Novel Molecular Mechanisms of Resistance to Somatostatin Analog Treatment in Pituitary Adenomas

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Novel Molecular Mechanisms of Resistance to Somatostatin Analog Treatment in Pituitary Adenomas

Nieuwe moleculaire mechanismen achter resistentie voor behandeling met somatostatine analoga bij hypofyse adenomen

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General introduction & Scope and aims of the thesis

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GENERAL INTRODUCTION

I. PITUITARY GLAND PHYSIOLOGY

General features

The pituitary gland is known as the “master gland” of the body, acting as the central endocrine regulator of growth, reproduction, metabolism, and response to stress [1]. The gland is situated underneath the hypothalamus within the sella turcica, and is connected to the hypothalamus and the brain via the pituitary stalk. The pituitary gland is widely vascularized by the hypophyseal portal system, which flows within the pituitary stalk. The portal system has a pivotal role in pituitary function, since it allows the hypothalamic releasing hormones to reach the pituitary gland and the pituitary secreted hormones to reach their peripheral target tissues. Briefly, the gland is composed of two main lobes, the anterior (adenohypophysis) and the posterior (neurohypophysis) lobe. A third lobe, the intermediate lobe, is rudimentary and ill-defined in human pituitary [2].

The different cell types of the anterior pituitary gland are distributed within the acini of the anterior lobe. These cells are named according to the specific hormone which they produce and include the following types: corticotrophs (adrenocorticotropic hormone, ACTH), thyrotrophs (thyroid stimulating hormone, TSH), gonadotrophs (luteinizing hormone, LH and follicle stimulating hormone, FSH), somatotrophs (growth hormone, GH), and lactotrophs (prolactin, PRL) producing cells. A small population of mammosomatotroph cells, that produce both GH and PRL, is also present. These hormone-producing cells are associated with non-hormone producing cells, named folliculo-stellate cells [2]. The secretion of the above mentioned specific trophic hormones is modulated in response to hypothalamic, intra-pituitary, and peripheral hormonal, as well as growth factor signals [3]. Pituitary cells are highly differentiated and are committed very early on during development to synthesize their unique hormone products. Therefore, during pituitary development, distinct cell types emerge from a common primordium and the process of cell differentiation follows a specific pattern and temporal sequence [4]. Up to date, a number of transcription factors have been identified that play important roles in the differentiation of pituitary hormone-producing cells. In this context, PROP1 is the earliest pituitary-specific transcription factor expressed during development and its deficiency can affect all hormone-producing cell types of the anterior lobe [5], while POU1F1 (pit-1), is the signature transcription factor for the somatotroph, lactotroph, and thyrotroph lineage [6]. Moreover, NEUROD1 or TPIT seems to be important transcription factors for the cell differentiation towards the corticotroph lineage [7, 8] (Figure 1).

Anterior pituitary hormones

Growth hormone

Growth hormone (GH) is a single-chain 191–amino acid polypeptide with a molecular size of 22 kilodaltons (kDa). GH is synthesized in and released by somatotroph cells, which represent the predominant cell type in the anterior pituitary. The half-life of GH in serum is 20 to 25 minutes [9] and the secretion rate in young adult men is approximately 600 mg/day [10]. GH exerts a broad spectrum of effects, mainly promotion of linear growth and regulation of metabolism. These effects can be direct on peripheral tissues, or mediated through the generation of insulin-like growth factor-I (IGF-I) by the liver [11]. The neuroendocrine control of GH secretion is primarily mediated by the action of two
hypothalamic hormones: GH–releasing hormone (GHRH) and somatostatin (somatotroph release inhibiting factor, SRIF). More recently, Ghrelin, a 28–amino acid peptide, produced primarily in the stomach, but also present in the hypothalamus, has been demonstrated to bind to a GH secretagogue receptor (GHS) endogenously expressed by the somatotroph cells and to stimulate GH secretion as well [12]. Moreover, GH secretion is also modulated via multilevel feedback mechanisms. Both GH and IGF-I exert feedback regulation by stimulating SRIF release and inhibiting GHRH release by the hypothalamus, resulting in the inhibition of GH secretion from the pituitary, while IGF-I acts also at the pituitary level by inhibiting basal and GHRH-stimulated GH secretion. GH secretion occurs in a pulsatile manner, mainly regulated via GHRH, with a mean of about 10 peaks/day [13, 14]. In human, approximately 70% of GH secretion occurs during the night, in association with deep sleep phase [15]. GH secretion is affected by feeding, and it is well known that obese people may have lower GH levels compared to normal-weight, while fasting enhances GH pulsatility [16].

**Thyroid-stimulating hormone**

Thyroid-stimulating hormone (TSH) is a 28-kDa heterodimeric glycoprotein hormone consisting of two subunits. The α-subunit is common to TSH, gonadotropins and chorionic gonadotropin (hCG), while the β-subunit is specific for TSH and determines its unique biological actions. TSH is secreted by thyrotroph cells, which represent about 5% of anterior pituitary cells and its half-life in human serum is approximately 30 minutes.
The primary neuroendocrine regulation of TSH is mediated through thyrotropin-releasing hormone (TRH), which is secreted from neurons situated in the hypothalamic paraventricular nucleus. On the contrary, as observed for GH, SRIF inhibits TSH secretion. The feedback regulation of TSH secretion occurs primarily in the pituitary and is mainly mediated by changes in peripheral thyroid hormone levels, namely thyroxine (T4) and its “active” form, triiodothyronine (T3). Elevate thyroid hormone levels reduce TSH secretory response to TRH and the effect of TRH on TSH synthesis. Moreover, thyroid hormone feedback also occurs on TRH-producing neurons.

TSH and thyroid hormones level concentrations are decreased by fasting, as a consequence of the reduction of both pulsatile and circadian TSH secretion, while acute physical activity induces a rapid increase in TSH levels.

Adrenocorticotropin hormone

Adrenocorticotropin hormone (ACTH) is produced by corticotroph cells, which represent around 20% of all anterior pituitary cells, by enzymatic cleavage of its precursor hormone, pro-opiomelanocortin (POMC) [22]. ACTH is essential for stimulating adrenocortical steroidogenesis and secretion, but plays also an important role in the maintenance of adrenal gland structure and size [22, 23]. Glucocorticoids are essential for response to stress, maintenance of vascular tone, cardiac contractility and endothelial integrity. Therefore, their production/secretion is tightly regulated throughout the hypothalamic-pituitary-adrenal (HPA) axis. Pituitary ACTH release is primarily stimulated by corticotropin-releasing hormone (CRH), synthesized in the paraventricular hypothalamus [24]. In addition, other factors, such as vasopressin, can directly stimulate ACTH release. Additional factors regulating POMC gene expression include serotonin, cytokines, catecholamines and, in particular, dopamine [22]. Glucocorticoids exert their inhibitory feedback by suppressing POMC expression (and subsequent ACTH secretion) in pituitary corticotrophs [25] and by inhibiting hypothalamic CRH and vasopressin secretion [26].

A “fast” feedback control system is always present in physiological conditions, such as exposure to moderate stress. Glucocorticoids exert their actions by binding to glucocorticoid receptors (GR), widely expressed throughout the body, and mineralocorticoid receptors (MR), whose expression is limited to peripheral mineralocorticoid target tissues (e.g. the kidney) [27, 28].

ACTH and glucocorticoid secretion follows a circadian rhythm, showing a typical peak in the early morning hours and a gradual decline during the day, interrupted by a second (lower) peak during the early afternoon [22].

Prolactin

Prolactin (PRL), a single-chain protein of 198 amino acids with a molecular size of 23 kDa, is synthesized in the lactotroph cells of the anterior pituitary, representing about 10-20% of all pituitary cells [29].

The main physiological functions of PRL are the stimulation of lactation and the modulation of maternal behavior, exerted by the activation of the prolactin receptor, which belongs to the type 1 cytokine receptor family [30]. Differently from what previously stated for the other anterior pituitary hormones, the regulation of PRL by the hypothalamus is mainly inhibitory, rather than stimulatory, and is mediated by the tonic secretion of dopamine. In this case, dopamine exerts its effect by binding to the type 2 dopamine receptor (D2R), known to activate inhibitory intracellular pathways. However, there is a number of hypothalamic peptides, known to stimulate PRL secretion, such as TRH, oxytocin, as well
as estrogens [29]. Moreover, PRL secretion is regulated through a short-loop feedback mechanism by the peptide interaction with its related receptors, endogenously expressed in the hypothalamic dopaminergic system [30]. As for GH, also PRL levels show a nocturnal rise, dependent on sleep [31]. Finally, PRL secretion is known to increase during lactation and in response to nipple stimulation [32].

**Luteinizing hormone and follicle-stimulating hormone**

Gonadotrophs represent about 10% of the cells in the anterior pituitary gland. Most gonadotrophs synthesize and secrete both luteinizing hormone (LH) and follicle-stimulating hormone (FSH), two glycoproteins with a common α-subunit and different β-subunits that confer specificity to each hormone. LH and FSH are secreted and tightly regulated in response to gonadotropin-releasing hormone (GnRH) pulsatility [33]. Indeed, differential GnRH pulse frequencies and amplitudes alter the secretion patterns of FSH and LH [34, 35], with increasing frequencies resulting in preferential secretion of LH, whereas decreasing frequencies result in greater FSH release. Moreover, in addition to GnRH and sex steroids, inhibins, activins, and follistatin are other important regulators of gonadotropin synthesis and secretion [36].

In a very simplified view, LH and FSH exert their effects on the ovaries and testes, leading to steroidogenesis and gametogenesis, thereby highlighting their critical role in reproductive function [37].

**II. PITUITARY ADENOMAS**

Pituitary adenomas (PAs) represent a heterogeneous group of tumors, which originate from cells of the anterior pituitary gland, with a reported prevalence of about 1:1000-1400 of the general population [38]. Moreover, they account for about 15% of primary intracranial neoplasms [39]. Mostly benign, PAs can severely affect the patient health status, either because of the associated hormonal secretion depending on the tumor phenotype, or due to the compression of critical adjacent structures, such as the normal pituitary cells, the optic chiasm, as well as important vascular pathways [40]. A number of different factors has been hypothesized to play a role in pituitary neoplasia initiation and proliferation, such as aberrant loss of tumor suppressors, overexpression of oncogenes, dysregulation of cell cycle and promotion of cellular proliferation, or even a gene mediated dysregulation of the physiological hormonal feedbacks [41]. Although in the recent years a number of studies focused on the familial pituitary syndromes resulting in the identification of specific gene defects, such as aryl hydrocarbon receptor interacting protein (AIP) mutation [42], they do not seem to have a major impact in most of sporadic adenomas.

Based on their size, pituitary adenomas are usually classified into micro- and macroadenomas. In macroadenomas, the maximum tumor diameter measured on MRI sequences is ≥ 10 mm. Epidemiological studies, taking into account all the different pituitary adenoma histotypes, report macroadenomas as about 40% of the entire pituitary adenoma population [38].

Being mostly monoclonal tumors, PAs may originate from all the different anterior pituitary cell sub-populations and are named based on their specific hormonal hypersecretion (or, as for the clinically non-functioning tumors, based on the absence of symptoms related to hormonal hypersecretion). The most frequent PA types are PRL-secreting adenomas, named prolactinomas (46-66% of all pituitary tumors), and the clinically non-functioning pituitary adenomas (NFPAs, 15-37%), followed by GH-secreting adenomas (about 10%) and ACTH-secreting adenomas (2 to 6%) [43-46]. On the contrary, TSH-secreting adenomas
(1-3%) and gonadotroph adenomas showing a clinically relevant LH and/or FSH secretion, are rarely encountered [47]. Noteworthy, positive immunostaining for pituitary cell types can be found in clinically non-functioning adenomas. This entity, classified as silent functioning adenoma, is often represented by tumors expressing gonadotroph cells [48]. In the present Thesis, we will focus our attention on three specific PA subtypes: TSH-secreting adenomas, ACTH-secreting adenomas and, mainly, GH-secreting adenomas. A brief description of the pathophysiological, clinical characteristics and the current medical treatment options for the above mentioned PA subtypes is given below.

**GH-secreting pituitary adenomas**

The presence of a GH-secreting adenoma (GHoma) is the most common cause (>90%) of acromegaly, a severe systemic condition characterized by elevated circulating levels of GH and insulin-like growth factor I (IGF-I) [3]. At diagnosis, the great majority of GHomas is represented by macroadenomas (~70%).

GH secreting-cell carcinomas are extremely rare and are diagnosed only in the presence of extracranial metastases, using rigorous criteria [49]. Based on their ultrastructural phenotype, GHomas can be classified into densely granulated or sparsely granulated adenomas. Densely granulated are generally slow growing tumors, presenting in patients older than 50 years. On the other hand, sparsely granulated adenomas usually present in younger patients and show a more rapid growth pattern [50]. In about 20-25% of cases, GHomas may co-secrete PRL. This may be due to the concomitant presence of GH and PRL secreting adenoma cells, also named mammosomatotroph adenoma cells or acidophil stem-cells [51] (Figure 2).

**Figure 2.** Schematic representation of a direct GH excess originating from the pituitary gland (A), together with a comprehensive list of its possible causes (B)

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Despite still being considered a rare disease, many recent reports have described a significant increase in the prevalence of acromegaly in the general population, up to 100 cases/million in different geographical areas [43, 44]. As for all hormone secreting-pituitary adenomas, signs and symptoms of acromegaly may be due to the elevated circulating GH and IGF-I levels, or to the local tumor growth. The latter can lead to visual field defects, hypopituitarism and headache. Prolonged exposure to elevated GH and IGF-I levels causes the typical features of acromegaly, such as enlargement of hands, feet, changes of facial features, and systemic symptoms like fatigue, arthralgia, sweating, sleep apnea, hypertension and diabetes mellitus [3]. Moreover, if not promptly and adequately treated, acromegaly may result in a number of comorbidities (cardiovascular, metabolic, osteoarticular and respiratory complications), ultimately associated with a 1.5-2 fold increased mortality compared to normal population [52, 53].

In this context, surgery, when performed by an expert neurosurgeon, remains the first-line treatment of GHomas, and results in a postoperative remission of the disease in about 60–80% of cases, depending on the size of the adenoma [54]. However, a relatively high number of acromegalic patients (about 40%), are unlikely to be controlled by surgery alone. In these cases, medical treatment with somatostatin analogs (SSAs) is the proposed first line approach. SSAs represent the mainstay of medical therapy, due to their well-established anti-secretory and anti-tumoral effect in GHomas [55]. However, several studies (including a very recent meta-analysis) have highlighted that about half of the patients does not achieve normalization of GH or IGF-I levels after long-term treatment with “classical” SSAs, and an even lower percentage of patients (30-40%) reach a complete biochemical control [56]. Interestingly, a very recent head-to-head superiority study, comparing the efficacy of octreotide and a recently developed SSTR panligand, pasireotide, in the treatment of naïve acromegalic patients, has demonstrated that the effect of the two drugs in the reduction of GH levels was superimposable, while pasireotide was more effective in lowering circulating IGF-I levels [57]. Moreover, dopamine agonists (in particular cabergoline, a selective D2R agonist) and the GH-receptor antagonist, pegvisomant, may represent other possible effective tools for the medical management of acromegaly, alone or in combination with SSAs [40].

Criteria for biochemical remission in acromegaly generally reported in literature include: normal IGF-I (≤ 1 x ULN, upper limit of normality), random GH < 2.5 μg/l and nadir GH after OGTT (oral glucose tolerance test) < 1 μg/l (being this latter more indicated for post-surgical evaluation of disease status). However, when using ultra-sensitive assays, cut-off levels for random GH and nadir GH after OGTT are < 1 μg/l and <0.4 μg/l, respectively [58].

ACTH-secreting pituitary adenomas

The presence of an ACTH-secreting adenoma in the anterior lobe of the pituitary gland, is the most common cause of Cushing’s syndrome (CS), a systemic condition characterized by a chronic state of hypercortisolism [59]. In pituitary dependent CS, also named Cushing’s disease (CD), the physiological negative feedback exerted by adrenal steroids is disrupted, thus contributing to the autonomous ACTH hyper-secretion from the adenoma. Moreover, ACTH and cortisol secretion seem to be inappropriately sensitive to CRH and arginine vasopressin (AVP) stimulation [60, 61]. ACTH-secreting adenomas are usually monoclonal, benign and slow growing microadenomas, despite the fact that they may show a more aggressive behavior (particularly with respect to tumor invasiveness and recurrence rate) compared to other PA histotypes [62]. Interestingly, some authors have hypothesized that CD might originate not only from ACTH-secreting adenomas of the anterior pituitary, but also from adenomas or adenomatous hyperplasia of corticotroph cells located in the intermediate lobe [63].
However, irrespective of the primary site of ACTH hypersecretion, the resulting state of hypercortisolism is associated with a number of peculiar clinical signs and symptoms and is furthermore correlated with a significant increase in patient morbidity and mortality [64-66]. More in detail, at the time of diagnosis the most common comorbidities affecting CD patients are: hypertension (60-80%), severe metabolic impairment (including dyslipidemia, obesity and diabetes mellitus), psychiatric disorders (major depression in 50-80% of cases), and osteoporosis (30-50%) [66]. Noteworthy, quality of life (QoL) in CD patients can remain compromised after disease remission, and this finding seems to be inversely correlated with a prompt and successful clinical intervention [66].

As already stated for other pituitary adenoma subtypes, the treatment of choice for ACTH-secreting adenomas is a selective adenomectomy carried out by an experienced neurosurgeon [67]. The overall remission rate after first surgical approach ranges between 60-90% and recurrence rates range between 10-35% [68, 69]. When neurosurgery fails or it is not feasible, radiotherapy, bilateral adrenalectomy or medical treatment are the remaining tools to achieve disease cure or, in most cases, disease control [67].

Current medical therapies for CD can be roughly classified into three different groups: adrenal blocking drugs, glucocorticoid receptor blocking agents and pituitary-directed drugs [70]. However, it is well recognized that the ideal therapy should target the primary cause of the disease (e.g. the ACTH-secreting pituitary adenoma) resulting in the control of the hormone hyper-secretion and, preferably, in a reduction (until the disappearance) of the adenoma mass. In this context, SSAs and DAs are nowadays the most commonly used pituitary targeting drugs in the medical management of CD [40]. In particular, a recent Phase III study has investigated the efficacy of a 6–12 months treatment with the SSTR panligand pasireotide in CD [71]. Using the highest tested dose (900 μg s.c. twice daily, up to 1200 μg), 29% of patients reached normal urinary free cortisol (UFC) levels after 6 months of treatment and the mean percentage change in baseline UFC after 12 months was –54.5%. Besides UFC reduction, patients showed tumor shrinkage and significant improvement in a number of disease related signs and symptoms, such as blood pressure, body weight and low-density lipoprotein cholesterol [71]. Based on these results, pasireotide is the first compound approved by EMA and FDA for the treatment of CD. As for DAs, Pivonello and colleagues in 2009 evaluated the efficacy of short- (3 months) and long-term (12–24 months) treatment with cabergoline in patients with CD unsuccessfully treated by surgery [72]. The authors reported a sustained normalization of UFC after 24-month treatment with titrated doses of cabergoline (up to 7 mg/week; median: 3.5 mg/week) in 40% of patients (8/20) and tumor shrinkage in 20% (4/20), with a good safety profile. Moreover, an improvement in hypertension and glucose intolerance was observed in the majority of patients, as well [72]. Combined treatment with cabergoline, pasireotide and the adrenal-blocking agent, ketoconazole, has been demonstrated able to normalize UFC in all the patients enrolled in a pilot study [73].

