 U/100 µl RNAse

inhibitor (HT Biotechnology Ltd., Cambridge, UK). The mixture was centrifuged at 14,000 rpm for 1 min to remove cell debris. After adding 100 µl pre-washed Dynabeads oligo(dT)$_{25}$ to the supernatant, the mixture was incubated for 5 min on ice. Thereafter, the beads were collected with a magnet, and washed three times with a Tris-buffer (10 mM Tris HCl, pH 8, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS), and once with a similar buffer from which LiDS was omitted. Messenger RNA was eluted from the beads in 50 µl of a 2 mM EDTA solution (pH 8) during 2 min at 65 °C. To avoid contamination by genomic DNA, the isolated polyadenylated [poly(A)$^+$] mRNA was subjected to a second purification by capturing the RNA on a fresh aliquot of pre-washed Dynabeads Oligo (dT)$_{25}$ and washing the captured RNA as above described. Complementary DNA was synthesized using the poly(A)$^+$ mRNA captured on the Dynabeads Oligo (dT)$_{25}$ in Tris-buffer (50 mM Tris-HCl, pH 8.3, 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$) together with 1 mM of each deoxynucleotide triphosphate, 10 U RNAse inhibitor, and 2 U avian myeloblastosis virus Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 20 µl. This mixture was incubated for 1 h at 42 °C. One-tenth from each cDNA library immobilized on the paramagnetic beads was used for each amplification. The amplification reaction mixture contained cDNA template, 0.5 U SuperTaq (HT Biotechnology Ltd., Cambridge, UK), 50 µM of each deoxynucleotide triphosphate (HT Biotechnology Ltd., Cambridge, UK), 5 pmol of each of a pair of oligonucleotide primers specific for human D_1-D_5 receptor subtypes or the hypoxantine ribosyl transferase (HPRT) in Tris-buffer (10 mM Tris-HCl, pH 9, 50 mM KCl, 2 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.1% Triton X-100 in a final volume of 50 µl. The sequences of the primers for D_1-D_5 and HPRT are listed in **Table 2**. The PCR reaction was carried out in a DNA thermal cycler (Perkin Elmer Cetus Instruments, Gouda, The Netherlands). After an initial denaturation at 94 °C for 5 min, the samples were subjected to 40 cycles of denaturation at 94 °C for 1 min, annealing for 2 min at 60 °C, and extension for 1 min at 72 °C. After a final extension for 10 min at 72 °C, 10-µl aliquots of resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly(A)$^+$ mRNA preparation for two DR subtypes, D_1 and D_5, whose genes are intron-less, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. Amplification of the cDNA samples with the HPRT specific primers served as positive control for the quality of cDNA. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in

Table 2: Specific oligonucleotide primers for dopamine receptors subtypes (D_1-D_5) and controls used in the RT-PCR study

Gene		Sequence (5'-3')	Size of PCR product (bp)
Dopamine receptors			
D_1	Forward	AACACCTCTGCCATGGACG	616
	Reverse	TGATGGCCACAGGGATGTAA	
D_2	Forward	GCGGACAGACCCCACTACAA	521
	Reverse	AAGGGCACGTAGAAGGAGAC	
$D_{2\ short/long}$ isoforms	Forward	CCATGCTGTACAATACGCGCT	$D_{2\ long}$: 599; $D_{2\ short}$: 512
	Reverse	GGCAATCTTGGGGTGGTCTTT	
D_3	Forward	CCCGCCCACATGCCTACTAT	1106
	Reverse	GAAGGCTTTCCGGAACTCGAT	
D_4	Forward	CCCACCCCAGACTCCACC	259
	Reverse	GAACTCGGCGTTGAAGACAG	
D_5	Forward	ACCTGTGCGTCATCAGCGT	921
	Reverse	TGCGATCGAAAGGACCCTC	
HPRT	Forward	CAGGACTGAACGTCTTGCTC	413
	Reverse	CAAATCCAACAAAGTCTGGCT	

the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of the DR subtypes and HPRT, 0.01 μg of human brain cDNA was amplified in parallel with the cDNA samples of each examined pituitary corticotroph tumor.

In vitro functional study: The cell culture was performed according to a previous report (25). Tumor tissue samples were placed in Hank's balanced saline solution (HBSS, Gibco BRL, Life Technologies Ltd., Paisley, Scotland, UK), supplemented with 5% human serum albumin (HSA, Cealb, CLB, Amsterdam, The Netherlands), penicillin (10^5 U/liter), and fungizone (0.5 mg/liter). After careful removal of blood clots, the samples were minced and washed several times with the HBSS+HSA. The minced tissues were enzymatically dissociated with dispase (1000 U/liter) for 1-2 hours at 37 °C. After removal of erythrocytes by centrifugation on a Ficoll gradient, the tumoral pituitary cells were plated in 24-well plates (Costar, Cambridge, MA,

USA) in 1 ml D-MEM (Gibco BRL, Life Technologies Ltd., Paisley, Scotland, UK) culture medium containing 10% vol/vol fetal calf serum and penicillin and/or streptomycin, and were incubated at 37°C in a humid CO_2 incubator for 24-48 h to allow them to attach at the bottom of the plate wells. Then, medium was removed and replaced with 1 ml fresh D-MEM culture medium, test substances were added and cells were re-incubated at 37°C for 72 h. Afterwards, medium was collected and stored at –20°C for the measurement of ACTH secretion. In all the experiments, bromocriptine and cabergoline were used as test substances and were added to the cell cultures at the concentration of 0, 10^{-12}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} M. In two cases (no. 5 and no. 8), the specificity of the drug effect was tested co-incubating the D_2 agonist with an excess (10 μM) of the D_2 antagonist sulpiride.

***In vivo* functional study**: The patients were treated with cabergoline. Cabergoline was administered at the initial dose of 1 mg/week and monthly increased of 1 mg/week until normalization of daily urinary cortisol excretion. Plasma ACTH and serum and urinary cortisol levels after 3-month treatment were compared with the baseline hormonal values. The mean of three different measurements of these hormones in three nonconsecutive days of the same week were considered for the baseline and post-treatment evaluation. During cabergoline treatment, blood and urinary samples were collected on the days when the patients did not take the drug. A 50% or greater decrease in daily urinary cortisol excretion was considered a significant clinical response to cabergoline. Moreover, patients who achieved a 50% or greater decrease with normalization of urinary cortisol levels were considered full responders whereas those who achieved a 50% or greater decrease without normalization of urinary cortisol levels were considered partial responders to cabergoline. Patients who achieved less than a 50% decrease of urinary cortisol levels were considered resistant to cabergoline treatment. After 3 months of treatment, two patients were taking 1 mg, three were taking 2 mg and the remaining five were taken 3 mg/week cabergoline.

Hormonal assays: ACTH levels were measured by immunoradiometric assay, using a commercially available kit. Serum and urinary cortisol levels were measured by radioimmunologic assay, using commercially available kits.

Statistical analysis: Data are expressed as mean±SE. The comparison between post and pre-treatment hormone values were performed by Analysis of Variance

followed by Newman-Keuls' test. The association between parameters was calculated by χ^2 test. Significance was set at 5%.

Results

Histological evaluation and Immunohistochemistry for pituitary hormones: Basophilic adenoma was documented in 12 (60%), basophilic hyperplasia in three (15%), chromofobe adenoma in four (20%) and acidophilic adenoma in one (5%) case. A positive immunostaining for ACTH was documented in all 20 cases (100%) in correspondence with the adenomatous or hyperplasic tissue. Conversely, a positive immunostaining for PRL was documented in 13 (65%) cases. However, PRL immunostaining was positive in scattered normal cells (acidophilic ACTH-negative cells) within the tumor tissue in 6 (46.2%), in defined areas of normal cells within or around the tumor tissue in 4 (30.7%), and in defined areas of tumor tissue (basophilic ACTH-positive cells) in 2 (15.4%) cases. The remaining case (7.7%) was characterized by a diffuse PRL immunostaining covering all acidophilic ACTH-positive tumor tissue. This case was diagnosed as a mixed ACTH/PRL-secreting pituitary tumor. No significant immunoreactivity was found for GH, TSH, FSH and LH in all cases; this was only represented by scattered cells around the tumor tissue, probably belonging to contaminating normal pituitary. The histological and immunohistochemical features for pituitary hormones of the tumor samples are listed in **Table 3** and **Table 4** for groups 1 and 2, respectively.

Receptor-ligand binding study: Specific binding of ^{123}I-epidepride was found at autoradiography in eight of 10 (80%) cases in group 1. It was localized in tumoral cells and in normal scattered cells or defined areas around tumoral tissue or within normal pituitary. The binding was homogeneously distributed in four (50%) and not homogeneously distributed within the tumor tissue in the remaining 4 (50%) cases. It was scored as moderately positive in three (37.5%) and strongly positive in the remaining five (62.5%) positive cases. The results of the R-LB study are summarized in **Table 3**. An example of a negative and a positive autoradiography of ^{125}I-epidepride binding are shown in **Fig. 1**.

Immunoblotting: A specific band of the expected molecular weight (68 KDa) of D_2 receptor was found at the immunoblot of glycoproteins derived from a corticotroph pituitary tumor (**Fig. 2**).

Table 3: Dopamine receptor expression in corticotroph pituitary tumors of patients of *Group 1*

Patient	Receptor-ligand binding study	Immunohistochemistry				RT-PCR study						
		Histology	ACTH	PRL	D2	D1	D2	D2s/l	D3	D4	D5	HPRT
1	+	basophilic hyperplasia	++	++	+	-	+	long	-	-	-	+
2	++	basophilic adenoma	+	-	+	ne	ne	ne	ne	ne	ne	ne
3	-	chromofobe adenoma	++	-	-	ne	ne	ne	ne	ne	ne	ne
4	+	chromofobe adenoma	++	+	++	ne	ne	ne	ne	ne	ne	ne
5	+	basophilic adenoma	++	++	+	ne	ne	ne	ne	ne	ne	ne
6	++	basophilic adenoma	+	-	++	-	+	short	-	-	-	+
7	++	basophilic adenoma	++	++	+	-	+	long/short	-	-	-	+
8	-	basophilic hyperplasia	++	+	-	-	-	-	-	-	-	+
9	++	basophilic adenoma	++	++	++	ne	ne	ne	ne	ne	ne	ne
10	++	basophilic adenoma	++	+	++	-	+	long/short	-	+	-	+

++ = strongly positive ; *+* = weakly positive ; *-* = negative ; ***ne*** = not evaluated

Table 4: Dopamine receptor expression in corticotroph pituitary tumors of patients of *Group 2*

Patient	Immunohistochemistry				RT-PCR study							*In vitro* ACTH secretion inhibition (%)	
	Histology	ACTH	PRL	D2	D1	D2	D2s/l	D3	D4	D5	HPRT	After bromocriptine	After cabergoline
1	basophilic adenoma	+	+	+	-	+	long	-	-	-	+	ne	ne
2	chromofobe adenoma	+	-	-	-	-	-	-	-	-	+	12	13
3	basophilic adenoma	++	+	++	-	+	short	-	-	-	+	ne	ne
4	acidophilic adenoma	++	++	++	-	+	long/short	-	-	-	-	43	57
5	basophilic adenoma	++	+	-	ne	ne	ne	ne	ne	ne	ne	15	14
6	basophilic hyperplasia	+	-	+	ne	ne	ne	ne	ne	ne	ne	ne	ne
7	basophilic adenoma	++	-	++	-	+	long	-	+	-	+	ne	ne
8	chromofobe adenoma	++	+	+	-	+	long	-	-	-	+	53	60
9	basophilic adenoma	++	+	+	-	+	long/short	-	-	-	+	ne	ne
10	basophilic adenoma	++	-	-	ne	ne	ne	ne	ne	ne	ne	ne	ne

++ = strongly positive; *+* = weakly positive; *-* = negative; *ne* = not evaluated

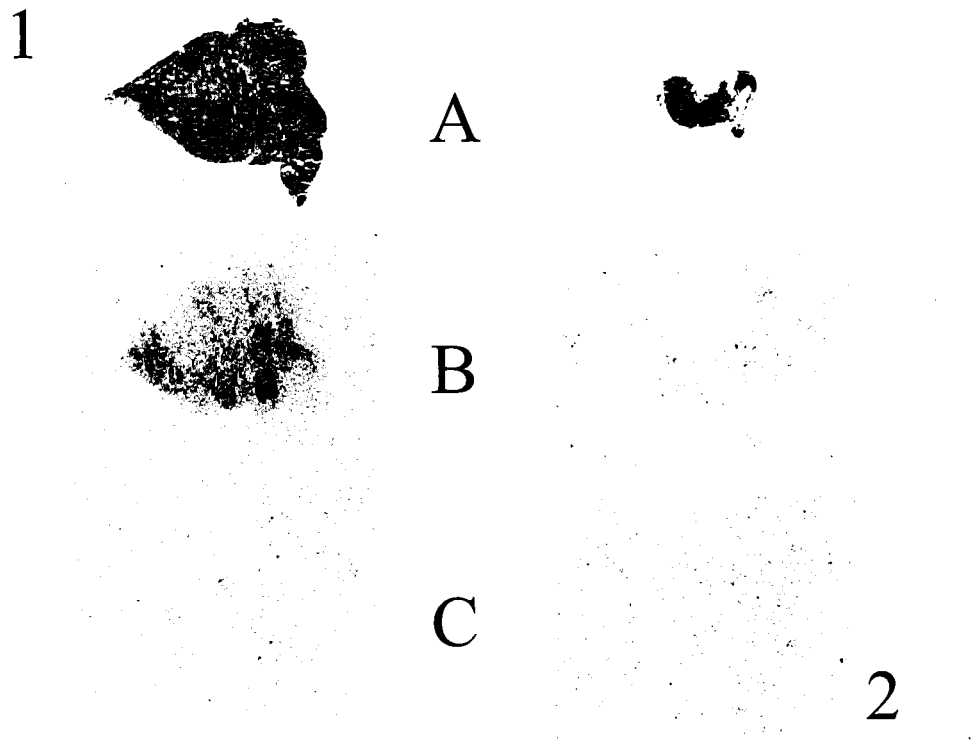


Figure 1: Expression of D_2 dopamine receptor subtypes by receptor-ligand binding study in 2 cases of human corticotroph pituitary tumors. Photomicrograph of D_2 receptor autoradiography. A, hematoxylin-eosin stained section; B, autoradiography showing total binding of ^{125}I-epidepride; C, autoradiography showing nonspecific binding (in the presence of 1 μM of cabergoline). The picture shows a positive (1, Case 2 of *Table 1* and *3*) and a negative (2, Case 8 of *Table 1* and *3*) case. The specificity of the binding is demonstrated by the complete disappearance of the radioactive signal in the presence of an excess of the high affinity D_2 agonist cabergoline.

Immunohistochemistry for D_2 receptor: Specific immunoreactivity for D_2 receptor was found in 15 (75%) cases, eight (80%) of Group 1 and seven (70%) of group 2. It was localized in ACTH- and ACTH/PRL-positive tumor cells and in normal PRL positive cells localized within tumor tissue or within normal pituitary.

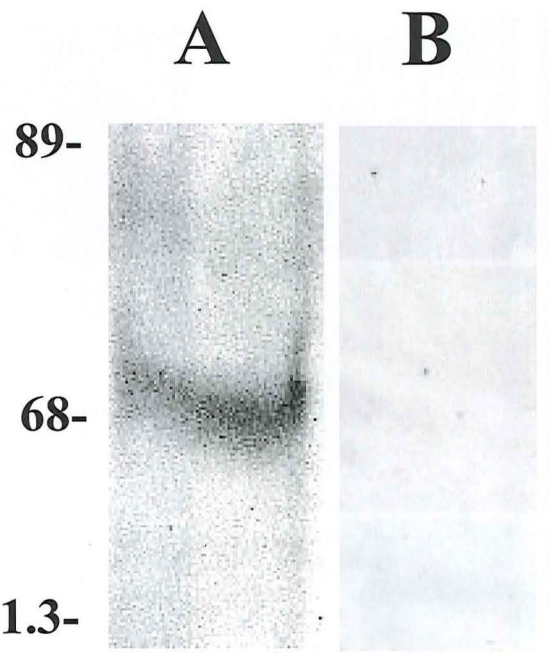
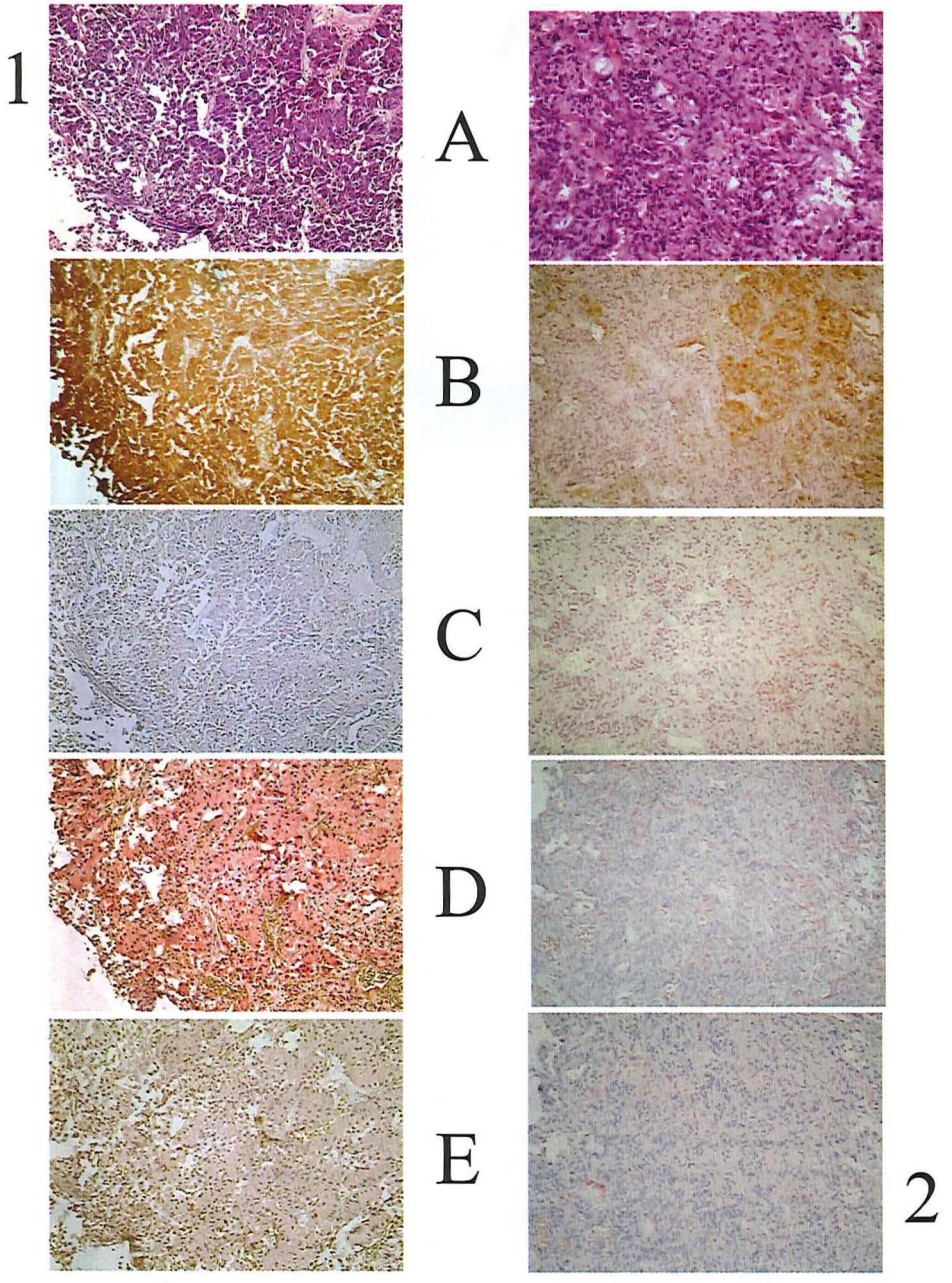


Figure 2: Immunoblot of glycoproteins derived from a human corticotroph pituitary tumor with D_2 receptor antibody. A-B: corticotroph pituitary tumor without (A) and with (B) the preincubation of the antibody with the antigen. The specificity of the immunoblot is demonstrated by the complete disappearance of the signal after the preincubation of the antibody with the antigen (D_2 receptor peptide).

The immunoreactivity was homogeneously distributed in seven (46.7%) and was not homogeneously distributed within the tumor tissue in eight (53.3) cases. It was scored as moderately positive in eight (53.3%) and strongly positive in the remaining seven (46.7%) positive cases. In group 1, a complete correspondence was found between the results of R-LB and the IHC studies. The results of IHC are summarized in **Table 3** and **Table 4** for groups 1 and 2, respectively. An example of a negative and a positive immunostaining for D_2 receptor is shown in **Fig. 3**.


1
A
B
C
D
E
2

Reverse transcriptase-polymerase chain reaction study: D_2 receptor was expressed in 10 of 12 cases (83.3%). D_{2long} was found in four (40%), D_{2short} in two (20%) and both D_2 isoforms in four (40%) positive cases. D_4 receptor was expressed in two cases (20%) associated to the expression of D_{2long} in one and to both D_2 isoforms in the remaining case. A complete correspondence was found between the results of RT-PCR and those of R-LB and IHC studies. No expression of other DRs were found. The results of RT-PCR study are summarized in **Table 3** and **Table 4** for groups 1 and 2, respectively.

***In vitro* functional study:** A significant and dose dependent inhibition of ACTH secretion was found in 2/4 cases (50%) of group 2 both after both bromocriptine and cabergoline administration. The inhibition rate was 43% in the first case and

Figure 3: Expression of D_2 receptor by immunohistochemistry in 2 cases of human corticotroph pituitary tumors. The immunohistochemical study has been performed on formalin-fixed and paraffin-embedded sections of the tumors. A, hematoxylin-eosin stained section; B, ACTH immunostaining; C, PRL immunostaining; D, D_2 receptor immunostaining performed with a specific polyclonal D_2 receptor antibody; E, D_2 receptor immunostaining after the preincubation of the antibody with the specific antigen. The picture shows a D_2 receptor positive (1, Case 7 of *Table 1* and *4*) and a negative (2, Case 5 of *Table 1* and *4*) case of ACTH-positive basophilic adenoma. ACTH immunostaining is similar in all cases whereas PRL immunostaining show a variable pattern in the different cases. In this picture PRL immunostaining is negative and scattered positive in case 1 and 2, respectively. The immunostaining for D_2 receptor show a homogeneous distribution in half and a dishomogeneous distribution within the tumoral tissue in the remaining half of cases. In this picture it was homogeneously positive and negative in case 1 and 2, respectively. The specificity of the immunostaining is demonstrated by the disappearance of the colorimetric signal after the preabsorption of the antibody with 100 nM of the specific antigen (D_2 receptor peptide). Magnification X40.

52% in the second one after bromocriptine and 53% in the first case and 60% in the second one after cabergoline administration, respectively. Both cases (100%) had a documented expression of D_2 receptor. No significant inhibition of ACTH secretion was found in the remaining two cases after either bromocriptine or cabergoline administration. The inhibition rate was 12 and 15% after bromocriptine and 13% and 14% after cabergoline administration for the two cases, respectively. No D_2 expression was found in either cases (100%). The specificity of the effect of bromocriptine and cabergoline was demonstrated by the block of this effect in one responsive case by the D_2 antagonist sulpiride (no. 8). No significant effect of sulpiride was demonstrated in one nonresponsive case (no. 5). The results of the *in vitro* study are summarized in **Table 4**. The individual ACTH response to bromocriptine and cabergoline in all 4 corticotroph pituitary tumor cultures are shown in **Fig. 4**.

***In vivo* functional study:** Significant inhibition of plasma ACTH and serum and urinary cortisol levels after 3 months of cabergoline treatment was found in six of 10 (60%) cases in group 1. However, a normalization of urinary cortisol was found in four of 10 (40%) cases, who were considered full responders, whereas a significant decrease without normalization of urinary cortisol was found in the remaining two cases, who were considered partial responders. A significantly different decrease in urinary cortisol levels was found between responder and resistant patients, who did not achieve a 50% or greater decrease of urinary cortisol levels after 3 months of cabergoline treatment (79.7±3.6% vs 27.7±7.8%; $p<0.05$). All cases responsive to cabergoline were associated to D_2 expression in the tumor tissue. Conversely, all cases not responsive to cabergoline were not associated with D_2 expression with the exception of one case (no. 6 of *Table 1* and *Table 4*), which was associated to a poor response to cabergoline treatment. A trend for a significant association between D_2 expression and clinical response to cabergoline ($\chi^2=3.35$, $p=0.067$) was found in the patients in the study. Among the four cases in which a normalization of ACTH and cortisol was obtained, three expressed D_{2short}, which was isolated in one case and associated with D_{2long} in the remaining two cases. The last case was associated to the expression of D_{2long} and D_4. The remaining case with a significant *in vivo* response to cabergoline and the only case not responsive to cabergoline but D_2 positive, expressed D_{2long} but not D_{2short} or D_4 receptors. The individual responses of urinary cortisol levels to the 3-month treatment with cabergoline are shown in **Fig. 5** and **Fig. 6.**

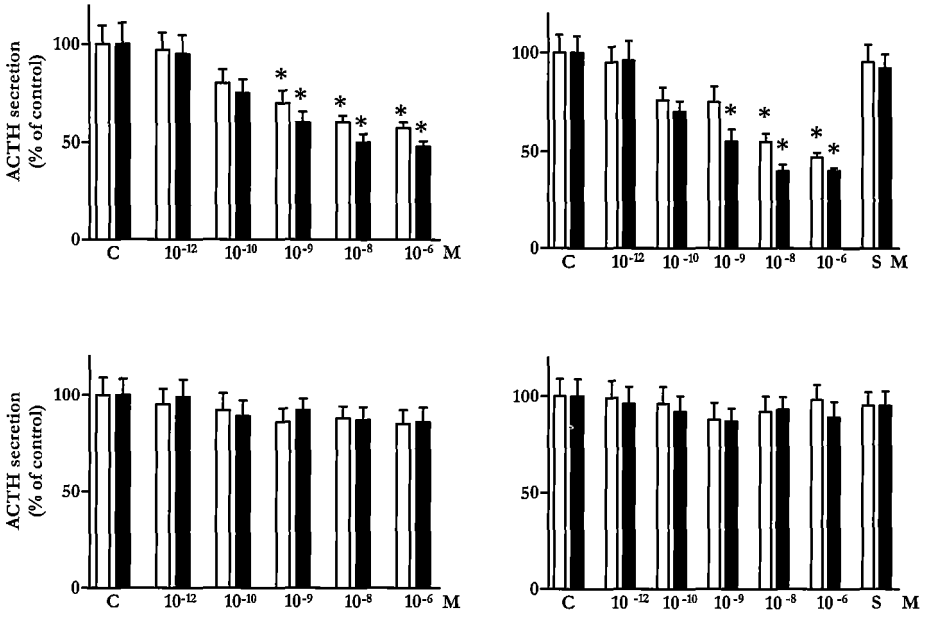


Figure 4: Effect of *in vitro* administration of bromocriptine (□) and cabergoline (■) on ACTH secretion in cell cultures derived by 4 different corticotroph pituitary tumors (Cases nos. 4 (A), 8 (B), 2 (C) and 5 (D) of *Table 1* and *4*). Corticotroph pituitary tumor cells were incubated in D-MEM supplemented with 10% foetal calf serum, penicillin/streptomycin during 72 hours in quadruplicate without (C) or with the drugs at the concentrations of 10^{-12}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} M and, in 2 cases (no. 5 and 8) with the drug at the dose of 1 μM co-incubated with an excess (10 μM) of the D_2 antagonist sulpiride (S). Values are expressed as secretion percentage (%) and are mean±SEM (n=4 per treatment group).

Discussion

The current study clearly demonstrated D_2 receptor expression in corticotroph pituitary tumors and the effectiveness of cabergoline treatment in controlling the cortisol hypersecretion associated to CD.

CD is a severe chronic disorder resulting from inappropriate and prolonged exposure to excessive endogenous adrenal cortisol secretion, secondary to altered pituitary ACTH secretion due to a corticotroph pituitary tumor (17); it represents the most common form of chronic endogenous hypercortisolism, accounting for

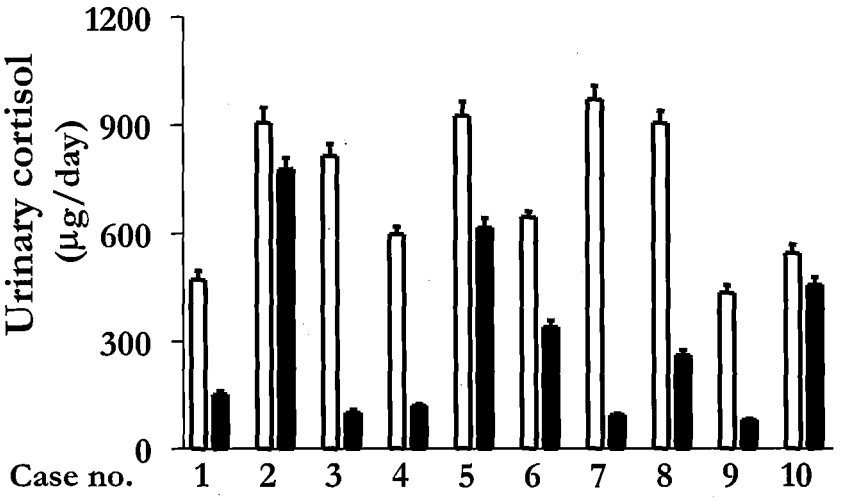


Figure 5: Effect of *in vivo* administration of cabergoline on urinary cortisol secretion in the 10 patients of *Group 2*. Cabergoline was administered at the initial dose of 1 mg/week in all patients and at the final dose ranging 1-3 mg/week after 3-month treatment. The dose has been monthly increased of 1 mg/week every month till normalization of urinary cortisol excretion. Values of daily urinary cortisol are expressed as absolute values in µg/day.

about 80% of cases of Cushing's syndrome (17). The neurosurgical resection of the pituitary tumor is the first line treatment of CD (26). However, transsphenoidal neurosurgery is successful in 70-80% of cases (27,28). Moreover, neurosurgical outcome is also affected by the lack of tumor evidence at transsphenoidal exploration in 15% and disease relapse after surgical remission in almost 10% of cases (26-28). Pituitary irradiation and total bilateral adrenalectomy represent second choice treatments in patients not cured by neurosurgery (17). However, although associated with a high success rate, these treatment modalities frequently result in secondary adverse events, such as hypopituitarism in the former and the development of a large and invasive pituitary tumor in the latter. In addition, adrenal insufficiency makes the patient life-long dependent on daily administration of after

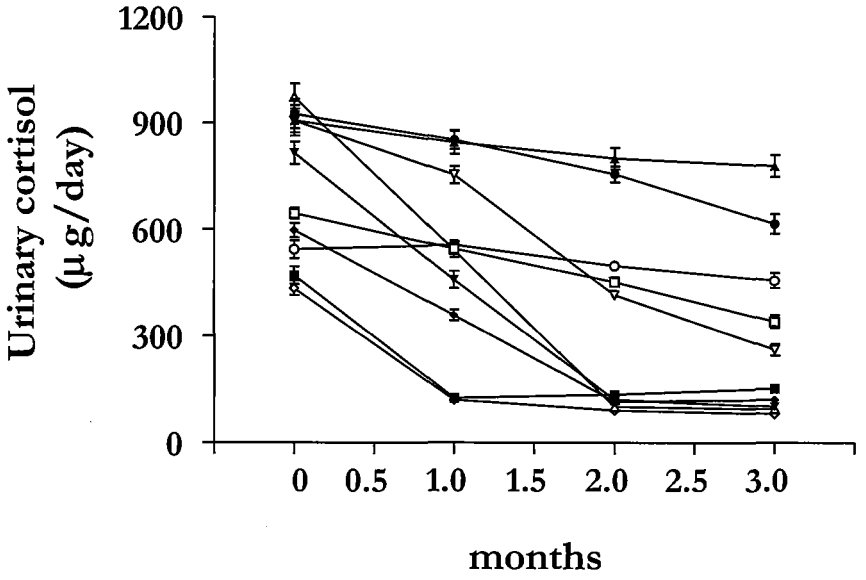


Figure 6: Individual values of urinary cortisol levels at baseline and during the 3-months cabergoline treatment of the 10 patients of *Group 2*. Cabergoline was administered at the initial dose of 1 mg/week in all patients and at the final dose ranging 1-3 mg/week after 3-month treatment. The dose has been monthly increased of 1 mg/week every month till normalization of urinary cortisol excretion. Values of daily urinary cortisol are expressed as absolute values in μg/day.

pituitary irradiation to obtain a rapid hormonal normalization before the definitive cure (16). Medical treatment is usually performed with adrenal blocking drugs, which do not act at level of the pituitary tumor (16). On the other hand, although several neuromodulatory drugs acting at pituitary level have been used in the treatment of CD, no single agent has ever demonstrated sufficient effectiveness to achieve a widespread clinical use in the management of the disease (29-31).

The only neuromodulatory drug that had a relevant, although controversial, role in the treatment of CD, was bromocriptine, the most widely used dopamine agonist. Bromocriptine was hypothesized to induce an inhibition of ACTH secretion and/or cell growth in corticotroph pituitary tumors, acting through DRs presumably expressed in the pituitary tumor cells. This hypothesis was based on the evidence

that DRs with an inhibitory function were demonstrated to be expressed in PRL-secreting pituitary tumors, where they induced a suppression of hormone secretion and inhibition of tumor growth in a great majority of cases (7-13). In corticotroph pituitary tumors, bromocriptine was found to induce significant inhibition or normalization of cortisol secretion in about 40% of cases after short-term treatment (32). However, controversial results with a success rate ranging from 0 to 50% were obtained by the different studies that tested the effectiveness of bromocriptine treatment in CD (16). Moreover, normalization of cortisol secretion and/or tumor shrinkage were only sporadically reported after long-term treatment with bromocriptine, demonstrating that only a subset of patients with CD was able to respond to chronic bromocriptine treatment (16,33,34). On the other hand, cabergoline was reported to induce significant shrinkage of a silent ACTH pituitary tumor (35), and a functioning ACTH-secreting pituitary tumor developed after bilateral adrenalectomy in a patient cured from CD (36). However, no study has ever evaluated the effectiveness of cabergoline treatment in CD. The current study evaluated the effectiveness of short-term treatment with cabergoline in a group of patients with CD, demonstrating a significant inhibition of cortisol secretion in 60% and a normalization of cortisol secretion in 40% of cases, suggesting that cabergoline is more effective than bromocriptine and is potentially useful in the treatment of CD.

The biological basis of the effectiveness of dopamine agonists in the treatment of pituitary tumors is represented by DR expression in the tumor. In the pituitary, the response to dopamine agonists is related to the activity of the D_2 receptor (37,38). This receptor belongs to the family of G protein-coupled receptors and acts through AMP cyclase enzyme inhibition (1,39). Alternative splicing of the gene encoding D_2 receptor leads to two different isoforms, the short isoform, named D_{2short}, and the long isoform, named D_{2long} (1,40). In normal human pituitary, D_2 receptor has been demonstrated to be expressed in more than 75% of the cell population, indicating that D_2 receptors are not expressed only in lactotroph and melanotroph, which does represent no more than 30% of the entire cell population of the normal pituitary gland (37). However, no study has systematically evaluated the co-localization of D_2 receptor with the different pituitary hormones in normal human pituitary gland, leaving only as a likely hypothesis the possible expression of D_2 receptor in non PRL and non MSH-secreting normal pituitary cells. A variable and heterogeneous expression of D_2 receptor has been recently demonstrated by *in situ* hybridization and IHC in 89% of all types of pituitary tumors and particularly in 69% of silent or functioning ACTH-secreting pituitary tumors (41). In PRL-secreting pituitary

tumors, the presence of a large amount of D_2 receptor explains the good therapeutical response to dopamine agonists, which induces PRL secretion inhibition and tumor shrinkage (42). Moreover, cabergoline has been clearly demonstrated to be more effective than bromocriptine in the treatment of this type of tumor (14,15). Resistance to dopamine agonists in PRL-secreting pituitary tumors may be explained by the absence or a small amount of D_2 receptor or its functional inactivity (43). D_2 receptor has also been demonstrated in GH-secreting pituitary tumors (44), although their response to dopamine agonists is not as marked as in PRL-secreting pituitary tumors, probably due to a difference in the abundance or functionality of the receptor (32,45). However, the relative resistance of these tumors to dopamine agonists was found to be overcome in a percentage of cases with the use of cabergoline (46,47). In clinically non-functioning pituitary tumors, D_2 receptor has been demonstrated in a large number of cases (48,49), although tumor shrinkage has been reported in only a minority of cases (34,50). However, in a recent study cabergoline was demonstrated to be more effective than bromocriptine at inducing tumor shrinkage (51). Dopamine agonist resistance in these tumors may be due to the same D_2 receptor abnormalities observed in PRL- and GH-secreting pituitary tumors. However, the expression of D_{2short} rather than D_{2long}, has been suggested to be associated with a better *in vitro* response to dopamine agonists (52).

As far as corticotroph pituitary tumors are concerned, no definitive evidence is available on D_2 receptor expression as one study demonstrated the absence of a significant and specific binding of radiolabeled spiperone, a dopamine antagonist, in two corticotroph pituitary tumors (8) whereas another study more recently demonstrated the D_2 receptor expression by *in situ* hybridization and IHC studies (41). However, experimental evidence in humans and rats supports the hypothesis of D_2 receptor expression and function in corticotroph pituitary tumors. Indeed, bromocriptine was demonstrated to suppress ACTH secretion from cultured human pituitary tumor cells by a dopaminergic mechanism (53) and to induce apoptosis in AtT-20 cells, a murine corticotroph pituitary tumor cell line (54). The current study represents the first clear demonstration of both D_2 receptor expression and D_2 receptor function in corticotroph pituitary tumors. This finding is strongly supported by the following evidence: 1) D_2 receptor expression has been evaluated at a molecular and cellular levels by different techniques, which permitted to demonstration of both transcription of D_2 receptor gene and translation in a D_2 receptor protein; 2) the comparison between the images obtained by R-LB or IHC studies and the histology clearly demonstrated the presence of D_2 receptors in tumor

cell cytoplasm or membranes, excluding that the D_2 receptor demonstrated at a molecular level might exclusively belong to normal pituitary cell infiltration within or normal pituitary tissue surrounding tumor tissue; 3) the *in vitro* effect of dopamine agonists on ACTH secretion inhibition in cell cultures obtained by D_2-positive corticotroph pituitary tumor samples, and the lack of this effect in cell cultures obtained by D_2-negative corticotroph pituitary tumor samples, demonstrated the expression of a functional D_2 receptor mediating the effect of dopamine agonists in these tumors; 4) the *in vivo* effect of cabergoline on cortisol secretion inhibition only in CD patients with a corticotroph pituitary tumors expressing D_2 receptor, definitely confirmed that D_2 receptor may mediate an important effect of dopamine agonists on ACTH and, consequently, cortisol secretion in corticotroph pituitary tumors.

The results of the current study support a possible therapeutic role of dopamine agonists in the treatment of CD. The expression of D_2 receptor in 80% of corticotroph pituitary tumors and the effectiveness of cabergoline treatment in 60%, with normalization of cortisol secretion in 40%, of patients with CD justify the possible use of cabergoline for controlling ACTH and cortisol hypersecretion associated to CD. The different successful rates observed with bromocriptine in past studies and with cabergoline in the present study may be related to the different molecular, biochemical and pharmacological characteristics of the two dopamine agonists, particularly to the higher specificity and affinity for D_2 receptor and the longer duration of action of cabergoline than bromocriptine (55,56). The evidence of a similar *in vitro* effect of bromocriptine and cabergoline in the four cases included in the study does not allow to draw definitive conclusions about the *in vitro* effects of the two drugs in corticotroph pituitary tumors due to the small number of cases evaluated. Moreover, different *in vitro* and *in vivo* behavior of the two drugs could be also not surprising considering that the *in vivo* effect of any drug is influenced by several factors that cannot be completely foreseen and reproduced in *in vitro* studies. Finally, it should be mentioned that the CD patients included in the current study were selected from two different endocrine clinics on the basis of the availability of tumor tissue, which excluded the smallest tumors, or on the basis of failure of neurosurgery with consequent persistence of CD, and/or recurrence of CD, which mainly included the tumors not easily removable by neurosurgery. In this view, the data presented in the current study do not necessarily represent the whole population of patients with CD. Cabergoline may be added at the list of therapeutic options and drugs, which can be adopted in different phases and conditions related to a complex disease like Cushing's syndrome (57).

In conclusion, the current study demonstrated the expression and function of D_2 receptor in corticotroph pituitary tumors. The presence of a functional D_2 receptor in 60% and the demonstration of effectiveness of a short-term treatment with the dopamine agonist cabergoline in normalizing ACTH and cortisol secretion in 40% of corticotroph pituitary tumors strongly support the possible therapeutic use of this drug in the management of persistent and/or recurrent CD.

