

**UNIVERSITÀ
DEGLI STUDI
DI PADOVA**

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Medicina DIMED

CORSO DI DOTTORATO DI RICERCA IN

SCIENZE CLINICHE E SPERIMENTALI

**CURRICOLO METODOLOGIA CLINICA E MEDICINA DELL'ESERCIZIO,
SCIENZE ENDOCRINOLOGICHE, DIABETOLOGICHE E NEFROLOGICHE**

CICLO XXXI

Tesi redatta con il contributo finanziario di Novartis Farma S.p.A.

***Personalized medical treatment
for pituitary adenoma***

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This thesis is based on the following articles, which are referred in the text by their Roman Numerals, First author, Journal and Year of publication (Arabic numerals characterize literature references, reported at the end of the text).

- I. Regazzo D, Gardiman MP, Theodoropoulou M, Scaroni C, Occhi G, **Ceccato F**. *Silent gonadotroph pituitary neuroendocrine tumor in a patient with tuberous sclerosis complex: evaluation of a possible molecular link*. *Endocrinol Diabetes Metab Case Rep*. 2018 Aug 23;2018. pii: 18-0086. doi: 10.1530/EDM-18-0086. eCollection 2018. PubMed PMID: 30159145
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A Prospective DXA Study. Horm Metab Res. 2017 Sep;49(9):660-666. doi: 10.1055/s-0043-115008. Epub 2017 Jul 17. PubMed PMID: 28718178.

- VI. Regazzo D, Losa M, Albiger NM, Terreni MR, Vazza G, **Ceccato F**, Emanuelli E, Denaro L, Scaroni C, Occhi G. *The GIP/GIPR axis is functionally linked to GH-secretion increase in a significant proportion of gsp(-) somatotropinomas*. Eur J Endocrinol. 2017 May;176(5):543-553. doi: 10.1530/EJE-16-0831. Epub 2017 Feb 8. PubMed PMID: 28179449.
- VII. Cannavò S, Ragonese M, Puglisi S, Romeo PD, Torre ML, Alibrandi A, Scaroni C, Occhi G, **Ceccato F**, Regazzo D, De Menis E, Sartorato P, Arnaldi G, Trementino L, Trimarchi F, Ferrà F. *Acromegaly Is More Severe in Patients With AHR or AIP Gene Variants Living in Highly Polluted Areas*. J Clin Endocrinol Metab. 2016 Apr;101(4):1872-9. doi: 10.1210/jc.2015-4191.
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- IX. Losa M, Bogazzi F, Cannavo S, **Ceccato F**, Curtò L, De Marinis L, Iacovazzo D, Lombardi G, Mantovani G, Mazza E, Minniti G, Nizzoli M, Reni M, Scaroni C. *Temozolomide therapy in patients with aggressive pituitary adenomas or carcinomas*. J Neurooncol. 2016 Feb;126(3):519-25. doi: 10.1007/s11060-015-1991-y. Epub 2015 Nov 27. PubMed PMID: 26614517.

Abstract

Introduction and Aim: Pituitary adenomas are common neoplasms, with a reported prevalence of about one case in 1000 subjects. Patients with pituitary adenomas show significant morbidity due to pituitary hormone hypersecretion or deficiencies, mass effects and infiltration of the surrounding tissues. Although transphenoidal surgery and radiotherapy are largely used to treat patients with pituitary adenomas, the overall long-term remission rate is not complete, beside side effects of surgery or brain irradiation. Therefore, medical treatments with pituitary-directed drugs are increasingly used in patients with secreting pituitary adenomas, especially when surgery fails or is not indicated, or awaiting for effects of radiotherapy. Somatostatin analogues (SSA) have been the mainstay of the medical treatment of GH-secreting adenomas, and nowadays are also used to treat ACTH-secreting pituitary adenomas, since these tumours express several types of somatostatin receptors (SSTR), with the prevalence of SSTR type 2 in the GH-secreting PA and of SSTR type 5 in the ACTH-secreting. Regrettably, 50% of patients with GH- secreting and 60% with ACTH- secreting pituitary adenomas do not respond to medical treatment with pituitary-directed drugs, or present only a partial hormonal reduction. Receptor desensitization, internalization and intra-cellular trafficking of SSTR could explain at least partially the lack of response, hence more data and knowledge about these cellular processes are urgently needed. Moreover, pituitary adenomas are not always benignant: some aggressive cases (up to 15-20% in all series) are characterized by rapid regrowth after first surgery, invasion of the surrounding structure, resistance to medical therapy, therefore the term Pituitary Neuroendocrine Tumor (PitNET) should be actually used.

The aims of this PhD project are to describe the role of medical treatment in patients with PitNET, in order to study the efficacy of available compounds; applicate the combination of medical treatment in clinical practice; analyse the differential effects (if existing) of medical treatment compared to surgery (considered the best curative treatment).

Materials and methods: Among our cohort of patients (120 with GH-, 134 with ACTH-, 171 with PRL-, 6 with TSH- secreting PitNET, 150 with non-secreting PitNET), we retrospectively and prospectively analysed clinical, radiological and pathological features of patient. Considering the treatment of aggressive PitNET or patients with Cushing's Syndrome, we focused our attention to everolimus, temozolomide (TMZ) and

metirapone (MET) treatment. In some case, primary cell culture were used to study the effect of medical treatment.

Results: Regarding medical treatment, we considered the use of everolimus, TMZ, cabergoline and MET.

1. In a patient with tuberous sclerosis complex (TSC) and silent gonadotroph PitNET we tested the efficacy of everolimus, observing a reduction of cell viability after an in vitro treatment of PitNET's derived primary cells. TSC analysis retrieved no disease-associated variants with the exception of the heterozygous intronic variant c.4006-71C>T found in TSC2: the computational tools predicted a gain of a new splice site with consequent intron retention, not confirmed by an in-vitro analysis of patient's lymphocyte derived RNA.
2. Regarding TMZ in aggressive PitNET, we conducted an Italian survey on 31 patients: 11 patients (35.5%) had reduction of the tumor during TMZ treatment, while 6 patients (19.4%) had progression of disease. Median follow-up after start of TMZ was 18 months. Seven patients presented disease progression. The 2-yr recurrence-free survival was 62% (95% C.I., 34 -99%). Seven patients died of progressive disease. The 2-yr and 4-yr survival rates were 90% (95% C.I., 77-100%) and 56% (95% C.I., 26-85%). Moreover, we treated a patient with a combined cabergoline+TMZ treatment, achieving excellent results.
3. Considering MET in patients with Cushing's Syndrome, patients were treated with a median dose of 1000 mg for 9 months. UFC and LNSC decreased quickly after the first month of treatment (-67% and -57% from baseline), with sustained UFC normalization up to 12 and 24 months (in 13 and 6 patients, respectively). UFC and LNSC normalized later (after 3-6 months) in patients with severe hypercortisolism (>5-fold baseline UFC). Regarding last visit, 70% and 37% of patients normalized UFC and LNSC, respectively. Body weight reduction (-4kg) was observed after UFC normalization. Severe side-effects were not reported, half female patients complained hirsutism, and blood pressure was not increased.
4. In patients with acromegaly, a significant proportion of patients developed Central Adrenal Insufficiency (CA) over time: while primary or secondary medical treatment did not contribute to the risk of CAI, repeated surgery or radiotherapy affected pituitary-adrenal axis. CAI was diagnosed in 18% of patients (10/57) after surgery, and in 53% (9/17) after radiotherapy (p=0.01).

Considering those aspects related to predict the effects of medical treatment with SSA in acromegaly, we studied the role of AIP-AHR and GIPR pathway. Considering AIP-AHR axis, involved in the detoxification

of endocrine disruptors and chemical pollutants, we observed that acromegaly is more biochemically severe and resistant to SSA treatment in patients living in highly polluted areas, especially if they also carry specific AHR and/or AIP gene variants. Moreover, we found a stimulatory effect of IGF-1 on GIP promoter support in GIPR-expressing somatotropinomas, suggesting a novel molecular pathway able to induce GH-secreting PitNET.

Conclusions: In this complex scenario, understanding the physio-pathology of PitNET is the beginning of personalized treatment. In clinical practice, a multidisciplinary team for the management of patients is fundamental, to suggest the correct treatment plan, tailored to the patient.

Riassunto

Introduzione e scopo: Gli adenomi ipofisari sono neoplasie frequenti, con una prevalenza di un caso ogni 1000 soggetti. I pazienti con adenoma ipofisario possono presentare segni e sintomi in correlazione alla secrezione autonoma (o deficitaria) di ormoni ipofisari, oppure possono presentarsi come “effetto massa” dovuto alla lesione occupante spazio in loggia ipofisaria. Sebbene la chirurgia e la radioterapia siano state molto utilizzate in passato, il controllo a lungo termine non è completo, sia in termini di secrezione che di lesione adenomatosa, esponendo comunque il paziente agli effetti collaterali dell'intervento o dell'irradiazione. Pertanto, la terapia medica è sempre più utilizzata, non solo nelle recidive post-chirurgiche, ma anche quando ulteriori interventi sono inefficaci, o in attesa degli effetti della radioterapia. Gli analoghi della somatostatina (SSA) sono stati per anni la principale terapia degli adenoma GH-secernenti, e al giorno d'oggi vengono utilizzati anche in quelli ACTH-secernenti, dato il loro effetto differenziale sui recettori della somatostatina (SSTR), soprattutto il tipo 2 nei GH-secernenti e il tipo 5 negli ACTH-secernenti. Purtroppo, fino al 50% dei pazienti non risponde in maniera soddisfacente alle terapie mediche, pertanto una maggior conoscenza della biologia cellulare ipofisaria è necessaria, per capire quale sia la strategia migliore per il paziente. Inoltre, in alcuni casi gli adenomi non sono sempre benigni (circa il 15-20% delle principali serie descritte in letteratura), caratterizzandosi per la resistenza alle terapie convenzionali, l'invasione dei tessuti locali o la rapida crescita. In tali casi, il termine Tumore Neuroendocrino Ipofisario (PitNET) viene recentemente proposto in letteratura. Lo scopo di questa tesi di dottorato è di studiare gli effetti delle terapie mediche in pazienti con PitNET; per sviluppare nuove strategie terapeutiche, per capire l'efficacia dei farmaci disponibili e per testare la loro combinazione.

Materiali e metodi: I pazienti che sono seguiti presso l'ambulatorio ipofisi dell'Unità Operativa di Endocrinologia dell'Azienda Ospedaliero-Universitaria di Padova (120 con PitNET GH-secernenti, 134 ACTH-secernenti, 171 PRL-secernenti, 6 TSH-secernenti e 150 PitNET non funzionanti) sono stati seguiti in uno studio retrospettivo e prospettico. I dati clinici, biumorali, di terapia, radiologici e patologici sono stati raccolti e analizzati. Tra le varie terapie mediche, maggior risalto è stato dato all'everolimus e alla temozolomide (TMZ) nei PitNET aggressivi e al metirapone (MET) in pazienti con Sindrome di Cushing. In casi selezionati sono state allestite linee cellulari derivanti dall'adenoma dei pazienti (primarie).