**TSH-secreting adenomas**

Despite the recently described significant increase in their incidence and prevalence [74], TSH-secreting pituitary adenomas (TSHomas) are the most rare subtype of pituitary tumors, representing about 0.5–3% of all pituitary adenomas. TSHomas are benign tumours, which usually present at diagnosis as macroadenomas (70-80%) [75]. The report of TSH-secreting carcinomas is anecdotal [76, 77].

The majority of TSHomas (72%) secretes TSH alone. However, in about 25% of cases they co-secrete other anterior-pituitary hormones, in particular GH and PRL (15 and 10% of cases, respectively).
TSHomas may result in a rare form of central hyperthyroidism, characterized by elevated circulating thyroid hormones (free-T3 and free-T4) in presence of a normal or inappropriately high TSH level. The sustained and often long lasting high circulating TSH levels may often result in an increased thyroid volume and vascularization, thus increasing the risk of misdiagnosis (primary vs. secondary hyperthyroidism) in the absence of a proper clinical evaluation [77].

The signs and symptoms of hyperthyroidism can be often associated to symptoms related to the tumor compression of the surrounding anatomical structures (e.g. visual field defects, headache, hypopituitarism). Moreover, most patients have a long history of thyroid dysfunction and, due to the above mentioned high risk of incorrect diagnosis, about 30% of TSHoma patients had inappropriate thyroidectomy or radio-iodine thyroid ablation during their clinical history [78, 79].

As for most of pituitary tumors, surgery is still considered the first-line treatment, with an estimated cure rate around 30-40% [60, 61]. Anti-thyroid drugs (methimazole or propylthiouracil), together with the non-selective beta-blocker propranolol, can be temporarily used to control the signs and symptoms of hyperthyroidism and to restore thyroid hormone levels before surgery [59]. The rather disappointing results of surgery (especially for the frequently detected macroadenomas) mean that about two third of TSHoma patients need additional treatment, such as radiotherapy or medical treatment, during their clinical history [40]. In this light, TSHoma patients show a satisfactory response to medical treatment with SSAs, which are known to induce biochemical control in about 80-90% and tumor shrinkage in 30-40% of cases [40].

III. PATHOPHYSIOLOGICAL BASIS FOR SOMATOSTATIN ANALOG TREATMENT IN PITUITARY ADENOMAS

Somatostatin and somatostatin receptors

Somatostatin (SRIF) is a cyclic peptide present in mammals in two biologically active isoforms, consisting of 14 (SRIF-14) and 28 (SRIF-28) amino acids (Figure 3). SRIF acts in the central nervous system as a neurotransmitter with both stimulatory and inhibitory effects [80], while at the pituitary level and in the periphery the peptide mainly exerts inhibitory effects on hormone/peptide secretion, as well as on cell growth and differentiation [81].

Up to date, five human SRIF receptor subtypes (ssts) have been cloned and characterized [82, 83]. The transcript of the sst2 gene can be present in two splice variants that differ only for the length of the cytoplasmic portion of the receptor (sst2A and sst2B). Human tissues express almost exclusively the unspliced variant sst2A, whereas both forms are present in rodents [84]. Somatostatin receptors (SSTRs) belong to the seven transmembrane segment receptor superfamily and functionally couple to G proteins [83, 85].
Figure 3. Amino acid sequences of the two biologically active somatostatin (SRIF) forms, SRIF-14 and SRIF-28 (Brazeau et al. 1973)

SRIF binding to SSTR subtypes activates a series of second messenger pathways, resulting in the inhibition of calcium channels and adenylate cyclase activity, which ultimately leads to inhibition of hormone secretion [86, 87]. Briefly, the effect of SRIF/SSAs in the inhibition of adenylate cyclase activity has been demonstrated for the ligand-mediated activation of all different SSTR subtypes, while the inhibition of calcium channels has been described after sst₁, sst₂, and sst₅ receptor activation [88, 89]. Stimulation of other second messengers, such as phosphotyrosine phosphatases (PTPs), plays a role in SRIF- and SSA-mediated control of cell growth, and seems to be activated by SRIF binding to all different SSTR subtypes [83, 89, 90]. More in detail, in pituitary tumor cells, SRIF and its analogues may exert their anti-proliferative action by acting on the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway (via sst₂), or throughout the inhibition (sst₅ and sst₂) or activation (sst₁ and sst₂) of the mitogen-activated protein (MAP) kinase pathway [89, 91]. Moreover, apoptosis has been recently highlighted as another possible anti-tumoral effect of SRIF and SSAs, mediated through their binding to sst₃ and sst₅ [92, 93].

A schematic representation of the complex signaling pathways following SSTR ligand-mediated activation is depicted in Figure 4.

Noteworthy, the predominant SSTR-signaling pathway observed after ligand binding activation, depends on multiple factors, such as cell type specificity, SSTR subtype distribution in a given cell type, as well as on the variable expression of the different signaling elements [94, 95].

Somatostatin analogs

Despite its potent antisecretory effect observed in a number of different tumor tissues, together with its promoting effect on cell cycle arrest and apoptosis, endogenous SRIF is not a useful tool in clinical practice. This is mainly due to its short circulating half-life (< 3 minutes in human serum), which results in the need for parental administration, and to the post-infusion rebound observed for a number of “target” hormones (e.g. GH and insulin) [96, 97].
Therefore, synthetic SRIF analogs (SSAs) have been designed based on the primary SRIF structure, in order to overcome the above-mentioned disadvantages. The first compounds developed were octreotide [98] and lanreotide [99]. They are both octapeptides, developed by capturing the Cys–Cys bridge present in naïve SRIF and stabilizing the structure incorporating a D-Trp instead of a Trp. Therefore, these compounds are small molecules, with enhanced half-life compared to SRIF (e.g. ~ 2h for octreotide) and a lower clearance, resulting in a longer duration of action and a long-lasting biological activity. Both compounds show a preferential binding affinity to sst_2. Moreover, octreotide has a weak to moderate affinity for sst_3 and sst_5, while lanreotide shows a slightly more pronounced affinity to sst_5. Differently from endogenous SRIF, octreotide and lanreotide do not bind to sst_1 and sst_4 [100]. Finally, long-acting formulations of these molecules, which allow patients to undergo drug injection once a month or even less frequently, have been developed, tested, and approved already 15-20 years ago, and are nowadays routinely used in the clinical practice.

**Figure 4.** Schematic representation of SSTR signaling pathways

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**Legend:** Schematic representation of the intracellular signaling pathways modulated by somatostatin receptors. Antisecretory effects: SRIF (or SSA) binding to SSTRs activates G proteins and inhibits adenylyl cyclase (AC) activity, activates K⁺ channels, and inhibits Ca²⁺ channels. On the other hand, SRIF (or SSA) antiproliferative effect is mainly mediated through the activation of different phosphotyrosine phosphatases (PTPs), such as Src-homology phosphatase type 1 (SHP-1), type 2 (SHP-2) and density-enhanced phosphatase 1 (PTP₁). SHP-1 seems to activate mainly intracellular pro-apoptotic signals, via caspase activation, induction of p53/Bax and the inhibition (mediated by NF-κB activation) of JNK anti-apoptotic effects. PTP₁, activated by Src, leads to the dephosphorylation of intracellular mediators involved in cell cycle progression, such as the ERK and the PI3K/Akt pathways. This results in the upregulation of the cyclin kinase inhibitors p21cip1/waf1 and p27kip1 and the tumor suppressor gene Zac1. Activated pathway: light grey arrows; inhibited pathway: dark grey arrows.
However, since the above described drugs mainly target sst₂, and different SSTR subtypes are heterogeneously expressed in pituitary tumors (see next Chapter), researchers aimed to generate compounds with a more universal binding profile for SSTRs, similar to that of endogenous SRIF. To our knowledge, among a number of novel compounds tested in vitro and described in the recent literature (e.g. somatoprim, KE108) [101, 102], pasireotide is currently the only SSTR panligand that has been approved for clinical use by FDA and EMA in both CD and acromegaly.

Pasireotide is a stable cyclohexapeptide with a long half-life (~24h), which has been synthetized based on SRIF-14 structure and shows high affinity for multiple SSTRs (sst₅ > sst₂ > sst₃ > sst₁) [103]. However, despite the initial search for a compound able to closely mimic native SRIF, recent studies have demonstrated that pasireotide shows different functional properties compared to both SRIF-14 and the “classically” available SSAs when binding SSTRs, and particularly sst₂ [104-106] (Figure 5).

Figure 5. Amino acid sequences of octreotide, lanreotide and pasireotide

Somatostatin receptor expression in pituitary tumors

All different SSTR subtypes (except sst₄) are widely and heterogeneously co-expressed in the different PA histotypes [85, 107]. In the last decades, SSTR expression has been investigated at both mRNA and protein level, by use of different techniques (in situ hybridization, RT-PCR, western Blot, and immunohistochemistry) [108-111]. The recent availability of well-validated monoclonal antibodies, in particular for the detection of sst₂ and sst₅ (the mostly expressed SSTR subtypes in pituitary and neuroendocrine tumors), has given the pathologist novel and, most importantly, trustable tools [112, 113].

Considering all PA types, sst₂ and sst₅ are the most represented subtypes, followed by sst₁ and sst₃ [85, 97, 114] (Table 1).

Sst₂ and sst₅ are overexpressed in GH-secreting adenomas, where they are present in about 90% of cases, while sst₁ and sst₃ are identified in about 50%. Using quantitative mRNA assessment, sst₅ results to be the most abundant subtype, followed by sst₂ [115, 116], while the evaluation of protein expression, by use of immunohistochemistry, shows that sst₂ is more abundant than sst₅ [107, 110]. Few studies investigated the expression of SSTRs in TSHomas. However, also in this PA type, sst₂ and sst₅ are the most represented subtypes, at both mRNA and protein level [107, 117, 118].

The majority of ACTH-secreting adenomas predominantly expresses sst₅ and sst₂ at mRNA level (75%), followed by sst₁ (present in 60% of cases) [119-121]. Moreover, sst₅ is the most abundant SSTR expressed in corticotroph cells, at both mRNA and protein level [120, 121]. Noteworthy, sst₂ expression has been demonstrated to be downregulated by glucocorticoid exposure, while sst₅ (and dopamine D₂ receptor, D2R) seems to be less modulated by hypercortisolism [122, 123]. Therefore, higher levels of sst₂ close to those
observed in GHomas, can be found in corticotroph adenomas, after restoration of the eu cortisolemic state in CD patients [120].

In prolactinomas, sst1 (90% cases) and sst5 (80%) are notably represented. Sst2, is expressed in a lower percentage of adenomas (60%), and, most importantly, at lower levels [97, 124]. Finally, NFPAs and gonadotrophinomas, show a heterogeneous pattern of SSTR subtype expression [97], with sst3 being the most expressed receptor, followed by sst2, sst1 and, to a lesser extent, sst5 [115, 125].

Table 1. Somatostatin receptor expression in the different human pituitary adenoma types

<table>
<thead>
<tr>
<th>Pituitary adenomas</th>
<th>Somatostatin receptor subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sst1</td>
</tr>
<tr>
<td>GH-secreting</td>
<td>60%</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>TSH-secreting</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>ACTH-secreting</td>
<td>60%</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
</tr>
<tr>
<td>PRL-secreting</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>++</td>
</tr>
<tr>
<td>NFPA/LH, FSH-secreting</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

Legend: NFPA, non-functioning pituitary adenoma; n.a., not assessed. For each adenoma type, the prevalence (percentage, %) of somatostatin receptor subtype expression in the different cases is reported. In the row below, a rough quantitative estimation of receptor expression is described as follow: -: very low, negligible; +/-: low; +: mild; ++: moderate; +++: high.

New insights in somatostatin receptor signaling, trafficking, and interactions

In recent years, a number of studies have investigated the pathophysiological role of SSTRs, leading to novel insights with possible important clues for clinical management of pituitary adenomas (reviewed in [126-129]). Indeed, nowadays it is well known that the five SSTR subtypes share common signaling pathways, although particular SSTR subtypes can activate distinct signaling pathways, as well [130, 131]. Recently, Duran-Prado and colleagues [132] reported the first evidence for the existence of two novel sst5-truncated variants (termed sst5TMD4 and sst5TMD5) in pituitary adenomas, which are absent in the normal pituitary gland. The sst5TMD4 variant was reported as particularly abundant in octreotide-resistant somatotropinomas and the authors speculated about its possible role in the attenuated response to SSAs observed in some pituitary tumors [133].
The intracellular pathways activated by SSTR activation appear different in different types of tumor cells and depend on the specific SSTR distribution pattern, signaling elements, as well as to receptor desensitization, internalization, and cross talk [94, 95]. Moreover, it has been suggested that different SSAs, in the same cell type, may elicit differential effects, due to the activation of different subsets of intracellular mediators. This phenomenon, also named biased agonism, seems to depend on the typical agonist–receptor interactions [106, 134]. Recent in vitro studies have demonstrated that octreotide and pasireotide modulate sst₂A receptor phosphorylation and trafficking in a clearly distinct manner, despite their approximately similar binding affinity to this SSTR subtype [105, 135]. Pasireotide resulted in a significantly lower internalization of sst₂ compared to both octreotide and naïve SRIF (both in vitro and in vivo) [104, 136], while it appeared more potent than octreotide in inducing internalization and signaling of sst₃ and sst₅ receptors [104]. The observed behavior of pasireotide as a partial agonist of sst₂, sheds light on the importance of the agonist-induced receptor conformation in affecting receptor signaling and regulation, more than agonist binding affinity alone.

In this context, it has to be reminded that like many other GPCRs, SSTRs undergo agonist-induced endocytosis following the agonist binding to the receptors. The activated receptor is then phosphorylated by G protein coupled receptor kinases (GRKs) and subsequently recruited by cytoplasmic proteins, named β-arrestins, determining uncoupling between the receptor and its related G proteins [137, 138]. The receptor/β-arrestin complex is then internalized by dynamin-dependent endocytosis. Therefore, β-arrestins seem to play a pivotal role in the desensitization–internalization process of GPCRs, including SSTRs [139-141]. Different SSTR subtypes display a differential interaction with β-arrestins. Sst₂ and sst₅ bind β-arrestin 2 with higher affinity than β-arrestin 1, resulting in a less stable receptor/β-arrestin complex and a faster recycling on cell membrane. On the contrary, sst₂ displays the same affinity for both β-arrestin 1 and 2, and is internalized into endosomes forming a tight SSTR/β-arrestin complex [126]. Moreover, while the recycling seems to be the most common process following the internalization of sst₂ and sst₅, degradation seems to be more common for sst₃ [142]. In this respect, very recently GPCR phosphatases have been highlighted as critical regulators of SSTR recycling. More in detail, protein phosphatase 1β (PP1β) has been identified as the phosphatase mainly involved in the regulation of sst₂ recycling, while protein phosphatase 1γ (PP1γ) seems to dictate the timing of sst₅ re-exposure on cell membrane [143]. Noteworthy, PP1β and β-arrestin1 have been demonstrated to exist as constitutive complexes that mediate sst₂ dephosphorylation at or near the plasma membrane [144]. Furthermore, other regulatory factors of this already very complex system are intracellular proteins such as ubiquitins, SNX-1, GASP, and NSF, that may lead the early endosome to either cell membrane or the lysosomal pathway [145-147] (Figure 6).

These above-mentioned new insights might be extremely important for the development of new therapeutic strategies targeting SSTRs with SSAs, especially for tumors that poorly respond (or even completely resist) to the “classical” SSA treatment schedules and/or in those cases that show escape from the effect of SSA after an initial response. The mechanisms behind such an escape from treatment (although more frequent in neuroendocrine tumors than in pituitary adenomas) have not been elucidated, yet. They could include receptor down-regulation as result of SSA-activated receptor trafficking [85, 148], or even genetic and epigenetic phenomena occurring during the natural history of the disease and resulting in the modulation of SSTR expression pattern and/or the modulation of the protein involved in the fine-tuned receptor trafficking [149].

The co-expression of different SSTR subtypes, or the presence of different intracellular components involved in their trafficking, could form the basis for the variability of
Figure 6. Simplified representation of SSTR desensitization and trafficking processes

Legend: After agonist activation, SSTRs are phosphorylated (mainly involving GRKs) and recruited by the cytoplasmic proteins β-arrestins that interrupt the coupling between the activated receptor and G proteins (desensitization process). β-arrestins also function as the link between the receptor and the components of the endocytic machinery, such as dynamin and clathrin. The internalized receptor is then directed to early endosomes in which it is dephosphorylated and dissociated from β-arrestins. The receptor is then directed to different intracellular compartments, leading to either recycling or degradation. Finally, the recycled receptor is back to the plasma membrane as functional (resensitized) receptor. The rate of recycled or degraded receptors seems to be influenced mainly by receptor/β-arrestin interaction and by other regulatory intracellular proteins, such as GPCR phosphatases, ubiquitins, and sorting proteins (e.g. GASP, SNX-1, NSF). GRK, G-protein-coupled receptor-kinase; PKC, protein kinase C; NSF, N-ethylmaleimide–sensitive factor; GASP, GPCR-associated sorting protein; SNX-1, sorting nexin–1; PP1β, protein phosphatase 1β; PP1γ, protein phosphatase 1γ.

SSTR internalization and recycling processes observed in different tumor cell types [85, 126, 150]. Moreover, it is known that SSTRs may act not only as monomers, but as homo- and heterodimers as well. Such receptor oligomerization may result in modified functional and pharmacological properties of the receptor complex [150-152]. In this context, since SSTRs and the dopamine type 2 receptor (D2R) are often co-expressed in endocrine tumors, and in particular in pituitary adenomas [129], SSTRs have been reported to physically interact with the D2R, forming heterodimers with enhanced functional activity [153]. This enhanced activity, seems to involve cellular events such as modified receptor internalization, trafficking, and signal transduction [154, 155] (Figure 7). In the light of these insights, new SSAs, which bind more than one SSTR subtype [153], as well as chimeric compounds binding both SSTRs and D2R, have been developed and are currently under evaluation, for treatment of SSTR and D2R co-expressing tumors [154].
Keeping all these data together, this suggests that not only the single receptor signaling, but also the cell types, the receptor cross talk, as well as the receptor trafficking processes, are important components determining the final effect of a given ligand.