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2

THE TREATMENT WITH DOPAMINE AGONISTS IN CUSHING'S DISEASE: COMPARISON BETWEEN SHORT-TERM AND LONG-TERM TREATMENT WITH CABERGOLINE

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Submitted for publication

ABSTRACT

The role of dopamine agonists in the treatment of Cushing's disease (CD) has been extensively debated and controversial results on the use of bromocriptine did not permit definitive conclusions. The aim of the current study was to evaluate and compare the effectiveness of short-term (3 months) and long-term (12-24 months) treatment with the more potent dopamine agonist cabergoline, and to compare the effectiveness of cabergoline with dopamine receptor expression in corticotroph pituitary tumors in 20 patients with CD unsuccessfully treated by neurosurgery. The study focused on the impact of cabergoline treatment on ACTH and cortisol secretion, and tumor size, as well as on the main complications of the disease, namely abdominal/visceral obesity, hypertension and glucose intolerance. Dopamine receptor expression was evaluated by standard and/or quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistocemisty (IHC). The responsiveness to short-term treatment was evaluated according to changes in urinary cortisol excretion: normalization was defined as full response, a $\geq$50% decrease without normalization was defined as moderate response, a $\geq$25% and <50% decrease was defined as poor response, whereas a <25% decrease was defined as resistance. The responsiveness to long-term treatment was defined by the persistence of normalized urinary cortisol secretion. At 3-months treatment, 15 (75%) patients were responsive (20% poorly, 20% moderately and 35% fully responsive) whereas 25% were resistant to the treatment. Body mass index, serum testosterone and estradiol levels significantly increased, whereas waist to hip ratio, systolic and diastolic blood pressure, fasting and post-glucose load glucose and insulin levels, HOMA index and PRL levels significantly decreased together with the decrease of plasma ACTH and serum and urinary cortisol levels. The prevalence of hypertension, glucose intolerance and gonadal or sexual dysfunction decreased in parallel with the normalization of cortisol levels. A significant improvement of blood pressure and a slight improvement in glucose tolerance and gonadal function was found both in responsive and resistant patients. Cabergoline treatment was continued in the 15 responsive patients. However, treatment escape was observed in 33% of them after 6 to 18 months. The remaining 10 patients were evaluated during long-term treatment: eight were followed for 24 months, whereas the remaining 2 were followed for 12 months, as at this time, cabergoline was withdrawn for intolerance. During long-term treatment, body mass index decreased, waist to hip ratio, serum glucose and insulin and HOMA index further decreased, whereas serum testosterone and estradiol further increased. Urinary cortisol as well as plasma and urinary cortisol levels, despite some fluctuations, remained within the

normal range. At the last follow-up, menstrual disorders in women decreased from 87,5% to 25%, whereas in the 2 men responsive to the treatment, sexual performance ameliorated. Moreover, the prevalence of hypertension and glucose intolerance decreased from 50% to 0% and from 62.5% to 30%, respectively. The D_2 receptor number in corticotroph pituitary tumor was the best predictor of the short-term responsiveness, whereas the presence of D_{2short} isoform was the best predictor of long-term responsiveness to cabergoline treatment. The presence of the D_{2long} only was associated to treatment escape, except when co-expressed with D_4 receptor. In conclusion, the results of the current study demonstrated that cabergoline treatment is effective in controlling cortisol secretion for at least for 1-2 years in more than one third of cases, with a significant improvement of abdominal/visceral obesity, blood pressure and glucose tolerance, gonadal and sexual function, being a useful treatment option in patients with CD unsuccessfully treated by neurosurgery.

Introduction

Cushing's disease (CD) is the most common form of chronic endogenous hypercortisolism, accounting for 80% of cases of Cushing's syndrome (CS) (1). It is caused by an increased cortisol secretion from the adrenal glands due to excess ACTH secretion by a corticotroph pituitary tumor (1). CS is a rare but severe disease associated with an increased mortality related to cardiovascular diseases which are mainly due to abdominal/visceral obesity, hypertension, impaired glucose tolerance, as well as thrombosis diathesis and, consequently, premature atherosclerosis (2). The first-choice treatment of CD is neurosurgery, aimed at the removal of the pituitary tumor (3). However, neurosurgery is effective in inducing immediate disease remission in around 60-80% and late disease remission in not more than 50-70% of patients, due to disease persistence or relapse in the 5 years after surgery (3). Pituitary radiotherapy and bilateral adrenalectomy are the alternative definitive therapeutic approaches to CD, but they are associated with long-term complications (3). Pharmacotherapy is not currently used in the treatment of CD, except for adrenal blocking drugs, used as transient treatment before definitive cure (3). However, although no drug has demonstrated a sufficient effectiveness in controlling cortisol secretion and inducing tumor shrinkage in CD, the dopamine agonist bromocriptine was reported to inhibit cortisol secretion in the short-term in a limited group of patients with CD (4-6). Recently, the dopamine agonist cabergoline was reported to effectively control cortisol secretion after short-term treatment in around 60% of patients, thereby being more efficacious than bromocriptine (7).

The aim of the current study was to evaluate the effectiveness of short-term and long-term treatment with cabergoline on ACTH and cortisol secretion, corticotroph tumor size and a number of complications of the disease, namely abdominal/visceral obesity, hypertension and impairment of glucose tolerance in patients with CD unsuccessfully treated by neurosurgery. In addition, the relationship between cabergoline effectiveness and the expression of dopamine receptors on the corticotroph tumors derived from neurosurgical resection in patients with CD was also studied.

Materials and Methods

Patients: Twenty patients (15 women and five males, age range, 24-60 years of age; mean, 39.4±11.7 years; median, 42 years) with persistent CD after unsuccessful neurosurgery entered the study after their informed consent had been obtained. The diagnosis of CD at the first admission and the confirmation of the persistence of CD after neurosurgery was based on: 1) increase in daily urinary cortisol excretion with inappropriately high plasma ACTH concentrations; 2) increase in basal serum cortisol concentrations with lack of the physiological circadian rhythm; 3) failure of urinary and serum cortisol suppression after low dose but greater than 50% decrease after high dose oral dexamethasone suppression test (8). The diagnosis of CD was supported by the evidence of a pituitary tumor at magnetic resonance imaging (MRI) of the pituitary gland or at the bilateral inferior petrosal sinus sampling (9,10). At the first diagnosis, 12 patients had pituitary microadenoma, five had macroadenoma, and the remaining three patients had normal pituitaries at MRI; the pituitary source of ACTH hypersecretion in these patients was confirmed by bilateral inferior petrosal sinus sampling. After neurosurgery, 13 patients turned out to have pituitary microadenoma, two had macroadenoma, and the remaining five had normal pituitaries at MRI. The histological and immunohistochemical study of the tumor removed by neurosurgery documented a corticotroph pituitary lesion (adenoma or hyperplasia) in all cases but one, definitely confirming the diagnosis of CD in these patients. The histological evaluation of the remaining case showed normal pituitary tissue: in this case the complete persistence of the clinical syndrome, the hormonal pattern and the pituitary tumor suggested the complete failure of neurosurgery. The patients' profile at study entry is shown in **Table 1.**

Study design: The study design was in accordance with the Helsinki Doctrine on Human Experimentation and it was approved by the local Ethical Committee. The study protocol included the evaluation of short-term (3 months) and long-term (12-24 months) effectiveness of cabergoline treatment on clinical, biochemical and radiological features, focusing on the complication of the disease, namely visceral obesity, hypertension and impaired glucose tolerance, and the comparison between cabergoline effectiveness and dopamine receptor expression in the corticotroph pituitary tumors.

Treatment protocol: Cabergoline (Dostinex, 0.5 mg, Pharmacia-Pfizer, Rome, Italy) was administered at the initial dose of 1 mg/week; the dose was progressively

Table 1: Patients' clinical profile at study entry

Patient (sex/age)	Plasma ACTH (ng/L)	Daily Urinary Cortisol (μg/24h)	Serum Prolactin (μg/L)	Pre-surgical Radiological findings	Surgical findings	Post-surgical Radiological fundings	Histological findings
1. f/49	54.0	471	22.0	microadenoma	microadenoma	microadenoma	basophilic adenoma
2. f/48	69.3	906	7.8	macroadenoma	macroadenoma	microadenoma	chromofobe adenoma
3. f/25	67.2	815	27.9	microadenoma	microadenoma	microadenoma	basophilic adenoma
4. f/35	78.1	598	30.2	microadenoma	microadenoma	no tumor	acidophilic adenoma
5. m/31	98.9	925	7.6	no tumor	microadenoma	no tumor	basophilic adenoma
6. f/42	52.0	645	30.1	no tumor	diffuse enlargement	no tumor	basophilic hyperplasia
7. m/45	43.3	971	10.2	microadenoma	microadenoma	microadenoma	basophilic adenoma
8. f/43	76.2	906	38.9	macroadenoma	macroadenoma	microadenoma	chromofobe adenoma
9. f/47	55.4	434	16.2	microadenoma	microadenoma	microadenoma	basophilic adenoma
10. f/26	99.1	544	15.2	macroadenoma	macroadenoma	macroadenoma	basophilic adenoma
11. f/38	71.1	560	21.2	microadenoma	microadenoma	microadenoma	basophilic adenoma
12. F/52	77.0	389	25.2	microadenoma	microadenoma	microadenoma	basophilic adenoma
13. f/55	101.0	443	44.4	microadenoma	microadenoma	no tumor	basophilic adenoma
14. f/29	78.0	467	12.2	macroadenoma	macroadenoma	macroadenoma	basophilic adenoma
15. m/30	32.9	333	33.3	microadenoma	microadenoma	microadenoma	chromofobe adenoma
16. m/18	59.4	299	31.4	microadenoma	diffuse enlargement	microadenoma	normal pituitary
17. m/31	78.8	441	7.8	microadenoma	microadenoma	microadenoma	basophilic adenoma
18. f/60	59.2	654	45.1	macroadenoma	macroadenoma	microadenoma	basophilic adenoma
19. f/46	71.2	444	19.3	no tumor	diffuse enlargement	microadenoma	basophilic hyperplasia
20. f/48	54.4	556	15.3	microadenoma	microadenoma	no tumor	basophilic adenoma

Normal ranges: plasma ACTH: 10-100 ng/L; urinary cortisol: 35-135 μg/24 h: serum prolactin: 5-25 μg/L

increased by 1 mg every month until normalization of urinary cortisol levels or a maximal dose of 7 mg/week had been reached. The clinical and biochemical evaluation was performed every month together with the evaluation of urinary cortisol levels till the normalization of cortisol levels, or the maximal dose of 7 mg/week had been reached, then it was performed every three months. The radiological evaluation was performed at 3, 6, 12 and 24-months after the starting of treatment.

Responsiveness to treatment: The evaluation of the treatment responsiveness was performed according to different criteria for short-term and long-term treatment. After short-term treatment, patients who achieved a normalization of urinary cortisol levels were considered full responders where those who achieved a $\geq$25% decrease without normalization of urinary cortisol levels were considered partial responders to cabergoline. In particular, the patients who achieved a $\geq$50% decrease were considered moderate responders, whereas the patients who achieved a $\geq$25% but <50% decrease of urinary cortisol levels were considered poor responders. Patients who achieved a <25% decrease of urinary cortisol levels were considered resistant to cabergoline treatment. After long-term treatment, the presence of urinary cortisol levels within the normal range was the only criterium to evaluate the responsiveness to treatment: patients who reached normalization of urinary cortisol levels and maintained urinary cortisol levels in the normal range till the end of the study (12-24 months) were considered responsive. Conversely, a re-increase of urinary cortisol levels after their normalization was considered an escape from treatment. Complete resistance to cabergoline treatment after short-term treatment as well as a treatment escape during long-term treatment, or evidence of a significant increase of tumor volume or the occurrence of severe adverse events during the treatment period was a criterium for cabergoline withdrawal and the exclusion of the patient from the study. The responsiveness to short term treatment with cabergoline treatment in terms of urinary cortisol changes in 10 out of the 20 patients of the study (no. 1-10 of Table 1) has been already described in a previous report (7)

Clinical study: The clinical study included the evaluation of typical symptoms or signs of the disease, mainly the regularity of menses in women and libido and sexual potency in men, body mass index and the waist to hip ratio, expression of general and abdominal or visceral obesity respectively, systolic and diastolic blood pressure and heart rate. The diagnosis of overweight or overt obesity was performed on the

basis of body mass index: a value between 25 and 30 defined overweight, whereas a value ≥30 kg/m^2 defined overt obesity (11,12) The diagnosis of hypertension was made if diastolic blood pressure was >90 mmHg (11,12).

Biochemical study: Biochemical studies included the evaluation of serum glucose and insulin levels, serum prolactin, serum testosterone or estradiol, together with plasma ACTH, serum and urinary cortisol levels. The diagnosis of glucose intolerance was made on the basis of fasting serum glucose levels and/or the response of serum glucose to glucose load during a standard oral glucose tolerance test (75 g glucose diluted in 250 ml saline solution, measuring serum glucose levels every 30 minutes for 2 hours): diabetes mellitus was diagnosed when fasting serum glucose levels was >126 mg/dL in two consecutive determination or ≥200 mg/dL two hours after the glucose load whereas an impairment of glucose tolerance was diagnosed when fasting serum glucose levels were between 110 and 126 mg/dL and between 140 and 200 mg/dl two hours after the glucose load. Insulin secretion and sensitivity were evaluated by measuring fasting insulin levels and the response of insulin to glucose load and measuring the HOMA index (fasting glucose (in mmol/L) x fasting insulin (in mU/L)/22.5), a marker of insulin resistance. The oral glucose tolerance tests were not performed in patients with fasting glucose levels higher than 140 mg/dL. The diagnosis of glucose intolerance was performed in accordance with previous reports (11,12). Serum insulin, testosterone, estradiol, prolactin as well as plasma ACTH, serum and urinary cortisol levels were measured by radioimmunologic or immunoradiometric assays.

Radiological study: Radiological studies included the evaluation of the size of the corticotroph pituitary tumors by magnetic resonance imaging (MRI) of the sellar region. MRI studies were performed on clinical 1.5T scanners, using T1 weighted gradient recalled-echo (repetition time 200-300 ms; echo time 10-12 ms; flip angle 90°, 4 signal averages) in the sagittal and coronal planes. The image acquisitions were repeated before and after administration of 0.1 mmoles of gadolinium chelate (diethylene-triamine pentacetate). The size of the pituitary tumor was determined by calculating the three diameters (longitudinal and transverse in coronal sections and antero-posterior in sagittal sections, and the volume, obtained by the following formula (longitudinal diameter x transversal diameter x antero-posterior diameter x $\pi/6$) (9). The radiological study was not performed in five patients, who had no detectable tumor at MRI at the study entry. A shrinkage of more than 25% was considered significant for the study.

Experimental study: Dopamine receptor subtypes (D_1-D_5) and D_2 receptor isoforms (D_{2long} and D_{2short}) expression, in terms of presence of transcriptional product, was evaluated by standard RT-PCR, whereas the relative amount of the transcriptional product and the protein product of the D_2 receptor was evaluated by quantitative RT-PCR (qRT-PCR) and IHC, respectively, on samples of corticotroph tumors removed from the patients at the time of neurosurgery. In detail, pituitary tumor specimens taken fresh directly at the time of tumor excision were fixed in 10% paraformaldehyde overnight and embedded in paraffin for the IHC study and/or quickly frozen on dry ice and stored in a freezer at -80°C for RT-PCR and qRT-PCR studies. The study included only samples in which the tumoral tissue (adenoma and/or hyperplasia) represented at least 90% of the section at the histological evaluation, to exclude the influence of normal pituitary tissue on the results of the studies. The IHC and standard RT-PCR study was performed according to a previous report (7). The methodology of qRT-PCR study were described in a previous report (13). The specific primers and probe for D_2 receptor were the following: (GenBank accession no. AF050737 coding sequence), sense (1062-1083), antisense (1177-1156), and probe (1080-1115). The dopamine receptor expression, evaluated by IHC and standard RT-PCR, in 10 out of the 20 patients of the study (no. 1-10 of Table 1) has been already described in a previous report (7)

Statistical analysis: The statistical analysis was performed by SPSS for Windows version 11.0 (SPSS Inc., Chicago, IL). The comparison between baseline and post-treatment parameters was performed by Friedman or Wilcoxon test. The association between parameters was calculated by χ^2 test. The correlation between parameters was performed by regression analysis calculating the Pearson or Spearman correlation coefficient. The multiple regression analysis was performed among the variables significantly correlated at linear regression analysis in order to identify the parameter considered the best predictor of the effectiveness of treatment. Data are expressed as mean±SE. Significance was set at 5%.

Results

Responsiveness to treatment with cabergoline

After 3-months treatment, 15 patients (75%) were responsive whereas the remaining 5 (25%) were resistant to treatment. In particular, four (20%) patients were poor responders, four (20%) moderate responders and seven (35%) full

responders to the treatment. Cabergoline was withdrawn in all five resistant patients after 3 months, except in one patient who refused alternative treatments and was treated for 12 months. Among the 15 responsive patients who continued cabergoline treatment, six of the eight (75%) partial responders normalized cortisol levels at 6-12 months treatment after increasing the dose of the drug. However, two of the seven (28.6%) full and three of the eight (37.5%) partial responders experienced a treatment escape, despite a further increase of cabergoline dose, between 12- and 18-months treatment, and stopped cabergoline treatment. Cabergoline was also discontinued at the 12-month follow-up in two (10%) patients who did not tolerate the treatment because of severe asthenia. Therefore, at the 24-month follow-up, eight (40%) patients were persistently controlled at a median dose of 3.5 mg/week. The profile of the urinary cortisol levels of all the patients during cabergoline treatment is shown in **Fig. 1**. The response to short-term and long-term treatment with cabergoline, in terms of inhibition of urinary cortisol levels in the individual patients is shown in **Fig. 2**.

Effectiveness of short-term treatment with cabergoline

Clinical evaluation: The clinical picture significantly improved in all responsive patients. The majority of responsive and none of the resistant patients reported a reduction of facial plethora, purple striae and ecchymoses. General asthenia and muscle weakness slightly improved in 40%, worsened in 13% and remained unchanged in the remaining 47% of responsive patients. A worsening of general asthenia and muscle weakness was found in all resistant patients. Body mass index significantly increased but waist to hip ratio significantly decreased: the prevalence of overweight or overt obesity changed from 60% and 20% to 55% and 25%, respectively. In responsive, but not in resistant patients, the distribution of fat mass was modified from an abdominal to a generalized pattern. Regularity of menses improved, as amenorrhea or oligomenorrhea was present in 53% and 40% before and 27 and 53% of women, respectively, after treatment. Similarly, libido and sexual potency improved. Sexual impairment was present in 80% of men before and improved in 75% of them during treatment. Systolic and diastolic blood pressure values significantly decreased whereas heart rate increased: the prevalence of hypertension decreased from 65% to 30%. Body mass index, waist to hip ratio, blood pressure and heart rate values before and after short-term treatment are summarized in **Table 2**.

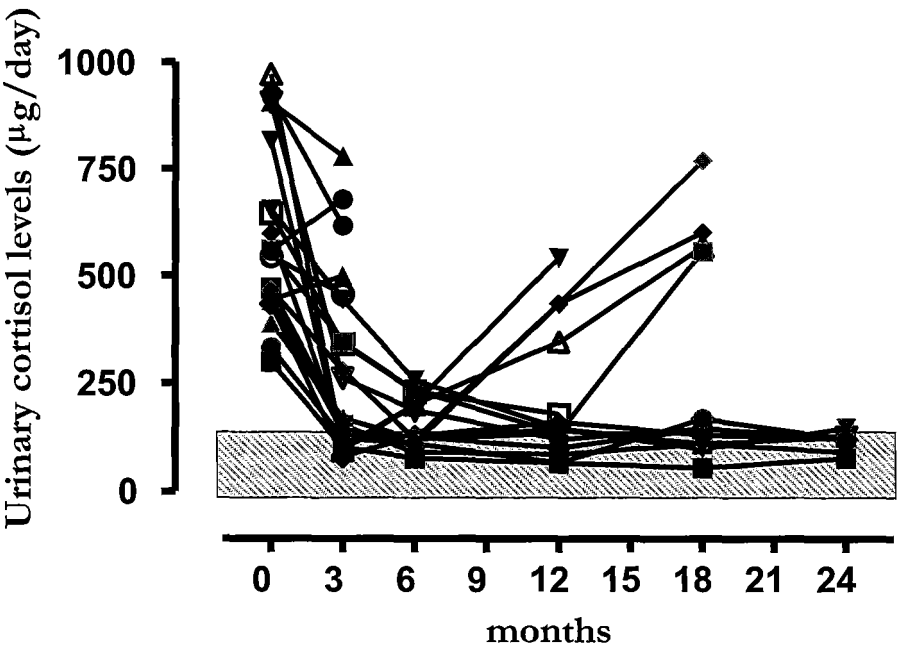

Figure 1: Urinary cortisol level during the entire period of treatment in all 20 patients treated with cabergoline. The shaded area show the normal range of urinary cortisol levels.

The changes of diastolic blood pressure values in the 15 patients responsive and the five resistant to short-term treatment are shown in **Fig. 3**.

Biochemical evaluation: Glucose tolerance significantly improved. Fasting serum glucose and insulin levels as well as HOMA index significantly decreased. A significant decrease of glucose and insulin peak levels after glucose load was also found. The prevalence of diabetes mellitus and impairment of glucose tolerance changed from 30% and 50% to 25% and 35%, respectively. An improvement in glucose tolerance was present in both responsive and resistant patients although it was more marked in the former than in the latter. In particular, in resistant patients, although fasting serum glucose and insulin and HOMA index were not significantly decreased, glucose and insulin peak after oral glucose load significantly decreased, with a consequent reduction of the prevalence of glucose intolerance. Glucose

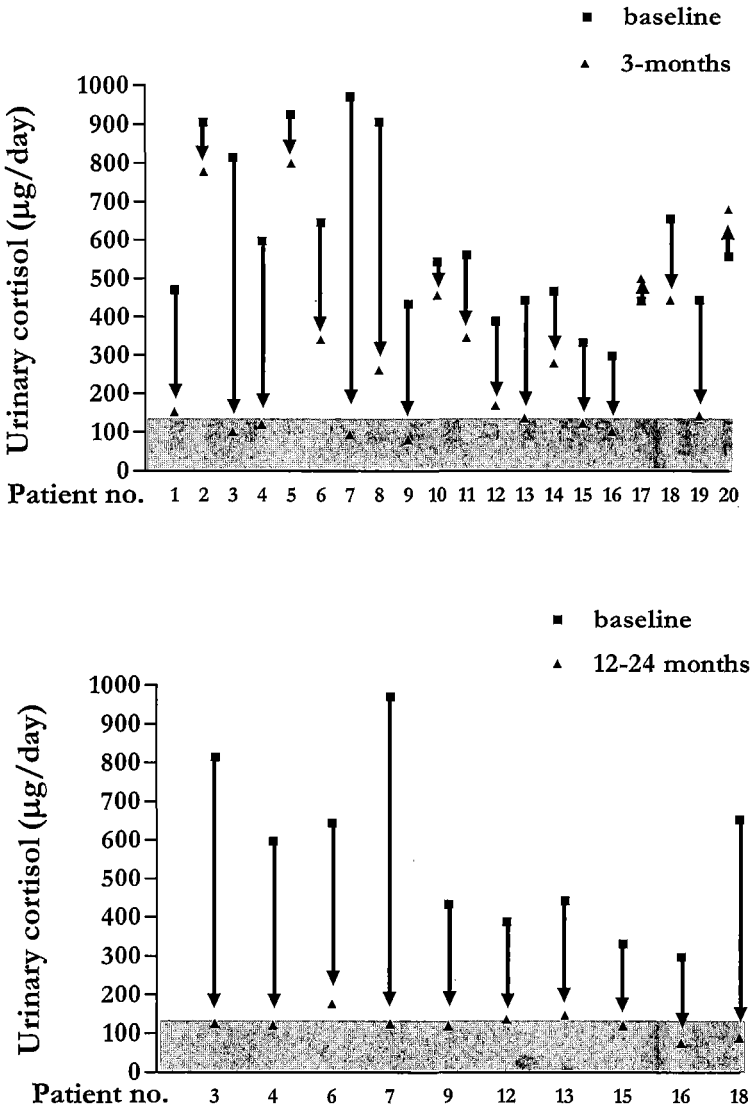

Figure 2: Changes in urinary cortisol levels after short-term (top) and long-term (bottom) treatment with cabergoline.

Table 2: Clinical, biochemical and radiological features of the 20 patients with Cushing's disease treated with cabergoline at baseline and after short-term treatment

Parameter	Baseline	3-months treatment	P
Body mass index (kg/m²)	27.6±0.7	28.1±0.6	0.019
Waist/hip ratio	1.09±0.03	1.08±0.03	0.013
Systolic blood pressure (mmHg)	146.8±3.0	136.3±2.3	0.000
Diastolic blood pressure (mmHg)	94.3±2.0	87.5±1.9	0.000
Heart rate (b/min)	68.0±1.5	71.7±1.9	0.005
Fasting serum glucose (mg/dL)	128.0±4.5	120.4±3.1	0.002
Fasting serum insulin (μU/mL)	11.9±1.0	11.0±1.0	0.000
HOMA index	3.77±0.40	3.29±0.33	0.001
Serum prolactin (μg/L)	23.1±2.6	2.0±0.2	0.000
Serum testosterone (μg/L)	2.4±0.2	2.9±0.5	0.042
Serum estradiol (ng/L)	27.1±2.3	44.5±2.9	0.001
Plasma ACTH (ng/L)	68.2±4.2	61.9±3.9	0.004
Morning serum cortisol (μg/L)	251.6±12.4	202.5±13.9	0.000
Urinary cortisol (μg/day)	590.1±46.7	304.5±52.5	0.000
Tumor volume (mm³)	310.3±81.1	299.2±82.8	0.345

Normal ranges: fasting serum glucose: 60-110 mg/dL; fasting serum insulin: 0-15 μU/mL: serum prolactin: 5-25 μg/L; serum testosterone: 3.0-9.0 μg/L; serum estradiol: 20-240 ng/L: plasma ACTH: 10-100 ng/L; morning serum cortisol: 50-200 μg/L; urinary cortisol: 35-135 μg/24 h. Serum testosterone levels refer exclusively to the male population as well as serum estradiol levels refer exclusively to female population. Serum estradiol levels were measured during the early follicular phase in patients with normal menstrual cycle (duration ≤40 days) and independently from the menstrual cycle in patients with olgoamenorrhea or amenorrhea.

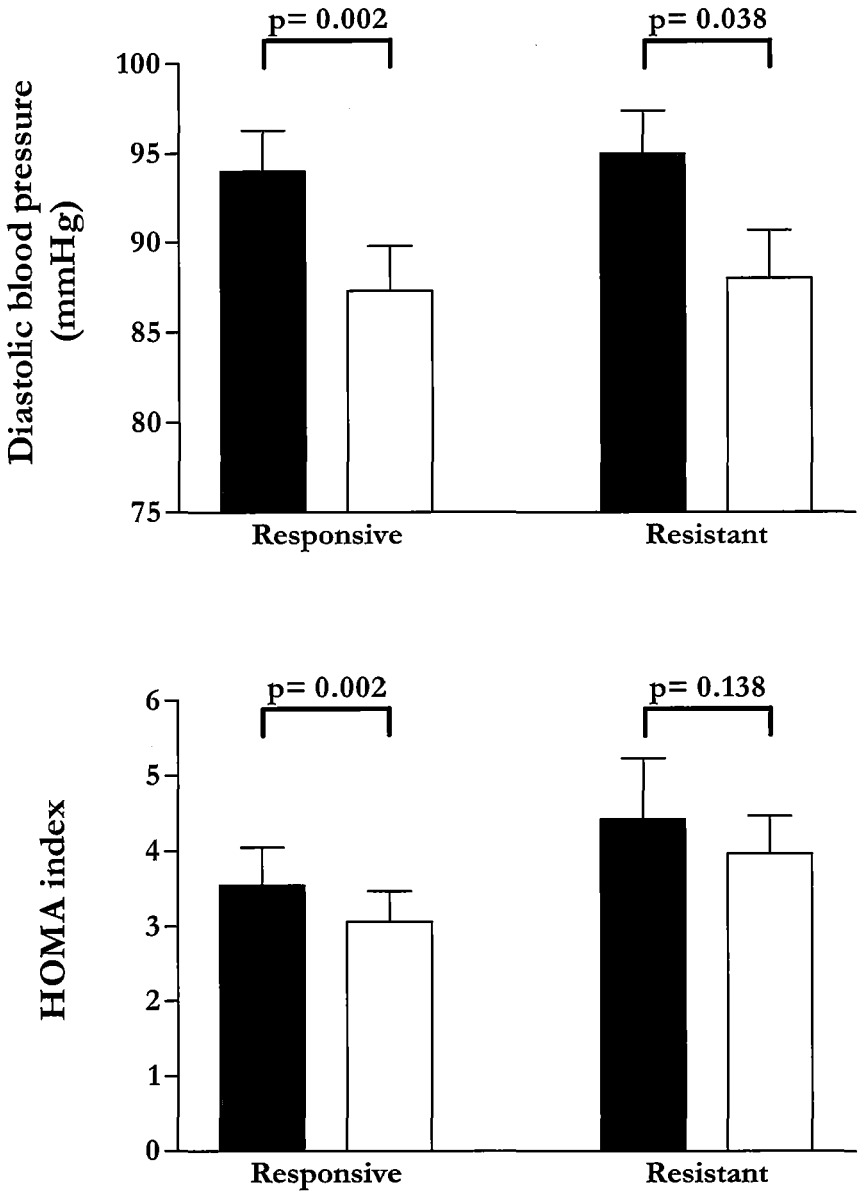

Figure 3: Change of diastolic blood pressure and HOMA index in responsive (n=15) and resistant (n=5) patients before (grey bar) and after (white bar) short-term treatment with cabergoline.

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tolerance completely normalized in three (20%) responsive patients and one (20%) resistant patient. A significant increase of estradiol and testosterone levels was found only in responsive, and a slight increase was found in resistant patients. Plasma ACTH as well as serum cortisol significantly decreased, in line with the changes in urinary cortisol excretion. As expected, the reduction of ACTH and cortisol levels was observed in responsive but not in resistant patients. The biochemical parameters before and after 3-months treatment are summarized in **Table 2.** A comparison in the change of HOMA index in the 15 responsive and the five resistant patients after short-term treatment is shown in **Fig. 3.**

Radiological evaluation: No significant change of pituitary tumor size was found after treatment in those patients with a detectable tumor before treatment. Similarly, the patient without a detectable tumor before treatment, still showed a similar radiological picture after treatment, without evidence of a clearcut tumor within in the pituitary gland.

Effectiveness of long-term treatment with cabergoline

Clinical evaluation: The clinical picture further improved during the 12-24 months of treatment with cabergoline in patients persistently controlled during the two years of treatment. In these patients, body mass index started to decrease after 6 months and waist to hip ratio further decreased during the entire period of treatment. The prevalence of overweight or obesity in the patients who reached two years of treatment decreased from 87,5% (overweight in 62,5% and obesity in 25%) at baseline to 62,5% (obesity in 0% and overweight in 62,5%) at the 24-month treatment. The distribution of fat mass was evidently modified from a abdominal to a generalized pattern in the majority of long-term responsive patients. General asthenia and muscle weakness further improved or started to improve after 6 months, changing their prevalence from 75% to 37,5%. Regularity of menses further improved, as the prevalence of amenorrhea and oligomenorrhea changed from 37,5% and 50% to 0% and 25% of women, respectively. Similarly, libido and sexual potency progressively improved in the two men who reached the two years of treatment. Systolic and diastolic blood pressure further decreased whereas heart rate further increased during the entire period of treatment, becoming stable and normal after 6-12 months of treatment. The prevalence of hypertension decreased from 50% to 0%. A slight re-increase of body weight and mass index and waist-to hip ratio, and a slight worsening of regularity of menses and sexual performance was

Table 3: Clinical, biochemical and radiological features of patients with Cushing's disease classified as long-term responsive to cabergoline treatment.

Parameter	Baseline (10 patients)	12-months treatment (10 patients)	24-months treatment (8 patients)	P
Body mass index (kg/m²)	28.2±0.9	28.0±0.8	27.1±0.7*	0.011
Waist/hip ratio	1.12±0.06	1.03±0.06**	1.01±0.06*	0.002
Systolic blood pressure (mmHg)	141.5±4.4	118.0±5.1*	123.1±4.4*	0.015
Diastolic blood pressure (mmHg)	91.0±2.5	75.0±3.8**	80.0±3.4*	0.002
Heart rate (b/min)	71.5±2.3	79.7±2.2*	76.9±2.1	0.368
Serum glucose (mg/dL)	128.2±8.1	115.0±3.2	106.0±5.2*	0.002
Serum insulin (μU/mL)	10.9±1.5	8.4±1.0**	7.2±1.0*	0.002
HOMA index	3.47±0.66	2.37±0.29**	1.88±0.29*	0.001
Serum prolactin (μg/L)	29.4±3.4	1.4±0.3**	0.8±1.5**	0.000
Serum testosterone (μg/L)	2.3±0.45	3.5±0.95	4.1±0.75	0.135
Serum estradiol (ng/L)	27.9±3.2	67.4±7.3*	66.3±6.2*	0.009
Plasma ACTH (ng/L)	62.4±6.1	45.4±3.6**	35.6±3.2*	0.002
Serum cortisol (μg/L)	236.2±17.6	160.2±6.0**	144.6±10.5*	0.002
Urinary cortisol (μg/day)	558.1±69.1	118.5±12.2**	115.4±7.8*	0.002
Tumor volume (mm³)	224.3±31.9	158.1±46.2*	133.7±56.7*	0.084

Normal ranges: fasting serum glucose: 60-110 mg/dL; fasting serum insulin: 0-15 μU/mL: serum prolactin: 5-25 μg/L; serum testosterone: 3.0-9.0 μg/L; serum estradiol: 20-240 ng/L: plasma ACTH: 10-100 ng/L; morning serum cortisol: 50-200 μg/L; urinary cortisol: 35-135 μg/24 h. Serum testosterone levels refer exclusively to the male population as well as serum estradiol levels refer exclusively to female population. Serum estradiol levels were measured during the early follicular phase in patients with normal menstrual cycle (duration ≤40 days) and independently from the menstrual cycle in patients with olgoamenorrhea or amenorrhea. The P column refers to the significance between the three time points of the evaluation; *.p<0.05 compared to baseline; **:p<0.01 compared to baseline

Table 4: Clinical, biochemical and radiological features of patients with Cushing's disease who experienced a treatment escape during the long-term treatment with cabergoline

Parameter	Baseline (5 patients)	6-months Treatment (5 patients)	12-months Treatment (5 patients)	18-months Treatment (4 patients)	P
Body mass index (kg/m²)	26.1±1.6	26.6±1.5	25.9±1.3	26.6±1.6	0.546
Waist/hip ratio	1.07±0.05	1.00±0.0.6*	0.99±0.06*	1.08±0.07	0.027
Systolic blood pressure (mmHg)	155.0±4.5	136.0±4.0*	139.0±3.7*	142.5±2.5	0.040
Diastolic blood pressure (mmHg)	100.0±4.2	89.0±2.9*	91.0±3.7*	95.0±2.9	0.027
Heart rate (b/min)	65.6±1.6	72.2±4.5	66.6±4.0	69.0±2.6	0.557
Serum glucose (mg/dL)	131.4±5.6	119.0±4.4*	119.8±3.5*	125.3±1.3	0.209
Serum insulin (μU/mL)	11.4±2.3	10.4±2.4	9.6±2.1*	8.2±2.5	0.029
HOMA index	3.72±0.82	2,99±0.69*	2.84±0.65*	2.53±0.77	0.038
Serum prolactin (μg/L)	22.7±4.4	2.2±0.4*	1.8±0.3*	1.1±0.1*	0.018
Serum testosterone (μg/L)	2.4	4.1	2.9	2.3	-
Serum estradiol (ng/L)	27.5±5.9	55.5±4.3	46.3±8.2	67.0±12.7	0.072
Plasma ACTH (ng/L)	74.0±3.6	57.5±5.0*	59.0±13.1	76.0±10.4	0.022
Serum cortisol (μg/L)	245.4±25.0	157.4±11.0*	200.6±22.9	250.3±23.3	0.112
Urinary cortisol (μg/day)	569.6±86.4	172.6±24.3*	377.8±68.1	623.0±49.5	0.019
Tumor volume (mm³)	277.8±92.0	-	268.7±85.5	-	0.465

Normal ranges: fasting serum glucose: 60-110 mg/dL; fasting serum insulin: 0-15 μU/mL: serum prolactin: 5-25 μg/L; serum testosterone: 3.0-9.0 μg/L; serum estradiol: 20-240 ng/L: plasma ACTH: 10-100 ng/L; morning serum cortisol: 50-200 μg/L; urinary cortisol: 35-135 μg/24 h. Serum testosterone levels refer exclusively to the male population as well as serum estradiol levels refer exclusively to female population. Serum estradiol levels were measured during the early follicular phase in patients with normal menstrual cycle (duration ≤40 days) and independently from the menstrual cycle in patients with oligoamenorrhea or amenorrhea. The P column refers to the significance between the four time points of the evaluation; *.p<0.05 compared to baseline.

found in patients who did not reach the two years of treatment because of a treatment escape between 6 and 18 month of follow-up. Body mass index, waist to hip ratio, blood pressure and heart rate values before and during long-term treatment in stably responsive patients and those who experience a treatment escape are summarized in **Table 3** and **Table 4**.

Biochemical evaluation: Glucose tolerance further improved during the 12-24 months of treatment with cabergoline in those patients persistently controlled during the two years of treatment. In these patients, serum fasting glucose and insulin levels, glucose and insulin peak after glucose load and HOMA index significantly decreased and then remained stable after 6-12 months of treatment. The prevalence of diabetes mellitus and impairment of glucose tolerance changed from 25% and 37,5% at baseline to 10% and 20%, respectively. Serum testosterone and estradiol levels progressively increased in men and women, respectively. Plasma ACTH as well as serum cortisol progressively decreased, in line with the changes in urinary cortisol excretion. The urinary cortisol levels, despite some fluctuations, remained normal. A re-increase of urinary cortisol together with serum ACTH and cortisol levels was observed in patients who had a treatment escape between 6 and 18 months of treatment. In these patients, glucose tolerance slightly worsened and serum testosterone and estradiol slightly decreased with the treatment escape. The biochemical parameters before and during the long-term treatment in patients stably responsive and those who experienced a treatment escape are summarized in **Table 3** and **Table 4**.

Radiological evaluation: A trend to a significant decrease of tumor volume was observed during the entire period of treatment. In particular, tumor shrinkage was observed in 50% of patients with a detectable lesion at baseline and responsive to cabergoline treatment. The shrinkage was observed after 6-month treatment in two patients and after 12-month treatment in the remaining two patients. All four patients demonstrated further tumor shrinkage at 12- and/or 24-month treatment. A stable tumor volume was found in the remaining 50% of patients. No change in tumor volume was observed in the seven patients in whom a treatment escape occurred. A slight increase of tumor volume was observed in the only resistant patient who continued treatment for 12 months. **Fig. 4** shows the radiological picture of two patients, one responsive and one resistant to cabergoline after 12 months of treatment.

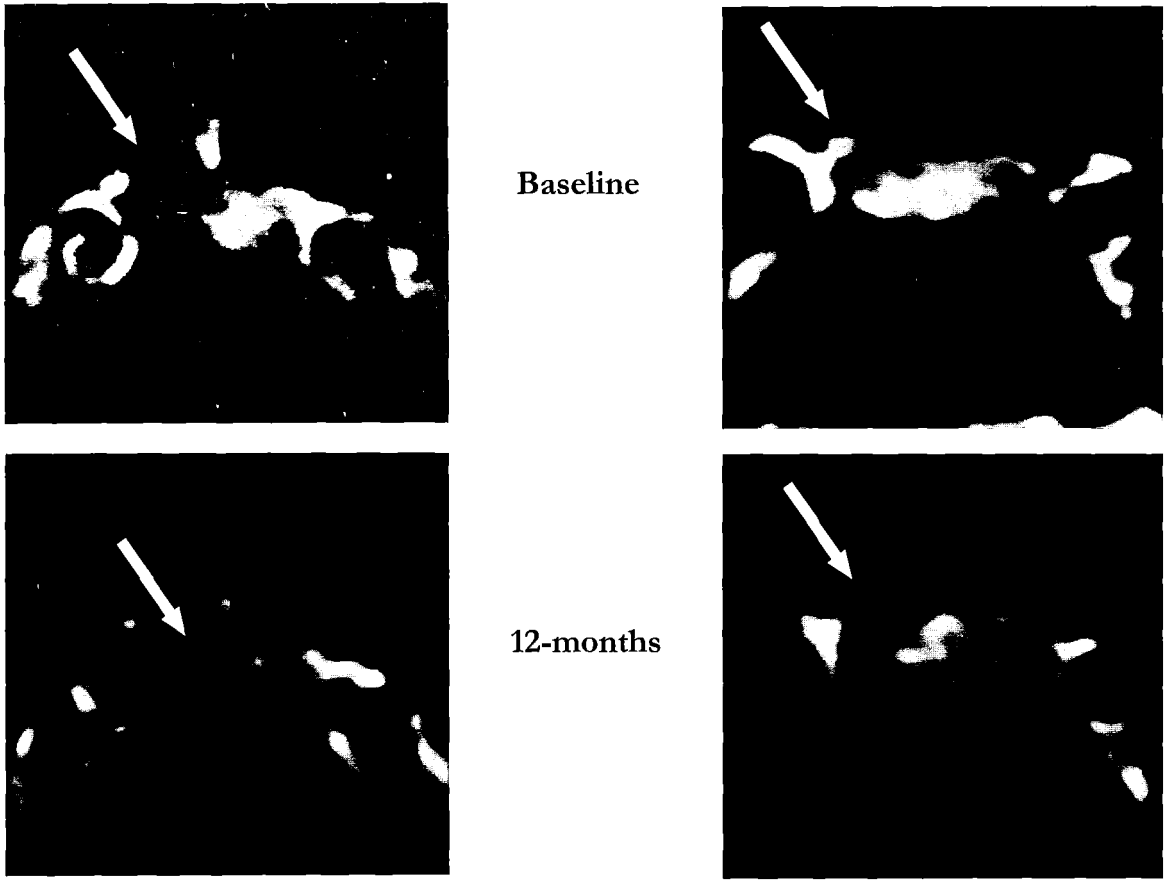

Figure 4: MRI of a patient responsive (left) and a patient resistant (right) to cabergoline treatment at baseline and after 12 months of treatment

Comparison with dopamine receptor expression

At the standard RT-PCR, D_2 was expressed in 14 (88%) whereas D_4 receptor in three (19%) of the corticotroph pituitary tumors. Particularly, the D_{2short} isoform was expressed in two (14,3%), D_{2long} in seven (50%) and both isoforms in the remaining five (35,7%) positive cases. D_4 receptor was associated to D_{2long} in two cases and to both D_2 isoforms in the remaining case. Moreover, D_2 receptor was not expressed only in the two resistant patients, whereas it was expressed in one and not evaluated in the remaining two resistant patients. D_2 was not evaluated also in one full and long-term responsive patient and one partial responsive patient who experienced a treatment escape. At the qRT-PCR, a variable relative number of D_2 receptor was found among the CD patients. The dopamine receptor and D_2 receptor isoforms as well as the relative amount of D_2 receptor in the different cases of corticotroph tumors are shown in **Fig. 5**. At IHC, a perfect concordance with the RT-PCR data was found. A significant association was found between D_2 receptor expression and short-term (χ^2=8.24, p=0.025) but not long-term (χ^2=2.14, p=0.467) responsiveness to cabergoline treatment. The D_{2short} expression was not significantly associated to short-term (χ^2=2.08, p=0.214) but only to long-term (χ^2=9.86, p=0.003) responsiveness to cabergoline treatment. D_{2short} expression was also significantly associated to the occurrence of significant tumor shrinkage at 1-year treatment (χ^2=7.33, p=0.018). Furthermore, the relative amount of D_2 receptor was directly correlated with the urinary cortisol change (r=0.816, p=0.000) and inversely correlated to the cabergoline dose (r=-0.611, p=0.004) after short-term but not after long-term treatment with cabergoline (r=-0.05, p=0.916 and r=-0.519, p=0.232, respectively. The resistance or escape to treatment was always associated to the absence of D_2 receptor or the presence of only D_{2long} isoform whereas the presence of D_{2long} isoform together with D_{2short} or D_4 receptor was never associated to treatment resistance or escape. The D_2 receptor amount was the best independent predictive parameter of the short-term responsiveness (B=0.93, p=0.000) whereas the expression of D_{2short} was the best independent predictive parameter of the long-term responsiveness (B=0.80; p=0000) to cabergoline treatment, either as normalization of cortisol secretion or tumor shrinkage.