Risultati: in termini di terapia medica abbiamo analizzato

1. In un paziente con sclerosi tuberosa e PitNET silente abbiamo testato l'efficacia dell'everolimus in colture primarie, osservando una generale riduzione della vitalità cellulare. Abbiamo poi riscontrato una nuova variante del gene TSC2, gli studi in silico predicono la ritenzione di un introne con perdita di un sito di splicing, che andrà confermato in ulteriori studi funzionali.
2. Considerando la terapia con TMZ in PitNET aggressivi abbiamo raccolto i dati di 31 pazienti provenienti da uno studio multicentrico italiano. 11 casi hanno presentato una riduzione del PitNET, con una mediana di terapia di 18 mesi. Il 90% e il 60% dei pazienti erano liberi da malattia a 2 e 4 anni dalla terapia con TMZ. Abbiamo poi trattato un paziente con TMZ e cabergolina, ottenendo ottimi risultati.
3. 31 pazienti con Sindrome di Cushing sono stati trattati per 9 mesi con 1000 mg di MET. I parametri ormonali (cortisoluria e cortisolo salivare notturno) si sono ridotti rapidamente già dopo un solo mese di terapia, normalizzando la secrezione di cortisolo fino a 12 e 24 mesi. I pazienti con ipercorticismo severo (>5 volte i valori normali al baseline) hanno raggiunto il controllo biochimico di malattia più lentamente, tuttavia il 70% dei pazienti normalizzava la cortisoluria all'ultima visita, con una riduzione media di peso di 4kg. In generale il MET era ben tollerato, senza importanti effetti collaterali.
4. Nei pazienti con acromegalia, lo sviluppo di insufficienza surrenalica centrale (CAI) non è trascurabile nel follow-up. Mentre la terapia medica non aumenta il rischio di CAI, il 18% dei pazienti (10/57) sviluppa iposurrenalismo dopo la chirurgia, mentre il 53% (9/17) lo sviluppa dopo la radioterapia.

Analizzando in vitro gli aspetti che potrebbero predire la efficacia della terapia con SSA nei pazienti con acromegalia, abbiamo studiato i pathway molecolari di AIP-AHR e del GIPR. L'asse AIP-AHR, coinvolto nella detossificazione di varie molecole interferenti endocrine e inquinanti chimici, si trova maggiormente mutato in pazienti acromegalici con malattia più severa e con minor risposta agli SSA, soprattutto se vivono in zone molto inquinate. Abbiamo inoltre scoperto un ruolo promuovente del recettore dell'IGF-1 nel recettore del GIP, coinvolto nella tumorigenesi ipofisaria e quindi nuovo aspetto da studiare nei PitNET GH-secerenti.

Conclusioni: Comprendere a fondo la fisiopatologia dei PitNET è l'inizio della personalizzazione della terapia medica, sempre più usata oggi. Nella pratica clinica quotidiana, pertanto, un team multidisciplinare è fondamentale per proporre al paziente il corretto piano terapeutico, personalizzato secondo le proprie caratteristiche biologiche.

Acknowledgements

This study was carried out during the years 2015–2018 at the Endocrinology Unit, Department of Medicine DIMED, University-Hospital of Padova. I wish to express my sincere gratitude to all those who contributed or participated in this project:

- Professor Carla Scaroni, my supervisor, for opening my eyes to the fascinating world of Endocrinology, especially in patients with pituitary and adrenal diseases. Without her input when I was attending the third year of the graduation in medicine I would never have the opportunity to start my career as a physician and a researcher. Through all these years her guidance has been most valuable, first in the degree in Medicine, then in the residency in Endocrinology and now in my PhD project. I am a lucky researcher because a farsighted person saw something brilliant in me, as the term “supervisor” indicate.
- Doctor Gianluca Occhi and Daniela Regazzo for giving me the opportunity to work in the Endocrine Laboratory, especially to collect clinical data and to try to connect them with molecular findings. Their support and encouragement to complete this project are highly appreciated.
- Professor Marco Boscaro for his valuable contribution to my growth in clinical research and in the complex area of human relationship management. His profound knowledge on endocrinology and his capacity to create a research group will help me to walk with my shoes, at least from tomorrow.
- All the researchers, residents and students that collaborate with me in the Pituitary and Adrenal office: Nora, Mattia, Marialuisa, Andrea, Laura, Silvia, Elisa, Chiara and all the group of the Endocrinology Unit.
- All the co-authors and collaborators who contribute to the present and previous works.
- I am also very grateful to all nurses for their her prompt assistance with various matters during this work.
- All the patients and their families who took part in the study. Without the effort of the participants this study would never have taken place.
- My family and friends, especially my wife Elena. I am very fortunate to have two wonderful princess as children, Marta and Laura. Their love has given me strength to work with this project and to get it done.
- Finally, I am grateful to the un-conditioned financial support of Novartis Pharma, without their effort this project would not have come to light.

1. Background

1.1 Pituitary Adenoma and Pituitary Neuroendocrine Tumor (PitNET)

Molecular basis of pituitary adenomas

Pituitary adenomas are increasingly recognised: their clinical prevalence is about 1/1000 inhabitants. Although considered benign, they induce significant morbidity due to pituitary hormone hypersecretion (prolactinomas, acromegaly, Cushing's disease), mass effects (i.e. hypopituitarism, visual loss and other neurological symptoms) or infiltration of surrounding tissues¹⁻⁵.

Trans-nasal-sphenoidal surgery (TNS) is the first line treatment for the major part of patients with pituitary adenomas, however the overall long-term remission rate after TNS is reported about 60-70%, because surgical failures or recurrences are far from uncommon, occurring in up to 30-40% of patients, and increase with time⁶⁻⁸. Radiotherapy is a therapeutic option, but it has not always proved effective, and any benefit can take from 2 to 10 years to become apparent, beside side effects of brain irradiation^{9,10}. Moreover, some pituitary adenomas are characterized by a clinical "aggressive" pattern, in particular with an invasive local growth and/or resistance to conventional therapies (considering surgery, radiotherapy or medical treatment)¹¹.

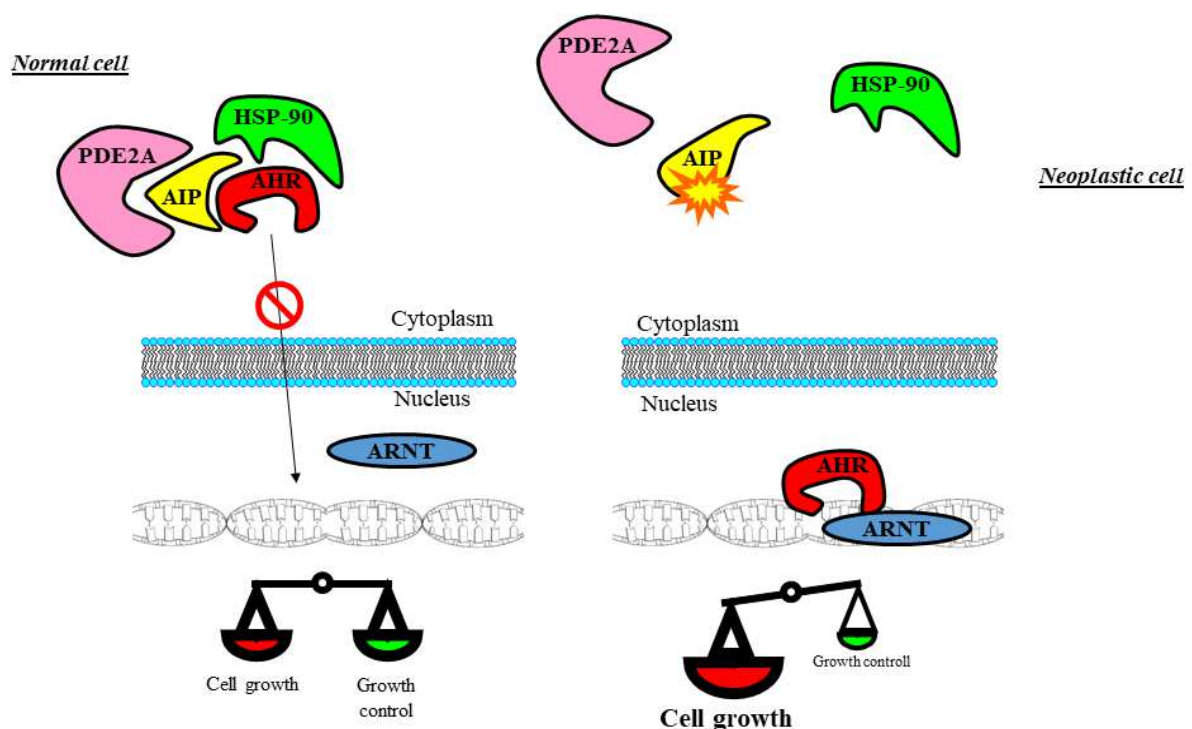
Medical treatments with pituitary-directed drugs are increasingly used in patients with secreting pituitary adenomas, especially when surgery fails or is not indicated, or while awaiting for effects of radiotherapy^{7,12}. Whether more or less aggressive, pituitary adenomas pose a serious therapeutic challenge for endocrinologists and neurosurgeons, and their treatment depends on functional phenotype and proliferation characteristics: current drugs act through endogenous receptors modulating the physiological pathways involved in the control of hormone secretion and growth.

The characterization of cellular signalling mechanisms in patients with familial tumours (less than 5% of pituitary adenoma) represent an invaluable tool for a rational approach to sporadic neoplasms, which are more frequently diagnosed. Most inherited pituitary adenomas are due to germline mutations inactivating menin (in the MEN1 syndrome) or aryl hydrocarbon receptor (AHR)-interacting protein (AIP); these inherited pituitary adenomas are frequently more aggressive than their sporadic counterpart, and are diagnosed in 40% of cases in families with Familial Isolated Pituitary Adenoma (FIPA)¹³⁻¹⁵. Some patients with typical features of MEN-

1 syndrome (parathyroid, pancreas and/or pituitary tumors) did not harbour mutations in *menin*, and are termed as MEN1-like. In about 10–20% of patients with MEN1-like features, a novel germline mutation in the *CDKN1B* gene, encoding for the cyclin-dependent kinase inhibitor p^{27KIP1}, was associated with the development of pituitary adenomas¹⁶. Recently, X-linked acrogigantism (X-LAG) has been described as a new syndrome of pituitary gigantism, caused by microduplications on chromosome Xq26.3, encompassing the gene *GPR101*, which is highly upregulated in pituitary tumours¹⁷.

AIP-AHR is a crucial pathway in the somatotroph cell¹⁸, as reported in figure 1. AIP down-regulation at somatic level may occur during the progression of some GH-secreting pituitary adenomas, leading to acromegaly¹⁹. Other mutations at somatic levels have been recognized, in the past, that activate *GNAS1* (Gsp- α , subunit of the heterotrimeric stimulatory G protein that gives to somatotroph cells a growth advantage)²⁰. Recently, somatic mutations in *USP8* (a deubiquitinase gene, involved in the reversible post-translational protein modification regulating the fate and function of various proteins in eukaryotic cells) have been also recognized in ACTH-secreting pituitary adenomas (characterizing Cushing’s Disease, CD)^{21,22}.

Figure 1: AIP-AHR pathway. In case of AIP mutation, Phosphodiesterase 2A (PDE2A) and heat shock protein 90 (HSP 90) are released in the cytoplasm, enabling AHR and AHR Nuclear Translocator (ARNT) to promote cell growth.



Inactivating mutations of the AIP gene, encoding for a co-chaperone cytoplasmic protein involved in the regulation of AHR activity and in many other intracellular interactions, have been found in 40% of patients with isolated familial somatotropinomas^{14,15,23}. Moreover, mutations in AIP gene are involved in the resistance to Somatostatin Analogs (SSA) in patients with acromegaly^{24,25}. Recently, a novel close relationship with environmental factors (endocrine disruptors and pollution) has been proposed. Increased prevalence of acromegaly was demonstrated in a district of the province of Messina (Sicily, Italy), identified as high-risk for health (HR) area by the Italian government on the basis of high atmospheric concentrations of pollutants and endocrine disruptors, as non-methane hydrocarbons and volatile organic compounds. The prevalence of acromegaly in this polluted area reached 210-300 cases per million inhabitants; the relative risk of developing acromegaly was 8-10 fold higher than a reference area with low industrial density (and therefore a reduced pollution and air contamination of endocrine disruptors)²⁶. Preliminary in vitro studies showed that long-term incubation with some endocrine disruptors, as phenol and bis-(2-ethylhexyl)-phthalate, increases energy content and proliferation of normal pituitary cells, in rats as well as in humans²⁷. In addition, the long-term benzene exposition increased GH synthesis in GH3 cells (a murine model of acromegaly), and this effect was associated with decreased AIP and increased AHR expression, with a potential impairment of the sensitivity to SSA²⁸. The AHR pathway has a key role in cellular detoxification mechanisms and several studies suggest that it is implicated in tumorigenesis, not only in the pituitary²⁹.