**Figure 7.** Simplified representation of SSTR-D2R heterodimerization and SSTR heterologous desensitization processes

**Legend:** Schematic representation of possible interactions between SSTRs and D2 receptor. Like the majority of GPCRs, SSTRs and D2 can also interact at the cell membrane, when co-expressed. It is well known that these receptors can act as heterodimers after agonist binding. Kidd et al. suggested a possible explanation for the intracellular pathway following the activation of the heterodimer represented by an upregulation of p21WAF1/CIP1, via c-Jun N-terminal phosphorylation and a concomitant inhibition of Ki-67 transcription. However, they observed that a different dimer complex composition can lead to a decrease in p21 transcription and to an increase in p53. It could also be hypothesized that receptor dimerization can influence the single receptor phosphorylation and β-arrestin interaction, resulting in the modulation of receptor desensitization, recycling, and degradation [155]. Moreover, recent evidences suggested a possible heterologous phosphorylation of both sst, and D2R by co-expressed phospholipase C (PLC)-coupled receptors (e.g. cholecystokinin (CCK) or bombesin (BBS) receptors) that may result in the modulation of receptor desensitization and internalization [156]. JNK, c-Jun N-terminal kinase; PKC, protein kinase C; PLC, phospholipase C; DAG, di acyl glycerol; PIP2, phosphatidylinositol bisphosphate; IP3, inositol trisphosphate.

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SCOPE AND AIMS OF THE THESIS

Based on the above-described findings and the increasing need for a better understanding of the pathophysiological basis that determines the responsiveness to SSA treatment in the different pituitary adenoma histotypes, we aim to address the following research questions in the present thesis:

• Is the evaluation of sst$_2$ protein expression in GH-secreting adenomas a good marker to predict the biochemical responsiveness to adjuvant SSA treatment in acromegalic patients? Is standard immunohistochemistry using newly available monoclonal antibodies a reliable method? These questions are addressed in the experiments described in Chapter 2 of the thesis.

• Does the balance between the expression of different SSTR subtypes drive the response to SSAs in TSH-secreting adenomas? Do TSH-secreting adenomas co-express SSTRs and D2R? Can chimeric compounds (binding both SSTRs and D2R) play a role in the medical treatment of patients with TSH-secreting adenomas? Chapter 3 describes the experiments addressing these research questions in TSH secreting pituitary adenomas.

• Recently, the SSTR panligand pasireotide has become available in clinical practice. However, the role of the individual SSTR subtypes in determining the efficacy of pasireotide in suppressing GH secretion is not yet defined. In Chapter 4 we aim to identify (in vitro) a subset of GH-secreting tumors, which are better responders to this novel compound compared to the classical SSAs, based on adenoma SSTR expression.

• β-arrestins and GPCR kinases (GRKs) are well known modulators of G-protein coupled receptors (GPCRs). In Chapter 5 we address the question whether β-arrestins and GRKs are differentially expressed in pituitary adenomas and to which extent these molecules influence the anti-secretory action of SSAs in GH-secreting adenoma cells in vitro and after acute exposure in vivo. Moreover, in the experiments described in Chapter 6, we investigate whether β-arrestin mRNA expression can be a useful additional marker (besides sst$_2$) to predict responsiveness to long-term SSA treatment in acromegaly.

• Dopamine agonists and SSAs are currently being evaluated for their efficacy in controlling hypercortisolism in patients with Cushing’s Disease (CD). Only a subset of patients with CD appears to respond well to these drugs and escapes from treatment have been reported as well. Since β-arrestins are involved in cell surface GPCR regulation and desensitization processes, we addressed the following issues in Chapter 7: a) do ACTH-secreting adenomas express β-arrestins? B) is β-arrestin expression affected by the exposure to high levels of glucocorticoids and is the effect of glucocorticoids on β-arrestin expression reversible?

• Chapter 8 provides a synthesis of the data we have generated and highlights the perspectives for future research in order to better understand sensitivity and resistance to GPCR-based medical therapies in patients with pituitary adenomas and other types of neuroendocrine tumors.
REFERENCES


Immunoreactivity score using an anti-sst2a receptor monoclonal antibody strongly predicts the biochemical response to adjuvant treatment with somatostatin analogs in acromegaly

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Abstract

Context: Somatostatin receptor subtype 2 (sst2A) protein expression has been demonstrated to positively correlate with somatostatin analog treatment outcome in GH-secreting adenomas. Recently, a new rabbit monoclonal anti-sst2A antibody (clone UMB-1) has been validated as a reliable method to selectively detect sst2A protein levels in formalin-fixed tissues.

Objective: The aim of the study was to establish whether the evaluation of sst2A protein levels, assessed with a routine reproducible immunohistochemistry protocol using UMB-1 antibody, may predict the successful adjuvant therapy with somatostatin analogs in acromegalic patients.

Design, Setting, and Patients: Thirty-six acromegalic patients from our referral hospital were evaluated retrospectively. Sst2A expression analysis was performed by immunohistochemistry in 25 patients and by quantitative RT-PCR in 26 patients. Sst2A immunoreactivity was evaluated using an immunoreactivity score (IRS), which takes into account both the percentage of positive cells and staining intensity.

Interventions: Patients with persistent disease after surgery (n = 26) were treated with somatostatin analogs for a median duration of 6 months.

Main Outcome Measure: GH and IGF-I levels were measured before and after postoperative treatment.

Results: Sst2A IRS showed a significant positive correlation with both GH (P = 0.039) and IGF-I (P = 0.001) suppression by octreotide. Sst2A IRS was negatively associated with IGF-I levels reached after treatment (P = 0.001), and patients that achieved IGF-I normalization showed significantly higher sst2A IRS compared to the group that was not normalized (P = 0.002). A sst2A IRS of at least 5 showed a sensitivity of 86% and a specificity of 91% in predicting IGF-I normalization during adjuvant octreotide treatment.

Conclusion: Sst2A IRS with the anti-sst2A antibody UMB-1 represents a valid tool in the clinical practice to identify acromegalic patients likely to be responders to adjuvant therapy with the currently available somatostatin analogs.
Introduction

The clinically available somatostatin analogs (SSAs), octreotide and lanreotide, preferentially bind the somatostatin receptor subtype 2A (sst2A), and the GH-lowering effect of these drugs has been positively correlated with both the level of mRNA and protein receptor expression (1–3). Sst2A protein has been mainly evaluated by immunohistochemistry (IHC) using different polyclonal antibodies (4–6). However, more recently, an IHC protocol performed with an sst2A rabbit monoclonal antibody (UMB-1 clone) has been demonstrated to be as effective as the “gold standard” in vitro method to quantify sst2 levels in tumor tissues, namely autoradiography (7). In fact, using the UMB-1 monoclonal antibody, sst2A profile has been examined in a cohort of GH-secreting adenomas (8), the majority of patients being pretreated with octreotide, confirming its high selectivity and reliability in pituitary tumors (9). However, no data using UMB-1 antibody in predicting the outcome to SSA adjuvant therapy in acromegaly have been produced so far. In this study, we characterized sst2A expression in a large series of mainly SSA treatment-naïve pituitary adenomas from acromegalic patients, both at mRNA (quantitative RT-PCR) and protein (IHC with UMB-1 Ab) levels. Moreover, in a subgroup of these tumors, we correlated IHC finding [expressed as immunoreactivity score (IRS)] with hormonal responses to an acute octreotide test, as well as to postoperative medical therapy with long-acting SSAs. Our main aim was to establish whether the sst2A IRS, evaluated with a routinely reproducible IHC protocol, may have a high positive predictive value for the successful adjuvant medical therapy with SSA, and may, consequently, become a feasible tool in the clinical practice.

Patients and Methods

Patients, tumors, and assays

Thirty-six acromegalic patients (18 men, 18 women; age range, 19–70 yr) that underwent transsphenoidal neurosurgery were evaluated. Twenty-seven patients (75%) had a macroadenoma, and nine had a microadenoma. Eleven patients were treated before surgery with octreotide long-acting repeatable (LAR) (20–30 mg/4 wk). No patient had received radiotherapy before or during the study period.

Patients with persistent disease after surgery (n = 26) started adjuvant octreotide LAR treatment (median duration, 6 months; range, 3–78 months) with a starting dose of 20 mg/4 wk. After 3 months, in patients not adequately controlled (IGF-I above reference range), octreotide LAR was increased up to 30 mg/4 wk.

Treated patients were investigated for GH and IGF-I levels immediately before (basal values) and after (posttreatment values) postoperative treatment. Moreover, an acute octreotide test was performed in 21 patients, as reported before (10). Approval from the Medical Ethical Committee of the Erasmus MC and informed consent to use the tumor tissues for research purposes were obtained.

Both GH and IGF-I concentrations were determined by use of a nonisotopic, automatic chemiluminescence immunoassay system (Immulite; Diagnostic Products Corp., Los Angeles, CA). Not all parameters were available for each patient.

Quantitative RT-PCR

Sst2A mRNA expression analysis was performed in 26 tumor samples by use of quantitative RT-PCR, as previously described (1). The sequences, final concentrations, and PCR efficiencies of the hypoxanthine phosphoribosyltransferase (hprt) and sst2 primer-probe pairs have been described previously (1). Samples were measured on an ABI Prism 7900 Sequence Detection System (Perkin-Elmer, Foster City, CA) and normalized against the expression of the housekeeping gene hprt.
**Immunohistochemistry**

Before immunostaining, formalin-fixed paraffin-embedded tissues from 25 GH-secreting pituitary adenomas (see Table 1 for general and clinical characteristics of patients) were cut (5 μm), deparaffinized, and rehydrated. Tissue slides were heated in Tris-EDTA buffer (pH 9.0) for 20 min (microwave) for antigen retrieval and bathed in a 3% H₂O₂/PBS solution for 15 min at room temperature in the dark to quench endogenous peroxidase. After washing with Tris/HCl/Tween 0.5%, sections were incubated with the anti-sst2 primary antibody [rabbit monoclonal (SS-8000-RM, clone UMB-1), dilution 1:50; Biotrend, Köln, Germany] overnight at 4 °C. After several washes, two drops of horseradish peroxidase rabbit/mouse (Dako Detection System; Dako Netherlands, Heverlee, Belgium) were added to tissues and incubated for 30 min. Bound antibody was visualized with freshly prepared 100 μl of Dako Detection System twice for 5 min at room temperature in the dark. Staining was then stopped by rinsing with water. Slides were counterstained with hematoxylin and coverslipped. For negative controls, the primary antibody was omitted.

The adenomas were scored semiquantitatively on the basis of a well-established immunoreactivity scoring system (IRS) (11). The IRS is calculated by the product of the percentage of positive cells (4, >80%; 3, 51-80%; 2, 10–50%; 1, <10%; 0, 0%) and the intensity of the staining (3, strong; 2, moderate; 1, mild; and 0, no staining), which results in IRS scores between 0 (no staining) and 12 (maximum staining).

**Statistical analysis**

The data were statistically analyzed using SPSS software version 15.0 for Windows (SPSS, Chicago, IL). When data distribution was normal, means ± SE were used; otherwise, median values (median, range: minimum-maximum) were calculated. Between-group comparisons were analyzed by the Mann-Whitney U test, and correlation coefficients were calculated by the Spearman rank order R. Assessment of the predictive discrimination of sst2A IRS was made using the receiver-operating characteristic (ROC) curve. Differences were taken to be statistically significant at $P < 0.05$.

**Results**

**Short- and long-term response to octreotide treatment**

Basal (morning, overnight fasting) mean GH levels ($n = 31$) were $16.1 ± 3.6$ (1.2 to 76.4 μg/liter), and basal mean IGF-I levels [expressed as upper limit of normality range (ULNR), $n = 28$] were $3.02 ± 0.34$ (1.2 to 7.7 ULNR). Basal GH and IGF-I values were directly correlated ($r = 0.57; P = 0.004; n = 24$).

After postoperative therapy, the mean IGF-I decrease was $36.9 ± 7.1\%$. IGF-I normalized in 12 patients (responders), reduced more than 50% without reaching normalization in three patients (partial responders), and reduced less than 50% in the remaining 11 patients (poor responders). GH levels were reduced by more than 50% in 10 patients and less than 50% in the other 10, with a mean percentage suppression of $42.2 ± 10.7$. The percentages of GH and IGF-I decrease during treatment were significantly and directly correlated ($r = 0.55; P = 0.011; n = 20$).
Table 1. General characteristics, tumor size, and clinical data of the 25 patients investigated for sst2A IRS

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex, Age (yr)</th>
<th>Tumour size</th>
<th>Postsurgery OCT LAR</th>
<th>% IGF-I suppression after OCT LARb</th>
<th>IGF-I (ULN) after OCT LARa</th>
<th>% GH suppression after OCT LAR</th>
<th>% GH suppression at nadir (OCT test)</th>
<th>sst2 protein (IRS)</th>
<th>sst2 mRNA (HPRT)</th>
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<td>-a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
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<tr>
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<td>F, 70</td>
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<td>37 (0)</td>
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<td>-</td>
<td>-</td>
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<td>Macro</td>
<td>Yes</td>
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<td>2.5</td>
<td>-21</td>
<td>-</td>
<td>2</td>
<td>-</td>
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<td>23</td>
<td>M, 51</td>
<td>Micro</td>
<td>-a</td>
<td>-</td>
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<td>-</td>
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<td>24</td>
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<td>Yes</td>
<td>58 (2)</td>
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<td>50 (2)</td>
<td>0.7</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>-</td>
</tr>
</tbody>
</table>

Legend: F, Female; M, male; OCT, octreotide; ULN, upper limit of normal; -, not available. a Patients treated with SSA (also) before neurosurgery. b Data in parentheses indicate percentage of IGF-I decrease categorized. IGF-I was scored 2 when normalized during therapy, 1 when reduced more than 50% but not normalized, and 0 when reduced less than 50%. c Data in boldface indicate patients that normalized IGF-I during adjuvant SSA treatment.
The percentage of GH suppression during the acute octreotide test ranged from 31.3 to 95.0% (basal vs. nadir) and was significantly higher (P = 0.012; n = 14) in the group of patients achieving IGF-I normalization after adjuvant treatment, compared with patients who were not normalized.

**Correlation between sst2A mRNA and protein expression and GH lowering during octreotide test**

A heterogeneous expression of sst2A mRNA content was recorded in our adenoma samples, with an 18-fold difference between the lowest and highest levels measured. Similarly, sst2A protein expression evaluated by IRS was variable in the different samples. Seven tumors showed low IRS (IRS 1–2), five showed intermediate (IRS 3–4), 11 showed high-intermediate (IRS 6–9), and two adenomas received the maximum score (IRS 12) (Fig. 1). Neither the patients’ general characteristics (age, sex) nor tumor size (micro- or macroadenoma) and SSA treatment before surgery were significantly related to sst2A expression (both mRNA and protein), although a trend for lower sst2A IRS in samples from SSA-pretreated patients was observed (P = 0.086). Both sst2A mRNA and protein levels were inversely correlated with basal IGF-I values (r = - 0.54, P = 0.018; and r = - 0.50, P = 0.026, respectively). Sst2A IRS showed a trend for direct correlation with mRNA expression (r = 0.39; P = 0.097; n = 19).

The sst2A protein expression (IRS) was strongly and directly correlated with the percentage of GH suppression after sc 100-μg octreotide administration (r = 0.73; P = 0.003; n = 14; Fig. 2A). A similar correlation was observed for mRNA level as well (r = 0.64; P = 0.010; n = 15; Fig. 2B).

**Sst2A IRS and adjuvant treatment with long-acting SSAs**

No correlation between sst2A mRNA levels and GH and/or IGF-I lowering after adjuvant treatment with octreotide was found. Conversely, a significant positive association was found between sst2A IRS and both GH (r = 0.55; P = 0.039; n = 14) and IGF-I (r = 0.70; P = 0.001; n = 18) suppression by octreotide LAR administered as adjuvant treatment. In line with this, sst2A IRS was negatively associated (r = - 0.82; P = 0.001; n = 18) with the IGF-I levels (as ULNR) after treatment (Fig. 2C). It is noteworthy that the group of patients that achieved IGF-I normalization showed significantly higher sst2A IRS compared with the non normalized group (P = 0.002; n = 18) (Fig. 2D).

The prognostic profile of sst2A IRSs in predicting normalization of IGF-I on treatment with octreotide LAR is graphically represented in Fig. 2E. A sst2A IRS of at least 5 (computed cutoff by ROC curve analysis) showed a sensitivity of 86% and a specificity of 91% (positive predictive value, 86%; and negative predictive value, 91%) in predicting IGF-I normalization during adjuvant octreotide LAR treatment.
Figure 1.

Legend: Heterogeneous immunohistochemical expression of sst2A in somatotroph pituitary adenomas. Representative examples of low (A), intermediate (B), high-intermediate (C), and high (D) sst2A IRS are shown (magnification, x400). Photomicrographs represent adenomas from non-pretreated patients, except for panel B (pretreated patient). A predominant membranous staining was observed in all samples, without any significant difference between pretreated or SSA-naive patients. The IRS is based on the evaluation of two independent observers, who were blinded for the other observers' score and for the results of octreotide treatment. All slides were scored identically by the two independent researchers.
Figure 2.

A) Positive correlation between the percentage of GH suppression by acute sc octreotide administration in vivo and both sst2A IRS (P = 0.003; n = 14) and sst2A mRNA level (P = 0.010; n = 15).

B) Higher sst2A IRS strongly correlated with lower absolute levels of IGF-I (age and sex normalized, ULNR) after adjuvant treatment (P = 0.0001; n = 18).

D) The group of patients that achieved IGF-I normalization after adjuvant treatment with long-acting SSAs showed significantly higher sst2A IRS compared with the non-normalized group (P = 0.002; n = 18).

E) ROC curve analysis of sst2A IRS in predicting achievement of normal IGF-I levels in patients (area under the curve, 0.935; P = 0.002). The central dotted line indicates neutrality, and the arrow shows the most relevant sst2A IRS cutoff value. The sensitivity (y-axis) was plotted against the corresponding false-positive rate (1-specificity, x-axis).

Legend: A and B, Positive correlation between the percentage of GH suppression by acute sc octreotide administration in vivo and both sst2A IRS (P = 0.003; n = 14) and sst2A mRNA level (P = 0.010; n = 15). C, Higher sst2A IRS strongly correlated with lower absolute levels of IGF-I (age and sex normalized, ULNR) after adjuvant treatment (P = 0.0001; n = 18). D, The group of patients that achieved IGF-I normalization after adjuvant treatment with long-acting SSAs showed significantly higher sst2A IRS compared with the non-normalized group (P = 0.002; n = 18). E, ROC curve analysis of sst2A IRS in predicting achievement of normal IGF-I levels in patients (area under the curve, 0.935; P = 0.002). The central dotted line indicates neutrality, and the arrow shows the most relevant sst2A IRS cutoff value. The sensitivity (y-axis) was plotted against the corresponding false-positive rate (1-specificity, x-axis).
Discussion

Surgery still represents the first-line therapy in the majority of patients with acromegaly (12). However, in case of persistent disease, SSAs are the first choice for adjuvant medical treatment (10, 13–16).

A large number of in vitro and in vivo studies already demonstrated that sst2A receptor expression correlates with the effectiveness of SSAs, both in terms of biochemical and tumor growth control, in different subsets of GH-secreting adenomas (2, 17, 18). In particular, in recent years, a number of studies focused on the correlation between the response to SSA treatment and sst2A protein expression (evaluated by IHC), highlighting sst2A expression as a crucial factor for successful treatment (4–6, 8). However, most of these studies investigated the correlation between sst2A immunoreactivity and therapy outcome in patients treated with SSAs before surgery. In two of these studies, it is noteworthy that the authors already speculated about a possible role of long-term SSA treatment in affecting sst2A expression, reporting a significantly lower sst2A immunoreactivity in tumor samples of patients pretreated with SSAs, compared with untreated (6, 8).