Tolerance

No significant side effect was experienced in the patients with the exception of severe hypotension (80/50 mmHg) associated with asthenia and weakness in 2,

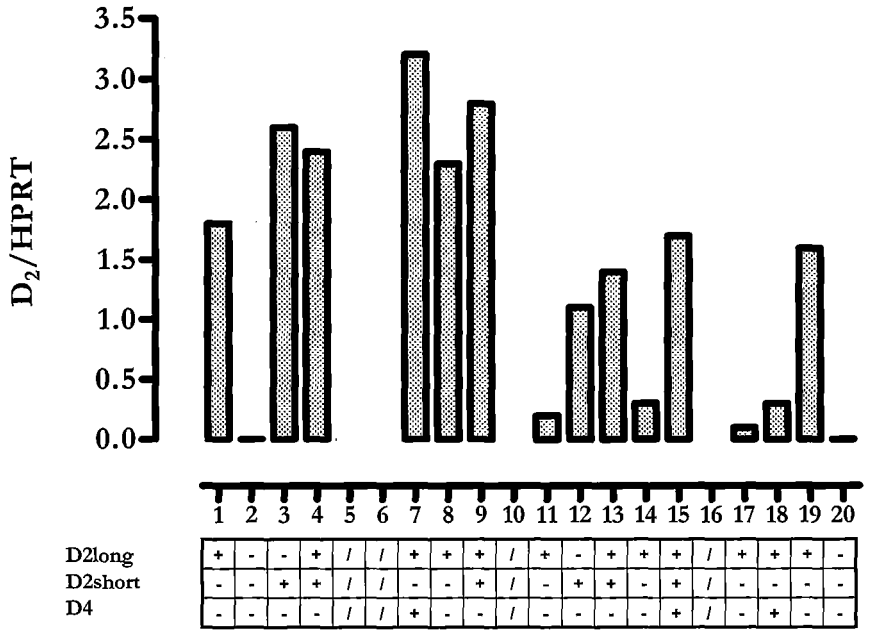

Figure 5: Relative amount of D_2 measured by quantitative RT-PCR and the correspondent dopamine receptor subtypes and D_2 isoforms as obtained at the standard RT-PCR in the 20 cases of CD.

who had already experienced this hypotensive crisis before the diagnosis of CD. Cabergoline was withdrawn in these patients at 12 and 18 months of treatment, respectively. A transient moderate asthenia and muscle weakness was registered in 4 patients, whereas a transient mild dizziness with nausea was reported by another patient during the first period of treatment. These side effects did not require treatment withdrawal. The patient who had hypotension was taking 7 mg of cabergoline per week, whereas the other patients experiencing side effects were taking from 3 to 7 mg of cabergoline per week.

Discussion

The current study is the first evaluating the long-term effectiveness of the treatment with the dopamine agonist cabergoline in CD. The results of the study demonstrate that: 1) 3-months treatment at a variable dose between 1 to 3 mg/week can control cortisol secretion in around 60% of patients; 2) 12-24 months treatment, at a variable dose between 1 to 7 mg/week, induces or maintains control of cortisol secretion in around 40% of patients; 3) cabergoline treatment stabilizes or decreases tumor size in the majority of responsive and not in resistant patients; 4) cabergoline treatment improves blood pressure, glucose tolerance, and gonadal and sexual function significantly in responsive as well as in resistant patients; 5) the dopamine D_2 receptor is expressed in around 80% of corticotroph pituitary tumors and is the best predictor of short-term responsiveness, whereas the presence of the D_{2short} isoform is the best predictor of long-term effectiveness of cabergoline treatment in patients with CD.

CD is a chronic disorder resulting from an inappropriate and prolonged exposure to increased cortisol secretion, secondary to increased ACTH secretion by a corticotroph pituitary tumor (1). CD is associated with increased morbidity and mortality due to cardiovascular disease, mainly related to the occurrence of visceral obesity, hypertension and glucose intolerance, which, together with dyslipidemia and thrombosis diathesis, cause premature atherosclerosis and increased risk of cardiac ischemia and stroke (2,11,14,15). These consequences of long-term exposure to excessive cortisol make CD a severe disease and require an as early as possible cure. Indeed, despite the persistence of cardiovascular risk factors (2,12) morbidity and mortality of patients cured from CD seem to be similar to those registered in normal population (2,15). Cure of CD is one of the major challenge in medicine. Neurosurgery is the first line treatment of CD. However, the transsphenoidal removal of the pituitary tumor is associated to a 60-80% success rate (8,16,17). Failure of neurosurgery is mainly due to a very small size of the microadenoma or the presence of multiple microadenomas or diffuse hyperplasia, not detectable at conventional imaging techniques or even at surgical exploration (8,16,17). Moreover, neurosurgical outcome is also affected by disease relapses during the 10 years after surgical remission in 10-20% of cases, reducing the final success rate of neurosurgery to around 50% of cases (8,16,17). Pituitary radiotherapy and bilateral adrenalectomy are second choice treatments in patients not cured by neurosurgery. However, these treatment modalities frequently result in important adverse events.

Indeed, radiotherapy is associated with a success rate ranging between 30 to 70%, but the cure of the disease is delayed up to 10 years at the cost of severe adverse events, mainly hypopituitarism (18). On the other hand, bilateral adrenalectomy is associated with a nearly 100% success rate, but it is related to a mortality rate of 2%, because of hypoadrenalism, which makes the patient life-long dependent on replacement therapy with glucocorticoids and mineralocorticoids, while it frequently induces the development of an invasive pituitary tumor during decades after surgery which is associated with skin hyperpigmentation, namely the Nelson's syndrome (19). Medical treatment plays a minor role in the treatment of CD, being usually performed before neurosurgery, or after pituitary radiotherapy in order to obtain a rapid hormonal normalization before the definitive cure (3). Medical treatment is usually performed with adrenal blocking drugs, which do not act at the level of the pituitary tumor (3). On the other hand, although several neuromodulatory drugs acting at the pituitary level have been used in the treatment of CD, no single agent has ever been demonstrated sufficiently effective to achieve a widespread clinical use in the management of the disease (20-22). The only neuromodulatory drug, which had a relevant although controversial role in the treatment of CD, was bromocriptine. Bromocriptine was hypothesized to induce an inhibition of ACTH secretion and/or cell growth in corticotroph pituitary tumors, acting through dopamine receptors presumably expressed in the pituitary corticotroph tumors. Indeed, bromocriptine induced a significant inhibition or normalization of cortisol secretion in about 40% of cases after short-term treatment (3,4,23,24). However, controversial results with a success rate ranging from 0 to 50% were obtained by the different studies (3). Moreover, normalization of cortisol secretion and/or tumor shrinkage were only sporadically reported after long-term treatment, demonstrating that only a subset of patients with CD responded to chronic bromocriptine treatment (3,5,6). On the other hand, the more recently developed and more potent dopamine agonist cabergoline was reported to induce a normalization of ACTH secretion and/or a significant shrinkage of different types of corticotroph tumors, namely two ACTH-secreting pituitary tumor associated to Nelson's syndrome (25,26) a silent ACTH (27), an aberrant ACTH-secreting (28) and a mixed ACTH and PRL-secreting pituitary tumor (29). Moreover, in a recent pilot study on 10 patients with CD, cabergoline has been demonstrated to induce a significant inhibition of cortisol secretion in 60% of patients with CD, suggesting a higher effectiveness of cabergoline than bromocriptine in the treatment of CD (7). However, no study has ever evaluated the effectiveness of long-term treatment with cabergoline in patients with CD.

The results of the current study have confirmed in a larger population of patients with CD that short-term treatment with cabergoline is able to induce an inhibition of cortisol secretion in around 75% of patients, although a full response with normalization of cortisol secretion was observed in 35%, and a partial response in the remaining 40%. The current study demonstrates a long-term success of cabergoline as well. Indeed, the study included 20 consecutive patients treated with cabergoline during the last 5 years. Among the 20 patients, 15 were treated for 1 year whereas 8 of them for at least 2 years. The results of long-term treatment with cabergoline demonstrated that among patients with an initial responsiveness, 33% experienced a treatment escape and 13% drug intolerance, requiring cabergoline withdrawal between 6 and 18 months of treatment. Only half of the number of patients with a short-term responsiveness continued to respond with stable normal cortisol levels for a period longer than one year, while more than one third of patients with CD respond to cabergoline with normal cortisol secretion for a as long as 1-2 years. It has to be outlined that although 25% of patients are completely resistant and an additional 35% withdrew cabergoline for treatment escape or intolerance despite normalization of cortisol secretion, 30% of patients partially responsive to short-term treatment became fully responsive with normalization of cortisol levels after increasing the cabergoline dose during long-term treatment, suggesting that the cabergoline dose and the period of treatment necessary to normalize cortisol secretion are extremely variable for each patient with CD.

An important finding of the current study is the demonstration that cabergoline treatment is able to significantly improve two of the main systemic complications of CD, namely hypertension and glucose intolerance. Indeed, blood pressure levels and the prevalence of hypertension significantly decreased already after short-term and nearly normalized in the majority of patients during long-term treatment. Similarly, serum glucose and insulin levels as well as the HOMA index, a parameter of insulin resistance, and the prevalence of impaired glucose tolerance or diabetes mellitus significantly decreased after short-term and even more decreased during long-term treatment. Moreover, the significant decrease of blood pressure values, and the slight decrease of glucose and insulin levels as well as the decrease in the prevalence of hypertension and glucose intolerance also in patients who were resistant or escaped from treatment, suggest that cabergoline might directly and positively influence blood pressure and glucose tolerance independently of the change in cortisol secretion. The direct effect of dopamine agonists on hypertension is largely documented and justified by the expression of dopamine receptors in the vascular system, where they mediate a dopamine induced relaxing effect, with a consequent

reduction of peripheral resistance (30). The direct effect of dopamine agonists on glucose tolerance is supported by the evidence that bromocriptine is able to improve glucose homeostasis in patients with type 2 diabetes mellitus, so that it has been proposed as an alternative medical treatment for the disease (31). The effect of bromocriptine on glucose homeostasis seems to be related to a potentiation of insulin-mediated hepatic glucose production or splanchnic glucose uptake. On the other hand, the possibility that dopamine agonists improve glucose homeostasis through inhibition of catecholamines release by the adrenal medulla has to be taken into account as well. The results of the current study are in agreement with these hypotheses and suggest that even when no effect on cortisol secretion was found, cabergoline may have a consistent impact on blood pressure and glucose intolerance. It is also important to outline that cabergoline is able to significantly improve gonadal and sexual function, ameliorating sexual performance in men and inducing the appearance of menses or menstrual cycle regularity in women with CD, in patients responsive and to a lesser extent also in patients resistant to cabergoline treatment. As bromocriptine was demonstrated to improve menstrual disorders in women with different forms of disorders of the menstrual cycle and gonadal and sexual function in men with gonadal failure or sexual impotence apparently not dependent from hyperprolactiemia (32,33), it is possible that cabergoline has both an indirect action, through the normalization of cortisol secretion, but also a direct action on gonadal and sexual function of patients with CD. The possibility that cabergoline induces such an effect through the suppression of PRL levels in these patients cannot be ruled out.

The biological basis of the effectiveness of dopamine agonists in the treatment of pituitary tumors is dopamine receptor expression on the tumor cells. The effectiveness of dopamine agonists in inducing inhibition of hormone secretion and shrinkage of tumor volume depends on the number of functional D_2 receptors expressed by the tumor in PRL- and GH-secreting pituitary tumors (34,35). Moreover, recently, the expression of D_{2short} rather than D_{2long}, has been suggested to be associated with a better *in vitro* and *in vivo* response to dopamine agonists in patients with clinically non-functioning pituitary adenomas (36,37). The results of the current study confirm that D_2 receptors are expressed, with an heterogeneous representation of the D_2 receptor isoforms, in the majority of corticotroph pituitary tumors removed from patients with CD and demonstrate that the effectiveness of cabergoline on ACTH and cortisol inhibition, as well as on tumor shrinkage is significantly correlated to the tumoral expression of D_2 receptor. The most interesting and innovative finding of the paper is represented by the demonstration

that the number of D_2 receptor is the most predictive parameter for the short-term responsiveness, whereas the expression of D_{2short} isoform is the most predictive parameter of long-term responsiveness to cabergoline treatment. Moreover, since treatment escape was always associated to the absence of D_{2short} and/or D_4 receptors and frequently associated to the presence of D_{2long} receptors, it is possible to hypothesize that D_4 and/or D_{2short} but not D_{2long} receptors are able to prevent treatment escape in corticotroph pituitary tumor.

The different success rate observed with bromocriptine in previous studies and with cabergoline in the present study may be related to the different molecular, biochemical and pharmacological characteristics of the two dopamine agonists, and particularly to the higher specificity and affinity for D_2 receptor and the longer duration of action of cabergoline (38,39). Alternatively, the superiority of cabergoline to bromocriptine could be explained hypothesizing that cabergoline rather than bromocriptine is able to induce apoptosis through D_{2short} or D_4 receptors, or that the drug is able to induce dimerization of the dopamine receptors, which is a phenomenon, which is known to be agonist-dependent and to potentiate the agonist actions (40). Finally, as dopamine receptors have been demonstrated to be expressed in the adrenal cortex and because cabergoline has been shown to exert a potent effect in modulating cortisol secretion by adrenal cortex cells *in vitro* (41), the possibility that cabergoline more than bromocriptine may induce *in vivo* a direct inhibition of cortisol secretion at the level of the adrenal, cannot be ruled out.

In conclusion, the current study demonstrated that cabergoline is effective in controlling CD in more than half of the patients during short-term treatment and in more than one third of the patients during long-term treatment, without major side effects. Moreover, cabergoline is able to control hypertension and glucose tolerance both in patients who responsive and those apparently not normalizing the cortisol secretion, protecting patients from the major cause of death, namely cardiovascular disease. Furthermore, cabergoline is able to improve gonadal and sexual function in both responsive as well as in resistant patients. The responsiveness to cabergoline treatment is mainly related to the number of D_2 receptors, but predominantly on the presence of D_{2short} isoform and/or the co-expression of D_2 and D_4 receptors. Therefore, cabergoline can be considered a useful tool in the management of persistent and/or recurrent CD.

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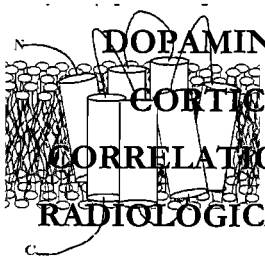
3

DOPAMINE RECEPTOR EXPRESSION AND DOPAMINE AGONIST EFFECTIVENESS IN CORTICOTROPH PITUITARY TUMORS: CORRELATION WITH CLINICAL, BIOCHEMICAL RADIOLOGICAL AND PATHOLOGICAL FEATURES OF PATIENTS WITH CUSHING'S DISEASE



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Submitted for publication

ABSTRACT

Dopamine receptors are expressed in the majority of corticotroph pituitary tumors and dopamine agonists are suggested to be effective in controlling the cortisol hypersecretion in a selective group of patients with Cushing's disease (CD). In order to characterize the tumors expressing D_2 receptors and the profile of patients which might respond to the treatment with D_2 agonists, the current study has been designed with the aim to correlate D_2 receptor expression and the effects of the D_2 agonist cabergoline to clinical, biochemical and radiological features of patients, as well as to pathological features of tumors removed form the patients during neurosurgery. The study included 72 patients with a diagnosis of CD. In all patients, the D_2 receptor expression as evaluated by immunohistochemistry (IHC) was correlated with the pathological features of the tumors and the characteristics of the patients, whereas in a subgroup of these patients, the D_2 receptor expression, evaluated by standard and/or quantitative RT-PCR, or the cabergoline responsiveness in terms of cortisol normalization, was correlated to the pathological features of the tumors and the characteristics of the patients. The results of the study demonstrated that D_2 receptor expression is significantly associated with the presence of neural pituitary tissue close to the tumors and/or nerve fibers within or surrounding the tumors, presumably expression of the tumor origin from the intermediate zone of the pituitary gland, as well as with the presence of a corticotroph hyperplasia rather than adenoma at the histological evaluation, and PRL staining within the tumor. Moreover, dividing the tumors according to the intensity of D_2 staining, those with the highest D_2 expression, are also associated with lower age, longer disease duration, a relative resistance to dexametasone and CRH and prevalence of hyperprolactinemia, together with an undetectable tumor at the imaging techniques and failure at neurosurgery. The initial response to cabergoline treatment was also associated with some of these characteristics of patients and tumors. However, the long-term responsiveness to cabergoline treatment was not significantly associated with any of these features, but only to the tumor expression of the short isoform of D_2 receptor and/or the expression of D_4 receptors in the tumor, equally deriving from the intermediate zone or the anterior lobe of the pituitary gland. In conclusion, the current study demonstrated that the corticotroph tumors expressing D_2 receptors derive from either the intermediate zone or the anterior lobe of the pituitary gland. Moreover, whereas the tumors originating from the intermediate zone generally express higher number of D_2 receptors and are associated to the best initial responsiveness to cabergoline treatment, those with the expression of short isoform of D_2, deriving from both the

intermediate zone and the anterior lobe of the pituitary gland are associated to a long-term responsiveness to cabergoline treatment.

Introduction

Dopamine D_2 receptors are expressed in the anterior and intermediate lobe of the pituitary gland, where they mediate the tonic inhibitory control of hypothalamic dopamine on prolactin (PRL) and melanocyte-stimulating hormone (MSH) secretion, respectively (1-3). However, the widespread expression of D_2 receptors throughout the entire pituitary gland suggests their expression and function in the majority of the pituitary cell types (4). Indeed, D_2 receptors were recently demonstrated to be expressed in corticotroph cells, including not only MSH-secreting cells of the intermediate zone but also the ACTH-secreting cells of the anterior lobe of the pituitary gland (5). The persistence of a functional D_2 receptor expression on tumor mammotroph and somatotroph cells led to the extensive therapeutic use of dopaminergic drugs in the treatment of PRL and GH-secreting pituitary tumors, where cabergoline was demonstrated to be more effective than bromocriptine in suppressing hormone secretion and inducing tumor shrinkage (6-12). The persistence of functional D_2 receptors has recently been also found in tumor corticotroph cells (13), supporting the observation of the effectiveness of bromocriptine in controlling ACTH and cortisol secretion in a selected group of patients with ACTH-secreting pituitary tumors (14-17). In these tumors, the dopamine agonist cabergoline was shown to be more effective than bromocriptine, as it was able to induce normalization of cortisol secretion in more than one third of patients, significantly improving the clinical picture related to Cushing's disease (CD) (13,18). The reason why the treatment with dopamine agonist is effective only in a subgroup of patients with CD is unknown. However, the responsiveness to bromocriptine was previously hypothesized to be associated with corticotroph tumors mainly deriving from the intermediate zone of the pituitary gland, which might be characterized by a specific hormonal pattern (14-17). The correlation with D_2 receptor expression in corticotroph tumors was not investigated so far. On the other hand, recently the responsiveness to cabergoline was demonstrated to be associated with the presence of dopamine receptors and the D_2 receptor number expressed in corticotroph tumors (13). The correlation with the hormonal pattern and, especially, the origin of these tumors in the pituitary gland was not investigated so far.

The current study was designed with a twofold purpose: 1) to correlate the expression pattern of dopamine and, mainly, D_2 receptors in corticotroph pituitary tumors with the clinical, biochemical, radiological and pathological features of CD in a large series of patients and 2) to correlate the effectiveness of treatment with the dopamine agonist cabergoline with the clinical, biochemical, radiological and

pathological features of a selected group of patients with CD, in order to delineate the profile of patients that might respond to medical treatment with cabergoline.

Patients and Methods

Patients: Seventy-two patients (57 females and 15 males, 18-80 years) with the diagnosis of CD entered the study after their informed consent had been obtained. Fifty-two patients were admitted to the Department of Internal Medicine of Erasmus Medical Center of Rotterdam, The Netherlands, and 20 were admitted to the Department of Molecular and Clinical Endocrinology and Oncology of "Federico II" University of Naples, Italy. All the patients were selected on the basis of the availability of clinical, biochemical and radiological features at the diagnosis and a pathological sample and report of the corticotroph pituitary lesion removed by neurosurgery. The 20 patients admitted to the Department of Molecular and Clinical Endocrinology and Oncology of "Federico II" University of Naples, Italy represent a selected group of patients which had persistence of CD after unsuccessful neurosurgery and were subjected to medical treatment with the dopamine agonist cabergoline. The diagnosis of CD was based on: 1) increase in daily urinary cortisol excretion with inappropriately high plasma ACTH concentrations; 2) increase in basal serum cortisol concentrations with lack of the physiological circadian rhythm; 3) failure of urinary and serum cortisol suppression after low dose but greater than 50% decrease after high dose oral dexamethasone suppression test or a decrease of serum cortisol of at least 70 μg/L in the 7 hours continuous intravenous dexamethasone suppression test, and/or a higher than 20% increase of cortisol levels after a corticotropin releasing hormone (CRH) stimulation test (19-22). The diagnosis of CD was supported by the presence of a pituitary tumor at magnetic resonance imaging of a pituitary gland or at bilateral inferior petrosal sinus sampling (BIPSS) (23,24). The histological and immunohistochemical study of the tumor removed during neurosurgery documented a corticotroph pituitary lesion (adenoma or hyperplasia) in all cases, definitely confirming the diagnosis of CD in the totality of patients.

Study design: The study protocol included four different objectives. The first step was the evaluation of D_2 receptor expression by immunohistochemistry (IHC) in the complete series of 72 cases included in the study. D_2 receptor expression was scored by a semiquantitative method as follows: absent, weak, moderate and strong. The cases were subsequently divided into four groups on the basis of D_2 receptor

expression as follows: group 1, cases with absent expression, group 2, cases with weak expression, group 3, cases with moderate expression, group 4: cases with strong expression of D_2 receptor. The second step of the study protocol was the correlation between the D_2 receptor expression, evaluated by IHC, and different clinical, biochemical and radiological features of the patients and different pathological features of the tumor samples. The clinical parameters included sex and age at the diagnosis of the disease as well as disease duration, and outcome of neurosurgery. The biochemical parameters included baseline urinary cortisol levels, serum PRL levels and the prevalence of hyperprolactinemia, cortisol suppression after dexamethasone test and cortisol stimulation after CRH test. The radiological parameters, represented by the pituitary MRI findings, included the presence or absence of a clear-cut adenoma and the size of the adenoma (micro or macroadenoma). The pathological parameters included the histological features (basophilic hyperplasia *vs* basophilic or non basophilic adenoma, the presence or absence of Crooke's cells surrounding the tumor, presence or absence of neural pituitary tissue close to the tumor and the immunohistochemical features. These included ACTH, MSH, PRL and neurofilament immunostaining, in order to characterize the corticotroph, the melanotroph and the lactotroph cells and the presence of nerve fibers in the pituitary tumor and gland. A semiquantitative score, dividing the staining intensity in absent, weak, moderate and strong was also used for the ACTH, MSH, PRL and neurofilament staining. The third step of the study protocol was the correlation between dopamine receptor expression (D_1-D_5 subtypes and D_2 receptor isoforms), evaluated by standard RT-PCR, or the D_2 receptor number, evaluated by quantitative RT-PCR and the clinical, biochemical, radiological and pathological features of the 21 and 17 cases in which the two different molecular studies were performed. The fourth step was the correlation between the effectiveness of the dopamine agonist cabergoline treatment and the clinical biochemical, radiological and pathological features in the 20 patients which entered the treatment trial. The correlation study was performed considering for each single correlation only the cases in which the parameter under study were available. In particular, the clinical evaluation was available for 100% of cases, whereas the biochemical evaluation was available in around 80% of patients, the radiological evaluation in around 70% of patients and the pathological evaluation in 100% of patients. The D_2 receptor expression of 20 of the patients included in this study have been already described in a previous paper (13), and the D_2 agonist responsiveness of 10 of these latter patients and additional 10 patients included in this study have also been already described in a previous paper (18), where no correlation study between D_2 receptor expression or D_2 agonist responsiveness and

clinical, biochemical, radiological and especially pathological features of the patients and/or tumors were performed. The study protocol was approved by the local Ethical Committees and it was in accordance with the Helsinki Doctrine on Human Experimentation.

Immunohistochemistry: The IHC study was performed on tissue samples fixed in 10% paraformaldehyde overnight and embedded in paraffin, according to a previous report (13). The formalin-fixed and paraffin-embedded tissue samples were cut in 5 μm thick sections. These sections were deparaffinized, dehydrated, exposed to microwave heating in citric acid buffer at 100°C for 15 min, rinsed in tap water followed by phosphate buffer solution (PBS) and subsequently incubated for 15 min in normal goat serum (1:10 dilution in PBS + 5% bovine serum albumin, BSA). The sections were then incubated overnight at 4°C with a rabbit anti-human D_2 receptor polyclonal antibody (Chemicon International, Temecula, CA, USA) in a dilution of 1:500. A standard streptavidin-biotinylated-alkaline phosphatase or -peroxidase complex (ABC kit, Biogenix, San Ramon, CA) was used to visualize the bound antibodies. Negative controls for the IHC included: *a*) omission of the primary antibody; *b*) preabsorption of the antibodies with the respective immunizing receptor peptide (at a concentration of 100 nM), both performed in sequential sections. Immunostaining for ACTH, MSH and PRL as well as GH, TSH, FSH and LH was also performed on sequential sections using specific antibodies at the standard dilution. Histological evaluation was performed on hematoxylin-eosin stained sequential sections. Positive and negative controls were represented by D_2 receptor immunostaining on sections of dopamine agonist sensitive and resistant PRL-secreting pituitary tumors, respectively and carried out in the same experiments on the corticotroph pituitary tumors. The specificity of the D_2 receptor antibody was tested by immunoblotting using a corticotroph pituitary tumor sample, documented in a previous report (13).

Reverse transcriptase-polymerase chain reaction: The RT-PCR study was performed in samples quickly frozen on dry ice and stored in a freezer at -80°C, according to a previous report (13). The study included only samples in which the tumor tissue represented at least 90% of the section at the histological evaluation, to exclude the influence of normal pituitary tissue on the results of the studies. Briefly, messenger RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS Oslo, Norway). Complementary DNA (cDNA) was synthesized using the poly A^+ mRNA captured on the Dynabeads Oligo $(dT)_{25}$ in a Tris-buffer together with 1 mM of

each deoxynucleotide triphosphate, 10 U RNAse inhibitor, and 2 U avian myeloblastosis virus Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK). The amplification reaction mixture contained cDNA template, 0.5 U SuperTaq (HT Biotechnology Ltd., Cambridge, UK), 50 μM of each deoxynucleotide triphosphate (HT Biotechnology Ltd., Cambridge, UK), 5 pmol of each of a pair of oligonucleotide primers specific for human D_1-D_5 receptor subtypes or the hypoxantine ribosyl transferase (HPRT) in a Tris-buffer. The sequences of the primers for D_1-D_5 including D_2 isoforms and HPRT have been previously published (13). The PCR reaction was carried out in a DNA thermal cycler (Perkin Elmer Cetus Instruments, Gouda, The Netherlands), according to the following protocol: initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and extension at 72 °C for 1 min, final extension at 72 °C for 10 min. Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation for two DR subtypes, D_1 and D_5, of which the genes are intron-less, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. Amplification of the cDNA samples with the HPRT specific primers served as positive control for the quality of cDNA. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of the DR subtypes and HPRT, 0.01 μg of human brain cDNA was amplified in parallel with the cDNA samples of each examined pituitary corticotroph tumor. The quantitative RT-PCR for D_2 receptor (D_{2short}+D_{2long}) was performed by using TaqMan Gold nuclease assay and ABI PRISM 7700 sequence detection system (The Perkin-Elmer Corporation; Forster City, CA, USA) for real time amplification, according the manufacturer's recommendations. The specific primers and probe for D_2 receptor were the following: (GenBank accession no. AF050737 coding sequence), sense (1062-1083), antisense (1177-1156), and probe (1080-1115). The amount of D_2 receptors mRNA was determined by meand of a standard curve generated in each experiment from known amount of genomic DNA. For the determination of the amount of HPRT mRNA, the standard curve was obtained by including dilutions of a pool of cDNA known to contain HPRT. The final amount of D_2 receptor mRNA was calculative as relative to the amount of HPRT mRNA and it is given in arbitrary units.

Treatment protocol: Cabergoline was administered at the initial dose of 1 mg/week and monthly increased by 1 mg/week till normalization of daily urinary cortisol excretion. Plasma ACTH and serum and urinary cortisol were evaluated

monthly. At the short-term (3 months) treatment, a $\geq$ 25% decrease of daily urinary cortisol excretion was considered a significant clinical response to cabergoline. Moreover, patients who achieved a $\geq$50% decrease with normalization of urinary cortisol levels were considered as full responders whereas those who achieved a $\geq$50% without normalization or >25% and <50% decrease of urinary cortisol levels were considered as partial responders to cabergoline. Patients who achieved a <25% decrease of urinary cortisol levels were considered to be resistant to cabergoline treatment. At the long-term (12-24 months) treatment a persistent normal urinary cortisol levels was considered a criterion for the responsiveness to the treatment.

Hormonal assays: ACTH levels were measured by immunoradiometric assay, using a commercially available kit. Serum and urinary cortisol levels were measured by radioimmunologic assay, using commercially available kits.

Statistical analysis: Data are expressed as mean±SD. The comparison between parameters were performed by Analysis of Variance followed by Newman-Keuls' test or Student *t* test. The association between parameters was calculated by χ^2 test. The correlation study was performed by regression analysis calculating the Pearson's or the Spearman's coefficient. A logistic multiple regression analysis including the parameters significantly correlated to D_2 receptor expression and/or D_2 agonist effectiveness in the correlation study was performed in order to evaluate the parameter(s) significantly and independently associated and best predicting the D_2 receptor expression and/or D_2 agonist effectiveness. Significance was set at 5%.

Results

Objective 1

Histological evaluation and Immunohistochemistry for pituitary hormones: A basophilic adenoma was documented in 52 (72.2%), basophilic hyperplasia in 11 (15.3%), a chromofobe adenoma in seven (9.7%) and an acidophilic adenoma in two (2.8%) cases. Two of the basophilic hyperplasias were classified as basophilic invasion of neurohypophysis. The presence of neural pituitary tissue or Crooke's cells was found in 24 (33.3%) and 14 (19.4%) cases, respectively. ACTH staining was documented in all 72 cases (100%) in correspondence with the adenomatous or hyperplastic tissue: it was homogeneous in 65 (90.3%) and heterogeneous or scattered throughout the tumor tissue in seven (9.7%) cases; it was weak in 12

(16.7%), moderate in 18 (25%) and strong in 42 (58.3%) cases. MSH staining was found in 56 (77.8%) cases: it was represented by scattered cells throughout the tumor in 18 (25%), and by a significant area of the tumor in the remaining 38 (52.8%) cases. Moreover, among the positive cases, MSH staining was weak in 12 (31.8%), moderate in 15 (39.5%) and strong in 11 (28.7%) cases. A total or partial overlap between ACTH and MSH staining was found in the majority of tumors. PRL staining was documented in 33 (45.8%) cases. However, it was represented by scattered or grouped normal cells (acidophilic ACTH-negative cells) within or around the tumor tissue in 16 (22.2%), and scattered or grouped tumor cells (basophilic ACTH-positive cells) in 15 (20.8%) cases. The remaining two cases (2.8%) were characterized by a diffuse PRL staining completely overlapping with the ACTH-positive tumor cells (mixed ACTH/PRL-secreting pituitary tumors). Moreover, among the positive cases PRL staining was weak in 8 (24.2%), moderate in 12 (36.4%) and strong in 13 (39.4%) cases. No significant immunoreactivity was found for GH, TSH, FSH and LH in all cases, being only represented by scattered cells around the tumor tissue, probably belonging to surrounding normal pituitary tissue. Neurofilament staining was found in 14 (19.4%) cases, although the number of spots were scarce in six (8.3%), intermediate in two (2.8%) and abundant in the remaining six (8.3%) cases. The presence of neural pituitary tissue close to the tumor and/or neurofilament immunostaining within or surrounding the tumors was found in 27 (37.5%), whereas the absence of both pathological features was found in the remaining 45 (62.5%) cases.

Immunohistochemistry for the D_2 receptor: A specific immunoreactivity for D_2 receptor was found in 59 (82.0%) cases. It was localized in ACTH- and MSH-, ACTH/MSH, ACTH/PRL- and MSH/PRL-positive tumoral cells and in all normal PRL positive as well as in a percentage of non PRL positive cells localized within tumoral tissue or normal pituitary. The immunoreactivity was homogeneously distributed in 25 (42.4%) and heterogeneously distributed within the tumoral tissue in 34 (57.6%) cases. It was scored as weakly positive in 14 (23.7%), moderately positive in 27 (45.8%), and strongly positive in the remaining 18 (30.5%) cases. Absent immunoreactivity for D_2 receptor was found in 13 cases (18.0%).

Objective 2

The summary of the comparison between D_2 receptor expression in the corticotroph pituitary tumors and the clinical, biochemical, radiological and pathological features of the patients is shown in **Table 1** and **Table 2.**

Table 1: Comparison between clinical, biochemical, radiological and pathological features of patients with D_2-positive and D_2-negative corticotroph tumors

Parameters	D_2-negative tumors	D_2-positive tumors	p
Clinical parameters			
Sex			
Male (%)	23.1	20.3	NS
Female (%)	76.9	79.7	NS
Age (yrs)	41.0±18.2	40.7±1.3	NS
Disease duration (yrs)	2.9±1.3	3.4±1.6	NS
Cure by neurosurgery (%)	69.2	59.3	NS
Biochemical parameters			
Urinary cortisol levels (μg/day)	372.8±144.5	441.4±161.3	NS
Cortisol suppression at DMX test (%)	91.5±45.9	71±14.2	NS
Cortisol stimulation at CRH test (%)	86.0±41.0	55.4±45.0	NS
Serum PRL levels (μg/L)	17.3±8.4	23.8±14.1	NS
Hyperprolactinemia (%)	15.4	44.1	NS
Radiological parameters			
Undetectable tumor (%)	23.1	50.9	NS
Size of the detectable tumor			
Microadenoma (%)	41.5	88.1	NS
Macroadenoma (%)	38.5	11.9	NS
Pathological parameters			
Tumor type			
Basophilic adenoma	61.5	74.6	NS
Basophilic hyperplasia	7.7	16.9	<0.05
Non basophilic adenoma	30.7	9.6	<0.05
Neural pituitary and/or neurofilament (%)	0	45.8	<0.05
Crooke's cells (%)	38.5	15.2	NS
ACTH staining (%)	100	100	NS
MSH staining (%)	61.5	81.4	NS
PRL staining (%)	0	28.8	<0.05

Table 2: Comparison between clinical, biochemical, radiological and pathological features of patients with different scores of D_2 expression in corticotroph tumors

Parameters	Group 1 D2-	Group 2 D2+	Group3 D2++	Group 4 D2+++	p
Clinical parameters					
Sex					
male (%)	23.1	21.4	22.2	16.7	NS
female (%)	76.9	78.6	77.8	83.3	NS
Age (yrs)	41.0±18.2	54.8±11.0*§	40.4±12.4	30.5±8.1	<0.001
Disease duration (yrs)	2.9±1.3*	2.9±1.0*	3.2±1.6	4.1±1.8	<0.001
Cure by neurosurgery (%)	69.2	71.4	63.0	44.4	NS
Biochemical parameters					
Urinary cortisol levels (µg/day)	372.8±144.5	384.0±108.9	469.5±186.9	445.1±143.4	NS
Cortisol suppression at DMX test (%)	91.5±45.9*	98.0±39.3*	83.9±22.2*	51.1±16.5	<0.001
Cortisol stimulation at CRH test (%)	86.0±41.0*	90.0±51.4*	50.3±44.2	35.2±30.6	<0.01
Serum PRL levels (µg/L)	17.3±8.4*	15.6±11.2*	23.3±10.3*	36.3±12.7	<0.001
Hyperprolactinemia (%)	15.4*	21.4*	48.2	77.8	<0.01
Radiological parameters					
Undetectable tumor (%)	23.1*	28.6	37.1	66.7	<0.05
Size of the detectable tumor					
Microadenoma (%)	41.5*§	85.7	85.2	94.1	<0.05
Macroadenoma (%)	38.5	14.3	14.8	5.9	NS
Pathological parameters					
Tumor type					
Basophilic adenoma	61.5	71.5	77.8	72.2	NS
Basophilic hyperplasia	7.7*	7.1*	14.8	27.8	<0.05
Non basophilic adenoma	30.7*	21.4*	7.4	0	<0.05
Neural pituitary and/or neurofilament (%)	0*§	7.1*§	44.4	77.8	<0.001
Crooke's cells (%)	38.5*	28.6	14.8	5.6	<0.05
ACTH staining (%)	100	100	100	100	NS
MSH staining (%)	61.5	71.4	77.8	94.4	NS
PRL staining (%)	0§	28.6	37.0	16.7	<0.05

The p values refers to the significance of the changes in the parameters between all groups, in case of numeric parameters, and to the difference between group 1 and /or 2 and group 4 in case the parameter is represented by a prevalence the prevalence, where the p values refers *p<0.05 *vs* group 4; §p<0.05 *vs* group 3

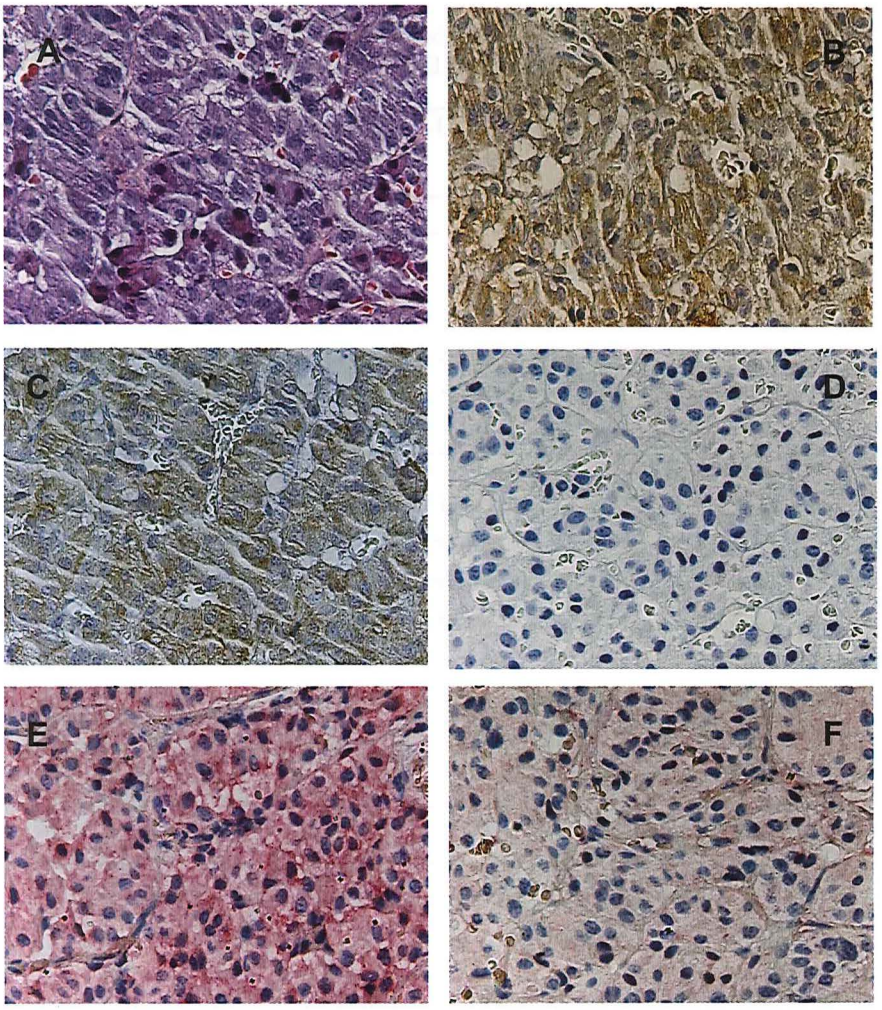

Figure 1: Examplary case of a corticotroph tumor presumably deriving from the anterior lobe of the pituitary gland.. A: histology; B: ACTH immunostaining; C: MSH immunostaining; D: neurofilament immunostaining; E: D_2 receptor immunostaining; F: D_2 receptor immunostaining *plus* peptide (displacement). The histology shows a basophilic adenoma, with weak MSH staining and absence of neurofilament staining. Magnification is 400X

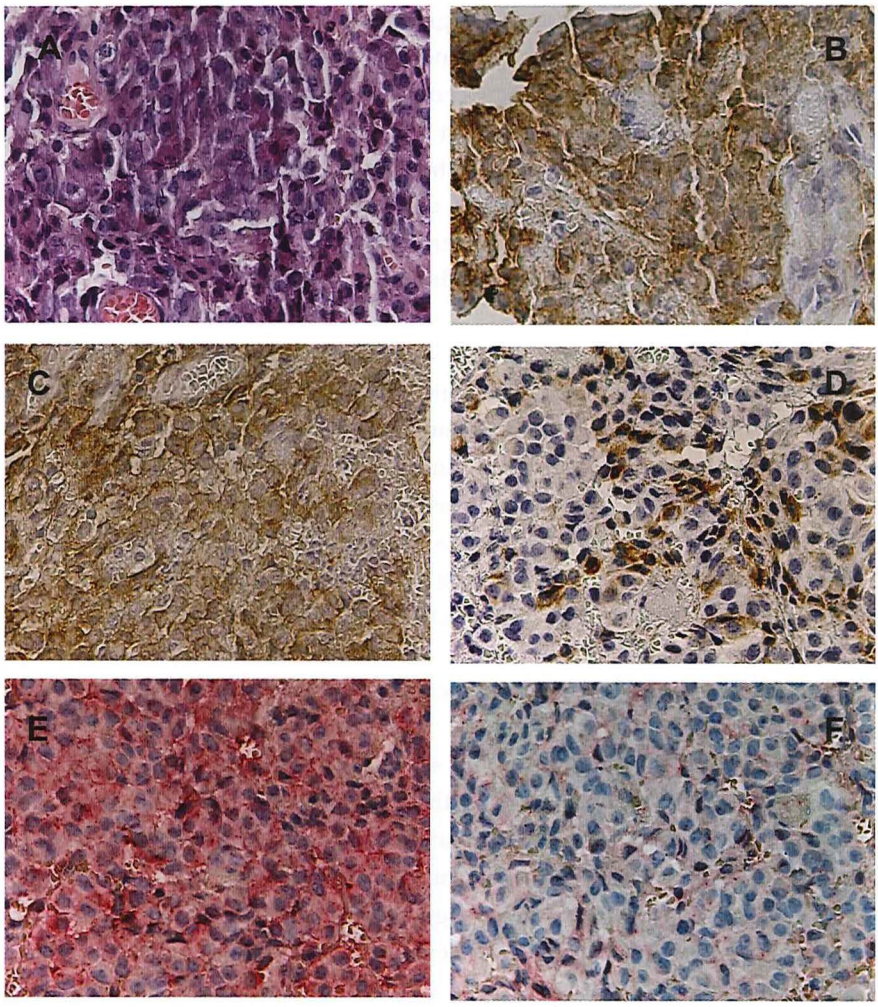

Figure 2: Examplary case of a corticotroph tumor presumably deriving from the intermediate zone of the pituitary gland.. A: histology; B: ACTH immunostaining; C: MSH immunostaining; D: neurofilament immunostaining; E: D_2 receptor immunostaining; F: D_2 receptor immunostaining *plus* peptide (displacement). The histology shows a basophilic adenoma, with strong MSH staining and presence of neurofilament staining. Magnification is 400X

Correlation with clinical data: No significant association was found between clinical parameters of patients with CD and D_2 receptor expression in corticotroph tumors. However, the comparison between the groups of patients with the different scores of D_2 receptor staining showed that age at diagnosis was significantly lower in patients of groups 3 and 4 compared to those of group 2, whereas disease duration was longer in patients of the group 4 compared to those of groups 1 and 2. Moreover, patients of group 4 had a higher, although not significantly, prevalence of neurosurgical failure than the patients of the other groups of the study.