Another interesting feature that could be used to characterize GH-secreting pituitary adenoma, and that could be used to personalize the therapeutic approach, is to study the response of GH to a Oral Glucose Tolerance Test (OGTT). In patients with active disease, a standard 75-gr OGTT fails to suppress serum GH levels <1 µg/L, however in approximately 30% of cases GH levels increase paradoxically³⁰. With the aim of clarifying the pathogenesis of pituitary adenoma in acromegaly, we recently focused our attention to the glucose-dependent insulinotropic polypeptide (GIP) receptor (GIPR), a member of the G-protein-coupled receptors superfamily (GPCR)³¹. Once activated by GIP, which is secreted³¹ by the K cells of the duodenum in response to mixed meals, GIPR transduces extracellular stimuli into intracellular responses by activating the cAMP pathway^{32,33}. In normal basal conditions, the glucose-dependent secretion of insulin in pancreatic β cells after GIP stimulation exemplifies this mechanism³⁴. When inappropriately expressed in the adrenal gland, the GIPR may instead result in the development of adrenal tumors, disrupting cAMP homeostasis by altering the cascade

normally triggered by ACTH³⁵, leading to the so-called food-dependent Cushing's syndrome³⁶. In 2011 we reported that nearly 30% of GH-secreting PitNET expressed GIPR at significantly higher levels than normal pituitary glands³⁷. Correlation of molecular findings with clinical data revealed that, in most cases, GIPR overexpression was associated with a paradoxical increase in GH after OGTT^{37,38}. Similar to what is observed in food-dependent adrenal CS, in GIPR-overexpressing GH-secreting PitNET, GH is inducible by meal³⁹. These acromegalic patients show abnormally high fasting and postprandial plasma GIP levels⁴⁰; this may be the consequence of a direct effect of GH, IGF-1 or both on GIP secretion. Based on these premises, the link between the GIP/GIPR axis and GH induction in GH-secreting PitNET primary cultures has never been studied.

Aggressive pituitary adenomas

Besides molecular characterization, pituitary adenomas are usually considered benign tumors¹, although some of them can exhibit an aggressive behavior. In such cases, their treatment plan is a challenge, and repeated surgery or additional medical treatment or radiotherapy is needed⁴¹. The crucial issue is to early identify this patients⁴², which may coincide only partially with the atypical adenomas described in the 2004 WHO histological classification (characterized by MIB-1 >3%, p53 immunoreactivity, high mitotic index^{11,43}), accounting only for 3-15% of cases in surgical reported series^{44,45}. In clinical practice, a close relationship among atypical adenomas and aggressive behavior is not always evident, since typical adenomas can show an aggressive behavior as well^{43,46}. Beside "atypical adenoma" definition, some patients with clinically aggressive pituitary adenomas are diagnosed among those with large or invasive masses, or considering those adenoma resistant to conventional treatments (neurosurgery, drug or radiotherapy), therefore presenting with earlier and more frequent recurrences, or even those defined as silent adenoma^{1,44-46}. In Table 1 are summarized the most relevant features that characterize aggressive pituitary adenomas.

In clinical practice, "Aggressive" definition is often used by way of "invasive" or "atypical", though they are not synonymous. Invasiveness is defined by radiological or surgical findings, and, on the other hand, aggressiveness is defined by clinical behavior, while atypical adenoma are defined on the basis of pathological report^{11,43,47}.

Table 1: definition of aggressive PitNET routinely used in clinical practice and during the multidisciplinary meeting. CSI: cavernous sinus invasion (Publication IV. Ceccato F, Acta Neurochir, 2017)

<i>feature</i>	<i>description</i>	<i>references</i>
tumor growth	tumor growth (>20% in one diameter) in pituitary MRI in the last year despite therapy (if any)	
invasive adenoma	MRI, computed tomography or surgical evidence of at least one: - CSI: encasement of internal carotid artery $\geq 45\%$ of the vessel circumference or when there was an involvement of at least three out of four cavernous sinus compartments (medial, superior, inferior, lateral) by the adenoma - sellar floor involvement - third ventricle involvement	48,49
giant adenoma	> 4 cm in adults	41,50,51
atypical adenoma	histological report of MIB-1 >3%, positive p53 immunoreactivity, high mitotic index	11
resistance to pituitary-directed pharmacological treatment	Uncontrolled hormone secretion despite two consecutive years of conventional pituitary-directed pharmacological treatment - PRL-secreting adenoma: cabergoline >2 mg/week - GH-secreting adenoma: >120 mg/month lanreotide autogel or > 30 mg/month octreotide LAR	3,8
silent adenoma	non-functioning PitNET with positive immunohistochemistry for ACTH, GH, PRL, TSH, LH or FSH.	44,45

The fourth edition of the World Health Organization classification of endocrine tumors has been published in late 2016. There are 3 main changes to the previous (2004) classification:

- the term "atypical adenoma" was completely eliminated due to the lack of definitive evidence in terms of a poor prognosis;
- the introduction of more precise cell lineage-based classification of pituitary adenoma that is defined based on lineage-specific transcription factors and hormones produced;
- the new term of pituitary neuroendocrine tumors (PitNET) with the elimination of the term "atypical adenoma"^{52,53}.

This recent classification is closer to the aggressiveness of the pituitary adenoma, and introduce the concept that a single clinical disease (i.e. acromegaly) could represent the final clinical manifestation of more than one different neuro-endocrine tumor (a GH-secreting PitNET). One year later, some authors proposed that a new classification of PitNETs into five grades based on invasion on MRI and immunocytochemical profile (as reported in table 2) is of prognostic value to predict postoperative tumor behavior and identifies those patients who have a high risk of early recurrence or progression⁴⁷.

Table 2: 5 grades of PitNET⁴⁷

Grade	Description
1a	Non-invasive
1b	Non-invasive but proliferative
2a	Invasive
2b	Invasive and proliferative
3	Metastatic

To conclude, an aggressive PitNET is a tumor with a low likelihood of being cured, especially with surgery alone. In such scenario, medical treatment may play a crucial role, to reduce or at least to stabilize the progression of PitNET. The development of new drugs relies on a better knowledge of the heterogeneous molecular abnormalities driving pituitary tumorigenesis, possibly focusing on intracellular pathways to be targeted with a low toxicity. Moreover, it is fundamental to discover new clinical or molecular findings to predict the aggressive behavior of PA, in order to identify those patients that require a close follow-up. Rather than new molecules, which development requires several years “from bench to bed”, there are increasing evidence that combination of available drug could be an effective strategy.

1.2 Medical Treatment in PitNET

Medical treatment in Cushing's Syndrome (CS)

For ACTH-secreting PitNET, called Cushing's Disease (CD), the treatment goals (with surgery, radiotherapy or medical therapy) are to normalize cortisol levels, reverse the clinical symptoms, and remove the secreting neoplasm⁶. Adrenal steroidogenesis inhibitors have been the mainstay of medical treatment for CD: the most used are ketoconazole and metyrapone. The available glucocorticoid receptor antagonist is mifepristone, is approved in the USA to control hyperglycemia secondary to hypercortisolism. There has recently been a lot of interest in using agents directly targeting the pituitary corticotroph cells to reduce ACTH secretion and, if possible, control the volume of pituitary adenomas. This is because corticotroph cells contain dopamine receptors (targets of bromocriptine and cabergoline), retinoic acid receptors, peroxisome proliferator-activated receptor gamma, or SSR, the target of the first drug developed and approved for Cushing's Disease (pasireotide)^{54,55}.

Available medical treatments for Cushing's Syndrome (CS) are:

- Ketoconazole (KET), used off-label to reduce hypercortisolism because of its effect to reduce the activity of cytochrome P450 adrenal steroidogenic enzymes, including 11 β -, 17 α - and 18-hydroxylase^{56,57}. Lowering cortisol in CD patients may trigger an ACTH response from the pituitary adenoma, with the risk of escape from treatment efficacy and a consequent secondary failure of medication. KET can be used as a temporary adjunct before a more definitive treatment, or after unsuccessful neurosurgery. One of the problems with ketoconazole is the paucity of published data on its use. Most studies concerned small samples, and no prospective clinical trials have been conducted so far^{56,57}. A reduction of cortisol levels was usually achieved with 600-800 mg/day, obtaining overall response rates of 53-88% in CS, and 45% in CD. In 2014 a multicenter retrospective study reported UFC normalization in 49% of patients, and in another 23% the UFC levels were reduced by at least 50%, using a median final dose of 800 mg/daily; on the other side a quarter of patients on long-term follow-up were treated inadequately. In 20% of cases the drug was administered prior to surgery, obtaining UFC normalization in 49% of cases. Hypertension, hypokalemia and diabetes improved in 40-50% of patients, but 20% of them abandoned the treatment due to poor tolerance⁵⁷.

- Metyrapone (MET) inhibits the conversion of 11-deoxycortisol into cortisol by 11 β -hydroxylase (CYP11B1), the final step in cortisol steroidogenesis, namely with a nadir in cortisol levels within 2 hours of its administration. Despite inducing a rise in ACTH, it is an effective long-term treatment when used 3-4 times a day⁵⁸⁻⁶⁰. Verhelst et al. reported that MET induced a remission of hypercortisolism in 20 out of 24 pituitary-irradiated patients with CD, and in 13 out of 16 patients with cortisol-secreting adrenal adenoma or carcinoma. Valassi et al. demonstrated UFC normalization in 57% of 23 patients treated with MET alone, and clinical control of the disease was achieved in 46% of them. In other studies, MET was administered to CD patients before therapy: a drop in mean serum cortisol was observed as soon as 48-72h later, and after 2 weeks the disease was controlled in 75% of patients. ACTH levels rose over 4 to 6 weeks, using a median dose of 2000 mg/d in weeks 1-3, which was raised to 2250 mg/d (range 500-6000) by week 6. In 2015 a large retrospective study in 13 University-Hospitals in UK reported that MET treatment is effective for short- and long-term control of hypercortisolemia in about 200 patients (considering both benign and malignant form of CS), achieving UFC normalization in 43% of cases after median 8 months (from 3 months to 12 years)⁶⁰. The optimal MET dosage has yet to be defined because it has never been the object of a rigorous clinical trial.
- Cabergoline (CAB) is an agonist of dopamine receptor type 2 (D2), which is expressed in about 80% of corticotroph tumors, and the in vitro and in vivo demonstration that dopamine agonists can reduce ACTH secretion, led to the introduction of this class of drugs in the treatment of CD^{12,61,62}. After 3 months of CAB, UFC levels had dropped by more than 25% compared to the baseline in 75% of the patients, even though a cortisol drop of 25% from baseline is a lower criteria than that of 50% used as a response cut-off in other clinical trials. 35% of patients were full responders and enjoyed a prolonged remission on median doses of 3.5 mg/week, without reporting no significant adverse events^{12,61,62}. A recent meta-analysis shows that CAB monotherapy is a reasonable alternative for subjects with persistent or recurrent CD after surgery, achieving controls of hypercortisolism in 39% of patients (124 cases in 6 observational studies), especially after long-term or high-dose treatment⁶³.
- Retinoic acid (RA) is able to reduced pro-opiomelanocortin synthesis and ACTH secretion in a murine tumoral corticotroph model, exerting also an antiproliferative action on adenoma cells^{64,65}. In a small prospective clinical trial, UFC levels dropped by at least 50% or normalized in 5 of 7 patients after 6

months of orally-administered RA (up to 80 mg daily). Interestingly, after an initial decline in ACTH levels during the first month of treatment, ACTH levels returned to the pretreatment range. In all patients, RA was generally well tolerated, and only mild conjunctival irritation, nausea, headache and arthralgia were observed⁶⁴. In 2016 a prospective study in 16 patients with CS confirmed a mean UFC reduction of 52%, achieving its normalization in 25% of cases (especially in those patients with mild hypercortisolism)⁶⁶. An in vitro study examined the effect of administering RA and bromocriptine, given the well-known permissive role of RA on the D2. In the pituitary corticotroph cell line AtT20, administering RA induced D2 and increased cell sensitivity to bromocriptine; and in corticotropinoma-derived primary cultures, the combined administration of RA and bromocriptine lowered the steady-state level of proopiomelanocortin more efficiently than either of the drugs alone⁶⁵.