In our study, we selected mainly patients naïve to SSA treatment to minimize the influence of pretreatment on sst2 expression, and we focused on the response to postsurgery adjuvant therapy to achieve clearer indications from a homogenous cohort of patients. Since its validation, the new rabbit monoclonal anti sst2A antibody UMB-1 appeared to be a very reliable method to selectively detect sst2A expression in paraffin embedded formalin-fixed tissues, facilitating the establishment of routine performance of sst2A IHC in human tumor samples, with a high quality of specific membranous staining (7, 9). Moreover, the evaluation of sst2A expression with a semiquantitative IRS system, which takes into account the intensity of the staining as well as the percentage of positive cells, resulted in a strong and convincing direct correlation between sst2A expression and IGF-I normalization, percentage IGF-I decrease, and lower IGF-I absolute values (as ULNR) after adjuvant treatment with SSAs. It is noteworthy that the predictive value of sst2A IRS was also confirmed when correlating sst2A protein levels with the amount of GH decrease after an acute octreotide test with a correlation coefficient and a significance comparable to that obtained by evaluation of sst2A mRNA in the same population, as already described (1, 18). However, despite the strong concordance observed for sst2A mRNA and protein data in predicting acute GH lowering, the correlation between sst2A mRNA and protein level (as IRS) in the same subjects was not statistically significant, as already observed by other authors in nonpituitary tumors (19).

This is the first study showing that a sst2A IRS of at least 5, comparable to that greater than 50% of moderately stained cells, which is easily visible with a low-power objective, results in an 86% positive (and 91% negative) predictive value to IGF-I normalization after a mean of 6 months adjuvant treatment with octreotide. This approach can be combined and may strengthen the already described positive predictive value of biochemical features explored by other authors (octreotide test and basal hormone assessment). In fact, in our study group, the amount of GH decrease after an octreotide test and the basal IGF-I values were also valuable predictors for IGF-I normalization after adjuvant treatment and, when combined with sst2A IRS (best predictor), could result in an improvement of the general predictive power. Interestingly, our finding of an inverse correlation between basal IGF-I values and sst2A IRS of the tumor could provide a reasonable pathophysiological explanation for the clinical observation of a lower rate of IGF-I normalization after SSA treatment in those patients with higher basal IGF-I values (20).

In conclusion, sst2A IRS using UMB-1 may represent a valid tool in clinical practice, due to the feasibility and reproducibility of a relatively low-cost method, as well as the
general availability of formalin-fixed samples, to identify those patients likely to be good responders, in terms of IGF-I normalization, to adjuvant therapy with the currently available SSAs. Moreover, in the light of the recent availability of other medical treatment-effective modalities for patients with acromegaly, such as GH-receptor antagonists and SSTR panligands, a trustable standardized evaluation of sst2A expression could be crucial to lead the best individualized medical approach, avoiding a delay in the establishment of an effective medical treatment, also in terms of health system costs.

Acknowledgments
We are indebted to Dr. E. J. Delwel, Dr. A. H. G. Dallenga, and Dr. J. H. van den Berge of the Department of Neurosurgery for their support. We thank P. M. van Koetsveld for his assistance in collecting the tissue samples.
REFERENCES


Balance between somatostatin and D2 receptor expression drives TSH-secreting adenoma response to somatostatin analogues and dopastatins

Federico Gatto, Federica Barbieri, Monica Gatti, Roberto Wurth, Stefan Schulz, Jean-Louis Ravetti, Gianluigi Zona, Michael D. Culler, Alexandru Saveanu, Massimo Giusti, Francesco Minuto, Leo J. Hofland, Diego Ferone and Tullio Florio

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Abstract

Context First-line therapy for thyrotropin-secreting pituitary adenomas (TSHomas) is neurosurgery, while medical treatment rests mainly on somatostatin analogues. Clinically available sst₂-preferring analogues, octreotide and lanreotide, induce normalization of hormone levels in approximately 90% of patients and tumour shrinkage in 45%.

Objective We evaluated somatostatin 1, 2, 3 and 5 and dopamine D2 receptor expression in tumour samples from three TSHomas, and the relationships between receptor expression, in vitro antiproliferative response and clinical data, including octreotide test and three months of therapy with octreotide long-acting repeatable (LAR). TSHoma cell proliferation was tested in vitro using octreotide, cabergoline and two chimeric compounds, BIM-23A760 and BIM-23A387.

Results All patients showed significant TSH lowering to acute octreotide test, but a hormonal response to long-term treatment was observed in only two patients, showing a high sst₅/sst₂ ratio. Patient 2, characterized by high expression of sst₂ and sst₁ and a relative lower expression of sst₅, experienced tachyphylaxis after prolonged octreotide treatment. In vitro, the somatostatin/dopamine receptor agonist BIM-23A760 caused the highest antiproliferative effect among those tested. Combined treatment with octreotide and cabergoline displayed an additive effect of magnitude comparable to that of the other chimeric compound (BIM-23A387). Octreotide resistance was confirmed in cells isolated from the non-responder patient, although it could be overcome by treatment with the chimeric compounds.

Conclusions A high sst₅/sst₂ ratio might be predictive of a positive outcome to long-term treatment with somatostatin analogues in TSHomas. Moreover, combined somatostatin and D₂ receptor targeting might be considered as a potential tool to improve the response rate in octreotide-resistant tumours.
Introduction

Thyrotropin-secreting pituitary adenomas (TSHomas) are a rare cause of hyperthyroidism and represent <1% of all pituitary adenomas, despite the increasing number of cases reported in recent years (1). The majority of TSHomas (70%) secrete TSH alone, while mixed adenomas, characterized by concomitant hypersecretion of other pituitary hormones, are found in about 16% [growth hormone, (GH)] and 10% [prolactin (PRL)] of cases (1). The primary therapeutic approach is neurosurgery, resulting in cure in one-third of patients. If surgery is contraindicated or declined, radiotherapy might be considered (2). As for medical treatment, somatostatin (SRIF) analogues (SSAs) are able to control hormone hypersecretion in 80–90% of cases (3) while significant tumour shrinkage is observed in about 45% (4). The therapy is based on the presence of SRIF receptors (SSTRs) on cell membranes, in particular subtypes 2 and 5, demonstrated by both in vitro studies and SSTR scintigraphy (5, 6). The currently available SSAs octreotide and lanreotide are sst2 selective compounds, with lower affinity to sst3 and sst5, exerting in vivo effects through the activation of these specific SSTR subtypes. However, in vitro, SSAs have been shown to activate distinct signaling pathways to exert their antisecretory and antitumoural effects in human pituitary adenoma cells (7, 8).

Recently, a correlation between long-term sst2-mediated inhibition of TSH secretion and the consistent expression of sst5 on cell membrane has been reported in few cases of TSH-secreting adenomas (9–11), in a way providing some clinical evidence to previously reported functional data, suggesting that sst5 expression influences sst2 internalization processes (12).

Dopamine (DA) receptor subtype 2 (D2) is also highly expressed in the pituitary gland (13) and has already been demonstrated to mediate the regulatory effects of hypothalamic DA on different pituitary cell populations (14). Moreover, previous studies have demonstrated the presence of D2 on TSH-secreting pituitary adenomas as well, representing the rationale for rather unsuccessful clinical trials with bromocriptine or cabergoline (2).

Co-expression of SSTRs and D2 in pituitary adenomas has recently been extensively reviewed (5, 15). The D2 receptor is associated with two or more SSTR subtypes, preferentially sst2 and sst5, with a high variability in receptor expression, because of tumour heterogeneity (16, 17). In the context of SSTR/D2 co-expression, combined treatment with SSAs and DA agonists has been demonstrated to be efficacious in subsets of pituitary adenomas (18).

Moreover, the effects of SSAs and DA agonists should be now re-evaluated according to recent insights about SSTR and D2 interactions in the cell membrane, leading the formation of SSTR/DR heterodimers (19, 20). Heterodimerization of these G-protein coupled receptors and the related cellular events open a new possible scenario in the field of treatments targeting SSTR-expressing tumours (15, 21–23). In this context, the use of chimeric compounds that bind SSTRs and D2 have provided interesting results in vitro in tumour cell lines (21, 24–26) and primary cultures of both GH-secreting and clinically nonfunctioning human pituitary adenomas (5, 22, 27).

In this study, we evaluated the expression of SSTRs and D2 in three TSH-secreting adenomas, correlating the expression level of the receptors with clinical data, including acute TSH response to octreotide test, as well as TSH and thyroid hormone concentrations after three months of therapy with octreotide LAR. Moreover, in accordance with the observed receptor pattern and the increasing evidence for SSTR and D2 interaction, isolated adenoma cells were tested in vitro with octreotide, cabergoline (alone or in combination) and two chimeric compounds, BIM-23A760 and BIM-23A387.
Patients, materials and methods

Patients
Three male patients with TSH-secreting adenoma were studied. General information, clinical characteristics, tumour size and pathology are summarized in Table 1.

Table 1. Clinical characteristics and tumour pathology, in a series of three TSH secreting adenomas

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age</th>
<th>Gender</th>
<th>Pathological staining</th>
<th>Tumour size</th>
<th>TSH (mU/l)</th>
<th>fT4 (pm)</th>
<th>TSH nadir (6 h) OCT test</th>
<th>Δ% vs basal TSH</th>
<th>OCT test mean values after 3 months of treatment</th>
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<tbody>
<tr>
<td>1**</td>
<td>41</td>
<td>M</td>
<td>TSH+ GH+ PRL+</td>
<td>macroadenoma</td>
<td>6·17</td>
<td>46·1</td>
<td>1·7</td>
<td>–81</td>
<td>0·65</td>
</tr>
<tr>
<td>2**</td>
<td>34</td>
<td>M</td>
<td>TSH+ GH– PRL–</td>
<td>macroadenoma</td>
<td>8·03</td>
<td>44·8</td>
<td>2·24</td>
<td>–66</td>
<td>29·5</td>
</tr>
<tr>
<td>3**</td>
<td>43</td>
<td>M</td>
<td>TSH+ GH+ PRL–</td>
<td>microadenoma</td>
<td>5·90</td>
<td>32·0</td>
<td>2·5</td>
<td>–62</td>
<td>0·5</td>
</tr>
</tbody>
</table>

Legend: Ki 67 ≤ 1% in all tumour samples. Normal range: TSH 0.2 – 4.2 (mU/l); fT4 12.0 – 21.9 (pmol/L).
* Concomitant treatment with methimazole.
** Patient 1 and 3 were treated with methimazole (few weeks) until TSH was lowered due to treatment with SSA.

Clinical data and receptor mRNA expression data of patient 1 have been already partially reported (10). Baseline and follow-up MRI examinations after 3 months of SSA treatment with octreotide LAR were performed to assess tumour shrinkage. All MRI evaluations were performed at San Martino Hospital (Genoa, Italy) and interpreted by local neuroradiologists. After preoperative treatment, all patients underwent surgery, and both fresh tumour samples and paraffin-embedded tissues were collected. Moreover, we set up primary cell cultures from fresh tumour samples to perform in vitro proliferation experiments. All patients signed a written consent to diagnosis, therapy and anonymous divulgation of medical data for scientific and research purposes, according to the local ethical committee.

Test compounds
The sst$_1$/sst$_3$/D$_2$ chimeric agonists, BIM-23A760 and BIM-23A387, were kindly provided by IPSEN/Biomeasure (Milford, MA, USA). Octreotide was a kind gift of Novartis International AG (Basel, Switzerland). All compounds were dissolved as 1 mM solutions in 0.01 M acetic acid containing 0.1% purified BSA (Sigma-Aldrich, Milano, Italy). Cabergoline (Pfizer Inc., New York, NY, USA) was prepared as 1 mM solution in 0.01 M acetic acid and 70% ethanol. Before experiments, a fresh aliquot of each compound was diluted with phosphate-buffered saline (PBS). Receptor affinities (Ki) of the compounds used are listed in Table 2.
Table 2. Ki of somatostatin (SRIF14) and of the agonists used in this study

<table>
<thead>
<tr>
<th>Ligand</th>
<th>sst&lt;sub&gt;1&lt;/sub&gt;</th>
<th>sst&lt;sub&gt;2&lt;/sub&gt;</th>
<th>sst&lt;sub&gt;3&lt;/sub&gt;</th>
<th>sst&lt;sub&gt;4&lt;/sub&gt;</th>
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<th>D&lt;sub&gt;2&lt;/sub&gt;</th>
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<tr>
<td>SRIF-14</td>
<td>2.3</td>
<td>0.2</td>
<td>1.4</td>
<td>1.8</td>
<td>1.4</td>
<td>–</td>
</tr>
<tr>
<td>Octreotide</td>
<td>1140</td>
<td>0.6</td>
<td>34</td>
<td>&gt;1000</td>
<td>7.0</td>
<td>–</td>
</tr>
<tr>
<td>Cabergoline</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>25</td>
</tr>
<tr>
<td>BIM-23A760</td>
<td>622</td>
<td>0.03</td>
<td>160</td>
<td>&gt;1000</td>
<td>42</td>
<td>15</td>
</tr>
<tr>
<td>BIM-23A387</td>
<td>293</td>
<td>0.2</td>
<td>77</td>
<td>&gt;1000</td>
<td>26</td>
<td>22</td>
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</tbody>
</table>

Hormone assays
Serum TSH and fT4 levels were measured by means of ultra-sensitive chemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). Normal ranges are 0.2 – 4.2 mIU/l for TSH and 12.0 – 21.9 pM for fT4.

Octreotide tolerance test
In all patients, an octreotide tolerance test was carried out as a routine clinical evaluation. A dose of 100 μg of octreotide was injected subcutaneously at 08:00, and blood samples for TSH and fT4 evaluations were collected every hour for 6 h.

Primary cultures of pituitary TSH-secreting adenoma cells
Primary cell cultures were obtained from fresh fragments of the pituitary TSHoma by mechanical disruption under sterile conditions, as previously described (28). Briefly, single-cell suspensions filtered through a 70-μm cell strainer (BD, Milano, Italy) were treated with human fibroblast antibody-coated microbeads (Miltenyi Biotec, Bologna, Italy) for the removal of fibroblasts according to the manufacturer’s specifications. Cells were cultured in Minimum Essential Medium (MEM) containing D-valine (Sigma-Aldrich) to avoid remaining fibroblast proliferation (22) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin/streptomycin and 2.5 μg/ml amphotericin B (all from Euroclone, Milano, Italy). Cells were maintained at 37° C in an atmosphere containing 5% CO<sub>2</sub>.

<sup>[3]H</sup>-Thymidine incorporation assay
TSHoma cells were starved for 24 h in 1% FCS medium prior to treatments, and then test substances were added for 24 h in the presence of <sup>[3]H</sup>-thymidine (2 μCi/ml; GE Healthcare, Milano, Italy) and 100 nM phorbol myristate acetate (PMA; Sigma-Aldrich). At the end of the labelling, cells were washed, harvested on glass fibre filters (Millipore, Milano, Italy) and extracted in 10% and 5% trichloroacetic acid, followed by 95% ethanol precipitation, and <sup>[3]H</sup>-thymidine uptake was measured in a scintillation counter according to standard protocols (29). In these experiments, octreotide, cabergoline (alone or in combination) and the two SRIF/DA chimeric compounds were tested at predetermined maximal concentrations (10<sup>-9</sup> M) (22). Each condition was tested in triplicate.

mRNA analysis
For D<sub>2</sub> and SSTR mRNA quantification, total mRNA was extracted from fibroblast-purified TSHoma cells using the RNAeasy Micro kit (Qiagen, Milan, Italy) and reverse-transcribed into complementary DNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. qRT-PCR was assessed in triplicate using primer and probes as previously described (22) on an ABI PRISM 7700
sequence detection apparatus (PE; Applied Biosystem, Paris, France), according to the manufacturer’s protocol. mRNA levels were normalized to β-glucuronidase (β-Gus) mRNA levels in the same reaction. The results were expressed as copy of gene/copy of β-Gus.

Immunohistochemistry
Prior to immunostaining, formalin-fixed paraffin-embedded TSH-secreting adenoma tissues were cut (5 μm), deparaffinized and rehydrated. Tissue slides were heated in Tris–EDTA buffer (pH 9.0) for 20 min (microwave) for antigen retrieval and bathed into a 3% H₂O₂/PBS solution for 15 min, at room temperature in the dark, to quench endogenous peroxidase. After washing with Tris/HCl/Tween 0.5%, sections were incubated with the following primary antibodies: anti-sst₂ (Epitomics, Burlingame, CA, USA; rabbit monoclonal, 1:50, overnight 4° C), anti-sst₅ (UMB-5; Epitomics, Burlingame, CA, USA; rabbit monoclonal, 1:10, 1 h at room temperature) and anti-D₂ (Santa Cruz Biotechnology, Santa Cruz, CA, USA; mouse monoclonal, 1:400, 1 h at room temperature). After several washes, two drops of horseradish peroxidase (HRP) Rabbit/Mouse (Dako Detection System, Dako Netherlands, Heverlee, Belgium) were added to tissues and incubated for 30 min. Bound antibodies were visualized with freshly prepared 100 μl of Dako Detection System (DAB) twice for 5 min at room temperature, in the dark. Staining was then stopped by rinsing with water. Slides were counterstained with haematoxylin and eosin and coverslipped. For negative controls, the primary antibody was omitted.

Statistical analysis
Results are expressed as mean ± SE. Statistical significance was determined by anova test for unpaired samples followed by Student’s t test. Differences were taken to be statistically significant at a probability level of <0.05. To minimize variation among different experiments, results are expressed as relative variation from control values.

Results

Octreotide test and clinical outcome after prolonged octreotide treatment
The octreotide test showed a significant decrease in serum TSH levels in all patients, with nadir values (6 h after SSA injection) ranging from -62 to -81 Δ % vs baseline (Fig. 1a and Table 1).

All patients started treatment with octreotide LAR (20 mg i.m. every 4 weeks) for at least three months. Two of three patients normalized TSH, fT4 and fT3 levels after 3 months of octreotide treatment (Fig. 1b and Table 1). Conversely, case 2 was completely nonresponsive to octreotide treatment, even after increasing the dose to 30 mg. Indeed, after three months of therapy, TSH values were almost four times higher than those reported at the time of diagnosis.

Moreover, in case 1 (responder patient), gadolinium-contrasted enhanced MRI scan showed impressive tumour shrinkage, with a greater than 50% reduction in tumour mass, compared with baseline. Conversely, the nonresponder patient did not show any tumour size modification at the MRI after three months of treatment (data not shown). MRI evaluation in case 3 showed unchanged appearances, possibly due to the size of the tumour already being very small at the first imaging.