Correlation with biochemical data: No significant association was found between biochemical parameters of patients with CD and D_2 receptor expression in corticotroph tumors. However, the comparison between the groups of patients with the different scores of D_2 receptor staining showed that the degree of cortisol suppression after a dexamethasone test was significantly lower in patients of group 4 compared to those of groups 1-3, whereas the degree of cortisol stimulation after a CRH test was significantly lower in patients of group 4 compared to those of group 1 and 2. Significantly higher PRL levels were found in patients of group 4 compared to those of groups 1-3, whereas the prevalence of hyperprolactinemia was significantly higher in patients of group 4 compared to those of groups 1 and 2.

Correlation with radiological data: No significant association was found between radiological parameters of patients with CD and D_2 receptor expression in corticotroph tumors. However, the comparison between the groups of patients with the different scores of D_2 receptor staining showed that the prevalence of a radiologically undetectable tumor was higher in patients of group 4 compared to those of group 1. In addition, the prevalence of a microadenoma was significantly higher in group 4 and 3 compared to those in group 1 and 2 and, consequently the prevalence of a macroadenoma higher, although not significantly, in patients of group 1 than in those of group 4.

Correlation with histological and immunohistochemical data: A significant association was found between some pathological features and D_2 receptor expression in corticotroph tumors. Indeed, the prevalence of basophilic hyperplasia was higher whereas the prevalence of non-basophilic adenoma was lower in tumors expressing D_2 receptors compared to those not expressing the receptor. Conversely, the prevalence of a basophilic adenoma was similar in the two types of tumors. The prevalence of neural pituitary tissue close to the tumors and/or neurofilament

staining within or surrounding the tumors was significantly higher in cases positive than in those negative for D_2 receptor, whereas the presence of Crooke's cells surrounding the tumors was similar in the two types of tumors. No significant association was found between D_2 receptor and ACTH, MSH staining; conversely, the presence of PRL staining in the tumor cells was significantly associated with D_2 receptor expression, all tumors negative for D_2 receptors being also negative for PRL staining. The comparison between the groups of patients with the different scores of D_2 receptor staining showed that the intensity of D_2 receptor staining was significantly and directly correlated with that of MSH staining ($r=0.82$; $p<0.01$) and inversely correlated with ACTH staining ($r=-0.65$; $p<0.05$). The prevalence of neural pituitary tissue and/or neurofilament staining was significantly higher in patients of group 3 and 4 than those of group 1 and 2. The prevalence of PRL immunostaining in the tumors was not significantly different between the 4 groups and especially between the patients of group 2-4. However, the intensity of the staining was significantly correlated with the intensity of D_2 receptor expression ($r=0.85$; $p<0.01$).

Two examples of corticotroph pituitary tumors, one likely deriving from the intermediate area, with the presence of neurofilaments within the tumor and a strong immunostaining for MSH, and another one likely deriving from the anterior lobe, without the presence neurofilaments and a weak positivity for MSH, are shown in **Fig. 1 and Fig. 2**, respectively.

Objective 3

Reverse transcriptase-polymerase chain reaction study: At the standard RT-PCR, D_2 receptor was expressed in 18 of 21 (85.7%) cases. The D_{2long} isoform only was found in nine (50%), D_{2short} only in three (16.7%), and both D_2 isoforms in 6 (33.3%) positive cases. D_4 receptor was expressed in four cases (19.0%), associated with the expression of D_{2long} in two and to both D_2 isoforms in the remaining 2 cases. No expression of other DRs were found. At the quantitative RT-PCR, a variable number of D_2 receptor was found in the 17 cases. A complete correspondence was found between the results of RT-PCR and those of IHC studies.

Correlation with clinical, biochemical, radiological and pathological data: No significant association was found between D_{2long} or D_{2short} isoform and any clinical, biochemical, radiological and pathological features of the patients. In

particular, the prevalence of neural pituitary tissue and/or neurofilament staining was similar in the tumors expressing and those not expressing the D_{2short} isoform, as well as in those expressing and those not expressing the D_{2long} isoform (**Fig. 3**). In particular, neural pituitary tissue and/or neurofilament staining were present in 33.3% of cases expressing the D_{2short} isoform and in 44.4% of cases not expressing the D_{2short} isoform of the D_2 receptor. On the other hand, the prevalence of the D_{2short} isoform of the D_2 receptor was similarly present in 42% of cases associated and in 55% of cases not associated with neural pituitary tissue and/or neurofilament staining. Conversely, all 4 cases expressing D_4 receptor displayed the presence of neural pituitary tissue and/or neurofilaments, a relatively weak ACTH staining, a relatively strong MSH staining and the presence of a significant PRL staining within the tumor tissue. A significantly higher number of D2 receptor was found in tumors associated than in those not associated to neural pituitary tissue close to and/or neurofilament staining within or around the tumor (2.1 ± 1.0 *vs* 0.6 ± 0.5, $p<0.05$).

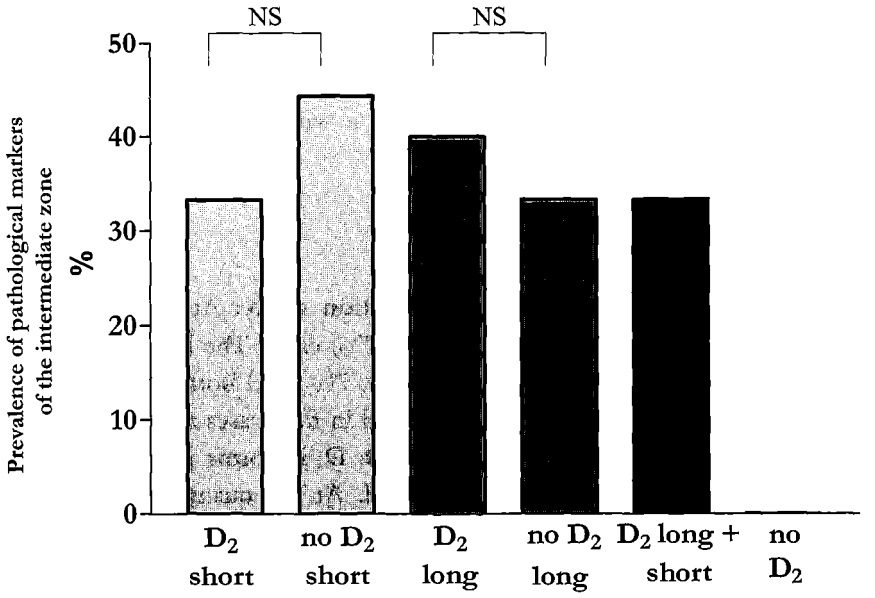

Figure 3: Prevalence of pathological markers of intermediate zone in the tumors with different expression of D_2 receptor isoforms.

Objective 4

Effectiveness of cabergoline treatment: An initial full response to 3-months treatment with cabergoline at the median dose of 3 mg/week was observed in 35%, whereas a partial response was observed in 40% and a resistance in 25% of patients. However, during the long-term follow-up, 75% of the partially responsive patients became fully responsive patients, but 33.3% of responsive patients had a treatment escape requiring drug withdrawal. A long-term and full response to cabergoline was observed in 40% of patients who started the treatment.

Correlation with clinical, biochemical, radiological and pathological features: Considering the short-term response to cabergoline treatment, a significantly lower cortisol response to dexamethasone (60.2±18.4 *vs* 92.4±24.3, $p<0.05$) and CRH (44.2±22.1 *vs* 80.2±29.4, $p<0.05$), as well as higher PRL levels (32.2±9.4 vs 19.2±7.1, $p<0.05$) were found in patients responsive compared with those not responsive to the treatment. A significant association was found between the response to cabergoline treatment and the presence of neural pituitary tissue and/or neurofilaments in the tumor ($\chi^2=6.7$, $p<0.05$, ***Fig. 4***). A significant correlation was found between the response to the treatment, in terms of percentage of cortisol inhibition, and intensity of D_2 receptor staining at IHC ($r= 0.75$; $p<0.01$) or number of D_2 receptor at the quantitative RT-PCR ($r=0.81$; $p<0.01$). No significant correlation was found between the response to treatment and the presence of D_{2short} or D_{2long} or D_4 receptors. Considering the long-term response to cabergoline treatment, no significant association was found between any clinical, biochemical, radiological and pathological parameter and the response to treatment. A significant association was found between the expression of D_{2short} isoform ($r=0.75$, $p<0.05$) but not D_{2long} in the tumors and the response to the treatment. D_4 receptor was always associated with a long-term responsiveness to cabergoline treatment. In particular, the long-term persistence of cortisol normalization was found in 42% of cases associated with neural pituitary tissue and/or neurofilament staining around the tumor, which expressed D_{2short} isoform and/or D_4 receptors, and in 38% of cases not associated with these pathological features, which also expressed D_{2short} isoform. Treatment escape was found in all cases not expressing D_{2short} or D_4 receptors and expressing only the D_{2long} isoforms of the D_2 receptor.

Logistic analysis: At multiple regression analysis, the best independent predictive parameters of D_2 receptor expression in the corticotroph tumors were the presence of neural pituitary tissue and/or neurofilament staining around the tumor (B=0.80,

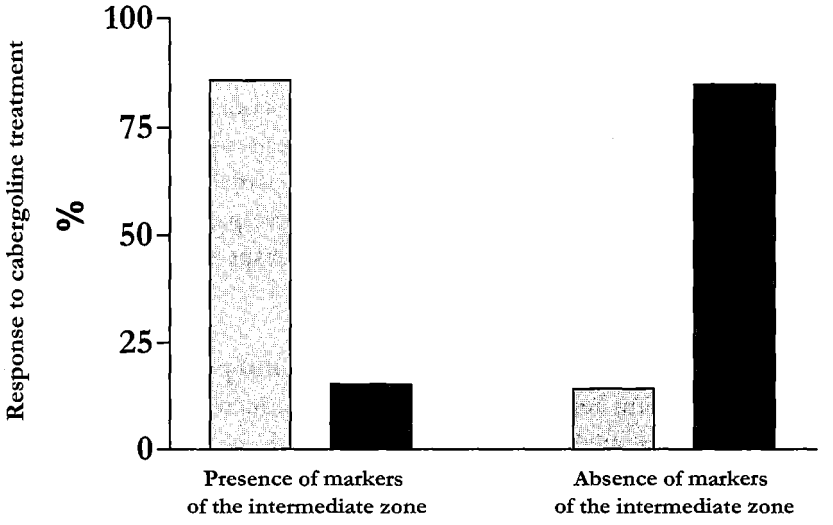

Figure 4: Prevalence of the presence or absence of the neural pituitary tissue and/or neurofilament staining in the corticotrophin tumors of patients fully responsive (light gray) or not fully responsive (dark gray) to short-term cabergoline treatment.

$p<0.001$), followed by the PRL staining within the tumors ($B=0.69$, $p<0.01$). The best predictive parameters of the short-term responsiveness to cabergoline treatment were the number of D_2 receptor ($B=0.93$, $p<0.001$), followed by the presence of neural pituitary tissue or neurofilament staining around the tumor ($B=0.71$, $p<0.01$), and the presence of hyperprolactinemia ($B=0.58$, $p<0.05$). Finally, the best independent predictive parameter of persistence of responsiveness to cabergoline treatment was the expression of the short isoform of D_2 receptor in the tumors ($B=0.80$; $p<0001$). In tumors presumably deriving from the anterior lobe, the PRL staining was the best clinical predictive parameter of D_2 receptor expression and responsiveness to cabergoline treatment, whereas in the tumors presumably deriving from the intermediate zone of the pituitary gland, the presence of hyperprolactinemia was the best clinical predictive parameter of the responsiveness to cabergoline treatment.

Fig. 5 represent a scheme of the distribution of D_2 positive and D_2 negative corticotroph tumors in the anterior lobe and intermediate zone of the pituitary gland

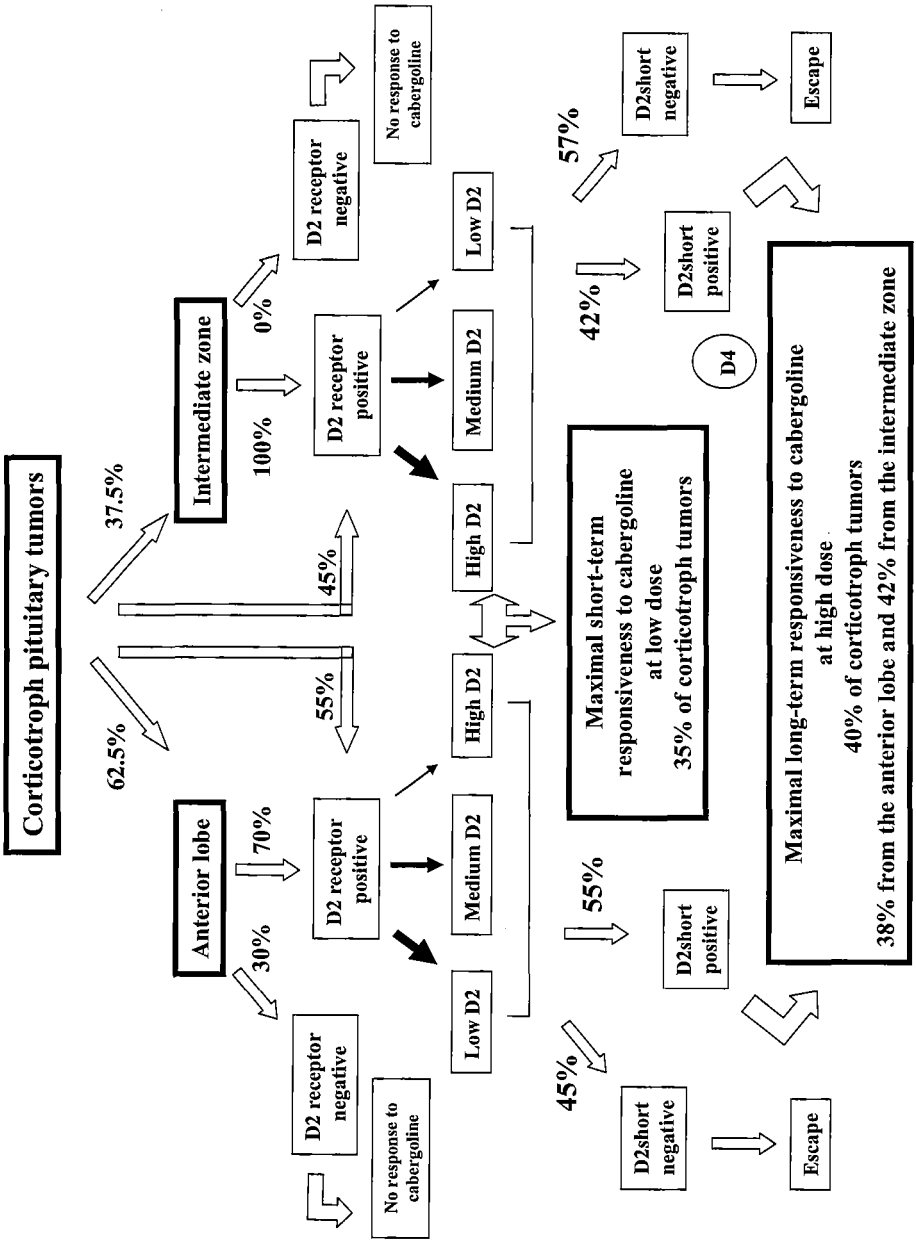

Figure. 5: scheme of the distribution of D_2 positive and D_2 negative corticotroph tumors in the anterior lobe and intermediate zone of the pituitary gland and their association with the dopamine receptor pattern and responsiveness to cabergoline treatment in the respective patients with Cushing's disease.

and their association with the dopamine receptor pattern and responsiveness to cabergoline treatment.

Discussion

The dopamine agonist cabergoline has been demonstrated to be effective in controlling hormone secretion and tumor size in around 40% of patients with CD unsuccessfully treated by neurosurgery, acting through the dopamine D_2 receptor

expressed in more than 80% of corticotroph pituitary tumors. The main question which arises from these observations is how to predict D_2 receptor expression and cabergoline responsiveness in patients with CD. The current study was designed with the purpose to give an answer to this question.

The results of the current study demonstrated that D_2 receptor expression in corticotroph pituitary tumors is not associated with any clinical, biochemical or radiological feature of patients with CD, but it seems to be significantly associated with the presence of neural pituitary tissue close to the tumor, or nerve fibers within or surrounding the tumors, which might be considered as pathological markers of a tumor origin from the intermediate zone of the pituitary gland. Starting with this assumption, the current study demonstrated that tumors originating from the intermediate zone represent around 45% of the D_2 expressing tumors and 0% of the not D_2 expressing tumors, suggesting that the tumor origin from the intermediate zone is sufficient to predict the D_2 expression while the tumor origin from the anterior lobe is not sufficient to exclude D_2 receptor expression. In other words, the tumors presumably deriving from the anterior lobe, which represented more than 60% of all corticotroph tumors, expressed D_2 receptors in 70% of cases, whereas the tumors presumably deriving from the intermediate zone, which represented less than 40% of all corticotroph tumors, expressed D_2 receptor in 100% of cases.

The different origin of corticotroph pituitary tumors is a controversial issue in endocrine pathology. It is well known, mainly from studies on experimental animals, that two populations of corticotroph cells can be recognized in the normal pituitary gland, the corticotroph cells of the anterior lobe, which process the precursor compound propiomelanocortin (POMC), almost exclusively release ACTH and stain mostly for ACTH and rarely also for MSH, and the corticotroph cells of the intermediate lobe, the melanotroph cells, which also process POMC but mainly releases MSH and stain for ACTH and/or MSH (25-28). The intermediate lobe is

the portion of the pituitary gland located between the anterior epithelial and the posterior neural lobe of the gland: its major characteristic is the relative lack of vascular supply, which remains at the periphery of the cells, and the presence of innervation, which arises from the hypothalamus and, passing through the neural lobe, forms synaptic connections directly to the cells (29,30). The most important innervation reaching the intermediate lobe is formed by the dopaminergic nerves, as also confirmed by the dopaminergic regulation of this area (31,32). However, the intermediate lobe is well defined in rats but not in humans, where it undergoes a progressive involution to a group of colloid-filled cysts that mingle with the neural lobe (33). Nevertheless, the melanotroph cell population of the residual intermediate zone has been recently demonstrated to express dopamine receptors, confirming the possible dopaminergic regulation of melanotroph cell growth and/or function in humans as well (5). These observations have suggested that two different types of corticotroph tumors may develop in the pituitary gland: a first type, which derives from the corticotroph cells of the anterior lobe, and a second type, which derives from the melanotroph cells of intermediate zone of the gland (15-17). An important differential characteristic would be the absence in the former and the presence in the latter of a dopaminergic regulation, and presumably, of dopamine receptors (15-17). Considering the pathological characteristics of the intermediate zone, the markers which might permit a distinction between tumors presumably deriving from the anterior lobe and those presumably deriving from the intermediate zone are the presence of neural pituitary tissue close to the tumors and/or the presence of nerve fibers within or surrounding the tumors. Theoretically, MSH immunostaining within the tumors should also be a marker of the melanotroph cells and not the corticotroph cells, but MSH was demonstrated to characterize the majority of corticotroph tumors independently of their origin (34). The results of the current study confirmed that MSH immunostaining cannot be considered a marker of the intermediate zone, as it was found in the great majority of tumors with a complete overlap with ACTH immunostaining. However, on the basis of the presence of neural tissue actually it was possible to differentiate the two tumor types. It is important to outline that neurofilaments are a specific, but not sensitive indicator of tumors localized in the intermediate zone, since they were present only in a portion of cases clearly associated with the neural pituitary tissue. On the other hand, their presence can be the only marker of the intermediate zone, when the tumor sample obtained at neurosurgery does not contain neural pituitary tissue, due to the loss of a portion of the tumor or the preservation of the complete neural pituitary.

The original hypothesis on the double origin of corticotroph tumors and their differential expression of dopamine receptors was based on the comparison of pathological features of the corticotroph tumors derived from a limited series of patients with CD and their responsiveness to treatment with the dopamine agonist bromocriptine (15-17). The hypothesis was also supported by the association between the responsiveness to dopamine agonist treatment and the presence of relative resistance to dexamethasone, the occurrence of hyperprolactinemia, and the persistence of hypercortisolism after neurosurgery, together with the evidence at the pathological examination of a corticotroph cell hyperplasia more than a well defined adenoma located at the border between anterior and posterior lobe of the pituitary gland (15-17). These observations supported the hypothesis that corticotroph tumors responsive to dopamine agonists probably resulted from dopamine depletion, which induced not only lactotroph hyperplasia with consequent hyperprolactinemia but also melanotroph hyperplasia with consequent hypercortisolism. The tumors deriving from these cells should be relatively resistant to dexamethasone, because melanotroph cells not regulated by peripheral glucocorticoids under physiological conditions, and resistant to neurosurgery, probably because they are frequently associated with corticotroph hyperplasia rather than adenoma (15-17).

The results of the current study, performed in a large series of patients, seem to be at least in partial agreement with this hypothesis. Indeed, D_2 receptor expression is always expressed in tumors derived from the intermediate zone, and significantly associated to basophilic hyperplasia, together with the presence of PRL immunostaining within the tumor. The lack of association between the different clinical, biochemical and radiological features presumably related to the intermediate zone and the D_2 receptor expression can be explained by the observation that most tumors deriving from the anterior lobe also expressed D_2 receptors, and represent more than half of D_2 positive corticotroph tumors. Moreover, it is interesting to notice that the cases with the highest expression of D_2 receptors were represented mostly by those deriving from the intermediate zone and are associated, beyond the pathological markers of the intermediate zone, with different features presumably related to this area, such as a relative resistance to dexamethasone and CRH and hyperprolactinemia, together with the undetectability of tumor at the imaging techniques, and in part to the failure of neurosurgery, possibly directly related to corticotrophin hyperplasia in the intermediate zone of the pituitary gland. These data suggested that tumors deriving from the intermediate zone represent those with

the higher number of D_2 receptors and, presumably, those with the greater response to dopamine agonists.

The results of the current study confirmed the existence of a correlation between the response to short-term treatment with cabergoline and the number of D_2 receptors in corticotroph pituitary tumors. Moreover, the responsiveness to short-term treatment with cabergoline was also significantly associated to the origin of the tumors from the intermediate zone: 75% of the responsive cases presumably were derived from the intermediate zone, and the remaining 25% from the anterior lobe. The patients responsive to cabergoline treatment also had a relatively lower response to dexamethasone and CRH, and increased PRL levels. These results suggested that the initial responsiveness to cabergoline treatment is mainly dependent on the receptor number on the corticotroph tumors, and that the source of ACTH in the majority of cases with a high D_2 receptor expression is probably derived from the intermediate zone of the pituitary gland. In other words, the majority of tumors deriving from the intermediate zone and a minority of tumors derived from the anterior lobe seem to have a significant D_2 receptor expression and, therefore, initial responsiveness to cabergoline treatment. However, part of patients initially responsive to cabergoline experienced an escape from treatment, whereas a percentage of patients not completely responsive to short-term treatment normalized urinary cortisol levels during long-term treatment with cabergoline. The most surprising result of the current study is the lack of correlation between the responsiveness to cabergoline treatment and the origin of the tumor from the intermediate area, during long-term treatment. Conversely, a significant association was found between long-term responsiveness to cabergoline treatment and the expression of the short isoform of the D_2 receptor in the corticotroph tumors: these tumors equally derived from intermediate zone or anterior lobe of the pituitary gland. On the other hand, all cases with D_4 receptor expression were long-term responsive to cabergoline treatment and all cases were presumably deriving from the intermediate area of the pituitary gland, suggesting that the expression of D_4 is a marker of the intermediate area and together with the short isoform of the D_2 receptor, predictive of the long-term responsiveness to cabergoline treatment.

In conclusion, the current study demonstrated that D_2 receptor is expressed in more than 80% of cases, among which 55% seem to derive from the corticotroph cells of the anterior lobe and 45% from the melanotroph cells of the intermediate zone of the pituitary gland. The tumors deriving from the intermediate zone are usually associated to a moderate or a strong D_2 receptor expression whereas those deriving

from the anterior lobe present the complete spectrum from absent to strong D_2 receptor expression. The tumors with a strong D_2 receptor expression and higher initial responsiveness to cabergoline treatment are mostly deriving from the intermediate zone of the pituitary gland. However, the persistent response to cabergoline treatment is not predictable on the basis of the origin from the intermediate zone of the pituitary gland or the number of D_2 receptors, but mainly on the basis of the expression of the short isoform of D_2 receptors, which equally occurs in tumors derived from the intermediate zone and the anterior lobe and/or the expression of D_4 receptor, which is a specific marker of the intermediate zone of the pituitary gland.

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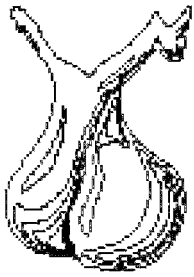

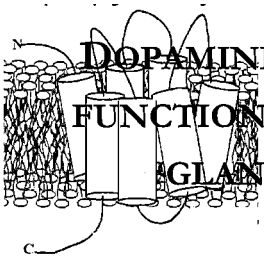

DOPAMINE RECEPTOR EXPRESSION AND FUNCTION IN HUMAN NORMAL ADRENAL GLAND AND ADRENAL TUMORS

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Journal of Clinical Endocrinology and Metabolism, 2004;89:4493-4502

ABSTRACT

Dopamine is known to play a role in the modulation of aldosterone and catecholamine secretion from the adrenal gland, where dopamine receptors (DR), in particular the DR type 2 (D_2), have been found to be expressed. DR expression has also been demonstrated in some types of benign adrenal tumors. The aims of the current study were to evaluate DR expression and D_2 localization in the normal adrenal gland and in different types of benign and malignant adrenal tumors, as well as to evaluate the *in vitro* effects of the dopamine agonists bromocriptine and cabergoline on hormone secretion in nontumoral adrenal cells. Adrenal tissues from 25 patients subjected to adrenal surgery for different diseases were studied. These included three normal adrenals; five adrenal hyperplasias; four aldosterone-secreting, two cortisol-secreting and two clinically nonfunctioning adrenal adenomas; two aldosterone-secreting, two cortisol-secreting and two androgen-secreting adrenal carcinomas; and 3 pheochromocytomas. In all tissues, DR and D_2 isoform (D_{2long} and D_{2short}) expression was evaluated by RT-PCR. D_2 localization was also evaluated by immunohistochemistry using a specific polyclonal antibody while D_2-like receptor expression was evaluated by receptor-ligand binding study, using the radiolabeled D_2 analog ^{125}I-epidepride. The effects of bromocriptine and cabergoline on baseline and ACTH and/or angiotensin II-stimulated aldosterone, cortisol and androstenedione secretion were evaluated in cell cultures derived from 5 different adrenal hyperplasia. At RT-PCR, both D_1-like and D_2-like receptors were expressed in all normal and hyperplastic adrenals. D_2 and D_4 were expressed in aldosterone- and cortisol-secreting adenomas, cortisol-secreting carcinomas and clinically nonfunctioning adenomas, whereas no DR was expressed in aldosterone and androgen-secreting carcinomas. D_2, D_4, and D_5 were expressed in pheochromocytomas. In all D_2 positive tissues both D_2 isoforms were expressed, with the exception of 1 case of aldosterone-secreting adenoma and the cortisol-secreting carcinomas, in which only the D_{2long} isoform was expressed. D_2-like receptor expression was confirmed at receptor-ligand binding study. At immunohistochemistry, D_2 was mainly localized in the zona glomerulosa and reticularis of the adrenal cortex and to a lesser extent in the zona fasciculata and medulla of normal and hyperplastic adrenal tissue. In the positive tumors, D_2 was localized in the tumoral cells. At the *in vitro* study, a significant inhibition of both baseline and ACTH-stimulated aldosterone secretion was found after high-dose cabergoline but not bromocriptine administration; and a significant inhibition of angiotensin-II stimulated aldosterone secretion was found after both bromocriptine

and cabergoline administration in the adrenal hyperplasias. In conclusion, the current study demonstrated that both D_1-like and D_2-like receptors are expressed in the normal adrenal gland and in a percentage of adrenal adenomas or carcinomas. Bromocriptine and cabergoline induce only a minor inhibition of the secretion of adrenal hormones in the nontumoral adrenal gland *in vitro*, not excluding, however, the possible effective use of dopamine agonists *in vivo* in the treatment of adrenal tumors.

Introduction

Dopamine is the predominant catecholamine neurotransmitter in the human central nervous system, but plays multiple roles in the periphery, as a modulator of cardiovascular and renal function, and endocrine regulation (1). The various actions of dopamine are mediated by five specific receptors (D_1-D_5), which can be subdivided in two different receptor families on the basis of their biochemical and pharmacological characteristics: D_1-like, including the D_1 and D_5, and D_2-like, including the D_2, D_3, and D_4 receptor (1). D_2 receptor exists in two different isoforms, the long (D_{2long}) and short (D_{2short}) isoforms (1). The different dopamine receptor (DR) subtypes have a differential distribution and play different roles in the various organs and tissues (1).

The dopaminergic system is known to regulate the renin-angiotensin-aldosterone system (2). D_1-like receptors are expressed in the renal juxtaglomerular apparatus where D_1-selective dopamine agonists stimulate renin secretion (3,4). Both D_1-like and D_2-like receptors have been reported to be expressed in the adrenal medulla where they modulate catecholamine release (5-7). Conversely, DR expression and function in the normal adrenal cortex are still a matter of debate. Indeed, the administration of the D_2 antagonist metoclopramide was shown to directly increase plasma aldosterone levels (8), without the mediation of any modulator of aldosterone secretion (9,10). However, dopamine or dopamine agonists, such as bromocriptine, did not modify basal plasma aldosterone levels (11), suggesting that aldosterone production is under maximal tonic dopaminergic inhibition. The presence of D_2-like receptors in the adrenal cortex has been evaluated by binding studies, which demonstrated their localization mainly in the zona glomerulosa of the adrenal cortex (12). Moreover, they were demonstrated to mediate the inhibition of angiotensin-stimulated, but not basal and ACTH-stimulated aldosterone secretion by dopamine agonists (13). Recently, both D_2 and D_4 receptors have been demonstrated by molecular studies to be heterogeneously expressed in all three zones of the adrenal cortex (14).

DR expression has not been extensively evaluated in adrenal tumors. D_2 and D_4 receptors have been recently demonstrated, by RT-PCR and *in situ* hydribization (ISH) in pheochromocytomas, the tumors deriving from adrenal medulla, and in benign aldosterone-producing tumors (6,14). However, no study has ever evaluated DR expression in benign cortisol- or sex hormone-producing or clinically non-functioning tumors as well as in malignant cortical adrenal tumors.

The aim of the current study was threefold: 1) to evaluate DR and D_2 isoforms expression by RT-PCR and D_2-like receptor expression by receptor-ligand binding (R-LB) study as well, in normal adrenal and adrenal hyperplasia and tumors; 2) to evaluate the localization of D_2 receptor expression by immunohistochemistry (IHC) study in normal adrenal and adrenal hyperplasia and tumors; and 3) to evaluate the *in vitro* effect of the dopamine agonists bromocriptine and cabergoline on baseline and ACTH and/or angiotensin II-stimulated aldosterone, cortisol and androstenedione concentration on cultured adrenal cells deriving from 5 different hyperplastic adrenals.

Patients and Methods

Patients: The adrenal samples were surgically removed from 25 patients (14 males, 11 females, age range: 34-66 years; mean: 48.5±0.7; median: 48.0) operated for renal or adrenal diseases after their informed consent had been obtained. Three of the 25 patients were subjected to monolateral nephrectomy for renal carcinomas, eight to adrenal tumorectomy, nine to monolateral adrenalectomy and the remaining five to bilateral adrenalectomy for adrenal diseases. The adrenal case load included three normal adrenals (deriving from the patients subjected to nephrectomy), five adrenal hyperplasias (deriving from patients with ACTH-dependent Cushing's syndrome), four aldosterone-secreting adrenal adenomas and two aldosterone-secreting adrenal carcinomas (deriving from patients with Conn's syndrome), two cortisol-secreting adrenal adenomas and two cortisol-secreting adrenal carcinomas (deriving from patients with ACTH-independent Cushing's syndrome), two androgen-secreting carcinoma (deriving from two female patients with virilizing syndrome), two clinically non functioning adrenal adenoma (incidentalomas) and three pheochromocytomas. The diagnoses were performed on the basis of clinical, biochemical, hormonal, radiological and pathological features of the respective cases.

Samples: Adrenal specimens were obtained, at the time of surgery, from the 25 patients. Samples of these specimens were taken fresh directly at the operation. A sample was fixed in 10% paraformaldehyde overnight and then embedded in paraffin for the IHC study. An additional sample was quickly frozen on dry ice, and stored in a freezer at -80°C for RT-PCR and/or R-LB study. In selected cases, a sample was also used for the establishment of adrenal primary culture.

Study design: In all 25 cases, DR expression was evaluated by RT-PCR, whereas D_2-like receptor expression was evaluated by R-LB study on adrenal cell membrane preparations in one case of normal adrenal, one case of adrenal hyperplasia, one case of cortisol-producing adenoma and one case of cortisol-producing carcinoma. D_2 receptor expression was evaluated also by IHC study in all 25 cases. In the three cases of adrenal hyperplasia, the *in vitro* effect of dopamine agonists bromocriptine and cabergoline was also evaluated. In two additional cases of adrenal hyperplasias, the effect of bromocriptine and cabergoline on angiotensin II-stimulated and ACTH-stimulated aldosterone secretion was also evaluated. The protocol was in accordance with the Helsinki Doctrine on Human Experimentation and it was approved by the Local Ethical Committees.

Receptor ligand-binding study: The frozen tissue samples were used for membrane R-LB study. The methodology of membrane isolation has been previously described (15). Briefly, the tissue samples were homogenized in a Polytron tissue homogenizer in ice-cold homogenization buffer [50 mM Tris-HCl (pH 7.7), 0.32 M sucrose, 1 mM phenylmethylsulfonylfluoride, 0.25 mM $caCl_2$] and centrifuged at 600 x g for 5 min at 4°C in order to remove nuclei and unbroken cells. The supernatant was then collected and centrifuged at 14,000 x g for 30 min at 4°C in an Eppendorf microcentrifuge. The pellet was washed once and resuspended in the same buffer. The protein content of membrane samples was determined by a spectrophotometric method. Membrane preparations (corresponding to 30-60 μg protein) of tissue sample were resuspended in binding buffer [50 mM Tris-HCl (pH 7.7), 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ $2H_2O$, 1 mM $MgCl_2$ $6H_2O$, 0.1% ascorbic acid] and incubated in a total volume of 100 μl at room temperature for 60 minutes with increasing concentrations of the D_2 analog (D_2 antagonist) ^{125}I-epidepride (Radiopharmaka, Seibersdorf, Austria) with and without excess (1 μM) of an unlabeled D_2 agonist cabergoline. After the incubation, 1 ml ice-cold Tris buffer was added to the reaction mixture, and membrane bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 x g in a microcentrifuge. The remaining pellet was washed twice in ice-cold Tris buffer, and the final pellet was counted in a γ-counter. Specific binding was taken to be total binding minus binding in the presence of 1 μM unlabeled cabergoline. Scatchard analysis was performed to measure the maximal binding (Bmax) and the dissociation coefficient (Kd) of the binding. Rat brain basal ganglia were used as controls in all the experiments. The experiment was performed three times for each case.

Immunohistochemistry: The formalin-fixed and paraffin-embedded tissue samples were used for the IHC. The methodology of IHC has been previously described (16). The tissue samples were cut in 5-μm-thick sections. The sections were deparaffinized, dehydrated, exposed to microwave heating (in citric acid buffer) at 100°C for 15 min, rinsed in tap water followed by phosphate buffered saline (PBS) and subsequently incubated for 15 min in normal goat serum (1:10 dilution in PBS + 5% bovine serum albumin, BSA). The sections were then incubated overnight at 4°C with a rabbit anti-human D_2 receptor polyclonal antibody (Chemicon International, Temecula, CA, USA) at a dilution of 1:500. A standard streptavidin-biotinylated-alkaline phosphatase or -peroxidase complex (ABC kit, Biogenix, San Ramon, CA) was used according to the manufacturer's recommendation to visualize the bound antibodies. Negative controls for the IHC included: *a*) omission of the primary antibody; *b*) preabsorbtion of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 nM). The immunostaining for the D_2 receptor and the negative controls were performed on sequential sections. Histological evaluation was performed on hematoxylin-eosin stained sequential sections. Positive and negative controls were immunostained for D_2 receptor on sections from a dopamine agonist-sensitive and a dopamine agonist-resistant PRL-secreting pituitary tumor, respectively; their immunostaining for D_2 receptor was carried out in the same experiments of the adrenals. The sensitivity of the D_2 receptor antibody had been previously tested performing an immunostaining with different dilutions of the antibodies (1: 100, 1:250, 1:500, 1:1000) on sections from a PRL-secreting pituitary tumor, choosing the dilution with the maximum of specific and the minimum of aspecific staining. The specificity of the D_2 receptor antibody was tested by an immunoblotting.

Immunoblotting: A frozen sample of a pheocromocytoma was used for the immunoblotting. Particularly, tumor tissue was suspended in a 10-vol ice-cold Tris-buffer (10 mM Tris-HCl pH 7.6, 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml soybean-trypsin-inhibitor, 50 μg/ml bacitracin), homogenized with a Polytron homogenizer at 900 rpm for 10 strokes and then ultracentrifuged for 1 h at 4°C at 100,000 x g. Membrane pellet was solublized in a lysis buffer (20 mM HEPES, 5 mM EDTA, 3 mM EGTA, 150 mM NaCl, 4 mg/ml dodecyl-B-D-maltoside) for 1h at 4°C and then ultracentrifuged for 1 h at 4°C at 100,000 g. Glycosylated proteins were purified from membrane pellet obtained after high-speed centrifugation, by wheat germ agglutinin (WGA) affinity chromatography: the pellet was resuspended in lysis buffer and cycled twice over a

0.5-mL WGA (Vector Laboratories, Burlingame, CA, USA) column equilibrated with lysis buffer. The column was washed with lysis buffer and eluted with lysis buffer containing 3 mM N,N',N"triacetyl-chitotriose (Sigma Chemical Co., St. Louis, MO, USA). The protein-containing fractions was determined with the Bradford assay standardized with BSA, pooled and stored at -80 °C. Starting material and WGA-purified membranes proteins were denatured and fractionated under reducing conditions on 12.5% SDS-PAGE, then transferred electrophoretically to Hybond C-extra nitrocellulose membranes (Amersham Life Science, Oakville, Ontario, Canada). After transfer, nonspecific binding sites were blocked by treating membranes with Tris buffered saline-Tween (TBS-T) containing 5% nonfat dried milk. After five washes with TBS-T, membranes were incubated for 16 h at 4°C with a 1:500 dilution of rabbit D_2 receptor polyclonal antibody (Chemicon International, Temecula, CA, USA) in TBS-T containing 1% BSA. Membranes were washed five times with TBS-T, and then incubated for 1 h at 22°C with 1:1000 dilution of horseradish peroxidase-linked anti-rabbit IgG (Amersham Life Science, Oakville, Ontario, Canada) and washed as before. The specificity of the antibody was confirmed pre-incubating the antibody with the respective immunizing receptor peptide (at a concentration of 100 nM). Immunoreactive bands were detected by chemiluminescence detection system (ECL western blot analysis system, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) according to manufacturer's protocol. The immunoreactive bands were visualized by autoradiography after 0.5 min exposure to Kodak Biomax film (Eastman Kodak Company, Rochester, NY, USA). Because the antibody recognizes both the native and the denatured forms of D_2 receptor, bands of 110, 68 and 47 KDa may be visualized by Western Blot. The expected band with the procedure used in our lab was the 68 kDa denatured form.