- Pasireotide (PAS) is a novel SSA approved by the EMA and FDA for treating adult patients with CD when surgery has failed, hypercortisolism has recurred, or surgery is not an option. Naïve somatostatin binds with a high affinity to all five subtypes of SSTR expressed on the target tissues: in corticotroph adenomas the membrane density of SSTR-2 is lowered by hypercortisolism, while that of SSTR-5 is unaffected, and this latter receptor is the target of pasireotide^{67,68}. In the first clinical trial, two weeks of treatment with PAS 600 µg bid sc. reduced UFC in 76% of cases, with a mean 45% reduction from patients' baseline levels⁶⁷. In a III-phase trial, 162 patients with CD were randomized to receive PAS 600 or 900 µg bid, stepping up the dosage after 3 and 6 months, up to the maximum of 1200 µg bid. Patients' UFC levels dropped quickly, with a median reduction of approximately 50% after 2 months of therapy, and remained stable in the first year, by the end of which 13% of patients in the 600 µg and 25% of those in the 900 µg group had normal UFC levels⁶⁸. PAS has proved effective not only in lowering UFC, but also in improving clinical signs and symptoms (blood pressure, body weight, lipid profile and anxiety disorders). Recently, a long-acting formulation of PAS (one-monthly injection, 10 30 or 40 mg) has been described in a phase III international prospective trial: it normalised UFC concentration in about 40% of patients with CD after 7 months, it had a similar safety profile to that of twice-daily subcutaneous PAS and therefore provides a convenient monthly administration schedule⁶⁹. PAS induce a worsening of glucose control (not only in diabetic patients) in up to 75% of patients, affecting the incretin system⁷⁰.

Combinations of drugs targeting different levels of the pituitary-adrenal axis may prove more effective while containing side effects and cost by reducing the average dose of each compound, but published reports addressing this aspect of CD treatment have only considered small series. The first study concerned 12 patients with persistent disease treated with CAB at an initial dose of 1 mg, raised to a maximum of 3 mg/week or until UFC levels returned to normal. After 6 months, 3 patients had normalized UFC levels with a mean dose of 2.5 mg. KET then was associated to the treatment with CAB in the other 9 patients, starting with a dose of 200 mg and increasing to a maximum of 400 mg/day, on the basis of the cortisol reduction obtained. Overall, 9 of the 12 patients (75%) achieved a normal UFC. Interestingly, the responders had lower baseline UFC levels than the non-responders⁷¹. Barbot et al. examined 14 patients with CD. The sample was divided into 2 groups: the first treated with CAB (n=6, maximum dose 3 mg/week), adding KET after 6 months; the second (n=8) with the same combination of drugs in reverse. Overall, UFC levels were normalized in 79% of cases, with no differences between the two treatment schedules. It is noteworthy that, despite normal UFC levels, 10/14 patients still had high late night salivary cortisol (LNSC) levels, presumably reflecting a subtle hypercortisolism that may be detrimental⁷². In another 80 days trial, 17 patients with CD were treated first with PAS 100 µg sc. bid, then with 250 µg sc. tid daily as of day 15 in patients with persistently high UFC levels. At day 28, CAB was added at a dose of 0.5 mg every other day, stepping up the dose to 1.5 mg every other day after 10 days if UFC levels remained abnormal. Then KET 600 mg daily was added at day 60 if UFC levels were still elevated. The disease was controlled with the first two drugs in 47% of patients, and in 88% after adding low-dose ketoconazole. Despite the small size of this series, the study clearly points to unexplored potential advantages of combination therapies⁷³. Every physician must bear in mind that both pasireotide and ketoconazole can prolong the Q-T interval, hence in association they should be used with caution in patients at risk of arrhythmia.

Considering cortisol-related comorbidities, currently few papers studied body composition changes in adult patients with CS after achieving remission. Three prospective works with different techniques (computed tomography, bioelectrical impedance analyses or magnetic resonance), reported a decrease of total body and fat mass in 7, 6 and 14 patients after remission⁷⁴⁻⁷⁶. Furthermore, three cross-sectional studies considering 37, 50 and 58 subjects evaluated with dual-energy X-ray absorptiometry (DXA) reported that fat mass was higher after stable remission of CS than in healthy controls⁷⁷⁻⁷⁹. Therefore, it is not yet clear whether body

composition is normalized after remission of hypercortisolism. Moreover, there are no published data regarding the effects of different treatments on body composition, as surgery (first-line treatment for CS, if possible) or pharmacological therapy (increasingly used, especially after surgical failure or waiting for radiotherapy's effect).

Medical treatment in GH-secreting or PRL-secreting PitNET

One of the first pharmacological options in GH-secreting PitNET are SSA, acting mainly through the somatostatin receptors type 2 (SSTR2) and type 5 (SSTR5) and to lesser extent dopamine-agonists, activating dopamine receptor type 2 (DRD2).

Treatment goals in acromegaly include normalisation of GH and IGF1 levels, tumour removal or at least significant reduction or stabilization of tumour size, preservation of normal pituitary function, reduction of comorbidities and improvement of quality of life^{3,7,30,80}.

Surgery is generally the first-line therapy for acromegaly, and it is the only therapy that may lead to lasting remission, especially in microadenomas (up to 80-90%). Radiotherapy in the form of conventional or stereotactic fractionated treatment is required for the treatment of a few patients with acromegaly⁸¹.

By contrast, medical treatment is quite often indicated as adjuvant therapy, and SSA is usually the first drug of choice⁷.

- SSA: Two SSAs are available and used worldwide: octreotide long-acting repeatable (LAR) and lanreotide Autogel: no blinded or randomised study that has compared octreotide LAR with lanreotide Autogel in *de novo* acromegalic patients has been published⁸¹. Some authors suggest that a switch between somatostatin analogues may be beneficial in individual patients, if i) the patient is a non-responder or ii) the patient develops subcutaneous nodules at the injection site and/or adverse gastrointestinal effects⁸². Presurgical lanreotide treatment have generated promising results, especially in larger adenomas⁸³. SSAs are established treatments for patients with acromegaly after unsuccessful pituitary surgery; meanwhile, primary therapy with these agents is recommended principally for a subgroup of patients with larger tumors when a surgical cure is unlikely, and additionally if surgery is refused or contraindicated⁷. In 2013, an international collaborative study considered lanreotide 120mg monthly as a primary treatment (PRIMARYS study) in 90 patients: after 12 months of treatment they observed a significant reduction in

tumor volume (at least 20% as per protocol) was observed in 63% of patients⁸³. To date, the normalization rates of GH or IGF-1 after first-generation SSAs (lanreotide or octreotide) is up to 55%⁸⁴. Recently, PAS has been proposed and compared to octreotide in a prospective trial, achieving a higher rate of acromegaly control^{85,86}. Future studies and extensive clinical experience (especially regarding PAS-induced diabetes) will determine the impact of this new SSA.

- Pegvisomant (PEG) is a 191-amino acid compound, initially developed as a new GH analogue (as a matter of fact most currently available assays cross-react with it), but it is an effective GH antagonist. PEG decreases IGF1 levels effectively in most patients, achieving IGF1 normalization in up to 90% of patients with 10–40 mg/day for 12–18 months^{87,88}. PEG does not reduce tumour size as SSAs, and a minor part of patients (2–3%) exhibited a significant increase in tumour size, therefore periodic MR is suggested⁸¹.
- CAB: Dopamine agonists were available for the medical treatment of acromegaly until the 80s. CAB is the most used, with a good safety profile, especially in mild cases. The use of CAB as a monotherapy after unsuccessful surgery has only been evaluated in small series: in a meta-analysis, the efficacy of CAB as a monotherapy (10 observational studies for 11 months, 160 patients, no randomized or placebo-controlled) to normalize IGF-I was 34 %. Normalization of both GH and IGF-I levels was observed for 39 % of the patients if CAB is combined to SSAs^{89,90}. However, little evidence is available in the literature regarding its use in acromegaly.

New data obtained on the processes involved in receptor desensitization, internalization, and intra-cellular trafficking could explain the lack of response to drugs, such as:

- ✓ Low (or absent) expression of SSTR receptor in the cell membrane
- ✓ High expression of truncated SSTR variants
- ✓ Cytoskeleton alterations, as cadherin or arrestin mutations, that interfere with intra-cellular trafficking of the SSTR
- ✓ Low or mutated AIP expression
- ✓ Low (or absent) expression of dopamine receptor DRD2 in the cell membrane
- ✓ Mutations in cyclin-dependent kinase inhibitors, atypical tumour suppressors genes playing a key role in cell cycle regulation, cell proliferation, and differentiation (i.e. p²⁷)^{30,91}.

Prolactin-secreting pituitary adenomas are generally characterized by an excellent response to dopamine-agonists (DAs, especially cabergoline), considering both prolactin secretion and tumor-size reduction⁸. Overall, up to 90-95% of patients with microprolactinomas and 70-80% of those with macroprolactinomas have normalized prolactin levels, reduced tumor sizes, and restoration of gonadal function with cabergoline⁹². Therefore, DA therapy is recommended as first-line treatment, also in patients with mass-effect symptoms⁸. Despite size and secretion, the term “insufficient response to cabergoline” may be better than “resistance to cabergoline”, because dopamine-agonist treatment was effective to reduce prolactin levels. In such cases, indication to cytoreductive surgery should be carefully considered and balanced, because complete surgical removal of a giant prolactin-secreting PitNET is challenging and tumour persistence after surgery occurs frequently⁹³.

Medical treatment in aggressive PitNET

In aggressive PitNET, whether definition used, other options needs to be considered after the failure of surgery, radiotherapy and conventional medical treatment.

Temozolomide (TMZ) is an alkylating agent that undergoes rapid chemical conversion in the systemic circulation at physiological pH to the active compound [5-(3-methyltriazeno)imidazole-4-carboxamide]. The cytotoxic effect of this active metabolite is accomplished through the methylation of guanine at the O⁶ position in DNA, causing formation of DNA adducts and subsequent autophagy, senescence, and apoptosis of neoplastic cells⁹⁴. TMZ is administered orally, has 100 % bioavailability, readily crosses the blood–brain barrier, is not cell-cycle specific, which is advantageous when treating relatively slow-growing tumors⁹⁵. The first case reports on the use of TMZ in pituitary adenomas or carcinomas were published in 2006^{96,97}, then several authors reported its efficacy^{11,98}, and now TMZ has a place in the therapeutic algorithm recently proposed by the European Society of Endocrinology to manage aggressive PitNET^{5,99}. Response to TMZ was initially reported to depend on the adenoma’s expression of 6 methylguanine DNA methyltransferase (MGMT), a DNA repair enzyme that has the potential to interfere with the action of TMZ⁹⁸. TMZ treatment is not effective in all aggressive PitNET, so other therapeutic options may be useful: in such scenario, in 2014 we described the association of PAS and TMZ in a series of patients with aggressive ACTH-secreting PitNET, achieving a significant improvement in both endocrine secretion and shrinkage of the adenoma¹⁰⁰.