Expression of SSTR subtype and D₂ mRNAs
To identify molecular determinants of the different responsiveness to octreotide treatment, we measured the mRNA content of sst₁₂₃₅ and D₂ in fibroblast-free purified TSHoma cells by qRT-PCR. Our patients were all treated with octreotide before surgery. However, there
is no evidence in the literature for quantitative changes in receptor expression in samples collected from pretreated patients. Indeed, the effect of SSA treatment is reported to mainly affect receptor localization (cytoplasmic and/or membranous) in cell compartments rather than receptor expression (30).

**Figure 1.**

Legend: (a) Inhibition of TSH secretion after acute octreotide test in three TSHoma patients. Data are expressed as percentage of the nadir vs control values. Significant suppression of TSH levels was observed in all patients studied. (b) TSH secretion during first-line treatment with octreotide LAR (20 mg/28 day i.m.). Results are expressed as TSH levels after three months of SSA treatment vs baseline evaluation (Δ% vs control). (c) Long-term TSH response is correlated to sst5/sst2 expression ratio. Single receptor expression was quantified by real-time PCR in fibroblast-free purified TSHoma cells (expressed as copy of gene/copy of β-Gus).

In two of three cases, sst5 was the most abundant receptor (2.15 and 11.84 copy/copy β-Gus, respectively), followed by sst2 (0.36 and 3.65 copy/copy β-Gus, respectively), with sst, and sst3 being almost undetectable (Fig. 2). On the other hand, case 2 showed a higher amount of sst2 than sst5 (15.57 vs 2.19 copy/copy β-Gus) and, surprisingly, very high expression of sst1 (6.53 copy/copy β-Gus) (Fig. 2). D2 was also significantly expressed (0.7, 0.64 and 0.34 copy/copy β-Gus) in all three cases, at values similar to those observed in clinically nonfunctioning pituitary adenomas (23).

Interestingly, comparing the in vivo response to long-term octreotide treatment (Fig. 1b) and the ratio between sst5 and sst2 expression level (Fig. 2), a mirror-like pattern was observed, with the responsive patients (cases 1 and 3) showing the highest sst5/sst2 ratio and the unresponsive case 2 the lowest (Fig. 1c).
Legend: mRNA evaluation of four of five SSTRs (subtypes 1, 2, 3 and 5) and dopamine receptor D2 quantified by real-time PCR in fibroblast-free purified TSHoma cells (expressed as copy of gene/copy of ß-Gus).

Expression of sst2, sst5 subtypes and D2 by immunohistochemistry
To validate mRNA expression data of the more relevant receptors (sst2, sst5 and D2), we performed immunohistochemical analysis on paraffin-embedded adenoma sections. Owing to problems in sample collection, only a small amount of tissue suitable for formalin-fixation was collected for patient 1, and this was used for essential immunohistochemical evaluation by the pathologist. Therefore, SSTR and D2 immunohistochemistry was carried out only on sections derived from patients 2 and 3. Immunohistochemical staining confirmed the pattern of receptor expression observed at mRNA level. Patient 2 showed strong sst5 immunoreactivity, both at the membrane and at the intracellular level, and the staining was much higher compared with patient 3 (Fig. 3c–d). On the contrary, sst2 was detected as strong signal in patient 3, displaying mainly membranous localization (Fig. 3f), while heterogeneous and less intense staining was observed in patient 2 (Fig. 3e). Both tumours were clearly positive for D2 receptor expression. Interestingly, patient 2 showed a predominant punctated perinuclear staining, likely dependent on the D2-long isoform expression, as reported (31). In patient 3, homogeneous intracellular positivity with areas of clear membranous localization was detected, possibly due to a relatively higher expression of the D2-short isoform (31).

Functional studies, [³H]-thymidine incorporation
The role of SSTRs and D2 in the control of TSHoma cell proliferation was evaluated in [³H]-thymidine incorporation experiments, using isolated fibroblast-free adenoma cell cultures. To enhance DNA synthesis in human pituitary adenomas, we evaluated the effect of the test compounds in cells pretreated with PMA, as previously reported (7). Under these experimental conditions, average [³H]-thymidine incorporation activity was approximately 800 cpm/10⁵ cells.

Considering all cases together, BIM-23A760 showed a statistically significant inhibitory effect on cell growth (P = 0.03) and was the compound with the highest antiproliferative effect among those tested (Fig. 4d). Conversely, octreotide (-12.3 ± 4.0, Δ% vs control, N.S.) and cabergoline (-13.1 ± 4, Δ% vs control, N.S.) seemed to be the less effective. Combined treatment with octreotide and cabergoline resulted in additive effects (-20.5 ± 6, Δ% vs control), although this did not reach statistical significance.

Individual analysis of the clinically responsive tumours (case 1 and 3) showed significant
Legend: IHC for sst₂, sst₅, and D₂ expression in two TSH-secreting adenomas (patient 2 left column, patient 3 right column). Pictures a–b, haematoxylin–eosin (H&E) staining (magnification, x 400). Pictures c–d, strong membranous and cytoplasmic sst₂ expression in patient 2 (c) and less intense staining in patient 3 (d) (magnification, x 400). Pictures e–f, strong sst₅ staining in patient 3, predominantly on cell membranes (f); lower expression (both membranous and cytoplasmic) in patient 2 (e) (magnification, x 400). Pictures g–h, patient 2 shows mainly a dotted D₂ positivity; patient 3, a more homogeneous staining (magnification, x 400). Negative controls (i, l) were performed omitting the primary antibody.
antiproliferative activity of octreotide (that reached statistical significance in case 1) that was potentiated by the co-administration of cabergoline (Fig. 4a,c). Accordingly, the administration of SRIF/DA chimeric compounds significantly inhibited DNA synthesis in both adenoma cultures (Fig. 4).

Figure 4.

![Graphs showing the effects of octreotide (oct), cabergoline (cab), their combination (oct + cab) and two chimeric SRIF/DA agonists BIM-23A760 and BIM-23A387 on \([\text{H}]\)-thymidine incorporation in the individual adenoma studied. Results are expressed as the mean ± SE per cent \([\text{H}]\)-thymidine incorporation vs control values (Δ% vs control). Statistically significant results are individually indicated in the graph (P values). a, b and c, individual analysis of the three TSHomas studied; d, statistical analysis of the results obtained pulling all the tumours together.]

**Legend:** Functional studies in primary cultures of TSHoma cells. Effects of octreotide (oct), cabergoline (cab), their combination (oct + cab) and two chimeric SRIF/DA agonists BIM-23A760 and BIM-23A387 on \([\text{H}]\)-thymidine incorporation in the individual adenoma studied. Results are expressed as the mean ± SE per cent \([\text{H}]\)-thymidine incorporation vs control values (Δ% vs control). Statistically significant results are individually indicated in the graph (P values). a, b and c, individual analysis of the three TSHomas studied; d, statistical analysis of the results obtained pulling all the tumours together.

Interestingly, chimeric compounds also reduced *in vitro* TSH secretion from these adenoma cell cultures (-68 and -63% of control, for BIM-23A760 and BIM-23A387, respectively), although the maximal inhibition was obtained with octreotide (-90%) (data not shown). Focusing on the clinically nonresponder patient (case 2), we confirmed *in vitro* the lack of sensitivity to octreotide (-4 ± 8, Δ% vs control, N.S.). Moreover, we observed a stronger difference between the antiproliferative effect observed after treatment with the chimeric compounds and the other tested molecules, BIM-23A387 being the most powerful and the only one able to reach a statistically relevant effect (Fig. 4b). Surprisingly, in this case, the combination of octreotide and cabergoline did not mimic the effects of the chimeric compounds, being as effective as octreotide alone (Fig. 4b).
Discussion

Owing to the rare incidence, few in vitro or in vivo studies have investigated the possible role of SRIF and DA agonists, as well as chimeric SRIF/DA analogues, in the control of hormone hypersecretion and tumour growth in TSH-secreting adenomas. Nowadays, the pharmacological approach to TSHomas rests mainly on the administration of the sst₂ preferring analogues, octreotide and lanreotide, which induce normalization of thyroid hormone levels in approximately 90% of patients, while significant tumour shrinkage is also observed in about 45% of cases (4, 32). Although these data suggest that sst₂ is the main receptor subtype involved in the beneficial effects of these drugs, the mechanism behind the heterogeneity of TSHoma response is not yet understood.

In recent years, it has been suggested that sst₅ expression on top of sst₂ might account for the long-term suppression of hormone hypersecretion (9) and tumour growth (11). In this context, we recently reported a case showing, for the first time, a significant antiproliferative and antisecretory response to octreotide, both in vivo and in vitro, in a tumour with a high sst₂/sst₅ mRNA level ratio, confirming the importance of sst₅ as a predictive factor for SSA treatment outcome (10).

Here, we report data from a larger study including in vivo and in vitro results from three patients. Although the number is still small, because of the rarity of TSHomas, several recent functional studies have included similar numbers of patients (33). We observed that all three patients evaluated showed a significant response to acute treatment with SSA in terms of serum TSH inhibition, but despite previous reports showing that the majority of TSHomas maintain sensitivity to SSA and that the octreotide test may be predictive of long-term efficacy (1), only the two patients with high sst₅/sst₂ ratio showed a significant response to long-term treatment. The patient showing a positive response to the acute octreotide test, but expressing relatively low levels of sst₅ in comparison with sst₂, rapidly developed tachyphylaxis during 3 months' treatment with SSA. These findings are in line with previously published data demonstrating that sst₅ expression may influence sst₂ trafficking, resulting in faster recycling of the internalized receptor (12, 34) and, possibly, as a consequence, a better response to prolonged treatment targeting sst₂.

However, our data differ from a previous report showing a better response (in terms of GH lowering) to octreotide treatment in GH-secreting adenomas displaying a higher sst₂/sst₅ mRNA ratio (35), highlighting the concept of possible differences in receptor function and interaction in different pituitary adenoma subtypes.

Moreover, the good response to octreotide test observed in patient 2 (nonresponder to long-term treatment) indicates preserved sst₂ receptor function, leading us to exclude the presence of genetic abnormalities affecting this SSTR subtype as an explanation for the lack of efficacy observed after long-term treatment.

Notably, tumour cells derived from the nonresponder patient expressed very high levels of sst₁ mRNA, while this subtype was almost undetectable in samples from the other two subjects. Recently, in vitro studies reported that, at cell membrane level, sst interacts with both sst₁ and sst₂, in different cell lines (26, 36). Even if the cellular events resulting from these interactions have not yet been clarified, we might also consider a possible negative effect of sst₁ on sst₂ activation by octreotide.

Finally, since their recent characterization, the presence of sst₂-truncated variants (sst₂TMD4 and sst₂TMD5) might be considered to explain the unexpected response to SSA treatment observed in pituitary tumours. However, the correlation between these two sst₂-truncated forms and the in vivo clinical response to SSAs is still debated, and their presence in TSHomas has not yet been demonstrated (37).

As mentioned earlier, significant tumour shrinkage after SSA treatment is observed in only
about 45% of TSHomas. Recently, interest in the role of D2 agonists in the treatment of human pituitary adenomas was renewed following the development of compounds with high affinity for both SSTRs and D2. In this study, for the first time, we tested the in vitro ability of two SRIF/DA chimeric agonists in inhibiting TSHoma cells proliferation and made a comparison with the effect induced by octreotide and cabergoline (alone and in combination). Taking all cases together, BIM-23A760, acting on sst2, sst5 and D2, was shown to have the highest antiproliferative effect. Combined treatment with octreotide and cabergoline mainly active on sst2 and D2, respectively, caused a clear additive effect, comparable to the effect reached with the other chimeric compound tested (BIM-23A387). In this context, combined treatment with SSA and DA, or the class of new chimeric compounds, should be considered as potential tools to improve the response rate of TSHomas to medical treatment, especially in terms of tumour mass control. Importantly, sensitivity in vivo to prolonged octreotide treatment was perfectly reproduced by the experiments in vitro, in which a significant reduction of DNA synthesis was observed in cases 1 and 3, while case 2 was nonresponsive, although sst2 down-regulation was not observed (at least on qRT-PCR and immunohistochemical analyses).

Although performed on only three adenomas, and further larger analysis is required to confirm these results, the data strongly suggest that the in vitro findings might also be useful in terms of predicting clinical outcome in vivo.

Interestingly, in cells isolated from the clinically nonresponder patient (case 2), expressing a low sst2/sst5 ratio and the same D2 amount compared with the clinically responder patients, we observed an even stronger effect of the two chimeric compounds, in terms of antiproliferative effect, compared with the other tested molecules, including the combination of octreotide with cabergoline. It is well known that differential expression of these receptors can alter the response to both DA and SSA in heterologous cell systems (19–21). In line with the concept of dynamic receptor interactions, it might be hypothesized that, in TSHomas expressing lower amount of sst5 than sst2, while a lower activity of sst2 may occur by itself, this latter subtype might better interact with D2, resulting in increased receptor cross-talk that leads to enhanced efficacy of chimeric SSTR/D2 molecules. Interestingly, as the effect of both chimeras in this tumour was even higher than the combination of octreotide and cabergoline, we can hypothesize that the effect of the chimeras could be more complex than the simple co-activation of SSTRs and D2. However, further studies will be necessary to address this issue. Taken together, these observations suggest that the therapeutic response of pituitary adenomas to DA and/or SRIF analogues may be closely dependent on the relative pattern of expression of both DA and SSTR subtypes (5, 15).

Finally, according to the receptor pattern observed in TSH-secreting adenomas, combination therapy with the already clinically available SSAs (sst2/sst5 selective compounds and SSTR panligand) and D2 agonists should be considered by clinicians as a rational therapeutic option in those cases likely to be treated pharmacologically and nonresponders to classical sst2/sst5 SSA monotherapy.

However, to better understand these complex mechanisms, there is a rising need of larger and well-designed studies, especially for such a rare pathology as TSH-secreting adenomas. The new generation of SRIF/DA chimeric compounds has been instrumental in in vitro studies, allowing us to investigate further the physiopathology of SSTR- and D2-expressing tumours. However, to make a further step, the availability of newer compounds will be crucial to translate these findings into clinical practice.

Acknowledgements
We sincerely acknowledge Anne-Laure Germanetti for performing the quantitative RT-PCR analysis and Marlijn Waaijers for performing IHC for somatostatin receptors.
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A head-to-head comparison between octreotide and pasireotide in the control of hormone hyper-secretion by primary cultures of GH-secreting pituitary adenomas and the role of somatostatin receptor expression

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β-arrestin 1 and 2 and G protein-coupled receptor kinase 2 expression in pituitary adenomas: role in the regulation of response to somatostatin analogue treatment in patients with acromegaly

Federico Gatto, Richard Feelders, Rob van der Pas, Johan M. Kros, Fadime Dogan, Peter M. van Koetsveld, Aart-Jan van der Lelij, Sebastian J. C. M. Neggers, Francesco Minuto, Wouter de Herder, Steven W. J. Lamberts, Diego Ferone, and Leo J. Hofland

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Abstract

Recent in vitro studies highlighted G protein-coupled receptor kinase (GRK) 2 and β-arrestins as important players in driving somatostatin receptor (SSTR) desensitization and trafficking. Our aim was to characterize GRK2 and β-arrestins expression in different pituitary adenomas and to investigate their potential role in the response to somatostatin analog (SSA) treatment in GH-secreting adenomas (GHomas). We evaluated mRNA expression of multiple SSTRs, GRK2, β-arrestin 1, and β-arrestin 2 in 41 pituitary adenomas (31 GHomas, 6 nonfunctioning [NFPAs], and 4 prolactinomas [PRLomas]). Within the GHoma group, mRNA data were correlated with the in vivo response to an acute octreotide test and with the GH-lowering effect of SSA in cultured primary cells. β-arrestin 1 expression was low in all 3 adenoma histotypes. However, its expression was significantly lower in GHomas and PRLomas, compared with NFPAs (P < .01). GRK2 expression was higher in PRLomas and NFPAs compared with GHomas (P < .05). In the GHoma group, GRK2 expression was inversely correlated to β-arrestin 1 (P < .05) and positively correlated to β-arrestin 2 (P < .0001). SSA treatment did not affect GRK2 and β-arrestin expression in GHomas or in cultured rat pituitary tumor GH3 cells. Noteworthy, β-arrestin 1 was significantly lower (P < .05) in tumors responsive to octreotide treatment in vitro, whereas GRK2 and SSTR subtype 2 were significantly higher (P < .05). Likewise, β-arrestin 1 levels were inversely correlated with the in vivo response to acute octreotide test (P < .001), whereas GRK2 and SSTR subtype 2 expression were positively correlated (P < .05).

In conclusion, for the first time, we characterized GRK2, β-arrestin 1, and β-arrestin 2 expression in a representative number of pituitary adenomas. β-arrestin 1 and GRK2 seem to have a role in modulating GH secretion during SSA treatment.
Introduction
Recently, many efforts in the field of pituitary adenomas research have been directed towards the characterization, besides membrane receptor expression, of new molecular determinants able to better explain the tumor variable response to G protein-coupled receptors (GPCRs)-targeting drugs and in particular to somatostatin analogs (SSAs) (1–3). These efforts were driven from both the increasing need to face the relatively high-cost of long-term biotherapies and from the recent clinical availability of new analogs (eg, pasireotide), with different receptor affinity, pharmacological profiles, and biological interactions compared with the “classical” and mostly-experienced drugs. In this context, the role of some intracellular molecules, such as β-arrestins and GPCR kinases (GRKs), which are involved in membrane receptor phosphorylation, desensitization, and trafficking in different cell models, has been pointed out as a possible main actor in the modulation of ligand activated-receptor response. Indeed, a number of in vitro studies elegantly demonstrated that somatostatin receptors (SSTRs) undergo agonist-induced desensitization and internalization (4, 5). These studies highlighted the GRK type 2 (GRK2) as one of the receptor kinases mainly involved in the homologous ligand-mediated receptor phosphorylation, the first step of the complex trafficking machinery. After GRK2-mediated phosphorylation, β-arrestins are recruited on cell membrane, determine uncoupling between the receptor and its related G proteins (desensitization process), and act as scaffold proteins, driving the receptor towards the endocytic machinery.

On the basis of the receptor affinity for the 2 different β-arrestins (1 and 2), Oakley et al (6) categorized GPCRs into 2 classes (A and B) characterized by peculiar differences in their trafficking dynamics. However, despite being first classified as a class B receptor, known to have slow recycling rate and stable β-arrestin linkage, SSTR subtype 2 (sst₂) has been demonstrated to efficiently recycle to the plasma membrane without entering any degradative pathway (7). sst₂ ligand-dependent phosphorylation, desensitization, and internalization processes have been extensively studied in transfected/silenced cell line models, being highlighted as fine-tuned agonist-dependent processes, where GRK2 and β-arrestins act as critical effectors. These insights led us to hypothesize that over- or underrepresentation of these molecules could play a role in modulating the response to SSTR-targeting drugs. The first aim of our study was to evaluate the quantitative expression of both β-arrestin and GRK2 mRNA (together with SSTRs, including sst₂ transmembrane domain 4 [sst₂TMD4], and dopamine type 2 [D2] receptor), in a representative number of pituitary adenomas, in order to characterize their expression profile in different adenoma histotypes. Moreover, our main aim was to evaluate the possible role of β-arrestin and GRK2 expression in driving the response to SSA in GH-secreting adenomas (GHomas). Therefore, within the GHoma group, we investigated the mutual correlations between the different intracellular molecules, as well as their relationship with the receptor profile. Finally, these data were correlated with the in vitro and in vivo short-term response to octreotide treatment, demonstrating a differential role of β-arrestin 1 and GRK2 in the modulation of the response of GH secretion to SSA.