RT-PCR: The frozen tissue samples were used for the RT-PCR. The methodology for the isolation of mRNA and the synthesis and amplification of cDNA has been previosulsy described (15). Messenger RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS Oslo, Norway) from a frozen tissue sample. The cells were lysed during 2 min on ice in a buffer containing 100 mM Tris-HCl (pH 8.0), 500 mM LiCl, 10 mM EDTA (pH 8.0), 1% LiDS, 5 mM DTT and 5 U/100 µl RNAse inhibitor (HT Biotechnology Ltd., Cambridge, UK). The mixture was centrifugated at 14,000 rpm for 1 min to remove cell debris. To the supernatant 100 µl prewashed Dynabeads Oligo $(dT)_{25}$ were added, and the mixture was incubated for 5 min on ice. Thereafter, the beads were collected with a magnet, washed three times with 10

mM Tris HCl (pH 8.0), 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS, and once with a similar buffer from which LiDS was omitted. Messenger RNA was eluted from the beads in 50 µl of a 2 mM EDTA solution (pH 8.0) during 2 min at 65 °C. To avoid contamination by genomic DNA, the isolated poly A^+ mRNA was subjected to a second purification by capturing the RNA on a fresh aliquot of pre-washed Dynabeads Oligo $(dT)_{25}$ and washing the captured RNA as above described. cDNA was synthesized using the poly A^+ mRNA captured on the Dynabeads Oligo $(dT)_{25}$ in a buffer containing 50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 10 U RNAse inhibitor, and 2 U avian myeloblastosys virus Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 20 µl. This mixture was incubated for 1 h at 42 °C. One tenth from each cDNA library immobilized on the paramagnetic beads was used for each amplification. The amplification reaction mixture contained cDNA template, 0.5 U SuperTaq (HT Biotechnology Ltd., Cambridge, UK), 50 µM of each deoxynucleotide triphosphate (HT Biotechnology Ltd., Cambridge, UK), 5 pmol of each of a pair of oligonucleotide primers specific for human D_1-D_5 receptor subtypes or the hypoxantine ribosyl transferase (HPRT) in a buffer of 10 mM Tris-HCl (pH 9), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.1% Triton X-100 in a final volume of 50 µl. The sequences of the primers for D_1-D_5 and HPRT are listed in **Table 1**. The PCR was carried out in a DNA thermal cycler with heated lid (Perkin Elmer Cetus Instruments, Gouda, The Netherlands). After an initial denaturation at 94 °C for 5 min, the samples were subjected to 40 cycles of denaturation at 94 °C for 1 min, annealing for 2 min at 60 °C, and extension for 1 min at 72 °C. After a final extension for 10 min at 72 °C, 10 µl aliquots of resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation for two DR subtypes, D_1 and D_5, whose genes are intron-less, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. Amplification of the cDNA samples with the HPRT specific primers served as positive control for the quality of cDNA. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive controls for the PCR of the DR subtypes and HPRT, 0.01 µg of human brain cDNA was amplified in parallel with the cDNA samples of each examined adrenal.

Table 1 : Specific oligonucleotide primers for dopamine receptors subtypes (D_1-D_5) and controls used in the RT -PCR study

Gene		Sequence (5'-3')	Size of PCR product (bp)
Dopamine receptors			
D_1	Forward	AACACCTCTGCCATGGACG	616
	Reverse	TGATGGCCACAGGGATGTAA	
D_2	Forward	GCGGACAGACCCCACTACAA	521
	Reverse	AAGGGCACGTAGAAGGAGAC	
$D_{2\ short/long}$ isoforms	Forward	CCATGCTGTACAATACGCGCT	$D_{2\ long}$: 599; $D_{2\ short}$: 512
	Reverse	GGCAATCTTGGGGTGGTCTTT	
D_3	Forward	CCCGCCCACATGCCTACTAT	1106
	Reverse	GAAGGCTTTCCGGAACTCGAT	
D_4	Forward	CCCACCCCAGACTCCACC	259
	Reverse	GAACTCGGCGTTGAAGACAG	
D_5	Forward	ACCTGTGCGTCATCAGCGT	921
	Reverse	TGCGATCGAAAGGACCCTC	
HPRT	Forward	CAGGACTGAACGTCTTGCTC	413
	Reverse	CAAATCCAACAAAGTCTGGCT	

Cell dispersion and cell culture: The fresh tissue samples were used for the establishment of cell cultures. The methodology of adrenal culture has been previously described (17). Adrenal tissue specimens were placed in Hank's balanced saline solution (HBSS, Invitrogen., Paisley, Scotland, UK), supplemented with Human Serum Albumin (HSA) 5% (Cealb, CLB, Amsterdam, The Netherlands), penicillin (10^5 U/liter), fungizone (0.5 mg/liter). After careful removal of blood clots, the specimens were minced and washed several times with the HBSS+HSA. The minced tissues were enzymatically dissociated with collagenase. After removal of erythrocytes by centrifugation on a Ficoll density gradient, the adrenal cells were plated in 24-well plates (Corning, Cambridge, MA, USA) in DMEM supplemented with 0.2% BSA. Viability of the cells was determined by trypan blue exclusion and was greater than 80% in every experiment. Cells were incubated at 37°C in a humid CO_2 incubator for 24 h with or without test substances in quadruplicate using

300.000 cells/ml. At the end of the incubation, 0.5 ml distilled water was added to each well, and the resulting suspension was collected and stored at –20°C for the measurement of aldosterone, cortisol and androstenedione secretion. Baseline and ACTH-stimulated and/or angiotensin II-stimulated hormone secretion was evaluated without and with bromocriptine and cabergoline administration. ACTH (ACTH 1-24, Synachten, Ciba-Geigy, Basel, Switzerland) was added to the cell culture at the final concentration of 10^{-10} M. Angiotensin II (Clinalfa, Laufelfingen, Germany) was added to the cell culture at the concentration of 10^{-6} M. Bromocriptine (Novartis, Basel, Switzerland) and cabergoline (Pharmacia, Milan, Italy) were used as test substances and added to the cell cultures at the concentration of 10^{-12}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} M for the experiment testing the baseline and ACTH-stimulated hormone secretion and at the concentration of 10^{-8} M for the experiments testing the angiotensin II-stimulated hormone secretion. Baseline aldosterone, cortisol and androstenedione concentrations (measured in the medium from untreated cells or from cells with ACTH alone) were compared to the respective hormone concentrations measured in the medium containing the different concentrations of bromocriptine and cabergoline.

Hormone assays: Aldosterone concentrations were measured by radioimmunoassay using a commercially available kit from Biochem Immunosystem (Bologna, Italy); cortisol concentrations were measured by radioimmunoassay using a commercially available kit from Diagnostic System Laboratories (Webster, USA); androstenedione concentrations were measured by a solid-phase, two site chemiluminescent enzyme immunoradiometric assay from Diagnostic Products Corporation (LA, USA).

Statistical analysis: Data are expressed in mean±SE. The comparison between pre and post-treatment values was performed by Analysis of Variance followed by Bonferroni test for correction for multiple comparisons. Significance was set at 5%.

Results

RT-PCR study: Both D_1-like (D_1 and D_5) and D_2-like (D_2 and D_4) receptors were found to be expressed in all cases of normal and hyperplastic adrenals. D_2 and D_4 were consistently expressed in both aldosterone- and cortisol-secreting adenomas, which also expressed, though not in all cases, D_1 or D_5. However, among the aldosterone-secreting adenomas, D_2 were not expressed in one of the four (25%)

cases. In addition, D_2 and D_4 were expressed in the two cases of cortisol-secreting carcinomas and in all the clinically non functioning adrenal adenomas, whereas no DR was expressed in the aldosterone- and androgen-secreting adrenal carcinomas. D_2, D_4 and D_5 were consistently expressed in the pheochromocytomas. In all cases positive for D_2, both D_2 isoforms were expressed, with the exception of the two cases of cortisol-secreting carcinomas and one of the three (33.3%) D_2 positive cases of aldosterone-secreting adenomas, in which only D_{2long} isoform was expressed. No D_3 receptor was found in any adrenal sample. The results of RT-PCR study are shown in **Table 2.**

Receptor-ligand binding study: Specific binding of ^{125}I-epidepride was found in all cases including a normal adrenal, an adrenal hyperplasia and a cortisol-secreting adrenal adenoma and carcinoma, with variable Bmax ranging from 22 to 40 fmol/mg protein and Kd ranging from 0.19-0.24 nM. The results of the R-LB study are shown in **Table 3.** Two examples of a ^{125}I-epidepride binding in a normal adrenal and a cortisol-producing adrenal adenoma are shown in **Fig. 1.**

Immunoblotting: A specific band of the expected molecular weight of D_2 receptor was found at the immunoblot of glycoproteins derived from the pheochromocytoma (**Fig. 2**).

Immunohistochemistry study: Specific immunoreactivity for D_2 receptor was found in all cases of normal adrenal and adrenal hyperplasias. In these tissues, the immunostaining was localized in all three areas of the adrenal cortex and in the adrenal medulla. However, it was strongly positive in the zona glomerulosa and reticularis of the adrenal cortex, moderately positive in the adrenal medulla and faintly positive in the zona fasciculata. Specific immunoreactivity for D_2 receptor was also found in three of the four (75%) aldosterone- (no. 9-11), the cortisol-producing and the clinically non functioning adrenal adenomas, as well as in the cortisol-producing carcinoma and in the pheochromocytomas but not in one case of aldosterone-secreting adenoma (no. 12), the aldosterone-secreting and the androgen-secreting adrenal carcinomas. A complete correspondence was found between the results of immunohistochemistry and RT-PCR study. Examples of D_2 receptor immunostaining in a normal adrenal, an adrenal hyperplasia, a cortisol-

Table 2: Dopamine receptor expression in normal adrenal and adrenal tumors

Case	Diagnosis	RT-PCR study						
		D1	D2	D2s/l	D3	D4	D5	HPRT
1	Normal adrenal	+	+	long/short	-	+	+	+
2	Normal adrenal	+	+	long/short	-	+	+	+
3	Normal adrenal	+	+	long/short	-	+	+	+
4	Adrenal hyperplasia	+	+	long/short	-	+	+	+
5	Adrenal hyperplasia	+	+	long/short	-	+	+	+
6	Adrenal hyperplasia	+	+	long/short	-	+	+	+
7	Adrenal hyperplasia	+	+	long/short	-	+	+	+
8	Adrenal hyperplasia	+	+	long/short	-	+	+	+
9	Aldosterone-secreting adenoma	-	+	long/short	-	+	+	+
10	Aldosterone-secreting adenoma	-	+	long/short	-	+	-	+
11	Aldosterone secreting adenoma	-	+	long	-	+	-	+
12	Aldosterone secreting adenoma	-	-	-	-	+	-	+
13	Cortisol-secreting adenoma	-	+	long/short	-	+	-	+
14	Cortisol-secreting adenoma	+	+	long/short	-	+	-	+
15	Non functioning adenoma	-	+	long/short	-	+	-	+
16	Non functioning adenoma	-	+	long/short	-	+	-	+
17	Aldosterone-secreting carcinoma	-	-	-	-	-	-	+
18	Aldosterone-secreting carcinoma	-	-	-	-	-	-	+
19	Cortisol-secreting carcinoma	-	+	long	-	+	-	+
20	Cortisol-secreting carcinoma	-	+	long	-	+	-	+
21	Androgen-secreting carcinoma	-	-	-	-	-	-	+
22	Androgen-secreting carcinoma	-	-	-	-	-	-	+
23	Pheochromocytoma	-	+	long/short	-	+	+	+
24	Pheochromocytoma	-	+	long/short	-	+	+	+
25	Pheochromocytoma	-	+	long/short	-	+	+	+
C	Human Brain	+	+	long/short	+	+	+	+

Table 3: Dopamine receptor expression in normal adrenal, adrenal hyperplasia and cortisol-secreting adrenal adenoma and carcinoma determined by Scatchard analysis of ^{125}I-epidepride binding on membrane homogenates.

Cases	^{125}I-epidepride binding	
	Bmax (fmol/mg protein)	**Kd** (nM)
Normal adrenal (no.1)	25±0.9	0.21±0.03
Adrenal hyperplasia (no. 4)	36±0.7	0.19±0.02
Cortisol-secreting adrenal adenoma (no. 9)	40±0.8	0.22±0.05
Cortisol-secreting adrenal carcinoma (no.14)	22±0.5	0.24±0.07
Rat brain basal ganglia	80±12.5	0.18±0.01

producing adrenal adenoma and a cortisol-producing adrenal carcinoma are shown in **Fig. 3**.

***In vitro* effectiveness of dopamine agonists**: A slight but not significant increase, at low dose (10^{-10} M), and a decrease, at high dose (10^{-6} M), of both baseline and ACTH-stimulated cortisol and androstenedione secretion was found either after bromocriptine or cabergoline administration in the cell cultures deriving from the adrenal hyperplasias. Conversely, a significant stimulation at low dose and a significant inhibition at high doses of both baseline and ACTH-stimulated aldosterone secretion was found after cabergoline but not bromocriptine administration (**Fig. 4**). A significant decrease of angiotensin-II-stimulated aldosterone secretion at the dose of 10^{-8} M was found either after bromocriptine or cabergoline administration, the cabergoline-induced however being significantly higher than the bromocriptine-induced inhibition (**Fig. 5**).

Discussion

The results of the current study demonstrated that: 1) D_1-like and D_2-like receptors are both expressed in the normal adrenal gland; 2) D_2 receptors are expressed in cells of all areas of the normal adrenal gland, although they are mainly localized in the zona glomerulosa and zona reticularis of the adrenal cortex and in the adrenal

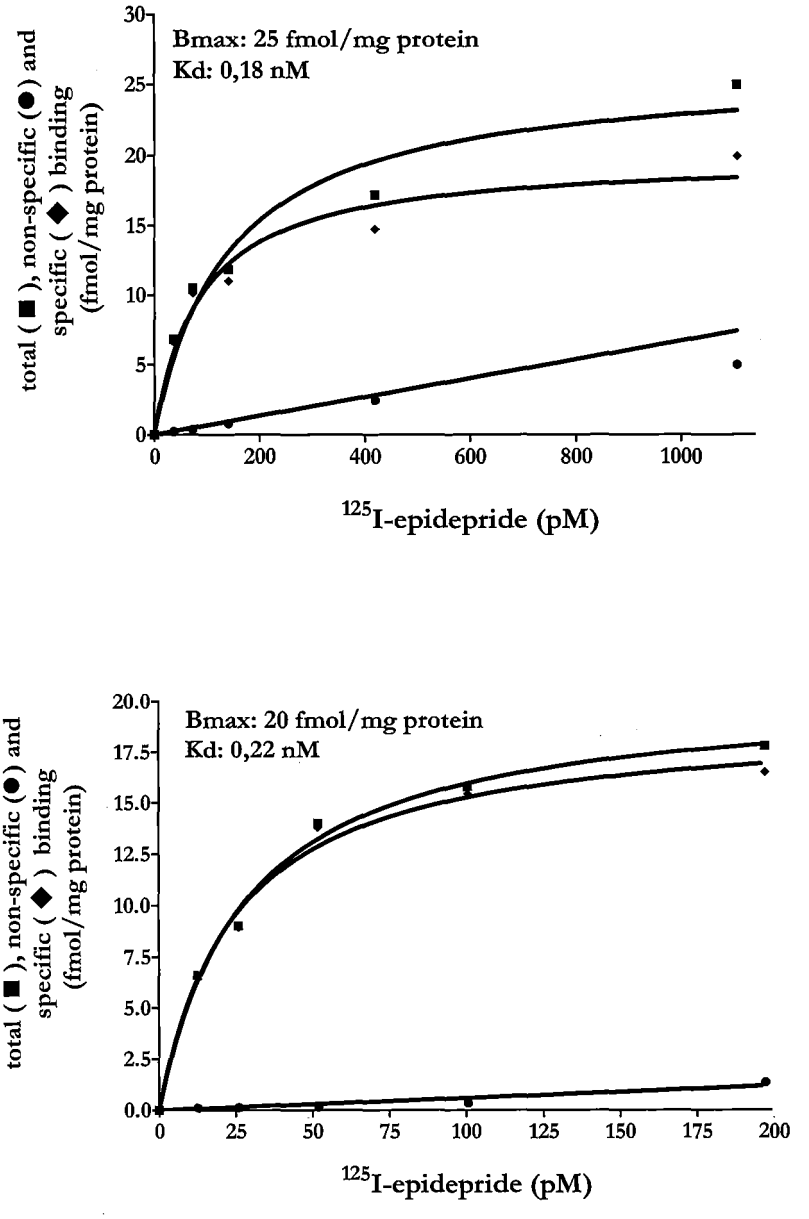


Figure 1: Binding of ^{125}I-epidepride to membrane homogenate preparation of a normal adrenal (up) and a cortisol-producing adrenal adenoma (down). ■, total binding; ●; non specific binding in presence of 1 μM of cabergoline; ♦; specific binding (total minus non specific binding.

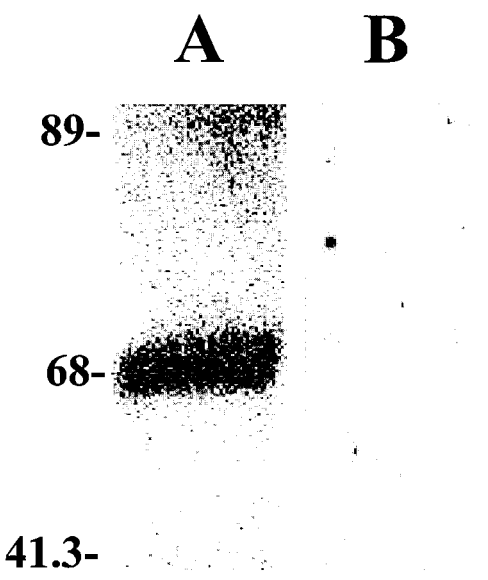
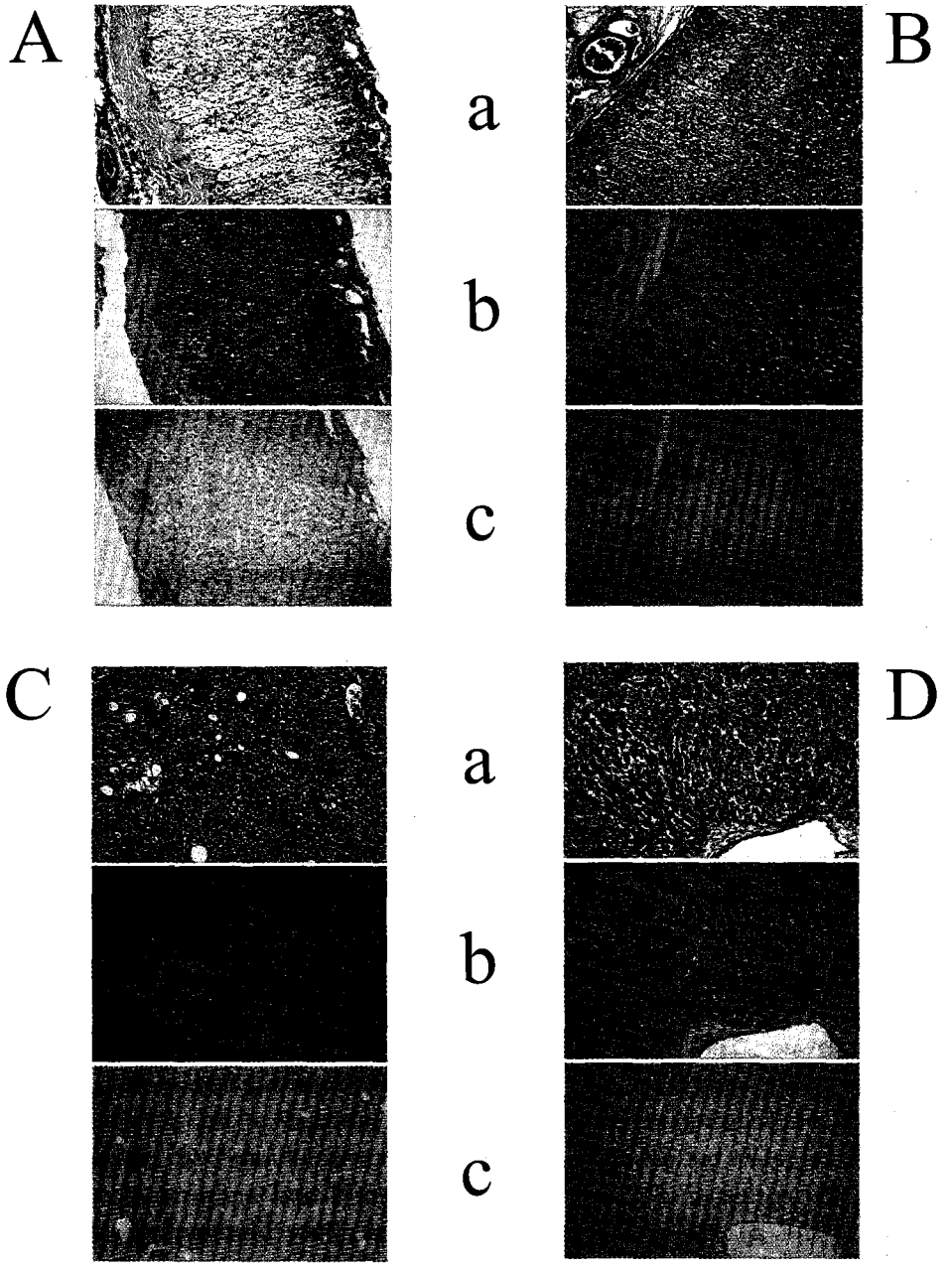


Figure 2: Immunoblot of glycoproteins derived from a human pheochromocytoma with D_2 receptor antibody. The figure show the results on the pheochromocytoma (no. 21) without (A) and with (B) the preincubation of the antibody with the antigen. The specificity of the immunoblot is demonstrated by the complete disappearance of the signal after the preincubation of the antibody with the antigen (D_2 receptor peptide).

medulla and scantly in the zona fasciculate of the adrenal cortex; 3) D_2-like receptors are expressed in different categories of adrenal benign tumors, such as aldosterone- and cortisol-secreting and clinically non functioning adenomas and in malignant tumors, such as cortisol-secreting carcinomas, as well as in pheochromocytomas; 4) a high concentration of the dopamine agonist cabergoline but not bromocriptine significantly inhibits both baseline and ACTH-stimulated aldosterone but not cortisol and androstenedione secretion; and 5) cabergoline is more effective than bromocriptine in inhibiting angiotensin-II stimulated aldosterone secretion.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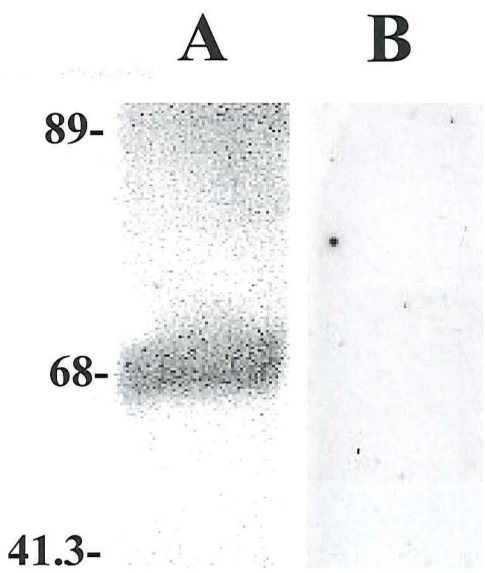

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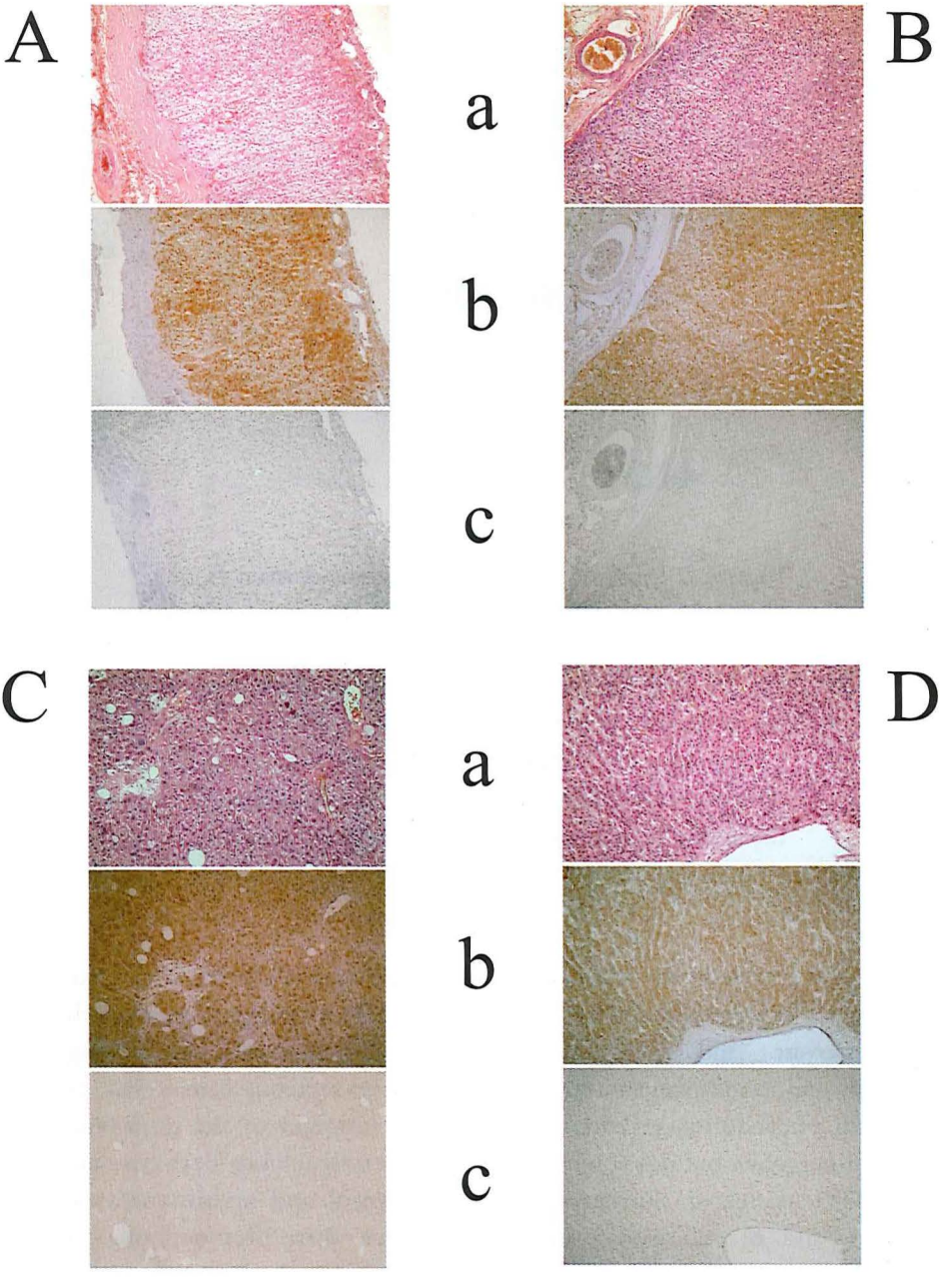
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DR expression in the adrenal gland has been first hypothesized after the evidence for a role of dopamine in the control of aldosterone secretion derived from *in vivo* studies in experimental animals as well as in humans. Indeed, the administration of the D_2 antagonist metoclopramide to both rats and humans was demonstrated to increase plasma aldosterone levels without influencing any stimulator of aldosterone release, an effect blocked by the intravenous injection of dopamine (8-10). However, the administration of dopamine or the dopamine agonist bromocriptine did not modify plasma aldosterone levels (11). These observations suggested that aldosterone production is under maximum tonic dopaminergic inhibition. Subsequent studies demonstrated that the sodium balance state is crucial for the effect of dopamine or dopamine agonists on aldosterone secretion. Indeed, dopamine and D_2 receptor agonists were shown to inhibit angiotensin II-stimulated and upright posture-induced increased aldosterone secretion in sodium-depleted, and not in sodium-repleted normal subjects (18,19). On the other hand, *in vitro* studies with isolated adrenal glomerulosa cells demonstrated that the activation of D_2 receptors resulted in a remarkable inhibition of angiotensin II-induced aldosterone secretion, whereas it did not influence basal and ACTH-induced aldosterone secretion (13). These studies suggested D_2 or D_2-like receptor

Figure 3: Expression of D_2 receptor by immunohistochemistry in 1 case of normal adrenal (A), 1 case of adrenal hyperplasia (B), 1 case of cortisol-producing adrenal adenoma (C) and 1 case of adrenal carcinoma (D). The immunohistochemical study has been performed on formalin-fixed and paraffin-embedded sections of the tumors. a, hematoxylin-eosin stained section; b, D_2 receptor immunostaining performed with a specific polyclonal D_2 receptor antibody; c, D_2 receptor immunostaining after the preincubation of the antibody with the specific antigen. A strong specific D_2 immunostaining is evident in the zona glomerulosa and reticularis of normal and hyperplastic adrenal as well as in the tumoral cells of cortisol-producing adenoma and carcinomas, whereas a faint but specific immunostaining is evident in the zona fasciculata of normal and hyperplastic adrenals. The specificity of the immunostaining is demonstrated by the disappearance of immunostaining after the preabsorption of the antibody with 100 nM of the specific antigen (D_2 receptor peptide). Magnification: X100.

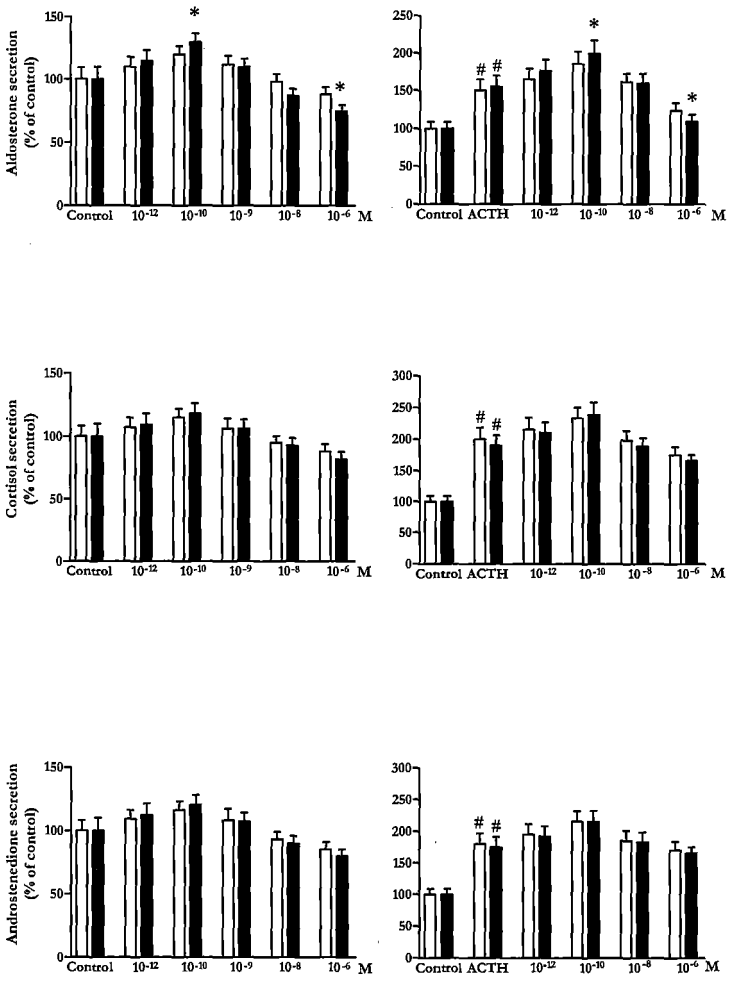

Figure 4: Effect of *in vitro* administration of bromocriptine (□) and cabergoline (■) on baseline (left) and ACTH-stimulated (right) aldosterone (up), cortisol (middle) and androstenedione (down) secretion in cell cultures derived by 3 different ACTH-dependent adrenal hyperplasia (no. 4-6). Values are expressed as secretion percentage (%) and are mean±SE (n=3 treatment; n=4 per treatment). (*= $p<0.05$ compared to control in baseline studies and to ACTH challenge in stimulated studies; #: $p<0.05$ compared to control in ACTH-stimulated studies).

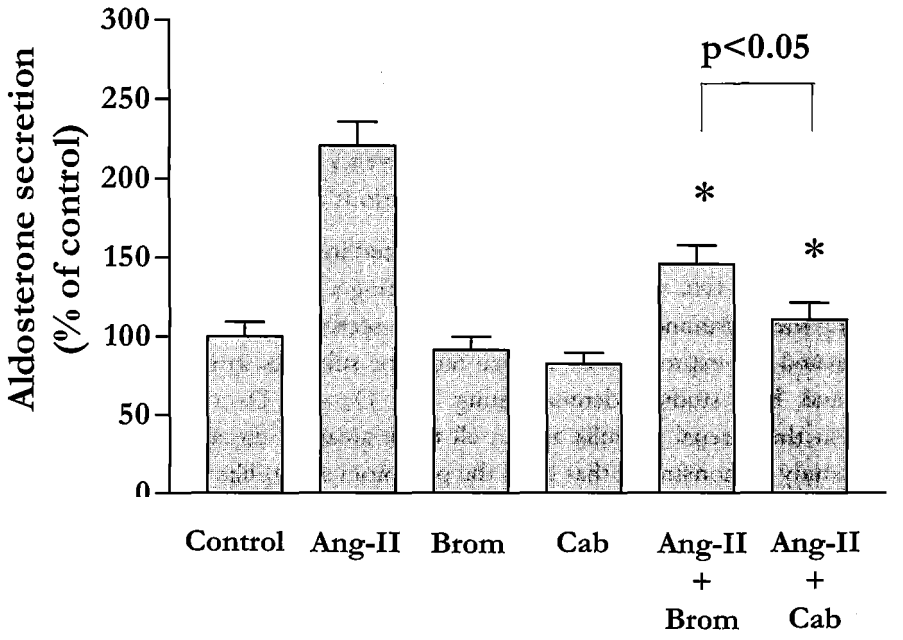

Figure 5: Effect of *in vitro* administration of bromocriptine and cabergoline on baseline and angiotensin-II-stimulated aldosterone secretion in cell cultures derived by 2 different ACTH-dependent adrenal hyperplasia (no. 7,8). Values are expressed as secretion percentage (%) and are mean±SE (n=2 treatment; n=4 per treatment). (*= $p<0.05$ compared to angiotensin-II stimulated aldosterone secretion).

expression in cells of the zona glomerulosa of the adrenal cortex and a selective functional interaction between dopamine and angiotensin II in the regulation of aldosterone secretion. No study has definitely evaluated the possible effect of dopamine or dopamine agonists on cortisol or androgen secretion by the adrenal gland.

DR expression in the adrenal gland has been subsequently confirmed by receptor ligand binding studies, demonstrating the presence of specific and saturable binding sites for radiolabeled dopamine antagonists such as spiperone, which binds both D_1-like and D_2-like receptors, and sulpiride, which selectively binds D_2-like receptors in the adrenal cortex of experimental animals as well as humans (2,12). These studies

demonstrated that both D_1-like and D_2-like receptors are expressed in the human adrenal cortex. On the other hand, the expression of D_1-like and D_2-like receptors in the adrenal medulla has been clearly demonstrated to mediate the dopamine regulation of catecholamine secretion (5-7). However, no study has evaluated the DR subtypes and D_2 isoforms expressed in the various areas of adrenal cortex.

The presence of D_1 receptor has been demonstrated in the zona glomerulosa of the rat adrenal gland by IHC and ISH studies, suggesting a possible role of this receptor in regulating the dopamine effects on aldosterone secretion (20). Moreover, a recent study evaluated the expression of D_2-like receptor subtypes in normal adrenals by RT-PCR and ISH studies, demonstrating that D_2 and D_4 receptors are both expressed in the adrenal medulla and in all three areas of the adrenal cortex (14). The same study demonstrated that D_4 is the predominant D_2-like receptor expressed in the adrenal cortex and that the zona glomerulosa is the area with higher density of D_2-like receptors (14). The results of the current study are in line with these latter ones, as demonstrated by the expression of both D_2 and D_4 receptors in the normal adrenal gland. In addition, the current study reported for the first time that both D_1 and D_5 receptors are expressed in the adrenal gland. Therefore, four of the five different DR are expressed in the human normal adrenal gland, suggesting the existence of a complex regulation of adrenal function by dopamine in physiological conditions. As far as the localization is concerned, the heterogeneous D_2 expression in the different areas as was demonstrated by level of D_2 gene product expression by ISH in a previous study (14), is confirmed in the present study by level of D_2 protein expression by IHC. Indeed, the expression of this DR subtype in all adrenal areas but mainly in the zona glomerulosa and reticularis and to a lesser extent in the zona fasciculata and adrenal medulla is clearly demonstrated. These findings suggested a possible role for dopamine in the regulation of the secretion of all different adrenal hormones. However, neither bromocriptine nor cabergoline, two different dopamine agonists, is able to induce a significant *in vitro* effect on both basal and ACTH-stimulated cortisol and androstenedione secretion. As far as aldosterone is concerned, as previously described (13), bromocriptine did not induce an effect on the secretion of this hormone, except a slight nonsignificant increase at low dose and a slight non-significant decrease of hormone secretion at high dose of the drugs, a secretion pattern, which was also observed for cortisol and androstenedione secretion. However, cabergoline induced a significant stimulation at low dose and a significant inhibition at high dose of baseline and ACTH-stimulated aldosterone secretion. On the other hand, both bromocriptine and cabergoline induced a significant inhibition of the angiotensin II-induced aldosterone secretion,

cabergoline being significantly more effective than bromocriptine. These differential effects of the two dopamine agonists may be explained by the different pharmacological characteristics of bromocriptine and cabergoline, which displays a higher affinity for D_2-like receptors (21,22). Moreover, because D_4 seems to play a role in the aldosterone secretion, a different effect of bromocriptine and cabergoline *via* D_4 receptors cannot be ruled out. The lack of a clearcut *in vitro* effect of dopamine agonists on hormone secretion in normal adrenal gland may be related also to opposite dopamine agonist effects mediated by two different DR expressed in the same cells. Indeed, opposite effects of dopamine antagonists on aldosterone secretion have been postulated to be mediated by D_2 and D_4 in a previous study (14). However, on the basis of the results of the present study, opposite effects of dopamine agonists mediated by a D_1-like and a D_2-like receptors may be also hypothesized to explain the minor effects exerted by bromocriptine and cabergoline on hormone secretion in normal adrenal gland. It must, however, be outlined that the results of the present study seem to confirm the concept that aldosterone production is under maximum tonic dopaminergic inhibition in normal conditions.

DR receptor expression in adrenal tumors has been poorly investigated. Indeed, D_2-like receptors have been found to be expressed in pheochromocytomas and in aldosterone-secreting adrenal adenomas (6,14). The current study is the first one evaluating DR expression in a wide series of various benign and malignant adrenal tumors. The expression of different DRs has been found in some categories of both benign and malignant adrenal tumors. In particular, aldosterone-secreting adrenal adenomas and pheochromocytomas express both D_2 and D_4 receptors and may also express D_5 receptors, suggesting that this latter receptor may be constitutively expressed in the zona glomerulosa as well as in the medulla of the normal adrenal gland and therefore plays a physiological role in aldosterone and catecholamine secretion together to the D_2-like receptors. Moreover, both cortisol-secreting adrenal adenomas and carcinomas express both D_2 and D_4 receptors, whereas D_1 receptors are also expressed in part of the adenomas. This suggests that D_1 may be constitutively expressed in the zona fasciculata of the normal adrenal gland where it may play a physiological role in cortisol secretion together with the D_2-like receptors. In addition, clinically non functioning adrenal adenomas express D_2 and D_4 receptors. However, neither aldosterone-secreting nor androgen-secreting adrenal carcinomas seem to express any DR in the series evaluated in the present study. Anyhow, DR, in particular D_2-like receptor expression, which is usually associated to inhibitory effects, raises the hypothesis of a possible effect of

dopamine agonists in the control of hormonal hypersecretion associated to some adrenal tumor types.

The last finding of the current study is represented by the evidence of the expression of both D_2 receptor isoforms (D_{2short} and D_{2long}), in all D_2 positive adrenal samples with the exception of one aldosterone-secreting adenoma and the cortisol-secreting carcinomas, which express only D_{2long} isoform. The D_2 isoforms derive from an alternative splicing of the D_2 receptor gene product (23). The affinity of dopamine and dopamine agonists for D_2 receptor isoforms is nearly identical, but the intracellular signaling pathways activated by the binding of the receptor with the ligand seems to be different for each isoform. Therefore, heterogeneous G protein coupling would allow a variable second messenger activation and, probably, different effects by the two D_2 receptor isoforms (24-26). In particular, the role of D_2 receptor isoforms has been recently studied in the different pituitary tumors. Indeed, D_{2short} isoform has been suggested to be associated with a more potent dopaminergic effect compared to D_{2long} isoform both in clinically non functioning (27,28) and ACTH-secreting pituitary tumors (29). A possible major role of D_{2short} rather than D_{2long} isoform in the control of hormone secretion in normal adrenal and adrenal tumors as well cannot be ruled out although this likely has some significance only or mainly in cortisol-secreting carcinomas, which is the only adrenal tumor expressing one isoform of the D_2 receptors.

In conclusion, the current study demonstrated that both D_1-like (namely D_1 and D_5) and D_2-like (namely D_2 and D_4) receptors are expressed in the normal adrenal gland and may be expressed in adrenal adenomas or carcinomas. Although bromocriptine and cabergoline are able to induce only a minor inhibition of adrenal hormones secretion in nontumoral adrenal gland *in vitro*, a potential use of dopamine agonists *in vivo* in the treatment of adrenal tumors cannot be excluded.