Medical treatment in non-secreting PitNET

Translational medicine is one of the modern cornerstones in “bench-to-bed” research. In such scenario, some genetic inherited alterations could represent a “simple” model of disease, useful to understand the pathophysiology of cellular transformation. As an example, Tuberous Sclerosis Complex (TSC) is an autosomal dominant multisystem hereditary cutaneous condition, characterized by multiple hamartomas that can be associated to endocrine system alterations^{101,102}. TSC is mostly caused by mutations of two tumor suppressor genes *TSC1* and *TSC2*, encoding for hamartin and tuberlin, respectively. In normal cells hamartin and tuberlin form a molecular complex involved in intracellular signaling pathways controlling cell growth and proliferation, including the mammalian target of rapamycin (mTOR) cascade. Therapeutic approach related to mTOR signaling, such as everolimus, may be used in some patients with pituitary adenoma^{101,102}. In nearly 10% of cases mutations are mosaic or intronic and a comprehensive genotype-phenotype correlation is still debatable. About two thirds of TSC cases are sporadic reflecting a high spontaneous mutation rate in these genes¹⁰³. Some reports addressing PitNET in TSC patients suggest the possible involvement of pituitary gland alterations in the pathological process of TSC¹⁰⁴. The development of pituitary tumors by nearly half of adult Eker rats (with spontaneous germline mutation of *TSC2*) further supports this possible association¹⁰⁵. Up to now, however, no molecular evaluation of PitNET in patients with TSC has been performed to establish the weight of this link and whether PitNET should be considered a clinical manifestation of TSC is far from being clearly understood.

In conclusion, there is still a considerable number of patients with PitNET without a personalized and effective medical treatment after surgical failure or recurrence: in these patients, a clinical and molecular characterization may guide medical treatment, in order to personalize their therapeutic plan.

2. *Aim*

The aim of this PhD project is to study the effects of medical treatment in patients with PitNET, in order to:

- Develop new medical treatment or strategies
- Study the efficacy of available medical treatment
- Apply the combination of medical treatment in clinical practice
- Analyse the differential effects (if existing) of medical treatment compared to surgery (considered the best curative treatment)

Different methodological approaches have been adopted during the 3 years of project, in order to address correctly the aims:

- A case-report study in the *in-vitro* everolimus-treated patient
- An observational planned study with decided doses of medical treatment to establish the effect of MET in patients with CS
- A cross-sectional study to analyse the effect of different approaches (medical treatment, surgery or radiotherapy) regarding the clinical picture of patients with acromegaly or CS.
- Literature review and retrospective analyses of our cohort of patients in case of aggressive PitNET
- *In-vitro* studies regarding cell-lines or primary-cell culture

3. Materials and methods

3.1 case identification and clinical analyses

Among our cohort of patients that are followed in the Pituitary Office of the Endocrine Unit of Padova (120 with GH-, 134 with ACTH-, 171 with PRL-, 6 with TSH- secreting PitNET, 150 with non-secreting PitNET), we retrospectively and prospectively analyzed clinical, radiological and pathological features.

We collected cross-sectional data from 97 patients with acromegaly and normal Hypothalamic-Pituitary-Adrenal (HPA) axis function at diagnosis of acromegaly, 38 males and 59 females, with a mean age of 60 ± 13 years (range 31-92). Their mean age at the time of their diagnosis was 46 ± 13 years (range 24-78) and they had a mean follow-up (as at December 2017) of 14 ± 10 years. We considered only those patients with a complete follow-up, discarding those cases with insufficient data or follow-up (at least 2 years of observation after diagnosis). Acromegaly was managed according to current international criteria^{3,80}. The diagnosis was based on clinical characteristics; inadequate GH suppression ($<1 \mu\text{g/L}$) after an oral glucose tolerance test (OGTT); and high IGF-1 levels for gender and age. Disease activity was judged on the last available visit. We considered the disease as “active” when randomly-measured serum GH was $\geq 1 \mu\text{g/L}$ or IGF-1 levels were below the upper limit of normality in patients with clinical symptoms of active acromegaly, and the GH nadir after OGTT was $\geq 1 \mu\text{g/L}$. Acromegaly was judged to be “controlled” when randomly-measured GH levels were $<1 \mu\text{g/L}$ and IGF-1 values were within normal range. Normal IGF1 levels were used to consider remission in patients treated with pegvisomant. Adenoma was classified by maximal diameter as micro-adenoma ($<10 \text{ mm}$) or macro-adenoma ($\geq 10 \text{ mm}$), and cavernous sinus invasion (CSI) was assumed from MRI evidence, diagnosed while during transsphenoidal surgery. SSA (octreotide LAR or lanreotide autogel), CAB or pegvisomant (that is only allowed in Italy for patients who have undergone surgery) were used as medical treatment. We assessed in all 97 patients basal morning (07.00 – 09.00 a.m.) serum cortisol at diagnosis, and then every 12-18 months during the follow-up, or whenever cortisol deficiency was clinically suspected (serum cortisol was checked back the next morning if too high or too low, considering pulsatile secretion). Adrenal insufficiency was diagnosed if $\text{F0} < 138 \text{ nmol/l}$, or if cortisol response to low-dose short synacthen test (LDSST) was inadequate^{106,107}.

To study the effect of MET in patients with CS, an *ad-hoc* designed observational trial (depicted in figure 2), has been created and studied. 31 consecutive patients with confirmed CS were enrolled and treated with MET for at least 4 consecutive weeks.

CS was diagnosed with at least two positive first line screening tests among UFC, LNSC and 1-mg dexamethasone suppression test (DST). We considered CD in those patients with positive ACTH immunostaining of the pituitary adenoma or ACTH pituitary/peripheral gradient >3 after CRH stimulation in petrosal sinus sampling or at least two of the following criteria: a) at least 80% decrease of serum cortisol after 8 mg DST; b) $\geq 50\%$ rise in ACTH or $\geq 20\%$ rise in cortisol levels after CRH stimulation test; c) MRI confirmation of a pituitary adenoma $\geq 6\text{mm}$; d) ≥ 6 months remission after pituitary surgery. The other ACTH-dependent CS patients were ectopic-CS. We considered adrenal CS in those patients with ACTH levels <10 ng/L and positive finding of an adrenal lesion.

All patients presented with hypercortisolism at baseline, defined as at least one among increased UFC levels or impaired cortisol rhythm with increased LNSC. The effectiveness of medical treatment was stratified considering baseline cortisol levels (either salivary or urinary) compared with upper limit of normality (ULN) in Padova's University-Hospital, as depicted in table 3.

Table 3: stratification of hypercortisolism severity according to cortisol levels at baseline visit.

	ULN	UFC nmol/24h	LNSC nmol/L
Normal	<1	<168	<2.6
Mild hypercortisolism	1-1.5	169-252	2.7-3.9
Moderate hypercortisolism	1.5-2.5	253-420	4-6.5
Severe hypercortisolism	2.5-5	421-840	6.6-13
Very severe hypercortisolism	>5	>841	>13.1

LNSC and UFC (two collections at each visit) were assessed after one month, 3 months (the core phase of the protocol), 6 months, 1 year and 2 years (for those subjects in the extension phase). Inclusion and Exclusion Criteria are depicted in table 4.

Figure 2: study design scheme

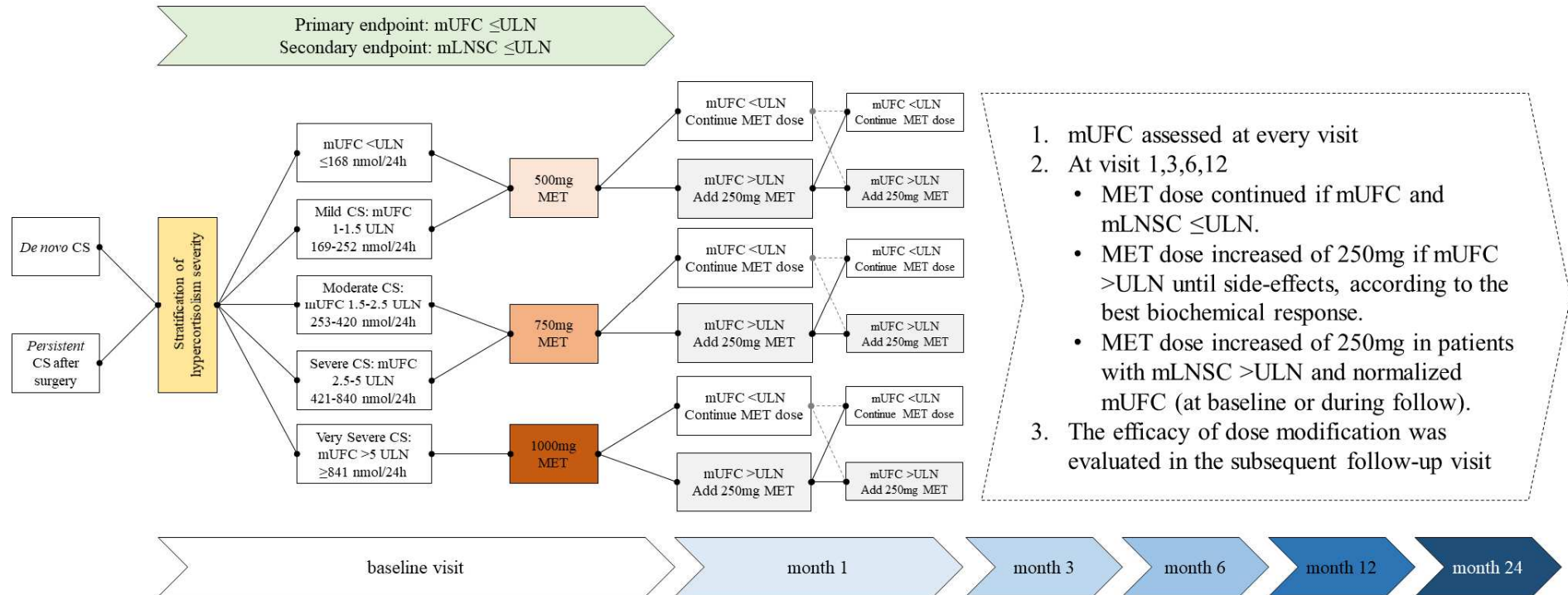


Table 4: inclusion and exclusion criteria for patients with CS.