Patients and Methods

Patients, tumors, and assays
Pituitary tumor samples were obtained by transsphenoidal surgery from a total of 41 patients (31 GHomas, 6 nonfunctioning pituitary adenomas [NFPAs], and 4 prolactinomas [PRLomas]). Diagnosis was established on the basis of clinical and biochemical
characteristics of the patients and afterwards confirmed by the pathology report on the basis of histology, as well as immunohistochemical evaluation of tumor samples. For all samples, directly after obtaining the tissue, a piece was snap frozen on dry ice and stored at -80°C until analysis. In 22 GHomas, from which enough material was available, part of the tissue was used for cell cultures. Moreover, the results of an acute octreotide test, performed as previously reported (8) as part of the routine clinical practice, were available for 19 acromegalic patients. In these subjects, we correlated the in vivo responsiveness to octreotide with the mRNA expression of both the receptors and intracellular proteins. Data from both the acute octreotide test and adenoma cell culture treatment were available in 13 patients (see Table 1). Detailed characteristics of the acromegalic patients included in our study are presented in Table 1.

All NFPA and PRLoma patients had a magnetic resonance imaging suggestive for a macroadenoma.

Histological examination of the 6 NFPA samples was negative for all the routinely tested pituitary hormones. No NFPA patient underwent presurgical medical treatment, either SSAs or dopamine agonists. All PRLomas were treated with titrated cabergoline dosages before surgery, resulting in the normalization of PRL levels in all cases, although without reaching a significant tumor shrinkage (reason for the following surgery). Approval from the Medical Ethical Committee of the Erasmus Medical Center and informed consent to use the tumor tissues for research purposes were obtained.

Human GH concentrations, from both patient blood samples and cell culture media, were determined by use of a nonisotopic, automatic chemiluminescence immunoassay system (Immulite; Diagnostic Products Corp). Not all parameters were available for each patient.

Table 1. General Characteristics, Tumor Size, and Octreotide Treatment Results of the 31 Acromegalic Patients Included in the Study

<table>
<thead>
<tr>
<th>Patient no.</th>
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<th>Age at diagnosis (yr)</th>
<th>Tumor volume</th>
<th>Presurgical treatment (OCT LAR)</th>
<th>GH suppression (% in vitro)</th>
<th>GH suppression (%) OCT test</th>
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follow Table 1
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**Legend:** Data in parentheses indicate percentage of in vitro GH suppression categorized. GH was scored 1 when reduced equal or more than 50%, and 0 when reduced less than 50%. F, female; M, male; OCT, octreotide; hyphen, not available.

**Quantitative PCR**

Quantitative PCR was performed according to a previously described method (9, 10). Briefly, to perform membrane receptor mRNA evaluation, poly A+ mRNA was isolated from adenoma tissues using Dynabeads Oligo deoxy-thymine nucleotides (dT)25 (Dynal AS). cDNA was synthesized using the poly A+ mRNA, which was eluted from the beads in H2O twice for 2 minutes at 65°C, using Oligo (dT)12-18 Primer (Invitrogen). For β-arrestin 1, β-arrestin 2, and GRK2 mRNA evaluation (intron spanning prime-probe sequences), total mRNA isolation was performed using a commercially available kit (Roche Applied Science). cDNA was synthesized from 500 ng of total mRNA, eluted in adequate amount of H2O to reach a 20-μL volume, using Oligo (dT)12-18 Primer. Samples were measured on an ABI Prism 7900 Sequence Detection System (PerkinElmer) for real-time amplifications, according to manufacturer’s protocol. The primer and probe sequences, the efficiencies, and the reaction conditions that were used for the detection of sst1, sst2, sst3, sst5, D2, and hypoxanthine–phosphoribosyl-transferase (hprt) have been previously described (10, 11). In addition, we also evaluated both human and rat β-arrestin 1, β-arrestin 2, and GRK2 mRNA expression, rat β-glucuronidase (β-Gus), and human sst5TMD4 (sst5-truncated form). Table 2 shows the relative primer-probe sequences and efficiencies values. The detection of human hprt and rat β-Gus mRNA served as controls (housekeeping genes) and was used to normalize membrane receptors, β arrestins, and GRK2 mRNA expression in human and rat samples, respectively.
### Cell dispersion and cell culture

Single-cell suspensions of the pituitary adenoma tissues were prepared by enzymatic dissociation with dispase as previously described in detail (12). For short-term incubation of monolayer cultures, the dissociated cells were plated in 48-well plates (Corning) at a density of 10^5 cells per well per 1-mL culture medium. After 3–4 days, the medium was changed, and 72-hour incubations without or with test substances were initiated. At the end of the incubation, the medium was removed and centrifuged for 5 minutes at 600g. The supernatant was collected and stored at -20°C until analysis. The choice for a 72-hour incubation was made on the basis of previous studies, in which we demonstrated that exposure of GH-secreting pituitary adenoma cells for 4–96 hours to octreotide showed a variable but in all instances during longer incubations statistically significant inhibition of GH release, which paralleled the sensitivity of GH secretion to octreotide in vivo (13). The culture medium consisted of MEM supplemented with nonessential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum, penicillin (1 x 10^5 U/L), fungizone (0.5 mg/L), L-glutamine (2 mmol/L), and sodium bicarbonate (2.2 g/L; pH 7.6). Media and supplements were obtained from Gibco Bio-Cult Europe (Invitrogen). Unfortunately, not enough tumor material was obtained to perform cell cultures for each tumor.

### Table 2. Primer-Probe Sequences for Human and Rat β-arrestin 1, β-arrestin 2, GRK2, human sst₅TMD4, and rat β-Gus

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<tr>
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**Legend:** fwd, foward; rev, reverse.
Cell line
The rat somatolactotroph pituitary cell line GH3 (CCL-82.1; American Type Culture Collection) was cultured in F-10 Nut Mix (1x) + Glutamax TM-1 medium, supplemented with 15% horse serum, 2.5% fetal bovine serum, and penicillin in a 5% CO₂ atmosphere at 37°C. Cells used in the current study did not exceed 20 passages. Medium was refreshed twice a week, and cell viability always exceeded more than 90% as measured by trypan blue staining. The cell line was confirmed to be mycoplasma free.

Media and supplements were obtained from Gibco Bio-Cult Europe (Invitrogen).

GH3 cell treatment for mRNA expression studies
To evaluate a possible direct effect of octreotide in the modulation of β-arrestin and GRK2 mRNA expression, GH3 cells were treated with octreotide, as described below. Cells were trypsinized, counted in a standard hemocytometer, and seeded at a density of 2 x 10⁵ cells (3-d experiment) or 1 x 10⁵ cells (7-d experiment) per well in 12-well plates (Corning) in 2-mL medium. After 72 hours, media were refreshed, and incubations were started without or with 2 different doses of octreotide (10⁻⁸ M and 10⁻⁹ M). At different time points (3 and 7 d), media were removed, and cells were lysed on ice with a buffer containing 100mM Tris-HCl (pH 8), 500mM LiCl, 10mM EDTA (pH 8), 5mM dithiothreitol, and 1% lauryl sulfate lithium salt (HT Biotechnology Ltd) and stored at -80°C until further analysis. For the 7-day experiments, medium and compound were refreshed at day 3. All experimental conditions were performed in quadruplicates.

Statistical Analysis
SPSS 15.0 for Windows (SPSS Inc) was used for statistical analyses. Receptor expression and intracellular molecule levels are expressed as mean ± SD (or as median [range]). Between group comparisons were analyzed by the Mann-Whitney U test, and correlation coefficients were calculated by the Spearman rank order R. Multiple linear regression analysis was performed with stepwise addition of the variables that had P values less than 0.1 in the univariate analyses. Differences were taken to be statistically significant at P < .05.

Results
Receptor expression levels
Mean mRNA expression levels of ssts and dopamine receptor D2 are shown in Figure 1, A–C. In general, our results were in line with previous data reported in literature (14–16). Within the GHoma group, the sst₅ was the most predominantly expressed sst receptor (relative expression, normalized to hprt), followed by the sst₂. D2 was the receptor expressed at the highest level among all receptors evaluated (Figure 1A). As expected, sst₅ TMD4 (sst₅-truncated form) expression was lower compared with the wildtype receptor. sst₃ was expressed at the highest level in the nonfunctioning adenomas, together with the sst₂. As for GHomas, D2 was the receptor expressed at the highest level (Figure 1B). As expected, D2 was highly expressed in most PRLoma samples, and sst₁ was the most predominantly expressed sst receptor (Figure 1C).
Characterization of β-arrestin 1, β-arrestin 2, and GRK2 mRNA expression in pituitary adenomas

β-arrestin 1, β-arrestin 2, and GRK2 mean mRNA expression levels, evaluated in the different adenoma types, are depicted in Figure 2, A–F. In all 3 adenoma histotypes, β-arrestin 2 was expressed at highest level, whereas low (or very low) β-arrestin 1 levels were detected in almost all samples analyzed (Figure 2, A–C). β-arrestin 2 and GRK2 expression was detected in all samples that were analyzed, whereas β-arrestin 1 mRNA levels were below the detection limit in 10 out of 26 (32%) GHoma samples. Conversely, β-arrestin 1 mRNA was detectable in all PRLoma and NFPAs samples included in the study. In more detail, we observed that β-arrestin 1 mRNA levels were significantly higher in NFPAs (mean 0.031 ± 0.0079, median 0.029), compared with both GHomas (mean 0.0087 ± 0.014, median 0.0048) and PRLomas (mean 0.0046 ± 0.0010, median 0.0046) (P < .0011 and P < .0095, respectively) (Figure 2D).

On the contrary, GRK2 was expressed in PRLomas at highest level (mean 0.38 ± 0.11, median 0.36) compared with GHomas (mean 0.16 ± 0.07, median 0.16; P < .0025) and NFPAs (mean 0.24 ± 0.07, median 0.25). The difference between PRLomas and NFPAs was not statistically relevant (P < .067). Moreover, GRK2 levels in the NFPA group were significantly higher compared with GHomas (P < .025) (Figure 2F). β-arrestin 2 mRNA expression was not significantly different between the 3 adenoma groups (GHomas: mean 0.88 ± 0.44, median 0.79; NFPAs: mean 1.16 ± 0.48, median 1.07; and PRLomas: mean 1.05 ± 0.22, median 0.97) (Figure 2E).
Figure 2.

Legend: Mean expression levels of β-arrestin 1, β-arrestin 2, and GRK2 in 3 different pituitary adenoma histotypes (26 GHomas, 6 NFPAs, 4 PRLomas) (A–C). Detailed comparison of β-arrestins and GRK2 mRNA expression between the 3 adenoma groups is depicted in D–F. Statistically relevant differences (P < 0.05) are reported in each graph. Values represent the mean ± SD per each intracellular molecule, assayed in duplicate. Expression levels are normalized against the housekeeping gene hprt.
β-arrestin and GRK2 correlation studies in GHomas

Within the GHoma group, we evaluated the mutual correlations between the different intracellular molecules and their expression related to the membrane receptor profile. The mRNA evaluation of β-arrestins and GRK2 was available in 26 samples, whereas the evaluation of both intracellular molecules and membrane receptor expression was available in 21 samples (see Supplemental Table, at the end of the Chapter). The statistically significant correlations are shown in Figure 3, A–D.

GRK2 mRNA expression was inversely correlated to β-arrestin 1 (Spearman’s r: -0.44, P < .023, n=26), whereas it showed a strong and direct correlation with β-arrestin 2 (Spearman’s r: 0.78, P < .0001, n = 26) (Figure 3, A and B). No (statistically significant) correlation was observed between β-arrestin 1 and β-arrestin 2 expression.

Moreover, neither β-arrestin 1 nor β-arrestin 2 was significantly correlated to any membrane receptor evaluated in our study (data not shown).

On the other hand, GRK2 mRNA expression was positively correlated to D2 (Spearman’s r: 0.55, P < .010, n = 21) and to the sum of the different sst expression (Spearman’s r: 0.56, P < .0089, n = 21) (Figure 3, C and D). No statistically relevant correlation was observed between GRK2 and any individual sst. Indeed, GRK2 is known to interact with different GPCR families and subtypes. In this context, a correlation between GRK2 and the amount of membrane receptors (eg, D2, the mostly expressed, or the sum of ssts), has to be expected, rather than a correlation with the individual GPCR subtypes.

Neither the patients’ general characteristics (age, sex) nor tumor size (micro- or macroadenoma) were related to β-arrestin 1, β-arrestin 2, or GRK2 mRNA expression.

In vitro and in vivo GH suppression by octreotide:
Correlation with β-arrestin 1, β-arrestin 2, and GRK2 mRNA expression

Primary cultures from 22 GHomas were incubated with or without 10^-8 M octreotide for 72 hours. The mean percentage GH suppression (vs control) was 40% (median 38%, range -5, 77%) (see Table 1). On the basis of the in vitro response to octreotide treatment, according to a previously accepted classification (17), tumors were divided into 2 groups, eg, responders (GH suppression vs control ≥ 50%, n = 7) and nonresponders (GH suppression vs control < 50%, n = 15).

As depicted in Figure 4A, β-arrestin 1 levels were significantly lower in the responder group, compared with the nonresponders (P = .018, n = 18). Conversely, GRK2 mRNA expression was higher in the responder group, compared with nonresponders (P = .041, n = 18), in line with what observed (and expected) for sst2 levels (also higher in the responder tumors, P = .049, n = 19) (Figure 4, C and D). β-arrestin 1 (Figure 4B) and sst5 (data not shown) expression were not significantly different between the 2 groups (P = .24, n = 18 and P = .596, n = 19, respectively).

Moreover, as previously described, in 19 patients that underwent an acute octreotide test, we correlated the in vivo responsiveness to octreotide with the mRNA expression of both the membrane receptors and GRK2 and β-arrestin 1 and β-arrestin 2. The mean percentage in vivo GH suppression was 73% (median 74%, range 31%–95%).

The in vitro and in vivo GH suppression rates showed a trend for a direct correlation (Spearman’s r: 0.54, P = .056, n = 13).

In line with in vitro data, we observed a statistically significant inverse correlation between β-arrestin 1 mRNA expression and percentage GH suppression after acute octreotide test (Spearman’s r: -0.74, P = .001, n = 16) (Figure 5A).

Conversely, both GRK2 and sst2 levels were directly and significantly correlated with a better response to acute octreotide administration (Spearman’s r: 0.54, P = .031, n = 16 and Spearman’s r: 0.64, P = .010, n = 15, respectively) (Figure 5, C and D).
Figure 3.

**GH-secreting adenomas**

**Legend:** Mutual correlations between the different intracellular molecules and their expression related to the membrane receptor profile have been analyzed within the GHoma group. Statistically relevant correlations (Spearman rank order R test) are depicted in A–D. Spearman's r and related P values are reported in each graph. mRNA expression levels are normalized against the housekeeping gene hprt. Total SSTR mRNA expression (D) has been computed by the rough sum of each sst expression.
Legend: Comparison of β-arrestin, GRK2, and sst mRNA levels between tumors responders (GH suppression ≥ 50%, n = 6) and nonresponders (GH suppression < 50%, n = 12) to in vitro octreotide treatment (dosage 10^{-8}M, 72-h incubation) (A–D). For sst mRNA comparison, tumor responders n = 4 and nonresponders n = 15; see Supplemental Table and Table 1 for details. Statistical significance was determined by the Mann-Whitney U test. The lower and upper bars represent the first and third quartiles, respectively. The lines across the box represent median value. The lines above and below the box represent the highest and lowest values. Statistically relevant differences (P < 0.05) are reported in each graph. Expression levels are normalized against the housekeeping gene hprt.
Figure 5.

Legend: Correlations between β-arrestin, GRK2, and sst2 expression and percentage GH suppression after acute octreotide test. Spearman's r and related P values (referring to octreotide test results as a continuous variable, as reported in Table 1), are reported in each panel. To achieve a clearer graphical representation, patient response has been stratified and depicted as tertiles (GH reduction < 60%, n = 3; between 60% and 80%, n = 7; > 80%, n = 6 [for sst, mRNA correlation, n = 5; see Supplemental Table and Table 1 for details]; computed cut-off values by use of SPSS software) in the related graphs. mRNA expression levels are normalized against the housekeeping gene hprt.
Again, β-arrestin 2 levels were not significantly correlated with percentage GH lowering after octreotide test (Spearman’s r: 0.11, P = .696, n = 16) (Figure 5B). Similarly, as for the in vitro data, sst5 mRNA expression did not correlate with the response to the acute octreotide test (Spearman’s r: 0.18, P < .516, n = 15) (data not shown). For clarity, patient response has been stratified and depicted as tertiles (GH suppression more than 80%, between 60% and 80%, <60%; computed cut-off values) in the related graphs (Figure 5). Noteworthy, multiple regression analysis of sst2, β-arrestin 1, and GRK2 mRNAs in predicting patient response to an acute octreotide test showed a very good overall predictivity for these 3 parameters (r²: 0.76; P < .008). The combination of the 3 variables resulted in a considerably higher r² value, compared with sst2, β-arrestin 1, or GRK2 alone. However, both sst2 and β-arrestin 1 mRNA expression still resulted as significant (but less powerful) predictors of acute octreotide test response at univariate regression analysis (r²: 0.29, P < .039 and r²: 0.39, P < .009, respectively).

**β-arrestin and GRK2 mRNA expression after octreotide treatment**

Considering the relevant correlations observed between β-arrestin 1, GRK2, and the response to octreotide treatment, we also aimed to investigate the effect of octreotide on β-arrestin and GRK2 mRNA expression. We firstly compared β-arrestin 1 and β-arrestin 2, as well as GRK2 levels between the group of patients pretreated with octreotide long-acting release (LAR) before neurosurgery (n = 7) and the SSA treatment naïve patients (n = 18). As shown in Figure 6A, no difference in β-arrestin 1, β-arrestin 2, and GRK2 mRNA expression between the 2 groups was recorded. Moreover, to further investigate this finding, we used the GH3 cell line as model. Basal β-arrestin 1, β-arrestin 2, and GRK2 mRNA expression levels were substantially in line with those observed in our human adenoma samples (Figure 6B). After both 3 and 7 days of octreotide treatment (at 2 doses, 10⁻⁸M and 10⁻⁹M), the mRNA expression of the intracellular molecules did not show any significant change from basal levels (Figure 6C), thus confirming the results observed in human GHoma samples.

**Discussion**

The clinically available SSAs, octreotide and lanreotide, represent the first line medical treatment for acromegaly (18). The widely demonstrated overexpression of SSTRs, in particular sst2, in GHoma cell membrane represents the rationale for SSA therapy. Indeed, a number of studies already highlighted sst2 expression (both at mRNA and protein level) as a good predictive factor for SSA efficacy in GHomas (17, 19).