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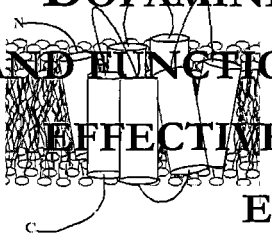
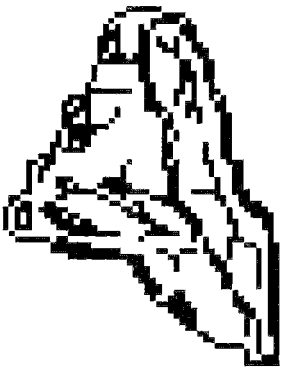


DOPAMINE RECEPTOR EXPRESSION AND FUNCTION AND DOPAMINE AGONIST EFFECTIVENESS IN CORTICOTROPH ECTOPIC TUMORS



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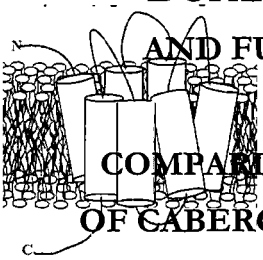

DOPAMINE RECEPTOR EXPRESSION AND FUNCTION IN ECTOPIC ACTH-SECRETING TUMORS: COMPARISON WITH THE EFFECTIVENESS OF CABERGOLINE TREATMENT IN ECTOPIC ACTH SYNDROME

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Submitted for publication

ABSTRACT

The presence of dopamine receptors has been postulated but never demonstrated in neuroendocrine tumors. Moreover, dopamine agonists have been used in the treatment of ACTH-dependent Cushing's syndrome (CS) due to pituitary tumors, but not in ACTH-dependent CS due to extra pituitary tumors, namely ectopic ACTH syndrome (EAS). The aim of the current study was to evaluate dopamine receptor and, particularly, dopamine D_2 receptor expression in neuroendocrine tumors from patients with EAS, and to evaluate the *in vivo* effectiveness of the dopamine agonist cabergoline in the treatment of post-surgical persistent EAS due to lung carcinoids. Six cases of ACTH-secreting tumors, including four lung carcinoids, one pancreatic and one thymic carcinoid were collected at surgery and used for the evaluation of D_2 expression by immunohistochemistry (IHC). The expression of dopamine receptor subtypes and D_2 receptor isoforms (D_{2long} and D_{2short}) was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) in three cases. These three patients had persistent EAS after surgery, and were treated with cabergoline at the dose of 3.5 mg/week (0.5 mg/day). Plasma ACTH, as well as serum and urinary cortisol levels, were evaluated monthly. The size of the residual tumor was monitored every three months and at the end of treatment period. The results of the IHC study demonstrated that D_2 receptor is expressed in five of the six cases (83.3%) of ACTH-secreting ectopic tumors. The results of RT-PCR study demonstrated that D_2 and D_4 receptors were expressed in three and two of the three cases evaluated, respectively. In particular, D_{2long} isoform was expressed in all three cases, together with D_{2short} in one case; D_4 was co-expressed with both D_2 isoforms in one case and only with the D_{2long} isoform in another case. The results of the treatment showed a significant decrease of plasma ACTH and serum and urinary cortisol after cabergoline administration in two of the three patients, in whom they normalized after 2-3 months. However, a treatment escape was demonstrated in one of these patients after 4 months whereas a stable control of hormone secretion was documented in the other responsive patient after 6 months of treatment. In conclusion, the results of the current study demonstrated that dopamine receptors are expressed in a majority of neuroendocrine tumors associated to EAS and that cabergoline treatment could be effective in controlling the ACTH and cortisol hypersecretion in a subgroup of patients with EAS.

Introduction

The ectopic ACTH syndrome (EAS) is a rare cause of chronic endogenous hypercortisolism, namely Cushing's syndrome (CS) accounting for approximately 15-20% of ACTH-dependent CS and 5-10% of CS (1-3). The most common causes of EAS are lung carcinomas and neuroendocrine tumors, including gastro-entero-pancreatic and, mainly, lung carcinoids (1-3). The diagnosis of EAS associated to neuroendocrine tumors is a challenge, since the similar clinical presentation and hormonal features make EAS often undistinguishable from pituitary-dependent CS, whereas the small size and/or the peculiar localization make tumors associated to EAS hardly detectable by conventional imaging techniques (2,4-6). The frequent somatostatin receptor (SR) expression has made SR scintigraphy (SRS) a common tool to identify occult carcinoids, although its real usefulness remains controversial (7,8). The treatment of choice of EAS associated with neuroendocrine tumors is the surgical removal of the tumor, but its success rate is limited due to persistent tumor remnants, especially in case of lung carcinoids (2,9,10). Therefore, pre-surgical lack of tumor localization or post-surgical persistence of tumor remnant frequently necessitates medical treatment, which is generally palliative and aimed at inhibiting adrenal cortisol secretion (11). Moreover, somatostatin analogs (SA) were found to be effective in controlling the carcinoid ACTH secretion (12). Dopamine receptors (DR) have been postulated to be expressed in neuroendocrine tumors (13). However, DR scintigraphy (DRS) is not routinely used for the localization of occult carcinoids, although it was experimentally demonstrated to visualize some types of neuroendocrine tumors (14). In addition, dopamine agonists (DA) have never been used in the treatment of EAS. The aim of the current study was to evaluate DR expression in ACTH-secreting extra-pituitary neuroendocrine tumors associated with EAS and to compare the DR expression with the *in vivo* effectiveness of the dopamine agonist cabergoline on the control of ACTH and cortisol secretion in three cases unsuccessfully operated and with persistent EAS after surgery. The study protocol has been approved by the local Ethical Commitees.

Patients and Methods

Patients: Six patients (2 females and 4 males, 30-50 years) with a diagnosis of EAS associated to ACTH-secreting neuroendocrine tumors entered the study after their informed consent had been obtained. The diagnosis of EAS was based on: 1) increase of daily urinary cortisol excretion with inappropriately high plasma ACTH

concentrations; 2) increase of basal serum cortisol concentrations with lack of the physiological circadian rhythm; 3) failure of urinary and serum cortisol suppression after low and high dose oral dexamethasone suppression test or intravenous dexamethasone suppression test; 4) absence of ACTH and cortisol response to CRH and/or DDAVP stimulation tests (1-4). The diagnosis of EAS was supported by the evidence of an extra-pituitary tumor at thorax or abdomen computed tomography (CT) and/or magnetic resonance imaging (MRI) and/or SRS (5-8). All patients were subjected to surgery for the removal of the tumor. The histological and immunohistochemical study of the tumor removed by surgery documented an ACTH-producing endocrine tumor in all cases, definitely confirming the diagnosis of EAS in the totality of patients. In particular, the final diagnosis was lung carcinoid in 4 cases (typical in 3 and atypical in 1), and pancreatic well differentiated endocrine carcinoma (pancreatic carcinoid) and a thymic carcinoid in the remaining two cases, respectively. After surgery, a clinical, hormonal and radiological remission of EAS was documented in 3 patients whereas disease persisted in the remaining 3 patients, all bearing a residual lung carcinoid (typical in 2 and atypical in 1).

Samples: Tumor specimens were obtained at the time of tumor excision by surgery. Samples of these specimens were taken fresh directly at the operation. They were fixed in 10% paraformaldehyde overnight and embedded in paraffin for the IHC study and/or quickly-frozen on dry ice and stored in a freezer at -80°C for RT-PCR study.

Study design: DR subtypes and D_2 isoforms (D_{2long} and D_{2short}) expression were evaluated by RT-PCR in three cases, while D_2 receptor expression and localization was evaluated by IHC in all 6 cases. The effectiveness of cabergoline treatment on ACTH and cortisol secretion was evaluated in the 3 cases of patients unsuccessfully operated with persistent EAS after surgery, corresponding to the three patients with lung carcinoids in whom both DR subtypes evaluation by RT-PCR and D_2 evaluation by IHC had been performed. The protocol was in accordance with the Helsinki Doctrine on Human Experimentation.

Immunohistochemistry: The IHC study was performed on tissue samples according to previous reports (15,16). Briefly, the formalin-fixed and paraffin-embedded tissue samples were cut in 5-μm-thick sections. These sections were deparaffinized, dehydrated, exposed to microwave heating in citric acid buffer at 100°C for 15 min, rinsed in tap water followed by phosphate buffer solution (PBS)

and subsequently incubated for 15 min in normal goat serum (1:10 dilution in PBS + 5% bovine serum albumin, BSA). The sections were then incubated overnight at 4°C with a rabbit anti-human D_2 receptor polyclonal antibody (Chemicon International, Temecula, CA, USA) in a dilution of 1:500. A standard streptavidin-biotinylated-alkaline phosphatase or -peroxidase complex (ABC kit, Biogenix, San Ramon, CA) was used to visualize the bound antibodies. Negative controls for the IHC included: *a*) omission of the primary antibody; *b*) preabsorption of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 nM), both performed in sequential sections. Immunostaining for ACTH (Neomarkers, Duiven, The Netherlands, dilution 1:100), Chromogranin A (Biogenix, Duiven, The Netherlands, dilution 1:100) and synaptophysin (Dako, Heverlee, Belgium, dilution 1:50) were performed on sequential sections. Histology was evaluated on hematoxylin-eosin stained sequential sections. Positive and negative controls represented D_2 receptor immunostaining on sections of dopamine agonists sensitive and resistant PRL-secreting pituitary tumors, respectively. The specificity of the D_2 receptor antibody was previously tested by immunoblotting using a corticotroph pituitary tumor sample (15,16). The intensity of immunostaining for ACTH, chromogranin A, synaptophysin and D_2 receptor was scored with the following semiquantitative method: -: absent, +: weak; ++: moderate; +++: strong immunostaining.

Reverse transcriptase-polymerase chain reaction: The RT-PCR was performed according to previous reports (15,16). Briefly, messenger RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS Oslo, Norway) from a frozen tissue sample. Complementary DNA (cDNA) was synthesized using the poly A^+ mRNA captured on the Dynabeads Oligo $(dT)_{25}$ in a Tris-buffer together with 1 mM of each deoxynucleotide triphosphate, 10 U RNAse inhibitor, and 2 U avian myeloblastosis virus Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK). The amplification reaction mixture contained cDNA template, 0.5 U SuperTaq (HT Biotechnology Ltd., Cambridge, UK), 50 μM of each deoxynucleotide triphosphate (HT Biotechnology Ltd., Cambridge, UK), 5 pmol of each of a pair of oligonucleotide primers specific for human D_1-D_5 receptor subtypes or the hypoxantine ribosyl transferase (HPRT) in a Tris-buffer. The sequences of the primers for D_1-D_5 and HPRT and the expected size of the products of the reaction have been described in previous reports (15,16). The PCR reaction was carried out in a DNA thermal cycler (Perkin Elmer Cetus Instruments, Gouda, The Netherlands) according to the following protocol: initial denaturation at 94 °C for 5

min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and extension for 1 min at 72 °C, final extension for 10 min at 72 °C. The resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation for two DR subtypes, D_1 and D_5, whose genes are intron-less, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. Amplification of the cDNA samples with the HPRT specific primers served as positive control for the quality of cDNA. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of the DR subtypes and HPRT, 0.01 μg of human brain cDNA was amplified in parallel with the cDNA samples of each examined pituitary corticotroph tumor.

Treatment protocol: Three patients were treated with cabergoline. Cabergoline was administered at the dose of 3.5 mg/week (0.5 mg/day) for 3-6 months. The clinical and hormonal evaluation was performed monthly whereas the radiological evaluation was performed every 3 months and/or at the withdrawal of the cabergoline treatment. The mean of three different values of these hormones measured in three non consecutive days of the same week were considered for the baseline and post-treatment evaluation. The evaluation of the responsiveness to cabergoline treatment was performed at 1 month follow-up. Patients who achieved a $\geq$ 50% decrease of daily urinary cortisol excretion was considered to show a significant clinical response to cabergoline. Moreover, patients who achieved a $\geq$50% decrease with normalization of urinary cortisol levels were considered full responders where those who achieved a $\geq$50% decrease without normalization of urinary cortisol levels were considered partially responders to cabergoline. Patients who achieved a <50% decrease of urinary cortisol levels were considered resistant to cabergoline treatment.

Hormonal assays: ACTH levels were measured by immunoradiometric assay, using a commercially available kit. Serum and urinary cortisol levels were measured by radioimmunologic assay, using commercially available kits.

Results

DR expression: At the IHC study, specific D_2 immunostaining was found in five (83.3%) of the six cases of ACTH-secreting carcinoids, particularly all four lung carcinoids and in the thymic carcinoid, but not the pancreatic carcinoid. D_2 immunostaining was strong and homogeneous in two cases of lung carcinoids, moderate in one lung carcinoid, and weak and heterogeneous in one lung carcinoid, as well as in the thymic carcinoid. At RT-PCR study, D_2 receptor was expressed in all three cases evaluated, whereas D_4 receptor was expressed in two of the three cases of lung carcinoid tumors. D_2 isoforms were heterogeneously expressed within the three different cases, being both D_{2long} and D_{2short} isoforms expressed in one case and only D_{2long} expressed in two cases of lung carcinoid. The results of the RT-PCR study were in agreement with the results of the IHC in the three cases in which both studied were performed. The results of the RT-PCR and IHC study are summarized in **Table 1**. An example of the IHC results in one case of lung carcinoid (no.4 of Table 1) is shown in **Fig. 1,** whereas an example of RT-PCR results in another case of lung carcinoid (no. 1 of Table 1) expressing both D_2 isoforms, is shown in **Fig. 2.**

DA effectiveness: A significant inhibition of plasma ACTH, as well as plasma and urinary cortisol levels, was found in 2 of the 3 patients with post-operative persistent EAS after 1 month, and a complete normalization of both ACTH and cortisol levels was found after 3 months of treatment with cabergoline. Conversely, no significant hormonal changes were found in the third patient, where treatment was stopped after 1 month. In one of the two responsive patients, ACTH started to rise again at 4-month, whereas cortisol started to re-increase at 5-month treatment and did not normalize despite the augmentation of the cabergoline dose from 0.5 to 1 mg/day. In this patient cabergoline treatment was stopped after 6 months of treatment. In the remaining responsive patient, ACTH and cortisol levels remained normal during the following 6 months of treatment, although with some fluctuation during the treatment period. This patient is at the 9-month follow-up and he is still controlled by cabergoline treatment. Plasma ACTH and serum and urinary cortisol levels in the three patients treated with cabergoline are shown in **Fig. 3.**

Comparison between DR expression and DA effectiveness: The patient long-term responsive to cabergoline treatment was bearing a carcinoid, expressing both isoforms of D_2 and D_4 receptors at RT-PCR study, and displaying a strong and

Table 1: IHC and RT-PCR results in the ACTH-secreting tumors deriving from patients with EAS

Cases	Histology	IHC			RT-PCR					
		Chromogranin	ACTH	D2	D1	D2	D2 isoforms	D3	D4	D5
1	Lung carcinoid	+++	+++	+++	-	+	S/L	-	+	-
2	Lung carcinoid	++	+	++	-	+	L	-	+	-
3	Lung carcinoid	++	++	+	-	+	L	-	-	-
4	Lung carcinoid	++	+	+++	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
5	Thymic carcinoid	++	++	+	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
6	Pancreatic carcinoid	++	+	-	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

IHC: +: weakly positive; ++ moderately positive; +++: strongly positive; -: negative; RT-PCR: +: positive; -: negative; S: short isoform;L: long isoform; *nd*: not determined.

homogeneous D_2 immunostaining at IHC study. The patient being responsive to the short-term treatment, but experiencing a treatment escape was bearing a carcinoid expressing only D_{2long} and D_4 receptors, but not D_{2short} isoform. In addition, this tumor showed a moderate D_2 immunostaining. The patient not responsive to cabergoline treatment was bearing a carcinoid tumor expressing D_{2long}, but not D_{2short} isoform and D_4 receptor, while displaying a weak and heterogeneous D_2 immunostaining at IHC study.

Discussion

EAS is a rare but severe syndrome frequently associated with the so called "neuroendocrine" tumors that are localized in the chest, as the lung "carcinoids", or derived from the gastro-entero-pancreatic system, as gastric, intestinal or pancreatic "carcinoids", recently defined as "endocrine tumors or carcinomas" (2,17,18). The most common tumor causing EAS is the lung carcinoid (3). The optimal treatment of EAS associated with neuroendocrine tumors, is surgical removal of the tumor and related resectable metastases (2,9). In particular, the primary treatment of EAS due to lung carcinoids is complete removal of the lung tumor and related lymph nodes (10). However cure of the disease is reached only in a minority of cases due to

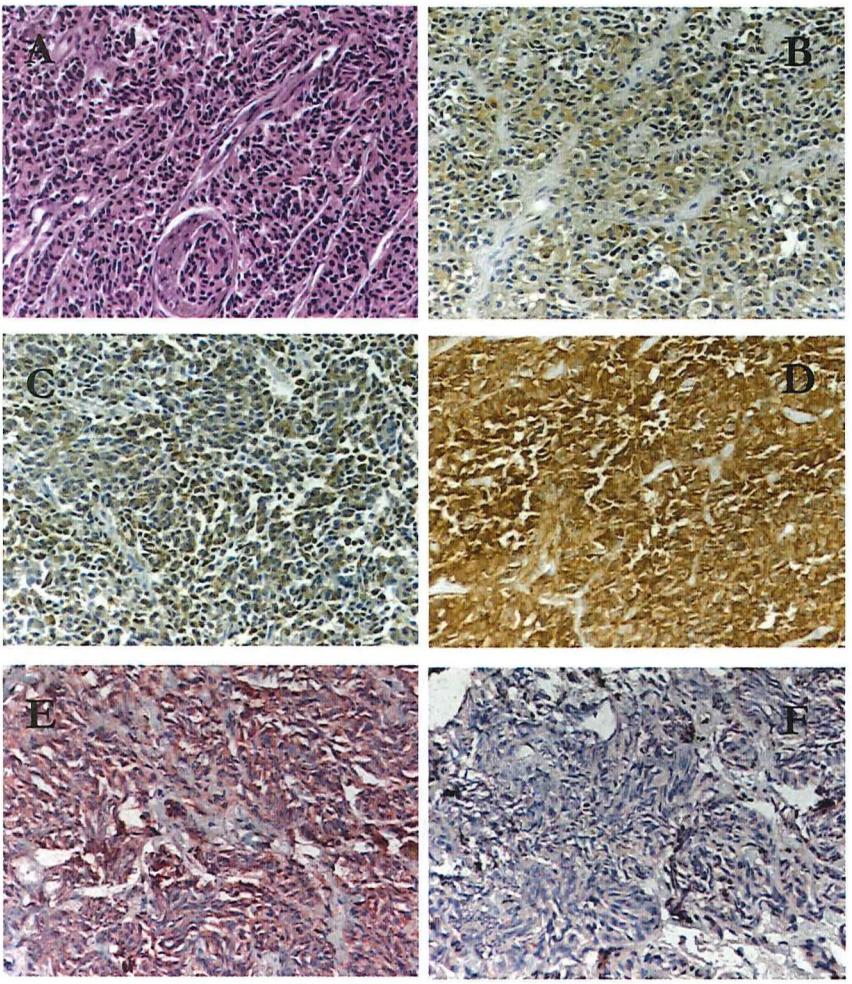

Figure 1: Example of the result of IHC study in a case of ACTH-secreting lung carcinoid (Case 4 of *Table 1*). The picture includes histology with hematoxylin eosin staining (A), ACTH (B), chromogranin A (C), synaptophysin (D) and D_2 receptor (E) immunostaining. Displacement of immunostaining after pre-absorption of the D_2 antibody with immunizing peptide, demonstrating specificity of the staining, is shown in photomicrograph F. The picture shows a diffuse immunostaining for chromogranin and ACTH within the lung tumor sample, confirming the diagnosis of ACTH-secreting carcinoid tumor. The carcinoid displays a significant immunostaining for D_2 receptor. Magnification: 200x

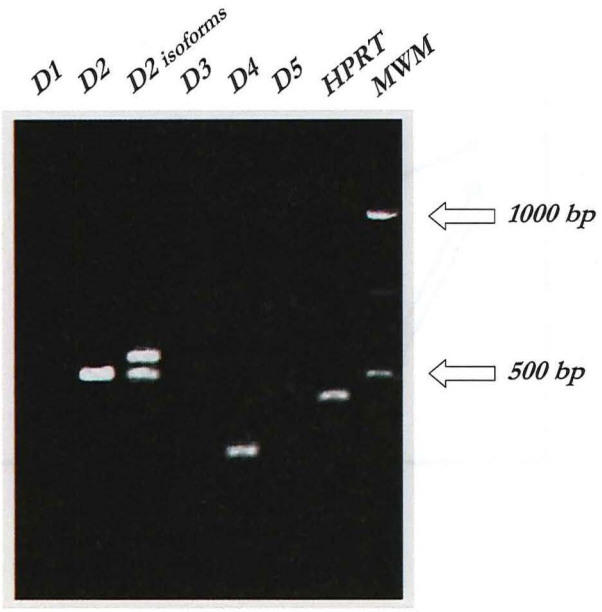

Figure 2: Example of the result of RT-PCR study for dopamine receptors in a case of ACTH-secreting lung carcinoid (Case 1 of ***Table 1***, Patient 1 of ***Fig. 3***). The electrophoresis gel shows a specific band for D_2 (D_{2long} and D_{2short} isoforms) and D_4 receptors and no band for the other dopamine receptors. The bands for D_2 and D_4 had the expected size of 521 (D_2), 599 (D_{2long}), 512 ($D_{2\,short}$) and 259 (D_4).

the following reasons: 1) the tumors are frequently undetectable, demonstrating the limits of the conventional diagnostic procedures; 2) the surgical removal of the tumor is often associated with persistent or recurrent disease, demonstrating the poor effectiveness of surgical procedures, and 3) the presence of metastases seriously complicates the management and worsens the prognosis of the malignant tumors (19-22). Therefore, surgery does not represent a curative treatment in the majority of patients. On the other hand, alternative treatments are generally palliative. Indeed, since chemotherapy is not effective except in aggressive tumors, treatments targeting the adrenal glands and aiming at normalizing cortisol secretion, are the most commonly used therapies when the source of ACTH secretion cannot

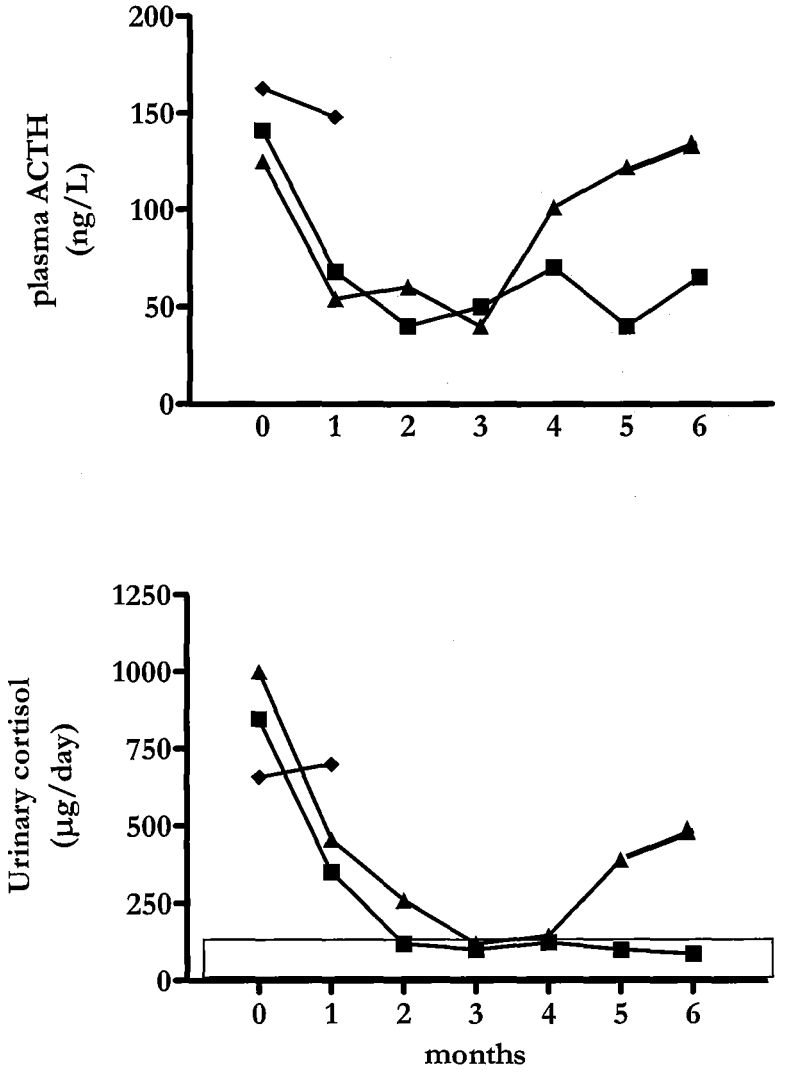

Figure 3: Plasma ACTH (top), and urinary cortisol (bottom) response to cabergoline treatment in three patients with persistent EAS due to an ACTH-secreting lung carcinoid. (■, patient 1); (▲, patient 2); (♦, patient) of *Table 1*.

be found, removed or treated (11). In this regard, a medical approach, as with adrenolytic drugs, or a surgical approach, as with bilateral adrenalectomy, is employed. However, both treatments cannot be definitive treatments, because they do not influence the causative tumor directly, although they prevent the complications related to CS. Moreover, the use of adrenolytic drugs is often associated with poor effectiveness and severe side effects and adrenalectomy is associated with irreversible hypocortisolism, which makes the patient life-long dependent on hydrocortisone replacement therapy (11). Furthermore, the specific treatment of malignant lung carcinoids depends on the dissemination and localization of the metastases, often requiring a multimodal therapeutic approach (20,22).

As have recently taken a central place in the management of EAS due to lung carcinoids (12). Indeed, carcinoids, being neuroendocrine tumors, frequently express SR (23). SR expression has been used either for the diagnostic or therapeutic approach of these tumors. SRS has been proposed as an important diagnostic procedure to visualize occult carcinoids associated to EAS and to predict the possible effectiveness of SA in the treatment of the disease (7,8,24). However, although short-term efficacy of octreotide in controlling ACTH and cortisol secretion has been described in many cases of EAS, reports of long-term effectiveness of octreotide are limited probably because of the frequent occurrence of treatment escape (12,25-27).

Despite the well known DR expression and role in pituitary tumors (15,28-34), the DR expression and its role in non-pituitary neuroendocrine tumors, and particularly in carcinoids, has never been extensively evaluated. In addition, the use of DRS for the localization of neuroendocrine tumors has never been described. Furthermore, despite the demonstrated DA effectiveness in the treatment of pituitary-dependent CS (15,35,36), the possible DA effectiveness in the treatment of EAS has never been tested.

DRs represent a group of five different G-protein coupled receptors (D_1-D_5), mediating the various central and peripheral actions of dopamine (37). These receptors can be subdivided in two different families on the basis of their biochemical and pharmacological characteristics: the D_1-like receptors, including D_1 and D_5, and the D_2-like receptors, including D_2, D_3 and D_4 receptor (37). DR subtypes have a tissue-specific distribution and play different roles in the various organs and tissues (37). The D_2 receptor seems to be the most widely expressed and most relevant DR in the endocrine system, as well as in endocrine tumors (37),

although D_4 has also been demonstrated in the normal pituitary and adrenal gland and in pituitary and adrenal tumors (15,16,37), where it might also mediate DA effects on hormone release and/or cell proliferation, in cooperation with D_2 receptor.

DRs, including both D_1-like and D_2-like receptors, as well as dopamine synthesis have been demonstrated in gastrointestinal neuroendocrine tumor cell lines (13). Moreover, dopamine synthesis has been documented in neuroendocrine tumors and, particularly, in gastrointestinal carcinoids, being the basis of the successful use of imaging techniques in the visualization of these tumors, as well as for pheocromocytoma (38,39). The current study is the first demonstrating DR expression in a significant proportion of ACTH-secreting ectopic tumors, including neuroendocrine tumors originating from the lung, thymus and the gastro-entero-pancreatic system and belonging to the class of the so called "carcinoids". D_2 and D_4 represented the only DR expressed in these tumors. These data suggest that DR may play a role in the biology of these tumors and/or form a target for the use of DA.

DA, mainly bromocriptine, have already been used with controversial results in ACTH-secreting pituitary tumors. However, an escape from treatment has been observed after long-term DA-treatment in a considerable number of cases (35,36). Recently, cabergoline has been demonstrated to be more effective than bromocriptine in the treatment of these tumors, suggesting a potent action of this long-acting DA, which has a higher affinity and selectivity for D_2 and, probably, D_2-like receptors (15). The current study demonstrates for the first time a possible effectiveness of DA, in particular cabergoline, in the treatment of ACTH-secreting ectopic tumors, causing EAS. Short-term cabergoline treatment significantly inhibited ACTH, and consequently cortisol secretion in two out of three cases with EAS due to a lung carcinoid. Hormonal suppression was maintained for a long-term follow-up in one of these two cases, without any change in the size of the tumor. It has to be pointed out that the only patient not responsive to cabergoline was bearing a tumor expressing only the long isoform of D_2, and the patient who experienced a treatment escape was bearing a tumor expressing the long isoform of D_2 and D_4 receptor. Conversely, the patient showing long-term responsiveness to cabergoline, was bearing a tumor expressing both isoforms of D_2 as well as D_4 receptors. Taken into consideration that three cases are not enough to draw final conclusions, it seems that the expression of short isoform of D_2 and/or the co-expression of D_4 may play a pivotal role in the effectiveness of DA in the treatment of these tumors associated to EAS. This hypothesis seems to be supported by

similar evidence found in the treatment of pituitary tumors (15,33). Alternatively, the responsiveness or the protection from treatment escape could be related to the different amount of D_2 and or D_4 expressed in the tumor. Indeed, it has to be pointed out that the patient being long-term responsive to cabergoline treatment had a tumor with a strong and homogeneous D_2 immunostaining. On the other hand, as only a qualitative and not a quantitative evaluation of DR by RT-PCR and a semiquantitative evaluation by IHC have been performed, this hypothesis cannot be confirmed or excluded on the basis of the results of the current study. Moreover, since no experience exists on the effectiveness of bromocriptine, no comparison can be performed between the effectiveness of bromocriptine and cabergoline in the treatment of carcinoid tumors and EAS. In addition, the well known pharmacokinetic and pharmacodynamic properties of cabergoline and the described potency of cabergoline in the treatment of pituitary tumors would suggest that it can be considered the best DA to be used in the treatment of EAS.

Finally, the possible effectiveness of DA, demonstrated in the present study, and the well described effectiveness of SA, demonstrated in previous studies, together with the demonstration of a synergistic cooperation between SR and DR and the potentiation of the correspondent SA and DA in transfected cell lines (40), suggest a possible synergism in the action of the two categories of drugs in the treatment of carcinoid tumors causing EAS. It should be noticed, however, that one of the two patients responding to cabergoline, had an escape from treatment after three months. Therefore, it needs to be determined in a larger series of patients with EAS, in which proportion of patients a long-term normalization of ACTH and cortisol levels can be achieved with carbergoline treatment.

In conclusion, DRs, mainly D_2 and D_4 receptors, are expressed in carcinoid tumors associated to EAS, where they can mediate a therapeutic effect of DA, particularly cabergoline, in the inhibition of ACTH and cortisol secretion, and in preventing the complications of glucocorticoids excess due to EAS. These data suggest that DA might be included in the therapeutical options, especially in combination with SA, of persisting EAS, mainly for patients with occult or non resectable tumors or patients with active disease before surgery or disease persistence after surgery, who are waiting for the definitive cure.

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2

EXPRESSION OF DOPAMINE AND SOMATOSTATIN RECEPTORS AND EFFECTIVENESS OF COMBINED TREATMENT WITH CABERGOLINE AND LANREOTIDE IN A CASE OF ECTOPIC CUSHING'S SYNDROME CAUSED BY AN ACTH-SECRETING LUNG CARCINOID TUMOR

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New England Journal of Medicine, 2005;352:2457-2458

Short Version

ABSTRACT

The current report describes a case of a patient with ectopic ACTH syndrome (EAS) due to a lung carcinoid tumor, which, after unsuccessful surgery, was successfully treated with a combination of a long-acting somatostatin analog and a long-acting dopamine agonist. A 35 years old male presented with a clinical syndrome suggestive of Cushing's syndrome (CS). Biochemical and hormonal tests suggested the diagnosis of EAS. Somatostatin receptor scintigraphy (SRS) displayed an abnormal uptake at the anterobasal region of the left lung. Computed tomography (CT) confirmed the presence of a lung tumor. The diagnosis of EAS caused by lung carcinoid tumor was made. The patient underwent chest surgery for the removal of the tumor, which was confirmed an ACTH-positive atypical carcinoid. However, after surgery, CS persisted. SRS still demonstrated the abnormal uptake in the left lung, whereas the chest CT was negative. As re-operation was considered no option, based on the positive SRS, the patient started a treatment with the somatostatin analog lanreotide. After 6 months, ACTH and cortisol secretion had normalized on a dose of 90 mg/month. However, after 12-month treatment, ACTH and cortisol levels escaped and lanreotide was stopped. The evaluation of somatostatin and dopamine receptor expression in a tumor sample demonstrated the D_2 and sst_5 receptor expression. Therefore, based on the documented D_2 expression by the tumor, a treatment with the dopamine agonist cabergoline was started. After 6 months, ACTH and cortisol secretion had normalized on a dose of 7 mg/week. However, after 12-month treatment, ACTH and cortisol levels escaped and cabergoline was stopped. In a final attempt of medical treatment, on the basis of the documented cooperation between D_2 and sst_5 receptors, a combined treatment with cabergoline and lanreotide was started. A rapidly-occurring and persistent normalization of ACTH and cortisol secretion was observed. After 12-month treatment plasma ACTH and urinary cortisol levels were still normal. The current is the first documented case of the effectiveness of a combined treatment with a somatostatin analog and a dopamine agonist in a patient with EAS associated to a lung carcinoid not responsive to the single agents during long-term treatment.

Introduction

The ectopic ACTH syndrome (EAS) is a rare cause of chronic endogenous hypercortisolism or Cushing's syndrome (CS), accounting for approximately 15-20% of ACTH-dependent CS and 5-10% of the totality of CS (1,2). The most common causes of EAS are lung carcinomas and neuroendocrine tumors, including thymic, pancreatic and, mainly, lung carcinoids (3,4). The major differences between EAS associated with a lung carcinoma and a lung carcinoid are the prognosis, which is more unfavourable in the former, and the diagnosis, which is more difficult in the latter. Indeed, in the case of lung carcinoids, the similar clinical presentation and endocrine features make EAS undistinguishable from pituitary CS (5,6). Moreover, the localization of a lung carcinoid is further complicated by the failure of conventional imaging techniques because of their small size and/or their location in the inner middle third of the lung, where differentiation from normal vessel is a challenge (7,8). The frequent expression of somatostatin receptor (SR) have made SR scintigraphy (SRS) a common tool to identify the occult lung carcinoids, although its real usefulness remains controversial (9-12). Conversely, dopamine receptor (DR) scintigraphy (DRS) is not routinely used for the localization of lung carcinoids, despite the fact that DR expression has been postulated in carcinoids (13) and DRS was demonstrated to visualize some types of neuroendocrine tumors (14). The treatment of choice of EAS associated with lung carcinoids is surgical removal of the tumor, but its success rate is limited due to persistent tumor remnants (15). Therefore, pre-surgical lack of localization of the tumor or post-surgical persistence of tumor remnant frequently necessitate medical treatment, which is generally palliative and aimed at inhibiting adrenal cortisol secretion (16). However, somatostatin analogs (SA) have been found to be effective in controlling the carcinoid ACTH secretion (17), whereas dopamine agonists (DA) have never been used in the treatment of EAS. SR and DR have been recently demonstrated to cooperate in their function, potentiating SA and DA effects (18). In the current report, we describe a case of EAS associated with a lung carcinoid, which was unsuccessfully treated by surgery and successfully treated by the combination of a long-acting SA, lanreotide, and a long-acting DA, cabergoline. The current report describes for the first time the DR expression and DA effectiveness, as well as the synergistic effect of a SA and a DA in the control of a lung carcinoid associated with EAS.

Case Report

A 35 year-old man was admitted to our Department with a clinical syndrome characterized by asthenia, headache, dyspnoea, sexual disturbances, polyfagia, polyuria and polydipsia. Physical examination revealed central obesity, muscle atrophy, moon face, and abdominal and thoracic purple striae. This clinical picture suggested a diagnosis of CS. The measurement of the blood pressure demonstrated systemic arterial hypertension. The laboratory examination revealed hyperglycaemia, hypercholesterolemia and hypokaliemia associated with metabolic alkalosis, hypocalcemia and hypophosphatemia. An oral glucose tolerance test demonstrated impairment of glucose tolerance. Baseline hormonal evaluation showed decreased serum TSH with normal thyroid hormones, decreased serum testosterone and increased urinary cortisol levels with greatly increased ACTH levels (**Table 1**). This hormonal pattern confirmed the diagnosis of CS, and indicated an ACTH-dependent CS. The dexamethasone test demonstrated a lack of cortisol suppression after low and a higher than 50% inhibition after high dose dexamethasone administration whereas there was an absent ACTH response to CRH administration. The main common tumoral and neuroendocrine markers, including chromogranin A and neuro-specific enolase (NSE) were negative. Pituitary magnetic resonance imaging (MRI) was negative. This combination of clinical, laboratory, hormonal and radiological features suggested the diagnosis of EAS. Therefore, after a standard chest and abdominal computed tomography (CT), which were negative, SRS by [^{111}In-DTPA-D-Phe^1]-octreotide (^{111}In-octreotide) scintigraphy was performed. The total body scan of SRS revealed an intense uptake of ^{111}In-octreotide in the anterobasal region of the left lung (**Fig. 1**). A high resolution chest CT scan, focused on the region with the pathological ^{111}In-octreotide uptake, confirmed the presence of a 1.5 cm lesion in the anterobasal region of the left lung, with the radiological characteristics of a carcinoid, without detectable pathologic lymph nodes (**Fig. 2**). On these bases a diagnosis EAS associated to an ACTH-secreting lung carcinoid was made.

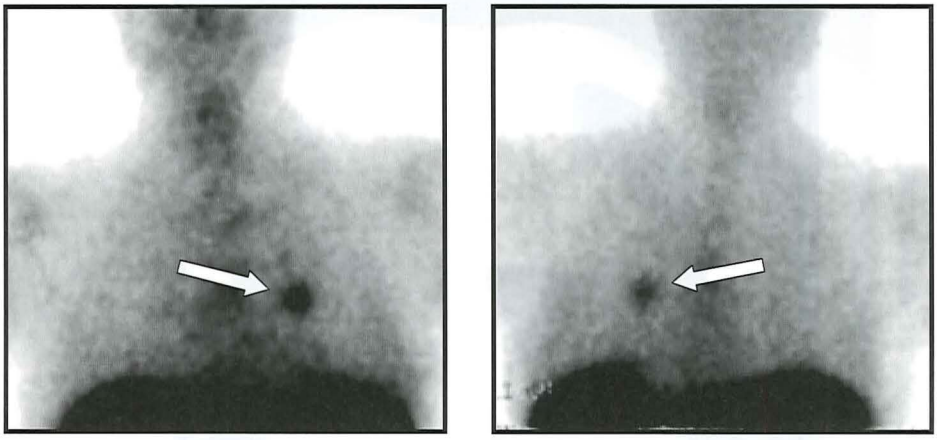
Three months after this diagnosis, the patient underwent chest surgery for the removal of the lung carcinoid. The surgical sample presented a 1.2 cm lesion in the superior lobe of the left lung. Histological examination of the removed lesion showed an atypical carcinoid (well-differentiated carcinoma) whereas immunohistochemical evaluation showed a chromogranin A-positive, NSE-positive, and ACTH-positive tumor confirming the diagnosis of ACTH-secreting lung carcinoid. At the time of surgery, a tumor sample was collected, fixed in

Table 1: Clinical, biochemical and hormonal parameters of the patient during the clinical course of the disease

	Systolic blood pressure mmHg	Diastolic blood pressure mmHg	Plasma Glucose (mg/dl)	Serum sodium (mmol/L)	Serum potassium (mmol/L)	Serum testosterone (µg/L)	Plasma ACTH (ng/L)	Serum cortisol 08.00 a.m. (µg/L)	Serum cortisol 08.00 p.m. (µg/L)	Urinary free cortisol (µg/day)
At the diagnosis	170	100	124	138	2.5	2.0	250	250	240	1800
After thorax surgery	150	95	110	139	3.3	2.4	120	220	184	900
After 6 month LAN	140	85	100	137	3.8	3.5	44	190	110	150
After 12 month LAN	150	95	115	139	2.9	2.4	112	230	170	510
After 6 month CAB	130	80	105	140	3.6	3.9	40	180	90	100
After 12 month CAB	140	90	115	138	2.9	3.4	95	240	155	460
After 6 month LAN+CAB	130	85	95	142	4.1	4.0	50	200	90	132
After 12 month LAN+CAB	120	80	100	139	3.8	3.9	40	180	80	90

LAN: lanreotide; CAB; cabergoline.

Normale values: plasma glucose: 60-110 mg/dl; serum sodium: 135-148 mmol/L; serum potassium: 3.5-5.3 mmol/L; serum testosterone: 3.0-9.0 µg/L; plasma ACTH: 10-50 ng/L; serum cortisol 08.00 a.m.: 50-200 µg/L; serum cortisol 08.00 p.m.: 50-90 µg/L; urinary free cortisol: 35-135 µg/day



A B

Figure 1: Somatostatin receptor scintigraphy with [^{111}In-DTPA-D-Phe^{1}]-octreotide. Planar image of the chest at 24 hrs from the injection at anterior (A) and posterior (B) views. The arrow indicates a pathological uptake in the antero-basal region of the left lung.

paraformaldehyde and embedded in paraffin for immunohistochemistry (IHC), and an additional tumor sample was collected, immediately frozen in dry ice and stored in a freezer at –80 °C for the reverse transcriptase-polymerase chain reaction (RT-PCR) studies.

During the month following surgery, despite a significant improvement of the clinical syndrome, blood pressure and glucose tolerance, as well as a normalization of kaliemia, calcemia and phosphatemia with correction of metabolic alkalosis, the patient still presented with symptoms and signs of CS, which tended to worsen over time. Three months after surgery a high resolution chest CT did not visualize the tumor remnant. A high resolution chest MRI was also negative. Hormonal evaluation revealed considerably decreased but still elevated plasma ACTH and urinary cortisol levels **(Table 1)**. The response to a dexamethasone test was similar to that observed at diagnosis. Therefore, the patient again underwent SRS, which showed persistence of ^{111}In-octreotide uptake in the same region visualized before. These findings suggested the diagnosis of persistent EAS due to a remnant of the carcinoid tumor.

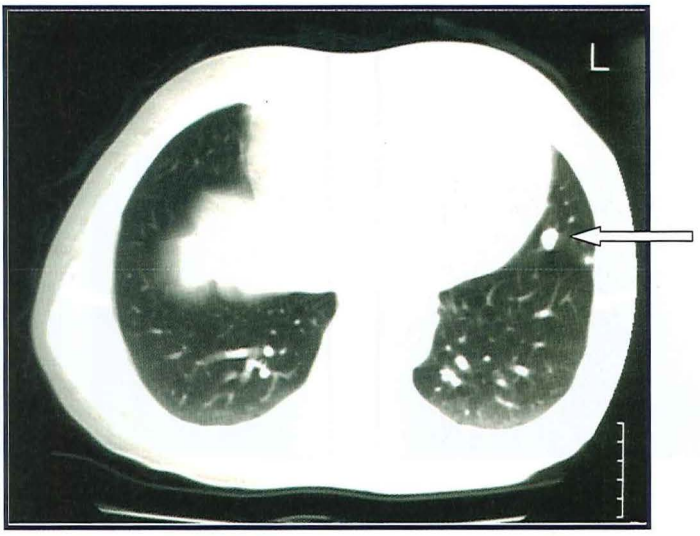


Figure 2: Computed tomography. Thorax scan of the basal region of the chest. The arrow indicates a round lesion of 1.5 cm diameter in the anterobasal region of the left lung.