Inclusion Criteria	Exclusion Criteria
Age >18 years at the baseline visit	Pregnancy, lactation
Biochemical evidence of CS	Previously pituitary radiotherapy.
In patients treated with MET before surgery with normal mUFC (mean of 3 UFC collections) or mild/moderate increase of mUFC at baseline, pseudo-Cushing's (functional hypercortisolism) exclusion was based upon increased mLNSC (mean of 2 LNSC collections) and unsuppressed serum cortisol after 1-mg DST	Patients who have a history of congestive heart failure (NYHA Class III or IV), unstable angina, sustained ventricular tachycardia, clinically significant bradycardia, advanced heart block, acute MI less than one year prior to study entry, or clinically significant impairment in cardiovascular function
Biochemical hypercortisolism at baseline before MET treatment (at least one among increased mUFC or mLNSC levels).	CS surgery: trans-nasal approach for CD, abdominal surgery for ACS or selected surgery (thoracic or abdominal) for EAS during MET treatment
At least 30 days post-surgery in those patients with a history of prior surgery.	Malignant disease.
Washout to prior current drug therapy, if the treatment was not tolerated or not able to normalize mUFC. The following washout periods were completed before baseline visit: ketoconazole: 2 weeks; pasireotide s.c.: 2 weeks; cabergoline: 4 weeks; mitotane: 6 months.	Patients with risk factors for torsade de pointes, i.e. patients with a baseline corrected QT interval >470 ms, hypokalemia, hypomagnesemia, family history of long QT syndrome, or concomitant medications known to prolong QT interval that could not be discontinued.
	Combination of another medical treatment to control hypercortisolism
	Patients who have had any previous MET treatment
	Radiotherapy while on MET treatment
	Impaired liver (history of liver disease such as cirrhosis, chronic active hepatitis B and C, ALT or AST >2 ULN, baseline total bilirubin >1.5 ULN) or renal function (serum creatinine >2.0 X ULN).
	MET discontinuation before 4 weeks of treatment

Regarding aggressive PitNET, we considered an aggressive pituitary adenoma when at least one of the items summarized in table 1 was met: tumor growth, invasive adenoma, giant adenoma, atypical adenoma, resistance to pituitary-directed pharmacological treatment, silent adenoma. All patients referred to Padova University-Hospital (mean follow up 6 ± 5 years), either in Endocrine or Neuro-surgery Unit.

We considered response to surgery in case of complete removal of the adenoma, evaluated with hormonal secretion and pituitary magnetic resonance imaging (MRI) 3-6 months after procedure. Response to radiotherapy was defined in case of radiological disappearance of adenoma at MRI and normalization of hormonal secretion (if present) at least 3 years after irradiation. Control of hormonal secretion and tumor growth were considered to define the response to standard dose of medical therapy (at least 2 consecutive years).

In aggressive PitNET the stratification of response could be cumbersome, therefore we decided to grade it considering a “pituitary-adapted” RECIST criteria. Patients who had a complete (disappearance of the tumor), or a partial response (decrease in tumor volume $>50\%$), or stable disease (reduction of tumor volume $<50\%$) were considered to have disease control. Disease progression was defined as an increment of tumor volume greater than 25% during treatment. Hormone response to TMZ treatment was categorized as normalization (complete remission of hormone hypersecretion), partial response (reduction of at least 50% compared to baseline but without normalization) and no response. Patients were censored at the date of the last follow-up, in order to calculate progression-free survival (PFS), disease control duration (DCD), and overall survival (OS) according to the Kaplan–Meier method.

3.2 *Laboratory medicine and imaging*

Clinical Evaluation

Clinical assessments were performed in the Endocrine Unit of the Padova University-Hospital. Participants were weighed and measured, wearing light clothing and no shoes, using a balanced beam scale and a vertical ruler: weight and height were recorded to the nearest 0.5 kg and 0.5 cm, respectively, then body mass index (BMI) was calculated (weight divided by height squared, kg/m²). Blood pressure was measured on the right arm three times in 5 minutes with a calibrated standard sphygmomanometer with the appropriate size cuff, after 5 minutes of resting in supine position according to the Korotkoff sounds, as recommended. Waist circumference was measured at the end of natural breaths at the midpoint between the top of the iliac crest and the lower margin of the last palpable rib. We considered hypertension if home systolic blood pressure levels were ≥ 130 mmHg or diastolic ≥ 85 mmHg (or if they were receiving antihypertensive treatment, a blood pressure $\geq 130/85$ mmHg during medical treatment was sufficient to up-titrate or modify the usual therapy). Hyperandrogenism was considered either clinical (hirsutism, defined as an excessive terminal hair in androgen-dependent areas in women and a modified Ferriman–Gallwey score ≥ 8 , alopecia and/or acne) and biochemical (not only testosterone, but also adrenal steroids precursors as 17-hydroxyprogesterone, androstenedione and DHEAS increased levels).

At each visit, clinical and anthropometrical data were collected, electrocardiography was assessed and routine hematologic/blood/urinary biochemical measurements were performed in the Laboratory Medicine Unit of the Padova University-Hospital. Clinical data were reported in the web-based database of the University-Hospital of Padova, used as an electronic Case Report/Record Form (eCRF). Adverse events (AEs), both drug-related or suspected drug-related, were collected at each visit and reported in the eCRF according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), version 4.0, as recommended by Ethics Committee.

Hormonal Evaluation

Hormones were measured in the same laboratory in the University-Hospital of Padova.

From 1982 to 2004 serum cortisol was measured by RIA (DIA-Sorin Diagnostics, Saluggia, Italy) with intra- and inter-assay coefficient of variation (CV) values of 5.4 and 9.6% respectively; since 2004 serum cortisol assays are performed with a commercial chemiluminescence immunoassay with declared intra/interassay CV less than 7% and less than 9%, respectively (Immulite, Siemens).

GH was measured with immunoradiometric assay (IRMA) and INSIK-5 (Sorin, Saluggia, Italy) with detection limits of 0.2 µg/L; IGF-1 was measured with radioimmunoassay (Nichols Institute, San Clemente, CA) for with a detection limit at least of 1.5 µg/L until 2003, then serum GH assays was performed with chemiluminescence by GH IMMUNOLITE 2000 (Siemens, the detection limit was 0.05 µg/L, intra- and interassay variation coefficients of 4.0% and 6.5%, respectively) and serum IGF-1 was measured by chemiluminescence with reagents supplied by DiaSorin Liaison (detection limit was 0.6 µg/L, with the intra- and interassay variation coefficients of 5.6% and 7.7%, respectively).

For 24-hour urine collection to measure UFC levels, the patients were instructed to discard the first morning urine void and to collect all urine for the next 24 hours so that the morning urine void on the second day was the final collection. The sample was kept refrigerated from collection time until it was analyzed: a 10 mL aliquot sample was taken and centrifuged at 3000 rpm for 10 min at room temperature. Two µL formic acid and 50 µL of deuterated internal standard solution (d4-cortisol and d7-cortisone) were added to 500 µL of urine supernatant or calibrators. The solution was vortexed for 30 seconds and centrifuged at 16000g for 5 minutes at room temperature. Twenty µL of the supernatant was added to 200 µL of 0.1% formic acid water solution and placed in the autosampler of the LC-MS/MS. UFC was measured, as is routine in our center, utilizing an Agilent HPLC series 1200 triple quadrupole mass spectrometer Agilent 6430 equipped with an Electrospray Ionization source in positive ionization mode (Agilent Technologies, Palo Alto, USA). The on-line cleanup/enrichment was carried out using a cartridge Zorbax Extend-C18 2.1x12.5 mm, 5 µm particle size and the HPLC separation by a 4.6x50 mm, 1.8 µm particle size, analytical column Zorbax Eclipse XDB-C18 (Agilent Technologies, Palo Alto, USA). Quantitative analysis was performed in the multiple reaction monitoring mode. The method was linear up to 625 nmol/L with a lower quantification limit of 5 nmol/L for

UFC, respectively. Within-run and between-run coefficients of variation were less than 5% and 6%, respectively. The mean recoveries were 106% for UFC. All the patients provided from two to five complete 24-hour urine collections, the average values from all of the collections were used in the final analysis. The reference range utilized in our department, determined using the LC-MS/MS method described above, is 16-170 nmol/24h for UFC¹⁰⁸.

Also LNSC is measured with a LC-MS/MS method. Patients were advised to soak for 2 or 3 minutes the absorbent cotton of Salivette ® device (Sarstedt, Numbrecht, Germany), samples were then stored at +4 °C. To avoid any source of food, blood, smoke or licorice contamination samples were collected at least 30 minutes before or two hours after taking a meal or drink and all participants brushed their teeth after saliva collection, and they were avoided smoking. After centrifugation we obtained at least 1 ml of saliva in all collections (repeating the procedures in few days if the patient did not provide adequate volume), then samples were stored at -20°C until assay. Each saliva sample or calibrator (300 µL), spiked with internal standard secondary mixed solution (30 µL), was applied to Oasis® HLB 1 mL solid phase extraction (SPE) cartridges (Waters, MA;USA), which had previously been equilibrated with 1 mL of methanol followed by 1 mL of water. After sample loading (250 µL), the washing step was performed with 500 µL water:methanol 80:20. 250 µL of methanol was then added and the eluate placed in the LC-MS/MS autosampler. The instrumentation consisted of an Agilent HPLC series 1200 with a column oven, an autosampler, a binary LC pump and a degasser together with an additional isocratic pump with a switching valve for on-line SPE and a triple quadrupole mass spectrometer Agilent 6430 equipped with an Electrospray Ionization (ESI) source, operating in positive ion mode (Agilent Technologies, Palo Alto, USA). On-line purification was carried out by a Zorbax Extend-C18 cartridge (2.1 × 12.5 mm, 5 µm particle size) and the HPLC separation by a Zorbax Eclipse XDB-C18 analytical column (4.6 × 50 mm, 1.8 µm particle size) (Agilent Technologies). Recovery tests. The mean recoveries of the three saliva samples were 101% for cortisol. The method was linear up to 55.4 nmol/L, low limits was 0.51 nmol/L. ULN for LNSC is 2.6 nmol/L¹⁰⁹.

Magnetic resonance

All patients had serial magnetic resonance imaging (MRI). For our purposes, we considered the images at diagnosis, after the latest surgical procedure, at the time of starting medical therapy, then every 3 to 6 months during the first year of treatment and then every 6-12 months thereafter, as clinically indicated. All comparisons of images showing treatment-induced dimensional changes were drawn between the baseline MRI and the one obtained after the last dose. The PitNET volume of the was measured on contrast-enhanced T1 sequences: the area of the lesion was drawn manually on each slice, then the sum of all the areas was multiplied by the thickness of the slice according to the formula: $\Sigma \text{Area} \times (\text{slice thickness} + \text{interslice gap})$. MRI scans were performed with a 1.5T or a 3T MRI (Achieva, Philips Medical Systems, Best, Netherlands), with a standard quadrature head coil. Each cerebral MRI underwent an operator-independent quantitative assessment performed with a free medical image viewer (Horos®) to automatically calculate the volume of the with PitNET.

Body composition

We measured body composition with DXA in all patients; we used the same Discovery W Hologic QDR 4500 C densitometer (Hologic Inc., Waltham, MA, USA) at baseline (during active hypercortisolism, before any treatment to reduce cortisol levels) and during remission. The mean precision error (coefficient of variation) was 0.6%; reproducibility was 1.2%. Total body and trunk/abdominal fat/lean mass were measured by DXA; whereas the R1-box was manually defined as DXA subregion 4 cm (or 3 pixels) slice at the top of iliac crest.

3.3 Molecular analyses

Primary cells everolimus treatments in non-functioning PitNET

Primary cells derived from PitNET were cultured in vitro (5000 cell/well in a 96 wells plate) and treated with everolimus (0.1 and 1 μ M, kindly provided by Novartis) for evaluating its pharmacological effect. After 72 hours treatment the conversion of the tetrazolium dye MTT (Sigma-Aldrich) to formazan was ascertained following manufacturer protocol. After media removal and the addition of DMSO, the solution's absorbance was measured at 550 nm – background subtraction set at 620 nm – with a microplate reader (Victor3 V 1420 Multilabel 206 Counter, Perkin Elmer). To search for the TSC disease-causative variant – and possibly associate it with pituitary adenoma development – patient's germline DNA was isolated from the peripheral blood and the genomic analysis of the entire *TSC1* (NM_000368.4) and *TSC2* (NM_000548.3) coding sequence and intronic boundaries was performed. Mutations were searched in dbSNP and ExAC databases, the computational tools MutationTaster and Human Splicing Finder were used to predict the function.