However, despite the promising results obtained using the new well-validated sst2 monoclonal antibody (20), we still face tumors that are resistant to SSA therapy despite high expression of this receptor (21), and the exact mechanisms underlying this discrepancy are not completely known yet (22).

All these findings led us to hypothesize a possible role of other molecules and/or cellular mechanisms, besides membrane receptor expression and/or mutual balance of receptor subtypes, driving SSA response in pituitary tumors, in particular the GHomas. In this context, as already mentioned, a number of in vitro studies highlighted a possible pivotal role for the receptor kinase GRK2 and β-arrestins (23).

In our study, for the first time, we characterized the mRNA expression levels of β-arrestin 1, β-arrestin 2, and GRK2 in a representative series of pituitary adenomas (n = 41), together with SSTRs and D2 expression.

The first clear finding was the low β-arrestin 1 mRNA expression observed in all the pituitary adenoma samples analyzed. Although β-arrestin 1 seems to be ubiquitously expressed,
Legend: Effect of SSA treatment on β-arrestin and GRK2 mRNA expression. (A) Comparison of β-arrestin 1, β-arrestin 2, and GRK2 mRNA levels between the group of patients pretreated with octreotide LAR before neurosurgery (pretreated, n = 7) and the SSA treatment naïve patients (not pretreated, n = 18). n.s., no statistically relevant differences detected. (B) Basal β-arrestin 1, β-arrestin 2, and GRK2 mRNA expression in GH3 cell line. Expression levels are normalized against the housekeeping gene β-Gus. (C) Relative β-arrestin and GRK2 expression (% vs control) after 72 hours of in vitro octreotide treatment (10⁻⁸M and 10⁻⁹M). As clearly depicted in the related graph, no statistically relevant changes in mRNA expression were detected in our cell model. The results of 7 days of treatment experiments (data not shown) were in agreement with the 72-hour experiment data. Values represent mean ± SD of 2 independent experiments, assayed in quadruplicate. arr, arrestin; OCTR, octretide.
and particularly high in the brain and the immune system (24), our observation is in line with previous reports describing a relatively low expression of β-arrestin 1 in normal pituitary samples compared with both other tissues and to β-arrestin 2 expression in the anterior pituitary (25, 26). Moreover, low β-arrestin 1 levels compared with both β-arrestin 2 and GRK2 were also detected in the rat GH3 cell line, as well as in normal rat pituitary tissue (data not shown). Therefore, low β-arrestin 1 mRNA seems to be a peculiar characteristic of both normal and tumoral pituitary tissues, although showing a peculiar cell/histotype specificity. In this context, GHomas and PRLomas showed a significantly lower β-arrestin 1 expression compared with NFPAs.

This finding could, at least partially, explain the clinical experience reporting a general good response to GPCR targeting drugs (eg, SSAs and dopamine agonists) in GH and PRL-secreting pituitary adenomas, compared with other tumors (eg, gastroenteropancreatic neuroendocrine tumors) expressing a comparable amount of target membrane receptors. Previous elegant in vitro studies already demonstrated that β-arrestin 1 plays an important role in sst2 desensitization upon agonist activation (27, 28). Moreover, other authors showed that sst2A, despite being first classified as a GPCR class B receptor, is efficiently recycled to the plasma membrane and does not enter any degradative pathway after ligand binding (7). This means that lower β-arrestin 1 levels could result in a higher amount of biologically active (less desensitized) receptor exposed on the cell membrane. In line with this hypothesis, our results show that lower β-arrestin 1 mRNA expression correlates with a better response to octreotide treatment, in terms of GH suppression, both in vitro and in vivo.

Conversely, we observed a direct and significant correlation between GRK2 mRNA expression and the effect of octreotide on GH secretion. Again, in vitro results were confirmed by the in vivo observations. This finding is in line with the inverse correlation that we observed between β-arrestin 1 and GRK2 mRNA expression. In this context, Penela et al (29) demonstrated that β-arrestin-mediated proto-oncogene tyrosine-protein kinase Src recruitment and subsequent GRK2 phosphorylation play a key role in GRK2 degradation. Moreover, previous studies reported an increase of GRK2 mRNA levels in several experimental situations characterized by an increased stimulation of GPCR, and this could be the case in low β-arrestin 1 tumors, where less receptor desensitization occurs. However, the relationship between GPCR signaling activity and cellular GRK2 levels is not straightforward, with other studies reporting an inverse correlation between GRK2 and GPCR activity. In addition, the effect of most GRK2 mRNA modulators seems to be cell-type specific, and more than a single second messenger contributes to its regulation (30). We observed that octreotide treatment per se did not result in the modulation of β-arrestins and GRK2 mRNA levels, neither in adenoma samples, nor in the GH3 cell line model. This finding lays for a peculiar β-arrestin/GRK2 ratio in the different adenomas, based on specific (but not drug related) pathophysiological features.

Noteworthy, β-arrestin 1 and GRK2 mRNA expression are not correlated to sst2 expression. These findings suggest the possible role of these intracellular molecules as independent (and additional) predictive factors for SSA treatment outcome in GHomas, besides sst2 expression. Indeed, as shown in the multiple regression analysis, the concomitant evaluation of sst2, β-arrestin 1, and GRK2 results in a better prediction of octreotide response compared with each single variable.

In this context, the recent availability of a new SSA, pasireotide, with a wider spectrum of sst affinity and different pharmacological profile and biological interactions compared with the classical analogs, contributed to the increasing interest in SSTR desensitization and trafficking machinery. Indeed, a number of in vitro studies have already demonstrated that pasireotide treatment results in a lower β-arrestin recruitment and different receptor
phosphorylation compared with both native somatostatin and octreotide (5, 31, 32). In line with the new findings presented in this study, we could speculate that the enhanced effect on GH suppression of pasireotide compared with octreotide observed in vitro in few tumor samples (10) and emerging from recent clinical trials (33, 34) could be related to an increased sst₂ activity (receptor less desensitized), more than to the activation of other receptor subtypes, namely sst₅.

In conclusion, for the first time, in our study, we characterized the quantitative mRNA expression of β-arrestin 1, β-arrestin 2, and GRK2, in a representative series of pituitary adenomas. Besides sst₂, β-arrestin 1 and GRK2 appear to play an important role in the modulation of SSA efficacy, at least on hormone secretion, in GHomas. Future studies aimed to confirm these findings at the protein level, focusing on the response to long term SSA treatment, could represent a real step towards a tumor-tailored treatment with SSAs in acromegaly.

**Supplementary Table.** mRNA expression of somatostatin receptors (ssts), β-arrestins and GRK2 in three different adenoma hystotypes (GH-secreting, NFPAs, PRLomas).

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**NFPAs**

|            | <     | 0.11780 | 0.1107 | <     | -    | 0.84  | 0.044 | 1.29  | 0.197  |
| 2          | 0.02970 | 0.13950 | 0.0055 | 0.00260 | -    | 0.46  | 0.023 | 0.84  | 0.14   |
| 3          | <     | 0.01320 | 0.0581 | <     | -    | 0.77  | 0.036 | 1.37  | 0.317  |
| 4          | 0.00140 | 0.05000 | 0.0262 | 0.00130 | -    | 0.21  | 0.029 | 0.79  | 0.212  |
| 5          | <     | 0.04300 | 0.2932 | 0.00260 | -    | 0.62  | 0.025 | 0.70  | 0.299  |
| 6          | 0.00865 | 0.15445 | 0.0687 | 0.00165 | -    | 0.36  | 0.028 | 1.95  | 0.297  |

**PRLomas**

|            | <     | 0.2732 | 0.0107 | 0.0007 | 0.2829 | -    | 2.7100 | 0.0045 | 1.0043 | 0.5355 |
| 2          | 2.9955 | 0.0110 | <     | 0.1215 | -    | 4.7543 | 0.0047 | 1.3772 | 0.3283 |
| 3          | 2.1400 | 0.0066 | <     | 0.2711 | -    | 0.1931 | 0.0033 | 0.8865 | 0.2722 |
| 4          | 0.1080 | 0.0540 | <     | 0.1023 | -    | 3.1200 | 0.0058 | 0.9293 | 0.3970 |

NFPAs, non-functioning pituitary adenomas; PRLomas, prolactinomas; <, below the detection limit (Ct values >40); -, not assessed; <sup>a</sup> mRNA expression is normalized to hprt levels
REFERENCES


β-arrestin expression as an additional marker to predict responsiveness to long-term somatostatin analog treatment in acromegaly
Glucocorticoids differentially modulate β-arrestin 1 and β-arrestin 2 expression in corticotroph tumor cells

Federico Gatto, Richard A. Feelders, Rob van der Pas, Peter M. van Koetsveld, Marica Arvigo, Fadime Dogan, Steven W. Lamberts, Diego Ferone, Leo J. Hofland
CHAPTER 8

General discussion & Future perspectives
CHAPTER 9

Summary
Samenvatting
Summary

Pituitary adenomas (PAs) represent a heterogeneous group of tumors, originating from cells of the anterior pituitary gland, with a reported prevalence of about 1:1000-1400 of the general population. In this context, studies conducted on autopsy and radiological series, although not reflecting the true prevalence of clinically relevant pituitary adenomas, reported an overall estimated prevalence of PAs in the general population of about 17%. Despite PAs are mostly benign, they can severely affect the patient’s health status, either because of the associated hormonal hypersecretion depending on the tumor phenotype, or due to the compression of critical adjacent structures, such as the normal pituitary cells, the optic chiasma, as well as vascular structures.

Somatostatin receptors (SSTRs) have been demonstrated to be widely (over)expressed in the different PA histotypes. Sst_2 and sst_5 are the most represented subtypes, followed by sst_1 and sst_3.

This peculiar SSTR subtype expression pattern represents the pathophysiological basis for the use of the “classical” somatostatin analogs (SSAs), which bind with high affinity sst_2 (and to a lesser extent sst_5) for medical treatment of patients with PAs, in particular for patients harboring GH- or TSH-secreting adenomas. However, the individual responses of patients to SSA treatment is very heterogeneous, also within patients harbouring the same PA histotype. Therefore, in the recent years a number of studies have focused on the investigation of different determinants, including patient clinical characteristics, imaging features, as well as histopathological and tumor molecular predictors, that could potentially affect the responsiveness to SSTR targeting.

In this Thesis, we aimed to deeper investigate a number of molecular predictors and mechanisms already proposed to play a role in resistance to SSA treatment, and to increase our knowledge on the pathophysiological basis of SSTR targeting in PAs, in order to identify new possible molecular effectors involved in the responsiveness of patients to SSA treatment.

As above mentioned, the “classical” SSAs, octreotide and lanreotide, preferentially bind to sst_2, and the GH-lowering effect of these drugs in acromegalic patients has been positively correlated with both tumoral mRNA and protein levels of this receptor subtype. Sst_2 protein has been mainly evaluated by immunohistochemistry (IHC) using different polyclonal antibodies. However, more recently, an IHC protocol performed with a sst_2 rabbit monoclonal antibody (UMB-1 clone) has been demonstrated to be as good as the “gold standard” in vitro method (autoradiography) to quantify sst_2 expression in tumor tissues. In Chapter 2 we characterized sst_2 protein expression in a large series of mainly SSA treatment-naïve pituitary adenomas from acromegalic patients, using immunohistochemistry with the UMB-1 antibody. We evaluated sst_2 immunoreactivity by the use of a semi-quantitative score system (IRS) and found a strong direct correlation between sst_2 IRS and IGF-I normalization after adjuvant treatment with SSAs. Moreover, we were able to provide a positive predictive value (86%, sensitivity 86% - specificity 91%), for IGF-I normalization after at least 3 months adjuvant SSA treatment, using sst_2 IRS assessed by standard immunohistochemistry. Therefore, in this study, we have confirmed the pivotal role of functional sst_2 protein expression in driving the response to SSA treatment in acromegaly. Moreover, we have demonstrated that the evaluation of sst_2 IRS, using immunohistochemistry technique with the UMB-1 antibody, may represent a useful tool in clinical practice to identify patients that are likely to be good responders to SSA adjuvant therapy.
However, as previously mentioned, PAs may express different panels of SSTRs. Therefore, in Chapter 3, we have evaluated the role of the heterogeneous SSTR co-expression on the cell membrane, as another factor possibly affecting the response to SSAs in patients with TSH-secreting adenomas. We have shown that, in this rare PA subtype, sst₂ and sst₅ are co-expressed at both mRNA and protein levels. All the patients included in the study showed a significant TSH lowering after acute octreotide test, but only two patients displayed a significant biochemical response to long-term SSA treatment. We observed that the two adenomas with a high sst₅/sst₂ mRNA ratio were those tumors showing a better response to long-term treatment. This data suggest that in TSH-secreting adenomas, differently from GH-secreting adenomas, sst₅ expression on top of sst₂ might favour the long-term efficacy of SSAs. The difference observed between TSH- and GH-secreting adenomas, points out the important concept of “cell type specificity”, whereby we can observe different receptor function and interaction in different tumor cell types.

In the light of the recent availability in the clinical practice of the new SSA panligand, pasireotide, in Chapter 4 we compared its direct anti-secretory effect to that of octreotide (a “classical” SSA) in a large number of primary cultures of GH-secreting adenomas, and we correlated these data with the SSTR expression in the different adenoma samples. We aimed to identify the presence, and the related SSTR expression pattern, of peculiar GH-secreting adenoma sub-populations in which the effect of one of the two compounds could be predominant. We observed that the overall effect of the two compounds in inhibiting in vitro GH secretion was superimposable. However, we showed that pasireotide was more potent than octreotide in 18% of samples, while octreotide was more effective in 15% of these pituitary tumors. Moreover, we found that adenomas with a lower sst₂ mRNA expression and a lower sst₂/sst₅ ratio are most likely to be better responders to pasireotide. Despite all these findings need to be further explored, and translated into a more clinical context, these data point out that the evaluation of SSTR expression in tumor samples could become a useful tool in the next future to select the best SSA for the adjuvant treatment of acromegaly, based on specific tumor characteristics.

As stated throughout this Thesis, the availability of new SSAs, with different receptor binding affinities and pharmacological properties compared to the “old” and most-experienced compounds, has increased the need for a better understanding of SSTR pathophysiology. In this context, based on previous elegant in vitro studies, GPCR kinases (GRKs) and, in particular β-arrestins (1 and 2) have been highlighted as two critical effectors in the regulation of SSTR desensitization and trafficking processes. Therefore, in Chapter 5 we have characterized, for the first time, GRK2 and β-arrestin mRNA expression in different pituitary adenomas histotypes (GH-, PRL-secreting and non-functioning adenomas). Interestingly, the expression of β-arrestin 1 was significantly lower in GH- and PRL-secreting adenomas, compared to non-functioning adenomas. Moreover, within the GH-secreting adenoma group, we have shown that lower β-arrestin 1 (and higher GRK2) mRNA levels correlate with a better response to octreotide treatment, in terms of GH suppression, both in vitro and after acute exposure in vivo.

These findings have been confirmed and strengthened by the study reported in Chapter 6. In this study, we correlated tumoral β-arrestin mRNA expression, evaluated in a large number of GH-secreting adenoma samples, with the responsiveness of patients to long-term SSA treatment (median follow-up 12 months). We observed that β-arrestin mRNA expression (both β-arrestin 1 and β-arrestin 2) significantly affected the responsiveness to long-term treatment with SSAs in acromegalic patients. Moreover, we also found that
β-arrestin 1 and 2 mRNA expression showed a strong inverse correlation with sst2 protein expression.

In our opinion, the data from Chapter 5 and 6 highlight β-arrestins as two novel molecular predictors for the biochemical response to SSA treatment in GH-secreting adenomas, since they affect both sst2 function (signaling and desensitization processes) and its expression on the cell membrane (internalization and recycling). Of course, these new findings need additional studies to be further confirmed, in particular investigating whether the evaluation of β-arrestin protein expression (by use of techniques routinely performed for clinical diagnosis) might represent a useful marker to predict responsiveness to SSA treatment as well.

Corticotroph adenomas of patients with Cushing’s disease (CD) represent a challenge for the medical therapy because these tumors do express SSTRs, however, a significant response to “classical” SSAs is scarce and disappointing. In Chapter 7, we have studied the expression of β-arrestin mRNA in ACTH-secreting adenomas and in a murine corticotroph model, the AtT20 cell line. Moreover, based on a recent study demonstrating that glucocorticoids induce β-arrestin 1 and repress β-arrestin 2 expression in non-neuroendocrine cell lines, we investigated whether the same modulation of β-arrestins occurs in corticotroph cells and whether this process is reversible after glucocorticoid withdrawal in vitro (comparable to restoration of normal glucocorticoid levels, in vivo). We observed that β-arrestin 1 mRNA levels are significantly higher (about ten-fold) in ACTH-secreting adenomas, compared to the GH-secreting adenomas. Moreover, we confirmed in both AtT20 cells and human corticotroph adenoma cells the role of glucocorticoids in the modulation of β-arrestins. Noteworthy, in AtT20 cells we observed that dexamethasone-mediated β-arrestin modulation was reversible upon glucocorticoid withdrawal.

In the light of the results observed in this study and the important role that β-arrestins play in the regulation of GPCR functions, we speculate that the glucocorticoid-mediated changes in β-arrestin expression, that likely occur during the natural history (and/or the clinical management) of CD, might play a role in mediating tumoral responsiveness to SSAs and/or dopamine agonists.

However, in order to confirm the above-mentioned hypotheses, functional studies using β-arrestin-knockdown/overexpression experiments are required.

Finally, a general discussion of the results described in the aforementioned chapters of the Thesis is presented in Chapter 8. Future developments of the reported new findings, together with new challenges for future studies, are discussed, as well.
Samenvatting

Hypofyse adenomen, afkomstig van cellen in de hypofyse voorkwab, vormen een heterogene groep van tumoren en komen in de algemene populatie voor met een prevalentie van ongeveer 1:1000-1:1400. Hoewel niet de werkelijke prevalentie van klinisch relevante hypofyse adenomen refooterend, rapporteren radiologische studies en studies met autopsie materiaal een geschatte prevalentie van hypofyse adenomen in de algemene populatie van ongeveer 17%. Ondanks het feit dat hypofyse adenomen in het algemeen goedaardig zijn kunnen zij de gezondheidstoestand van de patiënt ernstig schaden, ofwel door de bijkomende hormonale overproductie (afhankelijk van het adenoom fenotype), ofwel door druk op kritische aangrenzende structuren, zoals de normale hypofyse, de oogzenuw en/of bloedvaten.

Somatotatine receptoren (SSTR) komen verspreid tot (over-)expressie in de verschillende typen hypofyse adenomen. Somatostatine receptor type 2 (sst2) en type 5 (sst5) komen het meest frequent tot expressie, gevolgd door sst1 en sst3. Dit specifieke SSTR expressie patroon vormt de pathofysiologische basis voor het gebruik van “klassieke” somatostatine analogen (SSAs), die met een hoge affiniteit binden aan sst2 (en in mindere mate aan sst5), bij de medicamenteuze behandeling van patiënten met hypofyse adenomen. Dit betreft met name patiënten met GH- of TSH-producerende adenomen. De individuele respons van patiënten met hypofyse adenomen op behandeling met SSAs is echter zeer variabel, zelfs binnen een groep van patiënten met hetzelfde type hypofyse adenoom. Om deze reden hebben recente studies zich gericht op het zoeken naar verschillende kenmerken die voorspellend zijn voor een goede respons op behandeling met SSAs, zoals karakteristieken van de patiënt, beeldvormende eigenschappen van het adenoom, en histopathologische- en moleculaire kenmerken van het adenoom.