As a re-operation wasexcluded by the surgeon because of the negative radiological findings, and based on the positive SRS, a treatment with SA was planned. The acute test with subcutaneous octreotide (0.1 mg) did not suppress ACTH and cortisol levels. Despite this, the patient started treatment with lanreotide at 60 mg/month. After 3-months treatment, a significant decrease of plasma ACTH and an impressive decrease of urinary cortisol levels was observed together with an improvement of the clinical picture. Therefore, the dose of lanreotide was increased to 90 mg/month. After 6-months treatment, a normalization of ACTH and cortisol secretion was reached (**Table 1**). However, after 9-months treatment, an escape of plasma ACTH and urinary cortisol levels was observed and despite a further increase of the dose of lanreotide up to 120 mg/month, they were even higher at 12-month treatment evaluation (**Table 1**). No other major changes in laboratory and radiological examination were found. A SRS displayed an unchanged uptake pattern.

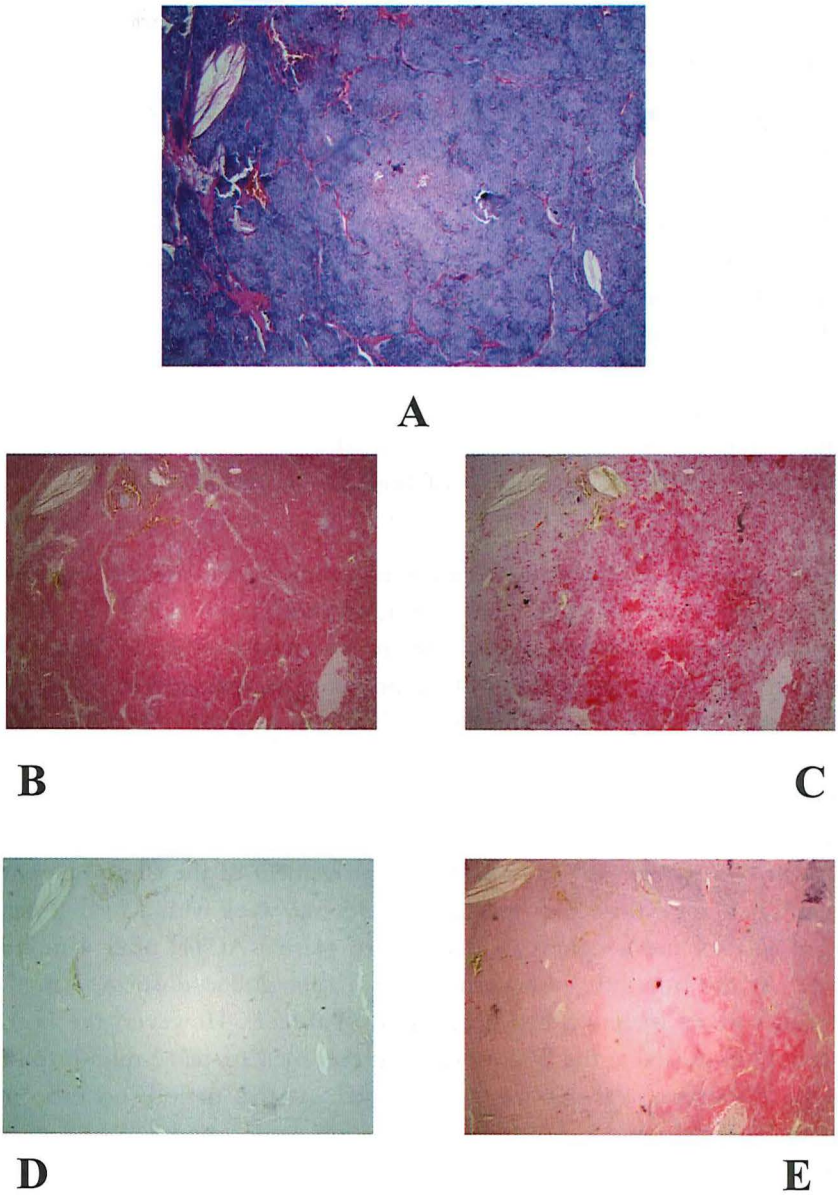


Figure 3: Immunohistochemistry in the carcinoid tumor. Histology (A), ACTH (B), chromogranin (C), sst_2 receptor (D) and D_2 receptor (E) immunostaining. The picture shows a diffuse immunostaining for chromogranin A and ACTH, confirming the diagnosis of ACTH-secreting carcinoid tumor. The carcinoid displays a significant immunostaining for D_2 receptor and not for sst_2 receptor.

However, due to the re-appearance of the clinical picture of CS, the treatment with lanreotide was stopped.

After lanreotide withdrawal, treatment with ketoconazole was started at the dose of 400 mg/day, then increased up to 800 mg/day till normalization of cortisol secretion was achieved. The patient was treated with ketoconazole for 6 months; thereafter hepatotoxicity occurred and the drug was stopped. During this period, IHC study, evaluating SR type 2 (sst_2) and DR type 2 (D_2) and RT-PCR, evaluating all SR and DR subtypes, was performed on the tumor sample collected at surgery. Beyond the positive immunostaining for chromogranin and ACTH, a significant, diffuse and specific immunostaining for D_2 but not for sst_2 was found throughout the tumor tissue (**Fig. 3**). The RT-PCR experiment demonstrated a weak SR type 1 (sst_1) and SR type 3 (sst_3) expression and strong SR type 5 (sst_5) expression as well as the expression of D_2, and particularly of both D_2 isoforms, and DR type 4 (D_4) (**Fig. 4**).

One month after ketoconazole withdrawal, re-testing of the patient demonstrated the clear recurrence of the disease. However, although plasma ACTH and urinary cortisol had increased, the radiological picture remained unchanged. Based on the evidence of DR and, particularly D_2 expression at the experimental studies, treatment with the long-acting DA cabergoline was planned. An acute test with cabergoline (1 mg) demonstrated a good tolerance to the drug , and a slight hormonal decrease after 24 hours. Therefore, cabergoline treatment was started at 1.0 mg/week. After 1-month treatment considerable urinary cortisol decrease but not of plasma ACTH as well an important improvement of the clinical picture was observed. Cabergoline dose was increased to 3.5 mg/week with a further decrease of urinary cortisol and a significant decrease of plasma ACTH after 4 months of treatment. At the 6-month evaluation, under a cabergoline dose of 7 mg/week, ACTH and cortisol secretion had normalized (**Table 1**). However, the hormonal values started to escape at the 9-month and further increase at 12-month follow-up (**Table 1**). Therefore, cabergoline was stopped. At this period, no other major changes in laboratory and radiological examination were found and SRS displayed an unchanged uptake pattern.

Finally, after 3 months wash out a combined treatment with lanreotide (120 mg/month) and cabergoline (7 mg/week) was attempted and normalization of ACTH and urinary cortisol levels was observed after 3 months of treatment and confirmed after 6 and 12 months of treatment (**Table 1**). Presently, after 18-months treatment, the patient is starting to demonstrate a slight increase of ACTH and

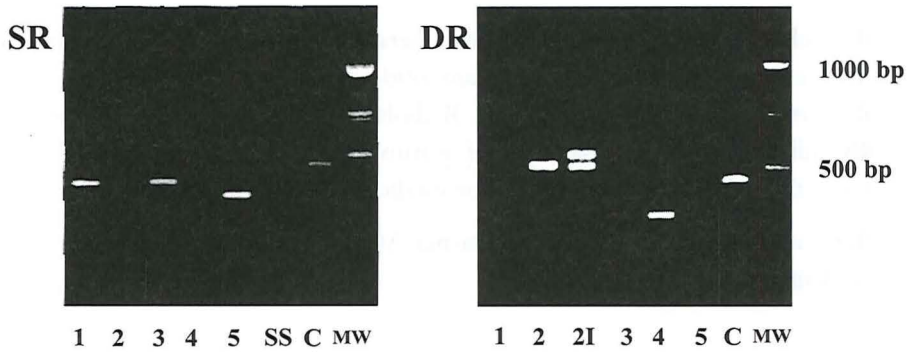


Figure 4: RT-PCR in a carcinoid tumor sample. Electrophoresis gel of the RT-PCR experiment showing the somatostatin receptors (SR) from 1 to 5 and the somatostatin (SS) and the dopamine receptors (DR) from 1 to 5, including the two isoforms (short and long) of the D_2 receptor (2I). The picture shows the positivity of the sst_1, sst_3 and mainly sst_5 and the strong positivity of both isoforms of D_2 and the D_4 receptors.

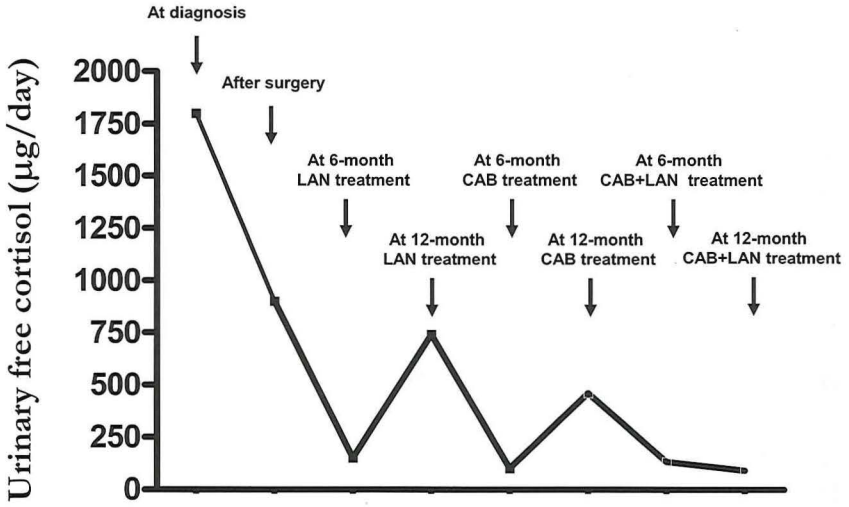


Figure 5: Urinary cortisol levels of the patient during the clinical course of the disease: at the diagnosis, after thorax surgery, after 6 and 12 months of lanreotide treatment, after 6 and 12 months of cabergoline treatments and after 6 and 12 months of combined treatment with lanreotide and cabergoline.

cortisol levels. A low-dose treatment with ketoconazole (200 mg/day) has been added to the combined treatment with lanreotide and cabergoline in order to keep cortisol secretion completely normalize. Radiological examination is still negative but SRS still shows the double area of positive uptake of ^{111}In-octreotide. The patient is at the moment well controlled by medical therapy.

The effect of different treatments on plasma ACTH and urinary cortisol levels is shown in **Fig. 5**.

Methods

RT-PCR: The methodology of RT-PCR has been previously reported (19-21). Messenger RNA was isolated using Dynabeads Oligo (dT)25 (Dynal AS Oslo, Norway) from a frozen tissue sample of the bronchial carcinoid. Complementary DNA (cDNA) was synthesized using the poly A+ mRNA captured on the Dynabeads Oligo (dT)25 using avian myeloblastosis virus Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK). The amplification reaction mixture contained cDNA template, 0.5 U SuperTaq (HT Biotechnology Ltd., Cambridge, UK), 50 μM of each deoxynucleotide triphosphate (HT Biotechnology Ltd., Cambridge, UK), 5 pmol of each of a pair of oligonucleotide primers specific for human somatostatin (sst_1-sst_5) and dopamine (D_1-D_5) receptor subtype, including both isoforms (short and long) of D_2 receptors or the hypoxantine ribosyl transferase (HPRT) in a buffer of 10 mM Tris-HCl (pH 9), 50 mM KCl, 2 mM MgCl2, 0.01% (wt/vol) gelatin, 0.1% Triton X-100 in a final volume of 50 μl. The sequences of the primers for somatostatin and dopamine receptors and HPRT have been previously reported (19-21). The PCR reaction was carried out in a DNA thermal cycler with heated lid (Perkin Elmer Cetus Instruments, Gouda, The Netherlands). Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A+ mRNA preparation for somatostatin receptors and D_1 and D_5, whose genes are intron-less, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. Amplification of cDNA samples with the HPRT specific primers served as positive controls for the quality of cDNA. To exclude contamination of the PCR reaction mixtures, reactions were also performed in the absence of DNA template in parallel with cDNA samples. As positive controls for the PCR reactions of the dopamine receptor subtypes and HPRT, 0.01 μg of human brain cDNA was amplified in parallel with the cDNA samples of the lung carcinoid.

IHC: The methodology of immunohistochemistry has been previously described 19,20,22). The tissue samples of the bronchial carcinoid were cut in 5 μm thick sections,. deparaffinized, and incubated with a D_2 receptor antibody (Chemicon International, Temecula, CA, USA) at the dilution of 1:500 or a sst_2 receptor antibody (Biotrend, Cologne, Germany) at the dilution of 1:3000. A standard streptavidin-biotinylated-alkaline phosphatase or -peroxidase complex (ABC kit, Biogenix, San Ramon, CA) was used to visualize the bound antibodies. Negative controls for the immunohistochemistry included: a) omission of the primary antibody; b) preabsorbtion of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 nm).

Hormonal assay: Plasma ACTH levels were measured using an immunoradiometric assay whereas serum and urinary cortisol were measured by a radioimmunological assay, using commercially available kits.

Statistical analysis: The comparison between the pre- and post-treatments parameters have been performed by Analysis of Variance for paired data. Significance was set at 5%.

Discussion

EAS is a rare but severe syndrome frequently associated with the so called "neuroendocrine" tumors that are localized in the chest, as the lung "carcinoids", or derived from the gastro-entero-pancreatic system, as gastric, intestinal or pancreatic "carcinoids", recently defined as "endocrine tumors or carcinomas" (2,17,18). The most common tumor causing EAS is the lung carcinoid (3). The optimal treatment of EAS associated with neuroendocrine tumors, is surgical removal of the tumor and related resectable metastases (2,9). In particular, the primary treatment of EAS due to lung carcinoids is complete removal of the lung tumor and related lymph nodes (10). However cure of the disease is reached only in a minority of cases due to the following reasons: 1) the tumors are frequently undetectable, demonstrating the limits of the conventional diagnostic procedures; 2) the surgical removal of the tumor is often associated with persistent or recurrent disease, demonstrating the poor effectiveness of surgical procedures, and 3) the presence of metastases seriously complicates the management and worsens the prognosis of the malignant tumors (19-22). Therefore, surgery does not represent a curative treatment in the

majority of patients. On the other hand, alternative treatments are generally palliative. Indeed, since chemotherapy is not effective except in aggressive tumors, treatments targeting the adrenal glands and aiming at normalizing cortisol secretion, are the most commonly used therapies when the source of ACTH secretion cannot be found, removed or treated (11). In this regard, a medical approach, as with adrenolytic drugs, or a surgical approach, as with bilateral adrenalectomy, is employed. However, both treatments cannot be definitive treatments, because they do not influence the causative tumor directly, although they prevent the complications related to CS. Moreover, the use of adrenolytic drugs is often associated with poor effectiveness and severe side effects and adrenalectomy is associated with irreversible hypocortisolism, which makes the patient life-long dependent on hydrocortisone replacement therapy (11). Furthermore, the specific treatment of malignant lung carcinoids depends on the dissemination and localization of the metastases, often requiring a multimodal therapeutic approach (20,22).

SAs have recently taken a central place in the management of EAS due to lung carcinoids (12). Indeed, carcinoids, being neuroendocrine tumors, frequently express SR (23). SR expression has been used either for the diagnostic or therapeutic approach of these tumors. SRS has been proposed as an important diagnostic procedure to visualize occult carcinoids associated to EAS and to predict the possible effectiveness of SA in the treatment of the disease (7,8,24). However, although short-term efficacy of octreotide in controlling ACTH and cortisol secretion has been described in many cases of EAS, reports of long-term effectiveness of octreotide are limited probably because of the frequent occurrence of treatment escape (12,25-27).

Despite the well known DR expression and role in pituitary tumors (15,28-34), the DR expression and its role in non-pituitary neuroendocrine tumors, and particularly in carcinoids, has never been extensively evaluated. In addition, the use of DRS for the localization of neuroendocrine tumors has never been described. Furthermore, despite the demonstrated DA effectiveness in the treatment of pituitary-dependent CS (15,35,36), the possible DA effectiveness in the treatment of EAS has never been tested.

DRs represent a group of five different G-protein coupled receptors (D_1-D_5), mediating the various central and peripheral actions of dopamine (37). These receptors can be subdivided in two different families on the basis of their biochemical and pharmacological characteristics: the D_1-like receptors, including D_1

and D_5, and the D_2-like receptors, including D_2, D_3 and D_4 receptor (37). DR subtypes have a tissue-specific distribution and play different roles in the various organs and tissues (37). The D_2 receptor seems to be the most widely expressed and most relevant DR in the endocrine system, as well as in endocrine tumors (37), although D_4 has also been demonstrated in the normal pituitary and adrenal gland and in pituitary and adrenal tumors (15,16,37), where it might also mediate DA effects on hormone release and/or cell proliferation, in cooperation with D_2 receptor.

DRs, including both D_1-like and D_2-like receptors, as well as dopamine synthesis have been demonstrated in gastrointestinal neuroendocrine tumor cell lines (13). Moreover, dopamine synthesis has been documented in neuroendocrine tumors and, particularly, in gastrointestinal carcinoids, being the basis of the successful use of imaging techniques in the visualization of these tumors, as well as for pheocromocytoma (38,39). The current study is the first demonstrating DR expression in a significant proportion of ACTH-secreting ectopic tumors, including neuroendocrine tumors originating from the lung, thymus and the gastro-entero-pancreatic system and belonging to the class of the so called "carcinoids". D_2 and D_4 represented the only DR expressed in these tumors. These data suggest that DR may play a role in the biology of these tumors and/or form a target for the use of DA.

DA, mainly bromocriptine, have already been used with controversial results in ACTH-secreting pituitary tumors. However, an escape from treatment has been observed after long-term DA-treatment in a considerable number of cases (35,36). Recently, cabergoline has been demonstrated to be more effective than bromocriptine in the treatment of these tumors, suggesting a potent action of this long-acting DA, which has a higher affinity and selectivity for D_2 and, probably, D_2-like receptors (15). The current study demonstrates for the first time a possible effectiveness of DA, in particular cabergoline, in the treatment of ACTH-secreting ectopic tumors, causing EAS. Short-term cabergoline treatment significantly inhibited ACTH, and consequently cortisol secretion in two out of three cases with EAS due to a lung carcinoid. Hormonal suppression was maintained for a long-term follow-up in one of these two cases, without any change in the size of the tumor. It has to be pointed out that the only patient not responsive to cabergoline was bearing a tumor expressing only the long isoform of D_2, and the patient who experienced a treatment escape was bearing a tumor expressing the long isoform of D_2 and D_4 receptor. Conversely, the patient showing long-term responsiveness to cabergoline, was bearing a tumor expressing both isoforms of D_2 and D_4 receptors.

Taken into consideration that three cases are not enough to draw final conclusions, it seems that the expression of short isoform of D_2 and/or the co-expression of D_4 may play a pivotal role in the effectiveness of DA in the treatment of these tumors associated to EAS. This hypothesis seems to be supported by similar evidence found in the treatment of pituitary tumors (15,33). Alternatively, the responsiveness or the protection from treatment escape could be related to the different amount of D_2 and or D_4 expressed in the tumor. Indeed, it has to be pointed out that the patient being long-term responsive to cabergoline treatment had a tumor with a strong and homogeneous D_2 immunostaining. On the other hand, as only a qualitative and not a quantitative evaluation of DR by RT-PCR and a semiquantitative evaluation by IHC have been performed, this hypothesis cannot be confirmed or excluded on the basis of the results of the current study. Moreover, since no experience exists on the effectiveness of bromocriptine, no comparison can be performed between the effectiveness of bromocriptine and cabergoline in the treatment of carcinoid tumors and EAS. In addition, the well known pharmacokinetic and pharmacodynamic properties of cabergoline and the described potency of cabergoline in the treatment of pituitary tumors would suggest that it can be considered the best DA to be used in the treatment of EAS.

Finally, the possible effectiveness of DA, demonstrated in the present study, and the well described effectiveness of SA, demonstrated in previous studies, together with the demonstration of a synergistic cooperation between SR and DR and the potentiation of the correspondent SA and DA in transfected cell lines (40), suggest a possible synergy of the combination of the two categories of drugs in the treatment of carcinoid tumors causing EAS. It should be noticed, however, that one of the two patients responding to cabergoline, had an escape from treatment after three months. Therefore, it needs to be determined in a larger series of patients with EAS, in which proportion of patients a long-term normalization of ACTH and cortisol levels can be achieved with carbergoline treatment.

In conclusion, DRs, mainly D_2 and D_4 receptors, are expressed in carcinoid tumors associated to EAS, where they can mediate the therapeutic effect of DA, particularly cabergoline, in the inhibition of ACTH and cortisol secretion, and in preventing the complications of glucocorticoids excess due to EAS. These data suggest that DA might be included in the therapeutic options, especially in combination with SA, of persisting EAS, mainly for patients with occult or non resectable tumors or patients with active disease before surgery or disease persistence after surgery, who are waiting for the definitive cure.

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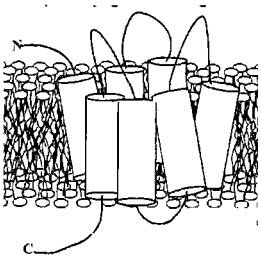
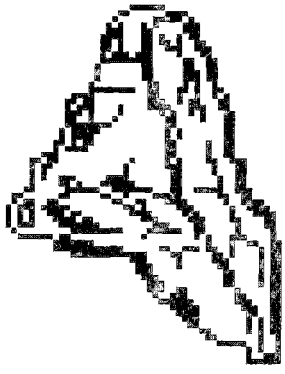


GENERAL DISCUSSION



GENERAL DISCUSSION

This thesis focuses on the role of dopamine receptors in the regulation of the human hypothalamus-pituitary-adrenal axis, either in physiological or in pathological condition, as well as on the potential role of dopamine agonists in the treatment of disorders of the hypothalamus-pituitary-adrenal axis, mainly in chronic endogenous hypercortisolism or Cushing's syndrome (CS).

Dopamine is the predominant catecholamine neurotransmitter in the human central nervous system, where it is involved in the regulation of cognition, emotion and locomotion, as well as in the regulation of the endocrine system. Dopamine is also an important neurohormone for the hypothalamus-pituitary system: it is produced by the hypothalamus and transported *via* the hypothalamus-pituitary vascular portal system into the pituitary gland, where it controls the synthesis and or secretion of different hormones. Dopamine exerts its function *via* five different receptors, which can be divided into two distinct families according to similar structural and functional characteristics, the D_1-like family, including D_1 and D_5, which mainly mediate a stimulatory action, and the D_2-like family, including D_2, D_3 and D_4 receptors, which mainly mediate an inhibitory action. The D_2 receptor has been indicated as the dopamine receptor mediating the totality of dopaminergic action at the level of the pituitary gland. Indeed, the expression and function of the D_2 receptor has been extensively demonstrated in the normal pituitary gland by different techniques, evaluating the transcriptional or the transductional gene products, either in experimental animals or in humans. In different studies, mainly performed in the rat pituitary gland, the D_2 receptor was found to be expressed in the anterior and in the intermediate lobe of the pituitary gland, and to mediate the inhibitory action of dopamine on prolactin (PRL) and melanocyte stimulating hormone (MSH) synthesis and secretion. This documented effect of dopamine suggested the predominant expression of D_2 receptors in the lactotroph cells of the anterior lobe and in the melanotroph cells of the intermediate lobe of the normal pituitary gland. On the other hand, no study in experimental animals or in humans has clearly investigated so far the expression of D_2 receptors in the different cell populations of the pituitary gland, *via* the demonstration of co-localization of D_2 receptors with the different pituitary hormones. Moreover, the pivotal role of dopamine in PRL and MSH release and the predominant D_2 receptor expression in lactotroph and melanotroph cells of the pituitary gland have been extrapolated from rat to humans, where, however, the morphology of the gland is different mainly for the absence of a well defined intermediate lobe, which is replaced by a cell cluster

localized among colloid-filled cysts between the anterior and the posterior lobe of the gland, in a so called intermediate area or zone. Recent studies have demonstrated that the D_2 receptor is expressed in more than 75% of the cell population, suggesting its presence in non-lactotroph and non-melanotroph cells of the human normal pituitary gland. The widespread expression of D_2 receptors in the pituitary gland is also suggested by its expression in different rat and/or human pituitary tumors, including not only the lactotroph or PRL-secreting, but also somatotroph or GH-secreting, thyrotroph or TSH-secreting, and recently, corticotroph or ACTH-secreting and clinically non functioning, gonadotroph or FSH/LH-secreting pituitary tumors. The possible expression of D_2 receptors in corticotroph cells of the anterior lobe of the pituitary gland is of particular interest, as the melanotroph cells of the intermediate zone, which express the receptor, are considered as a peculiar group of corticotroph cells: both populations are indeed able to produce either ACTH or MSH, the former predominant in the anterior lobe and the latter in the intermediate zone.

Chapter II of the thesis is focused on the demonstration of D_2 receptor expression by immunohistocehmistry (IHC) in the corticotroph cells of the human normal pituitary gland. The results of the study clearly confirmed that the D_2 receptor is expressed in a great majority of cell populations of the pituitary gland. Moreover, a comparison between the histology and the D_2 receptor immunostaining demonstrated that the receptor is homogeneously and strongly expressed in the intermediate zone, whereas heterogeneously expressed with variable intensity in the anterior lobe of the pituitary gland. Moreover, the comparison between D_2 receptor and ACTH, as well as MSH immunostaining, showed that both melanotroph and corticotroph cells do express the D_2 receptor, although with stronger intensity in the former compared with the latter cell population. This represents the first study clearly demonstrating D_2 receptor expression in the corticotroph cell population and suggests a possible physiological role of dopamine in the regulation of this cell population of the human normal pituitary gland. However, no major physiological effect of dopamine on corticotroph cells has been documented, either in experimental animals or in humans. Therefore, the possibility that dopamine participates in controlling growth or function of normal corticotroph cell populations remains unclear and requires further investigations. On the other hand, the results of this study suggest a potential therapeutic implication of dopamine agonists in the treatment of corticotroph pituitary tumors, in those cases in which dopamine receptor expression and function persists in the neoplastic corticotroph cells forming this type of pituitary tumors.

Chapter III.1 of the thesis is focused on the demonstration of D_2 receptor expression and function in corticotroph pituitary tumors. D_2 receptor expression has been previously investigated in a series of pituitary tumors of different origin, including a small series of silent or functioning corticotroph pituitary tumors. The current study is the first evaluating the expression of all types of dopamine receptors, including the D_2 isoforms, the D_{2long} and the D_{2short}, at the messenger and protein level, as well as the function of the dopamine receptors both *in vitro* and *in vivo* in a series of corticotroph pituitary tumors from patients with pituitary-dependent CS, namely Cushing's disease (CD). This study clearly demonstrated that D_2 is the predominant dopamine receptor subtype expressed in corticotroph pituitary tumors, as it is expressed in around 80% of cases, while the only other detectable dopamine receptor is the D_4 receptor, which was expressed in around 20% of cases. The expression of D_4 is always associated to that of D_2 receptors. Moreover, a heterogeneous expression of D_2 receptor isoforms was found among the tumors, the expression of D_{2long} alone or in combination with D_{2short} being more frequent than the selective expression of D_{2short} isoform. One of the most interesting findings of this study is the demonstration of the functionality of dopamine receptors in these tumors. The dopamine agonists bromocriptine and cabergoline, the former recognizing both D_1-like and D_2-like receptors, and the latter recognizing the D_2-like more than the D_1-like receptors, were able to significantly inhibit ACTH secretion from cultured human primary corticotroph pituitary tumor cells, in two cases expressing D_2 receptors, but not in two additional cases not expressing the receptors. These data supported the hypothesis of a direct effect of the dopamine agonists at the level of the tumoral corticotroph pituitary cells *via* the activation of D_2 and/or D_4 receptors. Moreover, cabergoline administered at the dose ranging from 1 to 3 mg/week for 3 months was able to significantly inhibit or normalize urinary cortisol secretion in 6 out of 10 patients with persistent Cushing's disease, who had previously been unsuccessfully treated by neurosurgery. The effectiveness of short-term cabergoline treatment in controlling cortisol secretion in about 60% of a small series of patients suggests that cabergoline might be a potential useful drug in the management of CD.

The possible use of treatment with dopamine agonists in the management of CD was previously hypothesized on the basis of the ability of the dopamine agonist bromocriptine to induce cortisol normalization and concomitant improvement of the clinical picture in around 40% of patients with CD after short-term treatment. However, the results of long-term treatment were rather disappointing, suggesting that bromocriptine is only sporadically effective in inducing a stable control of the

disease. The comparison between the previous results with bromocriptine and the current results with cabergoline suggests that cabergoline is more effective than bromocriptine in controlling CD after short-term treatment. A definitive conclusion on the potential use of cabergoline in CD required, however, the evidence of its effectiveness during long-term treatment.

Chapter III.2 of the thesis is focused on the evaluation and comparison between short- and long-term effectiveness of cabergoline treatment in patients with CD. This study evaluated the effect of cabergoline treatment during 1-2 years of treatment in 20 patients unsuccessfully treated by neurosurgery and persistence of CD, not only on ACTH and cortisol hypersecretion but also on the clinical symptoms and signs, tumor size as well as on the main complications of the disease, namely hypertension and impairment of glucose tolerance. The results of this study demonstrated that a differential response to cabergoline treatment frequently occurs in patients with CD. Indeed, a normalization of cortisol secretion was found in 35% of patients, a significant decrease without normalization of cortisol secretion in 40%, whereas no significant decrease of cortisol secretion occurred in the remaining 25% of patients after 3 months of treatment with cabergoline at the median dose of 3 mg/week. It is noteworthy that 75% of the patients partially responsive after 3 months completely normalized cortisol levels during the following 6-12 months of treatment. On the other hand, one third of patients initially responsive experienced a treatment escape during long-term treatment. Therefore, 40% of patients starting the treatment with cabergoline demonstrated a persistent control of cortisol secretion after 1-2 years of treatment. These data suggest that cabergoline is more effective than bromocriptine in controlling cortisol hypersecretion in patients with CD, although treatment escape occurs also with cabergoline in a small percentage of patients. The reason of the higher effectiveness of cabergoline compared to bromocriptine can be related to the different pharmacokinetic and pharmacodynamic characteristics of the two drugs: cabergoline is a long-lasting whereas bromocriptine is a short-lasting drug; moreover cabergoline displays a relatively higher affinity for D_2 receptors, and, probably, the entire D_2-like receptor family, which mediate the dopamine agonist effects on ACTH secretion and a relatively lower affinity for the D_1-like receptor family, which mediates the majority of side effects of the drugs. These characteristics make cabergoline more potent and better tolerated and, therefore, associated with a better compliance than bromocriptine. However, different mechanisms could explain the different effectiveness of cabergoline and bromocriptine as well. Indeed, since dopamine receptors have been demonstrated to homodimerize and heterodimerize with other

dopamine receptors with a consequent potentiation of the agonist action, it is possible that cabergoline may be more prone in inducing receptor dimerization than bromocriptine, therefore causing a more potent effect. Moreover, since dopamine agonists have been demonstrated to induce apoptosis, the possibility that cabergoline is able to induce dopamine receptor-mediated apoptosis better than bromocriptine cannot be excluded. Furthermore, cabergoline might bind and activate the two different D_2 receptor isoforms and/or D_4 receptor, heterogeneously expressed in corticotroph pituitary tumors better than bromocriptine, and *via* these receptors, induce more potent effects on ACTH and cortisol secretion compared to bromocriptine. The superiority of cabergoline to bromocriptine has been demonstrated in different pituitary tumors, such as PRL-secreting and GH-secreting pituitary tumors as well. The mechanism underlying treatment escape is not completely known. However, the demonstration of receptor desensitization, through agonist-induced receptor internalization and degradation, which have been demonstrated to be the basis of tachyphylaxis of somatostatin analogues in neuroendocrine tumors, could also be applicable to dopamine receptors in corticotroph pituitary tumors. Moreover, uncoupling from second messenger activation following long-term agonist exposure, may also provide a potential explanation for treatment escape. The reason why the phenomenon of the escape occurs in ACTH-secreting and not in PRL-secreting or GH-secreting pituitary tumors could be related to the different numbers and/or patterns of dopamine receptors, as well as the D_2 receptor isoforms in the different pituitary tumors. Indeed, the long-term responsiveness to cabergoline was significantly associated with the expression of the short, rather than the long isoform of D_2 receptor and/or the expression of D_4 receptors in the corticotroph pituitary tumors. It is important to outline that long-term treatment with cabergoline induced a significant improvement of the clinical picture and shrinkage of the corticotroph pituitary tumors in patients with persistently controlled ACTH and cortisol secretion, whereas a significant improvement of gonadal and sexual function as well as hypertension and impairment of glucose tolerance occurred in patients responsive, as well as in those not responsive or resistant to the treatment. These observations demonstrate that cabergoline may act on these parameters also independently from the inhibition of cortisol secretion. In summary, the results of this study demonstrate that cabergoline may be a useful tool in the treatment of CD, especially in patients unsuccessfully treated with the gold standard treatment, namely neurosurgery. The limit of medical treatment with cabergoline in CD is the persistence of its effectiveness in maintaining a stable control of the disease only in

around 40% of patients. Therefore, the choice to treat a patient with cabergoline should be considered in a selected group of patients with CD.

Chapter III.3 is focused on the predictability of D_2 receptor expression in corticotroph pituitary tumors in relation to the effectiveness of cabergoline treatment in patients with CD. In the 1980's, a series of studies from Rotterdam reported the observation that the patients with CD responsive to treatment with bromocriptine also demonstrated a relative resistance to cortisol suppression after dexamethasone administration at diagnosis, a relative resistance to cortisol normalization after treatment with neurosurgery, and concomitant hyperprolactinemia. The observation of a corticotroph hyperplasia instead of well-defined adenomas at the border between the anterior and posterior pituitary gland, suggested that the patients responsive to bromocriptine were represented by the group bearing a corticotroph lesion of the intermediate zone of the pituitary gland; this area is, indeed, mainly represented by melanotroph cells, the peculiar corticotroph cells which are not regulated by peripheral glucocorticoids, but by hypothalamic dopamine. This hypothesis also suggested that this type of corticotroph lesion was related to dopamine depletion, inducing corticotroph hyperplasia and, consequently, hypercortisolism, beyond lactotroph hyperplasia and hyperprolactinemia. This hypothesis was supported by the frequent observation of nerve fibers in the corticotroph tumors, a peculiar feature of the intermediate zone of the pituitary gland. In order to verify this hypothesis, the current study has evaluated the relation between D_2 receptor expression or the effectiveness of cabergoline treatment and a series of clinical, biochemical and radiological features in a large group of patients with CD, as well as a series of pathological features of the tumors removed from these patients at neurosurgery. This study demonstrated that corticotroph pituitary tumors removed from patients with CD presumably originated from corticotroph cells of the anterior lobe in 55%, and from melanotroph cells of the intermediate zone in the remaining 45% of cases. Among the whole group of the tumors, the majority are well defined basophilic adenomas whereas a minority are represented by basophilic hyperplasia, which seems to derive either from the anterior lobe or the intermediate zone of the pituitary gland. The expression of D_2 receptors was demonstrated in more than 80% of cases, including nearly all those originating from the intermediate zone and around 70% of those deriving from the anterior lobe. These observations suggest that the great majority of corticotroph tumors, independently from their origin express D_2 receptors and that the only ones not expressing the receptor derive from the anterior lobe of the pituitary gland. The results of the study demonstrated, on the other hand, that the

tumors expressing the highest number of D_2 receptors were mostly derived from the intermediate zone and were significantly associated with a specific characteristic of the CD patients. Indeed, the patients in whom a higher expression of D_2 receptors was found had a significantly lower age at diagnosis and a longer disease duration, a lower cortisol response to dexamethasone and CRH, higher circulating levels of PRL and prevalence of hyperprolactinemia, a higher prevalence of radiologically undetectable tumor and failure of neurosurgery, and a higher prevalence of corticotroph hyperplasia compared with those with a lower amount or absence of the expression of the D_2 receptor. These findings are in line with the original hypothesis and indicate that the tumors which express a high number of D_2 receptors are actually derived from the intermediate zone of the pituitary gland. The most important results of the current study, however, relate to the responsiveness to cabergoline treatment. Indeed, a significant correlation was found between the responsiveness to short-term treatment and the origin of the tumor from the intermediate zone as well as the number of D_2 receptor expressed in the tumor. However, this association was lost when the results of short-term were transposed to those of long-term treatment. The only significant association with the responsiveness of long-term treatment was represented by the tumor expression of the short isoform D_2 receptor or D_4 receptor in the tumors, without, however, any association with the tumor origin. In general, the results of this study demonstrate that the great majority of corticotroph pituitary tumors express D_2 receptors and potentially respond to cabergoline treatment; the initial responsiveness to cabergoline treatment, clinically observed in 75% of patients, is mainly associated with tumors derived from the intermediate zone of the pituitary gland but it is also observed in a certain percentage of tumors derived from the anterior lobe. However, the responsiveness to cabergoline treatment is maintained after long-term treatment only in a selected group of patients expressing the short isoform of D_2 receptor equally derived from the anterior lobe or the intermediate zone, or the D_4 receptor, mainly derived from the intermediate zone of the pituitary gland. These observations suggest that the clinical picture and the biochemical and radiological features of the patients might predict the initial responsiveness to cabergoline treatment, but only the pathological examination or the receptor characterization of the tumor might better predict the long-term responsiveness in patients with CD.

The data described in **Chapter III** of the thesis actually support the potential use of medical treatment with cabergoline in the management of CD. This represents an interesting finding, as CD is the only pituitary disease associated to a functioning pituitary tumor without any effective medical treatment acting directly on the tumor.

Indeed, CD is a chronic disorder associated with increased morbidity and mortality due to cardiovascular diseases, mainly related to the occurrence of visceral obesity, hypertension and glucose intolerance, which, together with dyslipidemia and thrombosis diathesis, induces premature atherosclerosis and increased incidence of cardiac ischemia and stroke. These observations make CD a severe disease and require an as early as possible cure. Therefore, cure of CD is one of the major challenges in medicine. Neurosurgery is the first line treatment of CD, but transsphenoidal removal of the pituitary tumor is associated with an average success rate of 75%. The failure of neurosurgery is mainly due to the very small size of microadenomas and the relatively frequent finding of multiple microadenomas or diffuse hyperplasia, not detectable using the conventional imaging techniques or even not detected at surgical exploration. Moreover, the neurosurgical outcome is also affected by disease relapse during the 10 years after surgical remission in 10-20% of cases, reducing the final success rate of neurosurgery to around 50%. Pituitary radiotherapy and surgical bilateral adrenalectomy are second choice treatments in patients not cured by neurosurgery. However, these treatment modalities frequently result in secondary adverse events. Indeed, radiotherapy is associated with a success rate ranging from 30 to 70%, but the disease cure is delayed up to 10 years at the cost of severe adverse events, mainly hypopituitarism. On the other hand, bilateral adrenalectomy is associated with a nearly 100% success rate, but it is affected by a mortality rate of 2%, it induces a permanent hypoadrenalism, which makes the patient life-long dependent on replacement therapy with glucocorticoids and mineralcorticoids, and it frequently induces the development of an invasive pituitary tumor associated with skin hyperpigmentation, namely the Nelson's syndrome, during decades after surgery. Medical therapy presently plays a minor role in the treatment of CD, being usually performed before neurosurgery or after pituitary radiotherapy in order to obtain a rapid hormonal normalization before the definitive cure. Medical treatment is usually performed with adrenal blocking drugs, which do not act at the level of the pituitary tumor. On the other hand, although several neuromodulatory drugs acting at the pituitary level have been used, no single agent has ever been demonstrated sufficiently effective to achieve a widespread clinical use in the management of the disease, being bromocriptine the only exception. In consideration of its demonstrated higher efficacy than bromocriptine, cabergoline represents, actually, the only valid medical possibility to control hypercortisolism associated with CD.

Chronic endogenous hypercortisolism, or Cushing's syndrome, however, may derive not only from an ACTH-secreting pituitary tumor, as in CD (pituitary-dependent

CS), but also from a cortisol-secreting adrenal tumor (adrenal-dependent CS) or an ACTH-secreting ectopic tumor (ectopic CS or ectopic ACTH syndrome). The obvious question after the demonstration of dopamine receptor expression in corticotroph pituitary tumors and the effectiveness of dopamine agonists in CD is whether dopamine receptors are expressed in adrenal and ectopic tumors and whether dopamine agonists might also be effective in the adrenal and ectopic forms of CS.

Chapter IV is focused on the evaluation of dopamine receptor expression and function in the human normal adrenal and in adrenal tumors. The expression of dopamine receptors has been previously demonstrated by receptor ligand studies in the normal adrenal gland both in experimental animals and humans. Moreover, dopamine agonists have been found to exert an inhibitory effect on catecholamine secretion and influence aldosterone secretion as well. The dopamine receptor has also been demonstrated in adrenal tumors deriving from the medulla, as well as in aldosterone-secreting benign tumors deriving from the cortex of the adrenal gland. However, no study has definitely demonstrated the specific pattern of dopamine receptors in the normal adrenal gland, as well as in the different adrenal tumors in humans. The results of this study demonstrated by RT-PCR that both D_1-like and D_2-like receptors are expressed in the human normal adrenal gland. In particular, D_1 and D_5, as well as D_2 and D_4 including both D_2 receptor isoforms, were found in the normal adrenal gland as well as in adrenal hyperplasia derived from bilateral adrenalectomy performed in patients with CD. Moreover, it was demonstrated by IHC that the D_2 receptor was moderately expressed in the medulla, which produces catecholamines, and strongly expressed in the *zona glomerulusa* and *zona reticularis* of the adrenal cortex, which produce mineralcorticoids, mainly aldosterone, and sex steroids, respectively, whereas it was faintly expressed in the *zona fasciculata* of the adrenal cortex, which produces glucocorticoids, mainly cortisol. This study also demonstrated that bromocriptine and cabergoline were able to induce a slight stimulation at low doses and an inhibition at high dose mainly of aldosterone, but also of cortisol and androstenedione *in vitro*, and that cabergoline was more potent than bromocriptine in both effects on primary cultures of adrenal cells deriving from adrenal hyperplasias. These findings suggested that dopamine may play a role in the regulation of hormone secretion or, possibly, cell growth in the adrenal gland, although this role has not been completely clarified yet. Finally, this study demonstrated that the majority of adrenal tumors maintain dopamine receptor expression. In particular, D_2 and D_4 receptors are usually expressed in benign aldosterone and cortisol-secreting tumors, as well as in non functioning tumors,

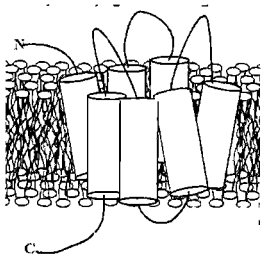
whereas D_2, D_4 and D_5 receptors are usually expressed in pheochromocytomas, derived from the medulla of the gland. D_2 receptors are also expressed in malignant cortisol-secreting tumors. Taking into consideration that the D_2-like receptors, which are associated with an inhibitory action, are mainly expressed in the adrenal tumors, dopamine agonists might exert a possible effect on hormone secretion and/or cell growth in these tumors. Moreover, the possibility that cabergoline is able to induce an inhibition of cortisol secretion in CD acting not only at pituitary but also directly at adrenal levels cannot be ruled out. On the other hand, it cannot be excluded that cabergoline may induce a cortisol inhibition also in cortisol secreting adrenal tumors and could be potentially useful in the treatment of adrenal-dependent CS.