Evaluation of GIPR in GH-secreting PitNET

Portions of the surgically removed specimens were fixed in 10% buffered formalin and then embedded in paraffin; standard sections stained with hematoxylin and eosin were used for diagnosis, whereas the presence of pituitary hormones was evaluated by standard immunocytochemical analyses. A second fragment for each tissue specimen was immersed in RNAlater (Ambion), kept at 4°C for 24 h and then stored at –20°C until RNA extraction. The remainder of each somatotropinoma was transferred to sterile cold complete culture medium and processed within 36 hours. The effect of GIP and SSA on GH secretion was examined in somatotropinoma-derived primary cells. Novartis Pharma AG kindly provided the SA Pasireotide (SOM230) and Octreotide (OCT), whereas GIP and Forskolin (FK) were purchased from Sigma-Aldrich. After seeding and incubation of primary cells at 37°C for 48–60 h, the medium was removed and replaced with 2% FBS DMEM containing GIP (100 nM), FK (10 μ M), OCT (100 nM) or PAS (100 nM). Cells were incubated for further 6 h (GIP and FK) or 24 h (OCT and PAS) before the medium was recovered and frozen. Immunofluorescence for GIPR expression was performed on GH-sec PAs on conventional sections after deparaffinization in xylene, rehydration through graded alcohols to water and antigen retrieval (Dako) in 10

mM sodium citrate buffer (pH 6.0) for 10 min at 96°C. Sections of a normal human pancreas and of a GH-sec PA incubated with non-immune serum were used as positive and negative controls respectively. GIPR expression was visualized with a rabbit polyclonal antibody (kindly provided by Prof. Timothy Kieffer, University of British Columbia, Vancouver, Canada; O/N, 4°C, 1:250) and Alexa Fluor 594-labeled donkey antirabbit IgG secondary antibody (Life Technologies, 1:250). The tumor specimens collected in RNA later were homogenized in a TissueLyser (Qiagen) in 1 mL of TRIzol reagent (Invitrogen) using a modified TRIzol protocol. RNA and DNA yields were determined on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was tested with the Agilent 2100 Bioanalyzer (Agilent Technologies). Genomic DNA in the RNA was removed by DNase, treating total RNA with Turbo DNA free kit (Ambion). RNA (500 ng) was reverse-transcribed with M-MuLV Reverse Transcriptase RNase H- (Euroclone, Pero, Italy) according to the manufacturer's recommendations. The possible interaction between the GH/IGF-1 axis and the synthesis and/or secretion of GIP was investigated with murine enteroendocrine cell lines STC-1 (ATCC CRL-3254). Cells were cultured at 37°C and 5% CO₂ in DMEM-low glucose (ECM0749, Euroclone) supplemented with 10% FBS, 3.7 g/L NaHCO₃, 2 mM l-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin. Twenty-four hours before the experiment, STC-1 cells (1.75 × 10⁵ cells/well) were seeded into 12-well plates. Cells were transiently transfected with 2 µL of Lipofectamine 2000 (Invitrogen) together with 1.6 µg of total DNA consisting of hGIP2.9kbluc (a kind gift from Prof. T Kieffer generated by cloning a 2.9-kb fragment of human GIP promoter (-2844 to +57 bp) upstream luciferase gene (17)) and pRL-TK (Promega) and incubated for 24 h. Cells were then treated for further 24 h with GH (from 1 to 100 ng/mL), IGF-1 (from 0.1 to 100 ng/mL), insulin (100 nM) or the combination of FK and IBMX (10 µM each) and the effect on GIP promoter activity was examined. All compounds used for cell treatments were purchased from Sigma-Aldrich.

3.4 Statistical analyses

Proportions and rates were calculated for categorical data; continuous data were reported as means and standard error or median and interquartile range (IQR). Groups were compared by chi-square test for categorical variables and by the Wilcoxon rank sum test for quantitative variables. Wilcoxon signed-rank test for paired samples was used to compare data at baseline and during MET treatment.

The database was managed and statistical analysis performed by SPSS 17 software package for Windows (SPSS, Inc., Chicago, IL, USA). Significance level was set as a $p < 0.05$ for all tests.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent was obtained from all individual participants included in the study.

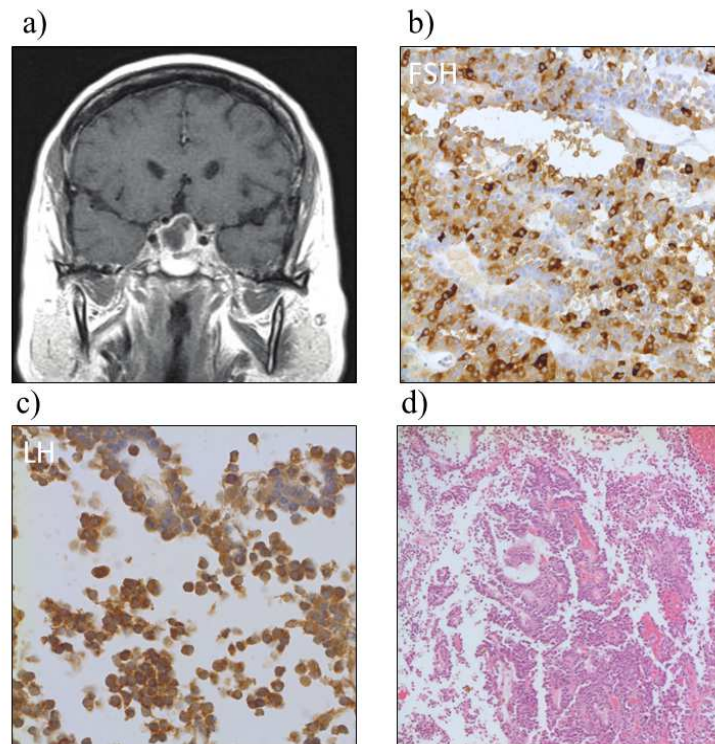
4. Results

4.1 *medical treatment with everolimus in non-secreting PitNET*

(Publication I. [Regazzo D, Endocrinol Diabetes Metab Case Rep 2018](#))

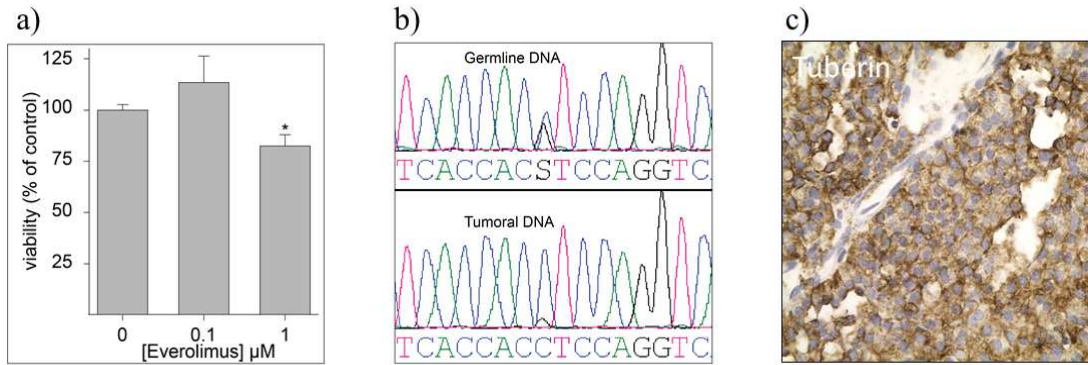
A 62 years old Caucasian woman presented TSC, diagnosed after the recognition of cognitive disability and neurobehavioral abnormalities of her only 32 years old son. She presented the common TSC brain lesions at MRI (i.e. cortical tuber and right retinal hamartoma) without other typical TSC-related manifestations (e.g. renal, pulmonary, cardiac or skin lesions) and no family history of TSC. During the last twenty years she refused medical care, however in April 2012 she complaint headache: MRI scan showed a PitNET (diameter 20x18x16 mm) with suprasellar extension, bilateral cavernous sinus invasion, shortened pituitary stalk and left optic nerve compression (Figure 3a). A hormonal study revealed normal thyroid and adrenal function, low gonadotropins (LH 3.5 U/L, range 11-61; FSH 13.5 U/L, range 35-150) and elevated prolactin (75.7-74-79 µg/L, range 5-25, probably due to shortened pituitary stalk). After 4 months she underwent transsphenoidal endoscopic neurosurgery with complete tumor resection. Histologic evaluation demonstrated a uniform PAS-positive basophilic adenoma with low proliferation index (MIB-1 <3%). Immunohistochemistry was positive for FSH and LH (figure 3b and 3c) and negative for ACTH, GH, prolactin and TSH. Clinical and histological findings were thus consistent with a silent gonadotroph PitNET.

Figure 3: a) Brain T1-weighted MRI after gadolinium injection: pituitary macroadenoma with suprasellar extension and bilateral cavernous sinus invasion; Immunohistochemical analysis of b) FSH (20X magnification) and c) LH (40X magnification) expression in the tissue slice of the silent gonadotroph PitNET d) and standard hematoxylin-eosin staining.



Given the therapeutic potential of everolimus in TSC¹⁰¹ and PitNETs¹¹⁰ – both conditions present in our patient – primary cells derived from her pituitary adenoma were cultured in vitro (5000 cell/well in a 96 wells plate) and treated with everolimus (0.1 and 1 μ M, kindly provided by Novartis) for evaluating its pharmacological effect. One μ M treatment induced a significant 20% decrease in cell viability ($p < 0.05$), confirming previous reported data¹¹⁰ (figure 4a). To search for the TSC disease-causative variant – and possibly associate it with pituitary adenoma development – patient’s germline DNA was isolated from the peripheral blood and the genomic analysis of the entire *TSC1* (NM_000368.4) and *TSC2* (NM_000548.3) coding sequence and intronic boundaries was performed. The analysis retrieved no disease-associated variants with the exception of the heterozygous intronic variant c.4006-71C>T found in *TSC2* (figure 4b) and not present neither in dbSNP nor in ExAC databases. The computational tools MutationTaster and Human Splicing Finder both predicted a gain of a new splice site with consequent intron retention that was not confirmed by an in-vitro analysis of patient’s lymphocyte derived RNA. In addition, molecular analysis on archived paraffin-embedded pituitary tumoral tissues failed to identify both loss of heterozygosity in *TSC2* locus (figure 4c), and protein expression reduction (figure 4c).

Figure 4: a) Effects of everolimus treatment on pituitary tumor primary cells viability; c) Electropherograms centered on the c.4006-71C>T variant both in germline (upper panel) and tumoral (lower panel) DNA; d) immunohistochemical analysis of Tuberlin expression (40X magnification) in the tissue slice of the silent gonadotroph PitNET (D93F12, XP® Rabbit mAb of Cell Signaling at 1:100 dilution after microwave antigen retrieval in 10mM citrate pH6.0).