Het doel van de studies beschreven in dit proefschrift is: 1) om een aantal reeds bekende voorspellende moleculaire kenmerken en –mechanismen voor resistentie voor behandeling met SSAs nader te onderzoeken, 2) om onze kennis te vergroten omtrent de pathofysiologische basis voor het gebruik van de expressie van SSTRs in hypofyse adenomen als doelwit voor medicamenteuze therapie en, 3) om mogelijk nieuwe moleculaire factoren te identificeren die betrokken zijn bij de respons van patiënten met hypofyse adenomen op behandeling met SSAs.

Zoals hierboven vermeld binden de “klassieke” SSAs met name aan sst2. Het GH-verlagende effect van deze medicamenten is in acromegale patiënten positief gecorreleerd met zowel tumor sst2 mRNA en -eiwit aantallen. Tot nu toe is het sst2 eiwit in hypofyse adenomen met name onderzocht door middel van immunohistochemie (IHC), gebruik makend van verschillende polyclonale antilichamen. Meer recent is echter een IHC protocol beschreven dat gebruik maakt van een konijn monoclonaal antilichaam (UMB-1 cloon) en dat even goed is om sst2 receptoren in tumor weefsel te kwantificeren als receptor autoradiografie, de “gouden standaard”. In hoofdstuk 2 hebben wij de sst2 eiwit expressie door middel van IHC met het UMB-1 antilichaam onderzocht in een grote serie hypofyse adenomen van acromegale patiënten die niet eerder medicamenteus behandeld zijn met SSA. De sst2 immuno-reactiviteit is gekwantificeerd met een semi-kwantitatief score systeem (IRS) en er werd een sterke directe correlatie gevonden tussen de sst2 IRS en het normaliseren van het circulerend IGF-I na adjuvant behandeling met SSAs. Daarnaast kon met deze methode een positieve voorspellende waarde (86% sensitiviteit en 91% specificiteit) berekend worden voor het normaliseren van IGF-I na minimaal 3 maanden van adjuvant behandeling met SSA. Middels deze studie hebben wij de essentiële rol van sst2 bij de respons van acromegale patiënten op behandeling met SSAs bevestigd. Daarnaast
hun wij aangetoond dat het bepalen van de sst_2 IRS, door middel van IHC met het UMB-1 antilichaam, een bruikbare techniek kan zijn om in de klinische praktijk patiënten te identificeren die waarschijnlijk goed reageren op adjuvant therapie met “klassieke” SSAs.

Zoals eerder vermeld komen naast sst_5 ook andere SSTR subtypen in hypofyse adenomen voor. In hoofdstuk 3 hebben wij daarom de rol van een heterogene SSTR expressie op de celmembrana, als een mogelijk andere factor die van invloed is op de respons op behandeling met SSAs, onderzocht in patiënten met een TSH-producerend hypofyse adenoom. In deze studie tonen wij bij dit zeldzame type hypofyse adenoom aan dat sst_2 en sst_5, gemeten op mRNA en eiwit niveau, gezamenlijk tot expressie komen in deze adenomen. Alle patiënten in deze studie lieten een significante daling van het serum TSH zien na een acute octreotide test, echter maar twee patiënten vertoonden een significante biochemische respons na langdurige SSA-behandeling. Wij konden aantonen dat de patiënten met adenomen met een hoge sst_2/sst_5 verhouding de beste respons lieten zien op langdurige SSA behandeling. Deze resultaten suggereren dat in TSH-producerende adenomen, anders dan in GH-producerende adenomen, sst_2 samen met sst_5 betrokken is bij een langdurige effectiviteit van behandeling met SSAs. Het gevonden verschil tussen TSH- en GH-producerende hypofyse adenomen ondersteunt een belangrijk concept van “celtype specificiteit”, waarbij een verschillende receptor functionaliteit en interactie waargenomen wordt in verschillende tumor celtypen.

Vanwege de recente beschikbaarheid van het nieuwe SSA pasireotide (een panligand) in de klinische praktijk hebben wij in hoofdstuk 4 het secretie remmend effect van pasireotide in een grote serie primaire kweken van GH-producerende hypofyse adenomen vergeleken met dat van octreotide (het “klassieke” SSA), en de gegevens gecorreleerd met de SSTR expressie in de verschillende adenomen. Het doel van deze studie was om het voorkomen van specifieke GH-producerende adenoom subpopulaties aan te tonen, waarin het effect van een van de twee stoffen het sterkst is. Wij vonden dat het gemiddelde effect van beide stoffen in de hele groep van adenomen gelijk is. Echter in 18% van de adenomen had pasireotide een sterker GH remmend effect dan octreotide, terwijl octreotide een sterker effect had in 15% van de adenomen. Bovendien vonden wij dat adenomen met een lagere sst_2 expressie en een lagere sst_2/sst_5 verhouding de meest waarschijnlijke kandidaten zijn voor een betere respons op pasireotide. Ondanks het feit dat deze bevindingen nader moeten worden onderzocht en vertaald moeten worden in een meer klinische context, tonen zij wel aan dat het onderzoeken van het SSTR expressie profiel in adenomen in de nabije toekomst een bruikbaar “gereedschap” kan worden om het best werkende SSA te selecteren voor adjuvant behandeling van acromegale patiënten.

Zoals in dit proefschrift uitgebreid besproken heeft de beschikbaarheid van nieuwe SSA met verschillende receptor bindingsaffiniteiten en -farmacologische eigenschappen in vergelijking tot de “klassieke” analogen, de behoefte aan een beter begrip van de SSTR pathofysiologie in hypofyse adenomen vergroot. In deze context, en op basis van fraaie eerdere in vitro studies, zijn GPCR kinases (GRKs) en in het bijzonder β-arrestines (1 en 2) naar voren gebracht als cruciale eiwitten die betrokken zijn bij regulatie van SSTR desensitisatie en trafficking processen. In hoofdstuk 5 hebben wij daarom GRK2 en β-arrestine mRNA expressie onderzocht in verschillende typen hypofyse adenomen (GH-, PRL-producerende hypofyse adenomen en klinisch niet-functionele hypofyse adenomen). In vergelijking met klinisch niet-functionele hypofyse adenomen bleek β-arrestine 1 expressie significant lager in de GH- en PRL-producerende adenomen. Bovendien vonden wij dat binnen de groep van GH-producerende hypofyse adenomen een lagere β-arrestine
1 expressie (en hoger GRK2) correleert met een betere respons op behandeling met octreotide, gemeten aan verlaging van de GH secretie na acute behandeling in vitro en in vivo.

Deze bevindingen werden bevestigd en verder onderbouwd door de studie beschreven in hoofdstuk 6. In deze studie hebben wij de β-arrestine expressie (zowel β-arrestine 1 als β-arrestine 2), gemeten in een groot aantal GH-producerende adenomen, gecorreleerd met de respons van patiënten op langdurige behandeling met SSA (mediane follow-up 12 maanden). Wij toonden aan dat β-arrestine mRNA expressie in het adenoom (zowel β-arrestine 1 als β-arrestine 2) een significante invloed heeft op de effectiviteit van langdurige behandeling van acromegale patienten met SSAs. Bovendien vonden wij dat β-arrestine 1 en β-arrestine 2 mRNA expressie sterk en omgekeerd gecorreleerd zijn met sst2 eiwit expressie in het adenoom. Naar onze mening tonen de resultaten van de studies in de hoofdstukken 5 en 6 een belangrijke rol voor β-arrestines in de respons van GH-producerende hypofyse adenomen op behandeling met SSAs, omdat deze moleculen niet alleen de sst, functie (signaaltransductie en desensitisatie processen), maar ook de sst, expressie op de tumor celmembraan (internalisatie en recycling) reguleren. Natuurlijk moeten aanvullende studies deze nieuwe bevindingen bevestigen. Bovendien moet onderzocht worden of het bepalen van β-arrestine eiwit expressie (middels technieken die routinematig worden gebruikt voor een klinische diagnose), een bruikbare merker is die gevoeligheid voor behandeling met SSA kan voorspellen.

Corticotrope adenomen van patiënten met de ziekte van Cushing (CD) vormen een uitdaging voor medicamenteuze therapie omdat deze adenomen wel SSTR bevatten maar de resultaten van behandeling met “klassieke” SSA tot nu toe teleurstellend zijn. In hoofdstuk 7 hebben wij de expressie van β-arrestine mRNA in ACTH-producerende hypofyse adenomen en in de muizen ACTH-producerende hypofyse tumor cellijn AtT20 onderzocht. Op basis van een recente studie waarin is aangetoond dat glucocorticoïden zowel β-arrestine 1 als β-arrestine 2 expressie beïnvloeden in niet neuro-endocriene celllijnen, hebben wij tevens onderzocht of eenzelfde beïnvloeding van β-arrestines door glucocorticoïden ook optreedt in corticotrope tumorcellen, en of dit proces reversibel is na het stoppen van behandeling met glucocorticoïden in vitro (vergelijkbaar met een herstel van normale glucocorticoïd spiegels in vivo). Wij vonden dat de hoeveelheid β-arrestine 1 mRNA significant hoger (ongeveer 10-voudig) is in ACTH-producerende hypofyse adenomen, in vergelijking met GH-producerende adenomen. Bovendien konden wij de rol van glucocorticoïden in de regulatie van β-arrestine expressie bevestigen in AtT20 cellen en in menselijke corticotrope adenoom cellen. Daarnaast bleek het effect van dexamethason-gemedieerde beïnvloeding van β-arrestine expressie reversibel na ontrekkning van behandeling met glucocorticoiden. Op basis van deze resultaten en vanwege de belangrijke rol die β-arrestines spelen in de regulatie van GPCR functie, hypothetiseren wij dat glucocorticoïd-gemedieerde veranderingen in β-arrestine expressie die mogelijk optreden bij het ontstaan van CD (en/of bij de klinische behandeling) een rol kunnen zouden kunnen spelen bij de gevoeligheid van de tumor voor behandeling met SSA en/of dopamine agonisten. Functionele studies met β-arrestine knockdown/overexpressie zijn echter noodzakelijk om deze hypothese te bevestigen.

Tot slot geeft hoofdstuk 8 een algemene beschouwing van de resultaten beschreven in de voornoemde hoofdstukken van dit proefschrift. Tevens worden verdere ontwikkelingen van de gerapporteerde bevindingen, alsmede uitdagingen voor nieuwe studies in dit hoofdstuk beschreven.
LIST OF PUBLICATIONS


11. Ferone D, Gatto F, Minuto F.
Pre-surgical treatment with somatostatin analogues in patients with acromegaly: the case for.

Immunoreactivity score using an anti-sst2A receptor monoclonal antibody strongly predicts the biochemical response to adjuvant treatment with somatostatin analogs in acromegaly.

Preoperative normalization of cortisol levels in Cushing's disease after medical treatment: consequences for somatostatin and dopamine receptor subtype expression and in vitro response to somatostatin analogs and dopamine agonists.

ß-Arrestin 1 and 2 and G protein-coupled receptor kinase 2 expression in pituitary adenomas: role in the regulation of response to somatostatin analogue treatment in patients with acromegaly.

Characterization and sub-cellular localization of SS1R, SS2R, and SS5R in human late-stage prostate cancer cells: effect of mono- and bi-specific somatostatin analogs on cell growth.

Clinical outcome and evidence of high rate post-surgical anterior hypopituitarism in a cohort of TSH-secreting adenoma patients: Might somatostatin analogs have a role as first-line therapy?

Emerging Targets in Pituitary Adenomas: Role of the CXCL12/CXCR4-R7 System.

Medical therapies in pituitary adenomas: Current rationale for the use and future perspectives.

Impact of pre-treatment with somatostatin analogs on surgical management of acromegalic patients referred to a single center
Endocrine. 2015 May. In press.
*: authors equally contributed

ß-arrestin expression as an additional marker to predict responsiveness to long-term somatostatin analog treatment in acromegaly.
Submitted
# PHD PORTFOLIO

**Name PhD student**  
Federico Gatto  

**Erasmus MC department**  
Internal Medicine, Endocrinology  

**Research School**  
Molmed  

**Promotor**  
L.J. Hofland  

**Copromotors**  
R.A. Feelders and D. Ferone  

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<td>Introductory Course on Statistics &amp; Survival Analysis for Research master/PhD students &amp; MDs, Molmed, Rotterdam</td>
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<td>Photoshop and Illustrator CS6 Workshop for PhD-students and other researchers, Molmed, Rotterdam</td>
<td>2014</td>
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<tr>
<td><strong>1.3 Oral Presentations (National and International Congresses, Conferences, Meetings, Courses)</strong></td>
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<tr>
<td>34th National Congress, Italian Society of Endocrinology, Sorrento, Napoli, Italy</td>
<td>2009</td>
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<tr>
<td>35th National Congress, Italian Society of Endocrinology, Montesilvano, Pescara, Italy</td>
<td>2011</td>
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</tr>
<tr>
<td>Targeting the Pituitary: Expert Knowledge Forum, Berlin, Germany</td>
<td>2011</td>
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<tr>
<td>Science Days Internal Medicine, Erasmus MC, Antwerp, Belgium</td>
<td>2011</td>
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<tr>
<td>15th Congress of the European Neuroendocrine Association (ENEA), Vienna, Austria</td>
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<tr>
<td>Targeting the Pituitary: Expert Knowledge Forum, Munich, Germany</td>
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<tr>
<td>95th Endocrine Society Meeting, San Francisco, USA</td>
<td>2013</td>
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<tr>
<td>36th National Congress, Italian Society of Endocrinology, Padova, Italy</td>
<td>2013</td>
<td>1.0</td>
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<tr>
<td>16th Congress of the European Neuroendocrine Association (ENEA), Sofia, Bulgaria</td>
<td>2014</td>
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<td>1.4 Poster Presentations (National and International Congresses, Conferences, Meetings, Courses)</td>
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<td>6th European Neuroendocrine Tumor Society (ENETS) Congress, Granada, Spain</td>
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<td>5th International Congress of the GRS-IGF Society, New York, USA</td>
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<td>14th Congress of the European Neuroendocrine Association (ENEA), Liege, Belgium</td>
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<td>93th Endocrine Society Meeting, Boston, USA</td>
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<td>Molmed Day 2011, Rotterdam, The Netherlands</td>
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<tr>
<td>Aspiring to excellence: Pituitary Expert Forum, Stockholm, Sweden</td>
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<tr>
<td>Aspiring to excellence: Pituitary Expert Forum, Vienna, Austria</td>
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<tr>
<td>Science Days Internal Medicine, Erasmus MC, Antwerp, Belgium</td>
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<tr>
<th>1.5 Presentations as Invited Speaker (National and International Congresses, Conferences, Meetings, Courses)</th>
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<tr>
<td>Up to Date in Neuro-Onco-Endocrinology, Genova, Italy</td>
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<tr>
<td>6th Italian Meeting of Hypothalamus-Pituitary Diseases, Napoli, Italy</td>
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<td>11th National Congress of the Italian association of Nuclear Medicine and Molecular Imaging, Torino, Italy</td>
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<tr>
<td>36th National Congress, Italian Society of Endocrinology, Padova, Italy</td>
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<tr>
<td>16th Congress of the European Neuroendocrine Association (ENEA), Sofia, Bulgaria</td>
<td>2014</td>
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<tr>
<td>Workshop: Controversies in the Diagnosis and Therapy of Pituitary Adenomas, Milano, Italy</td>
<td>2015</td>
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<th>1.6 Attending Other Meetings</th>
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<tr>
<td>8th European Neuroendocrine Tumor Society (ENETS) Congress, Lisboa, Portugal</td>
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<td>97th Endocrine Society Meeting, San Diego, USA</td>
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<tr>
<th>2. Teaching Activities</th>
<th>2012-2013</th>
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<tbody>
<tr>
<td>2.1 Supervising medical student</td>
<td>2012-2013</td>
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<td>2.2. Lectures at (Inter) National Endocrinology Courses</td>
<td>2010</td>
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<tr>
<td>Advanced Course on Neuroendocrine Tumors, Genova, Italy</td>
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<td>Course “Hypothesis Pituitary”, Montegridolfo, Italy</td>
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<tr>
<td>European Society of Endocrinology (ESE) Basic Endocrinology Course in Neuroendocrinology, Amsterdam, The Netherlands</td>
<td>2014</td>
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ABOUT THE AUTHOR

Federico Gatto was born on May 24th 1982, in Genoa (Italy). In 2001 he completed secondary school studies (Classic Lyceum) at the Liceo Classico Giuseppe Mazzini in Genoa. Thereafter, he attended medical school at the Università degli Studi di Genova (Genoa, Italy), where he graduated in July 2007, under the supervision of dr. D. Ferone, discussing a project entitled “Modulation of somatostatin and dopamine receptors and effects of the new somatostatin/dopamine chimeric compounds on the proliferation of LNCaP prostate cancer cell line”. In February 2008 he obtained the national licence to practice as a Medical Doctor. In March 2008 he started his residency in Endocrinology and Metabolic Diseases at the Department of Internal Medicine and Medical Sciences of the Università degli Studi di Genova, where he received his Board Certification in Endocrinology (50/50 magna cum laude) and was appointed with the title of Specialist in Endocrinology and Metabolic Diseases in March 2013. From September 2010 to March 2012 he worked as a research fellow at the Department of Internal Medicine of the Erasmus Medical Center, Rotterdam, under the supervision of Prof.dr. L.J. Hofland, where he started the research work presented in this thesis. After the end of his residency in Genoa (2013), dr. Gatto completed his research project at the Erasmus Medical Center, Rotterdam, again under the supervision of Prof.dr. L.J. Hofland. He is currently working as Clinical and Research post-graduate fellow at the Unit of Endocrinology, Department of Internal Medicine, of the Università degli Studi di Genova (Genoa, Italy). Since 2012 he is elected member of the ENEA (European Neuroendocrine Association) Young Research Committee (EYRC). He is member of the Editorial Board (Review Editor) of Frontiers in Cancer Endocrinology and Frontiers in Signalling and Translational Endocrinology journals, and he is reviewer for a number of internationally well-recognized peer-reviewed journals in the field of Endocrinology.
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Grazie a tutti gli “Italiani” di Rotterdam, che sono stati in questi anni una seconda famiglia.

Dimenticherò sicuramente di nominare qualcuno, e so già che me ne ricorderò non appena la tesi sarà andata in stampa.

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Con gli abbracci, le parole e l’esempio mi avete trasmesso quei valori e quei sentimenti che rendono ricco un uomo. Le lacrime che intravvedevo nei vostri occhi al momento dei saluti all’imbarco, dopo gli interminabili e numerosi viaggi verso l’aeroporto Malpensa, sono uno dei ricordi più dolci che porto con me.

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Ma in fondo, lo sai, lo diciamo sempre, tutti e due sappiamo qual è la risposta a tutto questo. La risposta è…