Chapter V is focused on dopamine receptor expression and function in endocrine tumors associated with ectopic CS. The ectopic tumors associated with CS are mostly represented by ACTH-secreting endocrine tumors localized in lung, thymus or gastroenteropancreatic tract and belong to the so called category of "carcinoids". **Chapter V.1** focused on the evaluation of dopamine receptors, mainly D_2 receptor, expression by RT-PCR and IHC, in six different ACTH-secreting endocrine tumors derived from the lung, thymus or pancreas and the effectiveness of cabergoline treatment in 3 patients with persistent ectopic CS despite the removal of the endocrine tumor from the lung. The results of the study demonstrated that D_2 receptor was expressed in more than 80% of these cases, with a heterogeneous expression of the two isoforms, whereas D_4 receptor was expressed in 1 out of 3 cases. Treatment with cabergoline at the dose of 3.5 mg/week was effective in normalizing cortisol levels in 2 of the 3 cases, although one of the responsive cases experienced a treatment escape after the third month of treatment. These findings demonstrated that the D_2 receptor is expressed in most ACTH-secreting endocrine tumors associated to ectopic CS and that cabergoline is potentially effective in controlling the disease in around one third of cases. This represents a new finding since residual ectopic tumors after primary surgery are often not detectable using the conventional imaging techniques and surgery cannot be useful as a secondary approach for the definitive cure of the disease. Indeed, endocrine tumors associated with EAS are actually not easily visualized with the conventional imaging techniques even at the time of the diagnosis of the disease. As the well known characteristic of these tumors is represented by their ability to express somatostatin receptors, scintigraphy with radiolabelled somatostatin analogues is currently used in an attempt to visualize these frequently "occult" tumors, although its real usefulness is still a matter of debate. Moreover, radiolabelled and non radiolabelled somatostatin

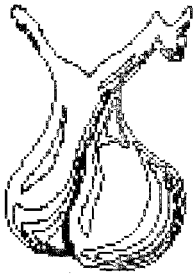
analogues, such as octreotide and lanreotide, have been used in the treatment of these tumors. The different reported trials demonstrated that somatostatin analogues can be effective in some cases in controlling the disease, although the patients are often affected by the phenomenon of treatment escape. Escape from somatostatin analogs therapy may be related to multiple mechanisms, including down-regulation of somatostatin receptors, uncoupling of second messenger activation and/or outgrowth of receptors negative clones. However, somatostatin analogues represent one of the most important currently available treatment modalities for this category of tumors. It is interesting to outline that somatostatin and dopamine receptors have been demonstrated to cooperate with a potentiation of the actions mediated by the respective agonists, *via* the phenomenon of receptor heterodimerization. In particular, a specific somatostatin receptor subtype, the sst_5 has been found to dimerize with the D_2 receptor transfected in a cell line and this was associated with a significant potentiation of the action of both a somatostatin analogue and a dopamine agonist. This finding suggests a possible effectiveness of the combined treatment with somatostatin analogues and dopamine agonists in endocrine tumors associated to ectopic CS expressing both categories of receptors. Indeed, **chapter V.2** describes a case of an atypical lung "carcinoid" expressing three different somatostatin receptors subtypes (sst_1, sst_3 and mainly sst_5) and two different dopamine receptor subtypes (D_2 and D_4) with persistent CS after surgery and a residual tumor not visualized by conventional imaging techniques, but clearly visible at the somatostatin receptor scintigraphy. In this case, the treatment with lanreotide controlled cortisol secretion for six months after which a treatment escape occurred. After subsequent treatment with cabergoline a similar behavior with six months effectiveness and following escape, occurred. However, thereafter, combined treatment with cabergoline and lanreotide was effective in controlling the disease for more than one year. This case represents an example of the possible dramatic potentiation in the effectiveness of these two types of drugs and the possibility that the combination of these compounds can be effective even when the single agents are not effective anymore. In general, **chapter V** demonstrated the relevance of dopamine receptors, beyond somatostatin receptors, and the possible use of dopamine agonists, alone or in association with somatostatin analogues, in the treatment of endocrine tumors associated to ectopic CS.

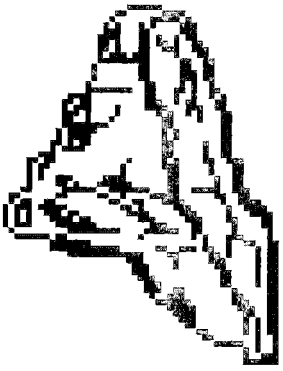
In summary, the dopaminergic system plays a pivotal physiological role in the regulation of the hypothalamus-pituitary-adrenal axis, as functional dopamine receptors are significantly expressed both in the corticotroph cells of the pituitary gland and in cells of the adrenal gland. The persistence of the dopamine receptors in

the ACTH-secreting tumors deriving from the pituitary gland and in tumors deriving form the adrenal gland, as well as the presence of the receptor in the extra-pituitary ACTH-secreting tumors suggest a possible usefulness of dopamine agonists in the management of CS.



SUMMARY





SUMMARY

The subject of the current thesis is the role of dopamine receptors in the regulation of the human hypothalamus-pituitary-adrenal axis in normal and in pathological conditions and the potential use of dopamine agonists in the treatment of the disorders of the hypothalamus-pituitary-adrenal axis, mainly in chronic endogenous hypercortisolism or Cushing's syndrome (CS).

The dopamine D_2 receptor was demonstrated to be expressed in corticotroph cell populations of the human normal pituitary gland by immunohistochemistry (IHC); D_2 receptor was found both in the corticotroph cells of the anterior lobe and in the melanotroph cells of the intermediate zone of the pituitary gland. The heterogeneity of D_2 receptor expression in the different cell populations suggests that melanotroph cells of the intermediate zone express a high number of the receptors compared to corticotroph cells of the anterior lobe of the pituitary gland (**chapter II**).

Dopamine D_2 receptor expression was demonstrated both by IHC and RT-PCR in more than 80% of corticotroph pituitary tumors obtained from patients with pituitary-dependent CS or Cushing's disease (CD). These tumors heterogeneously expressed the two isoforms (short and long) of the D_2 receptors as well as the D_4 receptor in a minority of cases, always in association with the D_2 receptor. The functionality of the D_2 receptor in the corticotroph pituitary tumors was demonstrated by the effectiveness of the dopamine agonists bromocriptine and cabergoline in inducing an inhibition of ACTH secretion *in vitro* in cell cultures derived from D_2 positive, but not in those derived from D_2 negative corticotroph tumors, and by the effectiveness of cabergoline in normalizing cortisol secretion after 3-months treatment in around 60% of patients with CD (**chapter III.1**). The effectiveness of cabergoline treatment was extensively evaluated in 20 patients with CD followed for 1-2 years. The evaluation of the clinical follow-up demonstrated that cabergoline at the median dose of 3 mg/week was able to induce a decrease of cortisol secretion after 3-months treatment in 75%, although a full response with a normalization of cortisol levels was found in 35% of patients with CD. On the other hand, continuation of cabergoline treatment in the responsive patients with increasing doses demonstrated that 75% of the partially responsive patients normalized their cortisol levels, whereas 33.3% of the responsive patients experienced a treatment escape. Therefore, overall 40% of patients starting cabergoline treatment can be stably and long-term controlled at a median dose of 3.5 mg/week after 1-2 years. The treatment with cabergoline was demonstrated to

be useful not only in normalizing cortisol secretion, but also in inducing tumor shrinkage in responsive patients and in improving gonadal and/or sexual function as well as hypertension and impairment of glucose tolerance, the main complications of the disease, both in responsive and non responsive patients. This demonstrates that at least part of the effects of cabergoline are not mediated by the inhibition of cortisol secretion (**chapter III.2**). The correlation between the clinical, biochemical, radiological and pathological features of CD patients on the one hand, and D_2 receptor expression in corticotroph pituitary tumors and/or cabergoline effectiveness in patients with CD on the other hand, were retrospectively evaluated in a large series of cases. The results demonstrated that not one feature was really able to predict D_2 receptor expression in the corticotroph tumors, although the tumors expressing the D_2 receptor had a significantly higher prevalence of histopathological characteristics suggesting their origin from the intermediate zone compared to those not expressing D_2 receptors, which seem to derive from the anterior lobe of the pituitary gland. Moreover, the group of tumors with the higher D_2 receptor expression, evaluated by a semiquantitative score at IHC or a quantitative score at RT-PCR, was significantly associated not only with the pathological characteristics, but also with some clinical, biochemical and radiological characteristics presumably associated with tumors derived from the intermediate zone, such as a relative resistance to the suppression by dexamethasone or the stimulation by CRH, the frequent occurrence of hyperprolactinemia, and the presence of corticotroph hyperplasia at histhological evaluation, rather than a well defined adenoma. Although these tumors showed the highest responsiveness to cabergoline at the initial treatment, the persistence of a stable control of the disease required the expression of the short isoform of the D_2 receptor, which is equally present in tumors of the intermediate zone and the anterior lobe and/or the expression of D_4, which is always expressed in the tumors derived from the intermediate zone of the pituitary gland (**chapter III.3**).

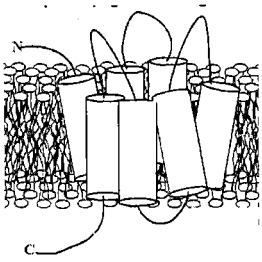
Dopamine receptors, including both the D_1-like (D_1 and D_5) and D_2-like (D_2 and D_4) receptors, were demonstrated by RT-PCR to be expressed in the human normal adrenal, where both isoforms of D_2 receptors are constantly expressed and D_3 receptor, also belonging to the D_2-like family, seems to be the only receptor never expressed. Moreover, D_2 receptors were demonstrated by IHC and found to be localized both in the adrenal medulla and in all three areas of the adrenal cortex, although the highest expression level was found in the *zona glomerulosa*, responsible for aldosterone secretion and the *zona reticularis*, responsible for sex steroid secretion. The evidence that dopamine agonists, especially cabergoline, induced a stimulation,

at the low dose, and inhibition, at high dose, mainly of aldosterone *in vitro*, suggests a role of the dopaminergic system in the regulation of the adrenal gland. The persistence of dopamine receptors, mainly D_2 and D_4 receptors, usually associated with inhibitory effects, in the majority of benign and in the cortisol secreting malignant adrenal tumors, suggests a possible usefulness of dopamine agonists in the treatment of these tumors and CS, and the possibility that cabergoline may induce a suppression of cortisol secretion in CD not only acting at the pituitary but also at the adrenal level (**chapter IV**).

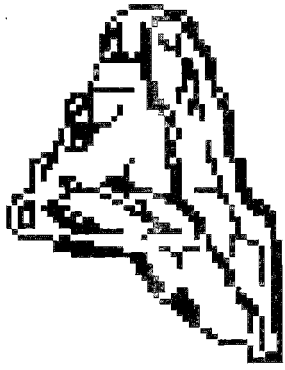
Dopamine receptors, and mainly D_2 receptors, were demonstrated by RT-PCR and/or IHC in the majority of endocrine tumors associated with the ectopic CS or ectopic ACTH syndrome. The D_2 receptor isoforms were heterogeneously expressed in these tumors, while the D_4 receptor was sporadically expressed. Moreover, treatment with cabergoline at the dose of 3.5 mg/week was able to control ACTH and cortisol secretion in two out of three patients, although a treatment escape was observed in one of these two responsive patients after the third month of treatment, whereas a persisting normalization of the cortisol secretion was still observed in the third patient after 6 months of treatment (**chapter V.1**). In an additional case, responsive for 6 months to the somatostatin analogs lanreotide at a dose of 120 mg/month, then responsive for the following 6 months to cabergoline at a dose of 3.5 mg/week, and followed by treatment escape and re-increase of cortisol levels after both treatments, the concomitant administration of both drugs was able to induce a persistent and stable normalization of cortisol secretion for more than one year, demonstrating that the combination of a somatostatin analog and a dopamine agonist may be more effective than the single agent in controlling cortisol secretion in ACTH-secreting endocrine tumors associated to CS. This phenomenon can be related to the demonstrated cooperation, *via* heterodimerization, of the two categories of receptors (**chapter V.2**). The presence of a functional D_2 receptor in corticotroph ectopic tumors supports the hypothesis of a possible use of dopamine agonists in the treatment of these tumors.

The sum of data included in this thesis demonstrates that dopamine receptors, and in particular, the D_2 receptor, are expressed in the majority of corticotroph pituitary and ectopic tumors, responsible of pituitary-dependent and ectopic CS, respectively as well as in the adrenal tumors, including the cortisol-secreting tumors, responsible of the adrenal-dependent CS. The presence of a functional D_2 and the demonstration of an effect of dopamine agonists on ACTH and/or cortisol secretion either *in vitro* or *in vivo*, supports the hypothesis of a physiological role in

the regulation of normal hypothalamus-pituitary-adrenal axis and a role of dopamine agonists in the treatment of the pituitary, adrenal and ectopic tumors associated to CS.



SAMENVATTING



SAMENVATTING

In dit proefschrift is de betekenis van dopamine receptoren in de regulatie van de normale en pathologische hypothalamus-hypofyse-bijnier as bij de mens onderzocht, alsmede de mogelijkheid om dopamine agonisten te gebruiken voor de behandeling van ziekten van de hypothalamus-hypofyse-bijnier as, met name chronisch endogeen hypercortisolisme ofwel Cushing's Syndroom (CS).
De aanwezigheid van de dopamine D_2 receptor werd in de ACTH-producerende (corticotrope) celpopulatie van de normale menselijke hypofyse door middel van immunohistochemie (IHC) aangetoond; D_2 receptoren werden aangetroffen in zowel de corticotrope cellen van de hypofysevoorkwab als in de melanotrope cellen van de hypofysemiddenkwab. De expressie van D_2 receptoren in de cellen bleek heterogeen te zijn en de resultaten van het onderzoek suggereren dat melanotrope cellen van de middenkwab een hoger aantal receptoren bevatten dan corticotrope cellen van de hypofysevoorkwab (**hoofdstuk II**).
Door middel van IHC en de reverse transcriptase polymerase ketting reactie (RT-PCR) kon worden aangetoond dat meer dan 80% van de corticotrope hypofyse tumoren, verkregen door middel van churgische verwijdering van de tumor bij patiënten met het hypofyse-afhankelijke CS ofwel de ziekte van Cushing (CD), D_2 receptoren bevatten. Deze tumoren bleken heterogeen in de expressie van de twee isovormen van de D_2 receptor (de korte en de lange variant), alsmede in een gering aantal in de expressie van de D_4 receptor. De functionaliteit van de D_2 receptor in corticotrope hypofyse tumoren werd aangetoond in vitro en in vivo. De dopamine agonisten bromocriptine en cabergoline remmen de ACTH afgifte door celkweken van D_2 receptor positieve, maar niet van D_2 receptor negatieve corticotrope hypofyse tumoren. Bovendien was 3 maanden behandeling met cabergoline effectief in het verlagen en/of normaliseren van de cortisol spiegel in ongeveer 60% van de patiënten met CD (**hoofdstuk III.1**). Vervolgens is de effectiviteit van behandeling gedurende 1-2 jaar met cabergoline uitgebreid bestudeerd in patiënten met CD. De klinische follow-up toonde aan dat cabergoline met een gemiddelde dosis van 3 mg/week na 3 maanden behandeling in staat was om een verlaging van cortisol spiegels te bewerkstelligen in 75% van de patiënten, hoewel een volledige respons met een normalisering van de cortisol spiegel werd waargenomen in 35% van de patiënten met CD. Voortzetting van behandeling met cabergoline in de patiënten die reageerden op de behandeling, toonde aan dat na verhoging van de dosis de cortisol spiegel alsnog normaliseerde in 75% van de oorspronkelijke partieel reagerende patiënten, terwijl 33.3% van de oorspronkelijk gevoelige patiënten ongevoeligheid voor het geneesmiddel ontwikkelden. Samenvattend bewerkstelligt

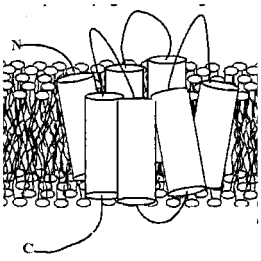
behandeling met cabergoline bij een gemiddelde dosis van 3.5 mg/week een landurige (1-2 jaar) normalisering van de cortisol spiegel in 40% van de patiënten met CD. De behandeling met cabergoline van patiënten met CD bleek niet alleen in staat de cortisol spiegel te normaliseren, maar induceerde tevens tumorverkleining in de gevoelige patiënten en resulteerde in zowel cabergoline gevoelige- als ongevoelige patiënten in een verbetering van gonadale en/of sexuele functie, alsmede in een verbetering van hypertensie en glucose tolerantie, zijnde de belangrijkste complicaties van de ziekte. Deze laatste resultaten tonen aan dat een deel van de effecten van cabergoline niet veroorzaakt wordt door een verlaging van de cortisol spiegel (**hoofdstuk III.2**). Vervolgens werd in een grote serie patiënten de correlatie bestudeerd tussen enerzijds de klinische, biochemische, radiologische en histopathologische kemerken van de patiënten met CD, en anderszijds de dopamine D_2 receptor expressie in de corticotrope tumoren en/of de effectiviteit van behandeling met cabergoline. De resultaten van deze studies toonden aan dat vrijwel geen enkel kenmerk een voorspellende waarde heeft voor wat betreft de expressie van D_2 receptoren. Daarentegen bleek dat tumoren die D_2 receptoren bevatten, in tegenstelling tot D_2 receptor negatieve tumoren, vaker histopathologische kenmerken hebben die een onstaan van de tumoren uit de middenkwab suggereren. D_2 receptor negatieve tumoren lijken voornamelijk afkomstig uit de hypofysevoorkwab. Daarnaast bleken tumoren met een hogere D_2 receptor expressie, onderzocht met een semi-kwantitatieve IHC methode en een kwantitative RT-PCR techniek, significant geassocieerd te zijn met niet alleen histopathologische kenmerken, maar ook met enkele klinische-, biochemische- en radiologische kenmerken die gerelateerd lijken te zijn aan tumoren onstaan uit de hypofysemiddenkwab. Deze kenmerken betreffen een relatieve resistentie voor remming van cortisol met dexamethason, en van stimulatie van cortisol met CRH, het frequent voorkomen van hyperprolactinemie en de aanwezigheid van hyperplasie van corticotrope cellen. Hoewel tumoren met de hoogste D_2 receptor expressie het best reageren op kortdurende behandeling met cabergoline, bleek dat patiënten met tumoren die de korte isovorm van de D_2 receptor bevatten het best langdurig reageren op behandeling. De korte isovorm van de D_2 receptor bleek in gelijke verhouding voor te komen in tumoren met een oorsprong in de hypofysemiddenkwab en de hypofysevoorkwab, terwijl de D_4 receptor altijd aanwezig is in tumoren van de hypofysemiddenkwab (**hoofdstuk III.3**).
Dopamine D_1-type (D_1 en D_5) en D_2-type (D_2 en D_4) receptoren werden met behulp van RT-PCR aangetoond in de normale menselijke bijnier. Beide D2 receptor isovormen waren altijd aanwezig, terwijl de D_3 receptor, behorende tot de

D_2-type familie, de enige dopamine receptor was die nooit werd aangetroffen. Met behulp van IHC werden dopamine D_2 receptoren aangetoond in zowel het bijniermerg, alsmede in alle drie cellagen van de bijnierschors. De hoogste expressie werd gevonden in de zona glomerulosa, verantwoordelijk voor aldosteron productie en de zona reticularis, verantwoordelijk voor de productie van sex steroïden. De bevinding dat dopamine agonisten, met name cabergoline, bij een lage dosis een stimulatie-, en bij een hoge dosis een remming van aldosteron secretie induceert, suggereert een rol van het dopaminerge systeem in de regulatie van de bijnierfunctie. De bevinding dat dopamine receptoren, met name D_2 en D_4 receptoren die gekoppeld zijn aan een remmend effect, tevens voorkomen in de meerderheid van benigne- en cortisol producerende maligne bijniertumoren, suggereert een mogelijk gebruik van dopamine agonisten in de behandeling van patiënten met deze tumoren. Daarnaast suggereren deze resultaten dat cabergoline in patiënten met CD niet alleen via een effect op de hypofyse tumor, maar tevens via een effect op het niveau van de bijnier een verlaging van de cortisol spiegel kan bewerkstelligen. De resultaten van het onderzoek naar dopamine receptoren in de normale bijnier en in bijniertumoren is beschreven in **hoofdstuk IV** van dit proefschrift.

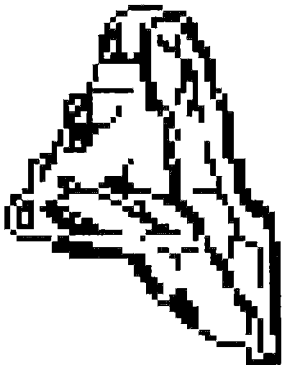
Onderzoek naar het voorkomen van dopamine receptoren in endocriene tumoren die door hun ACTH overproductie het ectopisch CS of ectopisch ACTH syndroom (EAS) veroorzaken, toonde vervolgens aan dat de meerderheid van deze tumoren D_2 receptoren bevatten. Er werd heterogeniteit gevonden in de expressie van de twee D_2 receptor isovormen, terwijl de D_4 receptor weinig frequent aanwezig was. Bovendien bleek dat behandeling met cabergoline in een dosis van 3.5 mg/week in staat was om de ACTH en cortisol spiegels in twee van de drie onderzochte patiënten met EAS te normaliseren. Een van de twee op cabergoline reagerende patiënten werd echter na drie maanden van behandeling ongevoelig voor het geneesmiddel. Daarentegen bleven in de derde patiënt de cortisolspiegels zelfs na zes maanden van behandeling met cabergoline genormaliseerd (**hoofdstuk V.1**). Een andere patiënt met EAS die na 6 maanden behandeling ongevoelig was geworden voor het somatostatine analoog lanreotide (120 mg/maand) werd vervolgens behandeld met cabergoline (3.5 mg/week). Na een normalisering van de cortisol spiegel gedurende 6 maanden in eerste instantie, trad er eveneens ongevoeligheid op voor cabergoline. Vervolgens werd een combinatiebehandeling met lanreotide en cabergoline gestart. Deze combinatiebehandeling bleek in staat om gedurende meer dan 1 jaar een blijvende normalisering van de cortisolspiegel te bewerkstelligen en toont aan dat de gelijktijdige behandeling met een somatostatine analoog en een dopamine agonist meer effectief is bij de behandeling van patiënten met ectopische endocriene ACTH-producerende tumoren dan de behandeling met

de geneesmiddelen afzonderlijk. Deze hogere effectiviteit van de combinatiebehandeling kan gerelateerd zijn aan heterodimeristaie van dopamine en somatostatine receptoren, resulterend in een sterkere activiteit van de hybride receptoren (**hoofdstuk V.2**). Het voorkomen van een functionele dopamine D_2 receptor ondersteunt de hypothese dat dopamine agonisten mogelijk gebruikt kunnen worden bij de behandeling van patiënten met EAS.

Alle gegevens van dit proefschrift samenvattend kan geconcludeerd worden dat dopamine receptoren, met name de D_2 receptor, voorkomen in de meerderheid van corticotrope hypofysetumoren en ectopische ACTH-producerende tumoren die in patiënten aanleiding geven tot het ontwikkelen van respectievelijk het hypofyse-afhankelijk CS en EAS. Daarnaast komen dopamine receptoren, met name de D_2 receptor, voor in bijniertumoren die het bijnier-afhankelijk CS verzoorzaken. De aanwezigheid van een functionele D_2 receptor, alsmede de aangetoonde effectiviteit van dopamine agonisten op de ACTH en/of cortisol productie in vitro en in vivo, ondersteunen de hypothese dat de D_2 receptor een fysiologische rol kan spelen in de regulatie van de normale hypothalamus-hypofyse-bijnier as. Tevens is er een mogelijke rol van dopamine agonisten in de behandeling van hypofyse-, bijnier- en ectopische ACTH-producerende tumoren die CS verzoorzaken.



ACKNOWLEDGEMENTS



ACKNOWLEDGEMENTS

It is always very difficult to put your thoughts and feelings into words in such an exciting and emotional situation. I am going to accomplish one of the most important events in my career and life, and I would like to mention all of the people who have contributed in making it possible for me. I want to be sure that I use the right words to convey my sincere gratitude for supporting and helping me reach such an important goal.

The present thesis is the result of a close scientific and personal collaboration between me, a young inexperienced Italian man in the field of research, coming from a just respectable research group of Naples, and the highly proficient and famous research group of Rotterdam, led by professor Steven W.J. Lamberts. I am very honoured and at the same time, very proud of the possibility to meet and work with this special group of people, running between Rotterdam and Naples, during the last seven years. I have learnt a lot from them since they have not only helped me become a better "scientist" but also a better "man". Together, we carried out an interesting research project, and this thesis represents the final outcome; however, the most important project we succeeded in finalizing is a life project called "friendship", which I hope will last forever. The tangible proof of this link is the fact that after these seven years, I have started to think of myself as an integral part of the Rotterdam research team, and, to feel a little Dutch voice in my soul. My experience in the Netherlands greatly enriched my mind and my heart. Nederland bedankt voor dit fantastische avontuur!!!

My first heartfelt "thanks" goes to my "promotor", Prof. Dr. Steven W.J. Lamberts. He is one of the greatest men and scientists in the world! His knowledge and experience in medical practice and research is enormous but he is still able to maintain a juvenile enthusiasm for every project, with a complete openness and availability for the young investigators; this is a rare virtue, which contributes in making him a unique person and scientist. He really played a major role in this important step of my career and life. Dear professor Lamberts, thanks for believing in such an immature, but willing investigator; thanks for having given me the opportunity to meet such interesting people and experienced scientists, to work in such a valid laboratory and to live in such a stimulating country; thanks for supporting me in all of the difficult moments, and always guiding me in the right direction, despite all of the drawbacks during the course; thanks for considering me as a promising investigator and giving me the opportunity to grow as a scientist and a man. On behalf of every young investigator, thanks for being so courageous in supporting and safeguarding the career of all deserving young people, based on meritocracy, even fighting against the world. Dear professor Lamberts, seven years

ago you were a great and unreachable myth for me, where I could only admire you from a distance, like Leonardo's "Gioconda." Meeting you and working with you has been a dream come true for me! I really hope that I have earned and deserved your consideration, and I hope I will not disappoint you in the future.

An immense gratitude and a friendly warm embrace goes to my "copromotor" Dr. Leo J. Hofland, the expert chief of the laboratory, where I spent two entire years and took my first steps in basic research. As time went on, he became a very special person for me. He was a great guide and supervisor for all of my work, and he spent a lot of time and energy teaching me how to face the basic research. He has given me treasures of knowledge to take home and keep with me forever. Dear Leo, how many times did your Dutch preciseness clash with my Italian flexibility?! However, I am now convinced that your preciseness has been a great challenge for me as well as my flexibility for you, and that the combination of the two gave us the best results in research and improved our personalities. I have to say that, beyond Dr. Hofland, the "scientist", I had the unique occasion to experience Leo, the "man". He always succeeded in making my daily life in Rotterdam as comfortable as possible. Dear Leo, thanks for all of the time we spent talking about my situation and for your comprehension regarding my difficulties and problems in Italy; thanks for all of the nice dinners organized at your place, where I frequently had the pleasure to spend some time with your lovely wife Nicolette and your splendid daughters Sandra, Ilse and Carlijn. I would like to thank your family for always making me feel at home. Dear Leo, I still remember the first time I met you and your family in Naples. We spent a wonderful day in Pompei where I had to act as a guide throughout the Roman ruins, but I was so excited that I did a bad job. At that time, I would never have thought of becoming a good friend of yours. However, one day, I promise, I will take you to Pompei again, but I will first prepare myself to be the best guide. My friend, I would like to give you a special "thanks" for all the personal support you gave me in finalizing my research project and my thesis, and a special "sorry" for all the troubles I gave you and for the precious time I stole from you. Dear Leo, like any good friends, we had some quarrels as well. I hope you can forgive me for any hard words that I have used this past months. At times, I had to release some stress and I needed to blow off some steam with a friend. Dear Leo, I will never forget what you did for me and my thesis, and above all, I will never forget our friendship, hoping that it will never end.

I would like to express my gratitude to all of the people from the laboratory, who taught me the technical procedures that were useful for finalizing the research project of this thesis. Thanks to Marlijn Waaijers, Diana Mooij, and Peter van Koetsveld. Your daily careful and experienced teaching and supervision have permitted me to perform successful experiments and to rapidly reach great results.

Dear Marlijn, Diana and Peter, thanks, without all of you this thesis would have never been realized. I would like to dedicate some extra words from my heart to the two ladies, with whom I had many nice conversations about Italian and Dutch customs during the daily "Koffie" and "Koffie Met" in the laboratory. Diana, you are one of the sweetest people I have ever met. I have to say that working close to you made me feel very warm, which is very important for somebody who lives in a foreign country far from his family and close friends. Marlijn, you are a very special person for me. My dear Marlijn, no words can be appropriate enough to express my immense gratitude and affection for you. You have taken care of me and of my daily life during the entire period I spent in Rotterdam. I will always remember your zeal in solving my complicate quarrel with the tax office. You were a friendly presence, always available in giving me support in any way by solving my problems inside and outside the laboratory. I think that the syntony and friendship that has spontaneously grown between us are extremely rare. You have definitely deserved a special place in my heart.

I would like to acknowledge prof. H.A.P Pols and prof. A-J van der Lely for their important support to my research project and all of the colleagues, who greatly contributed with their work or their knowledge in carrying on my research project and completing my thesis, including Dr. Wouter de Herder, Dr. Martin van Hagen, Dr. Ronald de Krijger and Dr. Max Kros. A special "thanks" goes to Wouter. He offered me a great part of the clinical data included in this thesis, and all of his experience in the clinical practice in the management of Cushing's syndrome. Dear Wouter, thanks for your strict collaboration, but also for all of your funny jokes. I cannot forget all the other people from the laboratory, including Liesbeth, Ellen, Babette, Frank, Joost, Lisa, Henk, Pauline, Carlotta, Piet, Jan-Willem, Joop, nice companions of my unforgettable days and nights in Rotterdam.

It is important for me to thank the three members of the "promotiecommissie", Prof. dr T.J. Visser, Prof. dr. F.H. de Jong, and Prof. dr. P. Jaquet. I am very honoured for the privilege to have such outstanding scientists in my doctoral committee. I would like to thank them for their kindness in allowing me to stay in Italy during the period of the examination of the thesis, and for its final judgment. Dear prof. Visser, thank you for always being very nice to me during the period I spent in the laboratory. Dear prof. de Jong, thank you very much for the constructive criticism and advice that significantly improved the quality of my thesis. Dear prof. Jaquet, thank you for your appreciation of my thesis and my research, and for demonstrating me your friendship in this delicate moment of my career.

This paragraph is dedicated to my special "paraninfen". The choice was very easy. I wanted two close friends, which could be symbols of the connection between Naples and Rotterdam, and the expression of my two souls: the Italian and the

Dutch ones. Diego Ferone and Virgil A.S.H. Dalm are simply the best I could have. Diego is one of my best Italian friends, and he has been part of my research team in Naples, but above all, he is the person with whom I started my adventure in the Netherlands. Virgil is one of my best Dutch friends, and he has been part of my research team in Rotterdam, but above all, he is the person, with whom I enjoyed the end of my adventure in the Netherlands. My dear Diego, you shared everything with me, the ins and the outs, the good and the bad, the enthusiasm and the frustration. You endured my happiness, my anger and my suffering like only a very good friend can do. I will always remember the days and the nights we spent together in the laboratory performing experiments, especially when a sudden irruption of the police scared us so much that it caused us to destroy all of the tubes and the experiments. I will also never forget our little escapes to Amsterdam to have some fun. My dear Diego, thanks for being beside me and for always being a good friend of mine; you are very important for me. Virgil, I enjoyed your company very much. The memories of our funny jokes in the laboratory still happen to make me laugh at times, as well as the memories of our personal secrets still allow me to feel the intriguing atmosphere of those moments we shared during my last period in Rotterdam. My dear Virgil, thanks for the fun, but also for the help that you spontaneously gave me in performing some important experiments for my research project after my departure. And thanks for taking complete care of the organization of my party. I hope that one day I will find the right way to compensate you.

It is very important for me to acknowledge my research group in Naples. I greatly thank prof. Gaetano Lombardi, who was a stepping stone for me in clinical research. I would like to thank him for giving me the opportunity to have this formative experience in Rotterdam. A special "thanks" goes to his wife Annamaria, a very dear and deep person, who has been one of the most enthusiastic "fans" of me and "sponsor" of my career.

It comes from my heart to give special thanks to my guide, prof. Annamaria Colao. She saved me from an uncertain future when I was still a young fellow, and she has always protected me during my formative period in becoming a doctor and a scientist. Her enthusiasm in research and her spirit to prevail against adversity had captured my attention right away. She has always given me the best support to work in a complicated environment and proceed in a difficult career. She has the virtue of being objective and never forgetting the final purpose of her work; the goal of building a valid team of investigators who can work together with harmony, and the desire in reaching important results in medical science. It is a pleasure for me to be part of this team and work beside such an outstanding woman and scientist. Of course, I cannot deny that her wonderful blue eyes contribute in creating such a pleasurable environment. Dear Annamaria, I will never forget the enthusiastic

period when we started to grow rapidly as a recognized research group. I will always remember the nights spent in the hospital playing and singing with all of our friends and colleagues during your duty. Dear Annamaria, thanks for your trust and admiration in such a naive guy with a troubled personality. I hope to have the possibility to work with you for the rest of my life.

I cannot forget to mention all of my colleagues in Naples. As they are a lot of people, I will just mention my strict coworkers Antongiulio, Mariagiovanna, Carla, Giovanni, Antonio, Cristina, Renata, Linda e Mariano, but my affection is addressed to everybody. I would like to give special regards to Antongiulio. He started his training in medical practice and research with me, and he immediately earned my respect and affection. I have always considered him as a brother rather than a simple coworker. He grew so much and so rapidly that he replaced me in an exceptional manner during the two years that I spent in Rotterdam. His strength and meticulousness allowed him to reach important goals very rapidly. What I appreciated much of this guy is that during all these years, he always stayed beside me throughout the highs and lows. Dear Antongiulio, thank you for your friendship and for your fidelity. I hope that you will always keep in mind that, beyond my apparent hardness, I will always support you and help you make your career as brilliant as possible. I cannot forget to thank Cristina for her invaluable help in the clinical management of patients with Cushing's syndrome. The contribution of Antongiulio and Cristina in the clinical research on Cushing's syndrome has been really important for finalizing this thesis. Finally, I want to send a big kiss to Carla, who has always made herself completely available and ready to help me in any situation, and to give a strong hug to Giovanni, who has opened his house for me so frequently during my recent escapes to Rotterdam, and facilitating my contact with the laboratory during this past year.

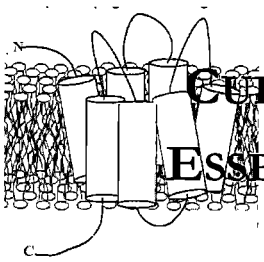
A special "thanks" goes to all my friends: their presence are always important in the crucial moments of life. In particular, I want to thank Carlo Alviggi for his effort in performing the best prenatal ultrasound scan of my two children for the realization of the cover of this thesis. I cannot forget to mention Luca Aiello for giving me a hand in the paging of this thesis and to Luciano De Venezia, and his coworker Luca Daniele, for having improved the quality of the pictures of this thesis, and mainly for having transformed my own personal idea in a beautiful cover for the thesis.

This is the most difficult and the most emotional paragraph! I think that no word can be appropriate enough for thanking the people who really make my life. No word can completely express my feelings for my family. My first embrace is for my lovely wife, Daniela. She is simply unique. Her infinite generosity and immense love for our family, together with her tenacity and passion for life, has made her a marvellous women and fantastic wife. She has changed my life enormously, and she

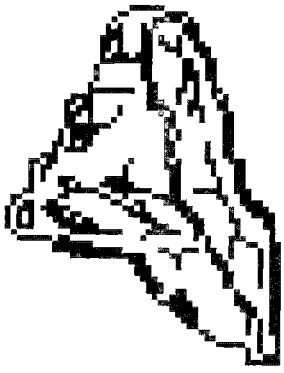
makes me a better man day by day. My darling, thank you very much for all of the support you have offered me during the last years, allowing me to finalize this thesis, and thanks for tolerating my outlets, my moodiness and my intricate personality. And, most important, thank you from the bottom of my heart for giving me such a wonderful family and for offering your heart every day to our exceptional children. Last, but not least, please trust my love. It is incommensurable and it is forever. What I can say to my angels! It is not a secret that my children, Désirée and Christian, are the most precious treasures of my life. I thank God every day for giving me the immense luck to have such jewels. They have enriched my soul so much to completely change my inner world, and my life, since the moment I received the news of their existence. Now, I could not live without them. The impish light of their eyes, their sweet or sly smiles, the funny expressions of their faces, their loving grasps, their sweet kisses and their lovely sounds have taught me the right way to look at life; they have completely filled my life. My sweet Désirée and my funny Christian, thanks for existing and giving me a tangible reason for being in this world. I hope you can feel all the love of your father.

Per mia madre e mio padre, non esistono parole che possano contenere e trasmettere la mia immensa gratitudine e il mio infinito amore. La loro completa dedizione, la loro costante presenza nei momenti di gioia e nei momenti di dolore, il loro continuo sacrificarsi in silenzio per permettermi di soddisfare i miei desideri e realizzare i miei sogni, e più importante, il loro costante amore mi hanno reso un uomo privilegiato e mi hanno permesso di raggiungere in modo equilibrato e maturo i migliori traguardi nella carriera e nella vita. Mamma, papà, grazie di esistere e di esserci sempre per me. Ringrazio il cielo di essere nato vostro figlio.

Looking back at the last seven years, I feel an intricate and intense tingle of emotions. Seven years ago, I dreamed of setting up a house to live with a nice family, and of building a career in medical research, with a perfect combination between the clinical and scientific counterparts. I thought these objectives were too ambitious to achieve altogether. Well! Today I am very happy to have reached both goals in a short time; I started a research career, and most of all, I have a wonderful family. This was only possible thanks to all of the people who have supported me during these years. And I have to admit, a dash of luck was needed because the combination between family and career is not always easy, and often requires a lot of sacrifice. I hope that the people of my working environment in Naples and Rotterdam can understand and forgive me for any faults in my engagements. Mostly, I pray every day in order that my family and my children will forgive me for my absence and lack of attention in such an important period of their lives. I hope they will understand that all I do has the final goal of making their future lives more cheerful. They will always hold a special place in my mind and my heart.



CURRICULUM VITAE AND ESSENTIAL BIBLIOGRAPHY



CURRICULUM VITAE

The author of this thesis was born on the 9th of January 1970 in Naples, Italy. Between 1983 and 1988 he attended the Scientific Lyceum in Naples. In 1988, he obtained the diploma of Scientific Lyceum with the maximal score (60/60). Between 1988 and 1994, he attended the medical school at the Faculty of Medicine of "Federico II" University of Naples. During his medical studies, he attended as pre-graduate fellow the Department of Molecular and Clinical Endocrinology and Oncology (chair: prof. Gaetano Lombardi) of the "Federico II" University, where he was involved in studies on pathogenesis, diagnosis and treatment of pituitary diseases. In 1994, he obtained the degree magna cum laude in Medicine, with special "Academic Mention" for the curriculum, whereas in 1995, he obtained the license to practice as a medical doctor in Naples, with the maximal score (90/90). Between 1994 and 1999, he attended the School of Specialization in Endocrinology and Metabolism (chair: prof. Gaetano Lombardi), and continued his attendance as post-graduate fellow in the Department of Molecular and Clinical Endocrinology and Oncology of the "Federico II" University of Naples, where he was mainly involved in studies on the pathogenesis, diagnosis and treatment of pituitary and adrenal tumors, focusing on Cushing's syndrome. In 1999, he obtained the post-graduate degree magna cum laude (50/50) in Endocrinology and Metabolism, and in the same year he was registered as a Specialist in Endocrinology and Metabolism in Naples. Between 1999 and 2002, he attended the doctorate of research in Neuropsychopharmacology (chair: prof. Lucio Annunziato) of the "Federico II" University of Naples, where he was involved in studies on the physiopathology of somatostatin and dopamine receptors in pituitary tumors. In 2003, he obtained the degree of Doctor in Neuropsychopharmacology.

Between November 1998 and December 2000, he was research fellow at the Department of Internal Medicine of the Erasmus University (Erasmus Medical Center) (chair: prof. Steven W.J. Lamberts) of Rotterdam, the Netherlands, where he carried on a project on the dopamine receptor expression and function in the normal and pathological hypothalamus-pituitary-adrenal axis.

Since January 2005 he is research fellow at the Division of Neuroendocrine Pathology (chair: Annamaria Colao) of the Department of Molecular and Clinical Endocrinology and Oncology, "Federico II" University, Naples, Italy

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Henslow:
...The natural condition is one of the insurmountable obstacles on the road to imminent disaster...

Fennyman:
So what do we do?

Henslow:
Nothing, Strangely enough, it all turns out well

Fennyman:
How?

Henslow:
I don't know. It is a mistery

Shakespear in Love
By Marc Norman & Tom Stoppard
1998

Erasmus

Federico II