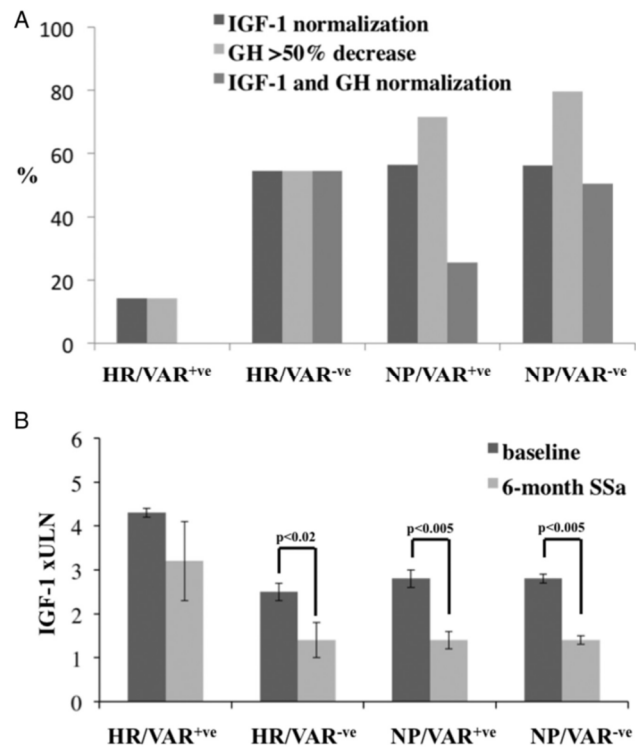


4.2 Resistance to SSA in GH-secreting PitNET

(Publication VII. Cannavò S, *J Clin Endocrinol Metab.* 2016; VI. Regazzo D, *Eur J Endocrinol* 2017)

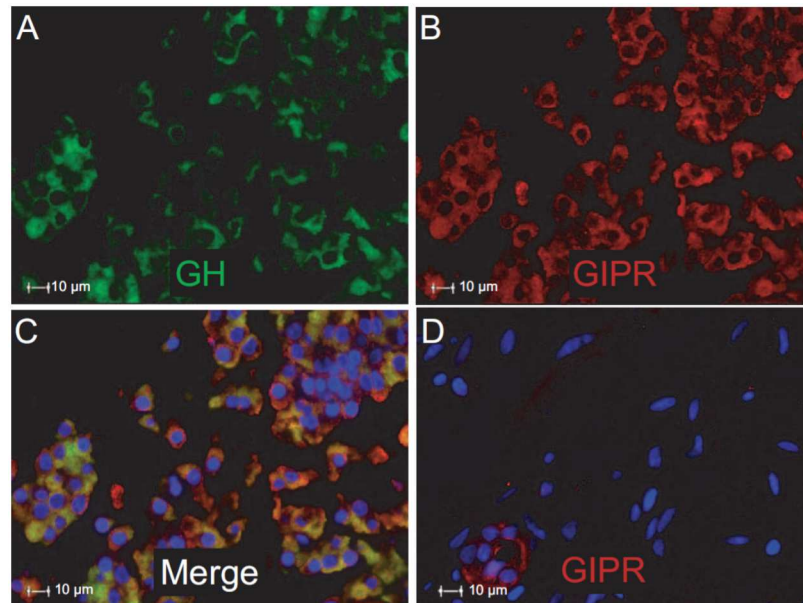
In a multicentric Italian collaborative study (supported by a grant of the Ministry of Education, University and Research of the Italian Government PRIN 2010/2011, cod- DII112000360001), 210 consecutive acromegalic patients (79 men; mean age 47 ± 10 years) were recruited at the Endocrinology Units of the University Hospitals of Messina (Sicily), Padua-Montebelluna (Veneto) and Ancona (Marche Region) up to 2015. Twenty-three of 210 patients had lived for at least 20 years before diagnosis in HR areas, reported in the list of the areas of national interest for environmental risk (SIN) identified by the Department of Environment of the Italian Government on the basis of data collected by the Regional Agencies for Environment Protection (ARPAs). Data regarding the responsiveness to SSA treatment was evaluated in 142/187 and 18/23 cases, respectively. Heterozygous variants of AIP gene were detected in 7 of 210 patients (3.3%), two familial and five sporadic cases. A p.R304Q mutation (c.911G>A) and a p.R304 nonsense mutation (c.910C>T) was found in three and one patients, respectively, and a nucleotide substitution in the donor splice site of intron 3 (IVS3+1 G>A) in one patient, and a p.R16H change (c.47G>A) in the remaining two cases. Overall, rs2066853 (c.1661G>A) AHR polymorphism was found in homozygosis in one patient and in heterozygosis in 46 cases (22.4%). Moreover, heterozygous rs4986826 (c.1708G>A) AHR change was detected in six cases with rs2066853 AHR polymorphism (2.9%), one in homozygosis and five in heterozygosis. Among the 23 patients from HR areas, seven showed AHR polymorphisms (30.4%) and two were found with AIP mutations (8.7%). Mean IGF-I levels and pituitary tumor diameter were higher in these nine patients than in the other 14. These patients showed significantly higher IGF-I levels and tumor diameter at diagnosis also in comparison with those from non-polluted areas, regardless of whether they were carriers of AHR and/or AIP variants. SSA treatment normalized both IGF-I and GH levels in none of the mutated patients that live in HR areas, suggesting that acromegaly is more biochemically severe and resistant to SSA treatment in patients living in HR areas, especially if they also carry specific AHR and/or AIP gene variants, as summarized in figure 5.

Figure 5: Panel A, the percentage of patients who normalized IGF-I levels or reduced GH concentrations >50% or normalized both IGF-I and GH levels, stratified on the basis of HR or NP areas where they lived for >20 years before diagnosis and on the basis of the occurrence (VAR+) or not (VAR-) of *AHR* and/or *AIP* variants. Panel B, the differences of IGF-I levels, expressed as x ULN at baseline and after 6 months of treatment in the four groups of patients.



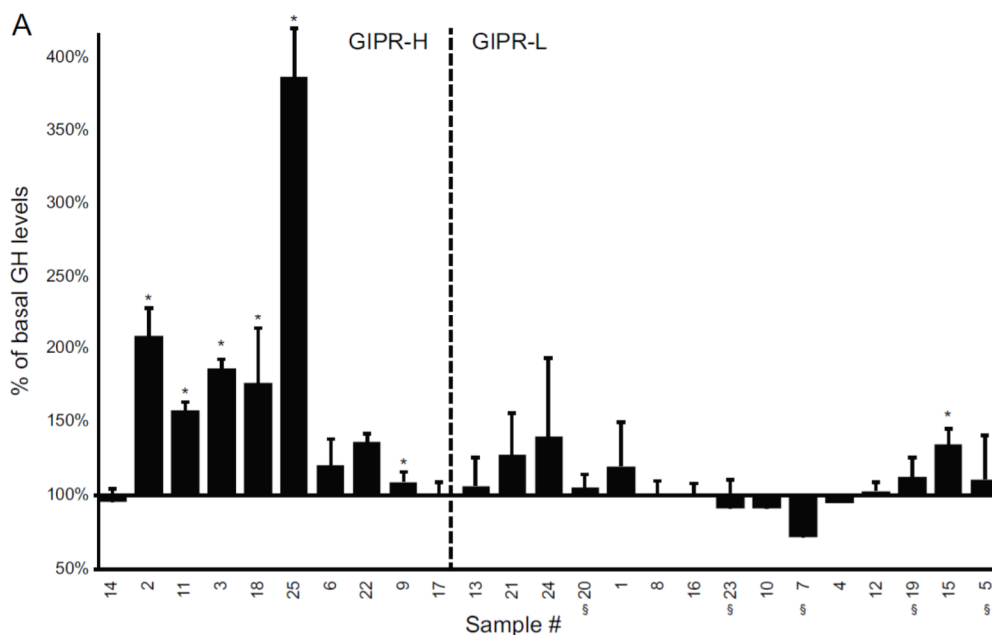
Considering GIP and GIPR pathway in GH-secreting PitNET primary culture, samples were divided into two distinct subgroups: the first comprises 15 samples expressing GIPR within the range for normal pituitaries (GIPR-L; from 1.6×10^{-4} to 1.7×10^{-2} , mean 2.9×10^{-3}), and the second comprises 10 samples expressing GIPR at significantly higher levels (GIPR-H; from 0.02 to 0.27, mean 0.13 ± 0.08). By immunofluorescence we confirmed the high GIPR expression in the adenoma sections in the latter cases, both membrane and cytoplasmic immunoreactivity. Co-localization of red and green staining in the same cell confirmed that GIPR is expressed in GH-secreting tumor cells (figure 6).

Figure 6: Representative immunofluorescence staining of a GH-secreting PitNET with high GIPR expression. (A) GH is visualized in the green immunofluorescent channel, whereas (B) GIPR in the red one. (C) Co-localization of red and green staining in the same cell confirms that GH-secreting tumor cells express GIPR. Cells are counterstained with Hoechst (blue) to mark nuclei. (D) Positive control is a section of a normal human pancreas. Immunofluorescent images are at 100× magnification.



To definitively confirm the link between GIP/GIPR and the GH-PI, GH-secreting PitNET derived primary cultures have been established and cells were treated with GIP. As shown in figure 7A, 8/10 GIPR-H cases significantly responded to GIP.

Figure 7: GH-sec PA-derived primary cultures responsiveness to different stimuli. (A) Cell cultures have been treated with 100 nM GIP or vehicle and GH secretion has been evaluated. The level of GH secretion was expressed as relative percentage to vehicle-treated tumor cells



4.3 Long-term medical treatment in acromegaly and risk of hypopituitarism

(Publication VIII. Ceccato F, Horm Metab Res 2016)

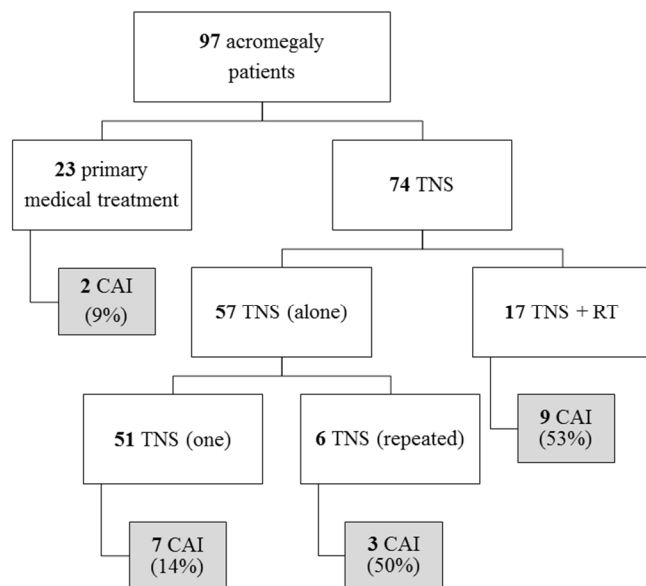
Our cohort of patients with acromegaly is described in table 5, sorted by the presence of Central Adrenal Insufficiency (CAI). The overall prevalence of CAI in our cohort was 22% (21 out of 97 patients), and it was found unrelated to age, gender, age at diagnosis, duration of follow-up or cavernous sinus invasion, although CAI patients had larger adenomas. Two of our patients were excluded from further analyses because presented CAI at acromegaly diagnosis.

Table 5: Clinical characteristics of acromegalic patients with and without central adrenal insufficiency (CAI). Data are shown as medians (IQR) or percentages.

Characteristic	With CAI (n = 21)	Without CAI (n = 76)	p
Age at acromegaly diagnosis (years)	41 (33-51)	49 (38-57)	0.107
Age at time of study (years)	64 (46-67)	60 (51-70)	0.673
Follow-up for acromegaly (months)	192 (84-264)	108 (60-204)	0.122
Female (%)	14/21 (67)	48/81 (59)	0.536
Adenoma max diameter (mm)	21 (15-23)	15 (10-16)	0.01
Pituitary macroadenoma (%)	11/13 (85)	44/65 (68)	0.324
Cavernous sinus invasion (%)	19/21 (90)	57/81 (70)	0.225
Pituitary TNS (%)	19/21 (91)	55/81 (68)	0.053
Pituitary TNS (without RT)	10/12 (83)	47/72 (65)	0.322
SSA (%)	14/21 (67)	50/81 (62)	0.802
SSA as primary treatment	2/2 (100)	19/21 (90)	0.397
SSA after TNS	12/19 (63)	31/55 (56)	0.605
Dopamine agonist (%)	4/21 (19)	17/81 (21)	1.000
Pegvisomant	5/21 (26)	15/81 (27)	0.935
Pituitary RT (%)	9/21 (43)	9/81 (11)	0.001

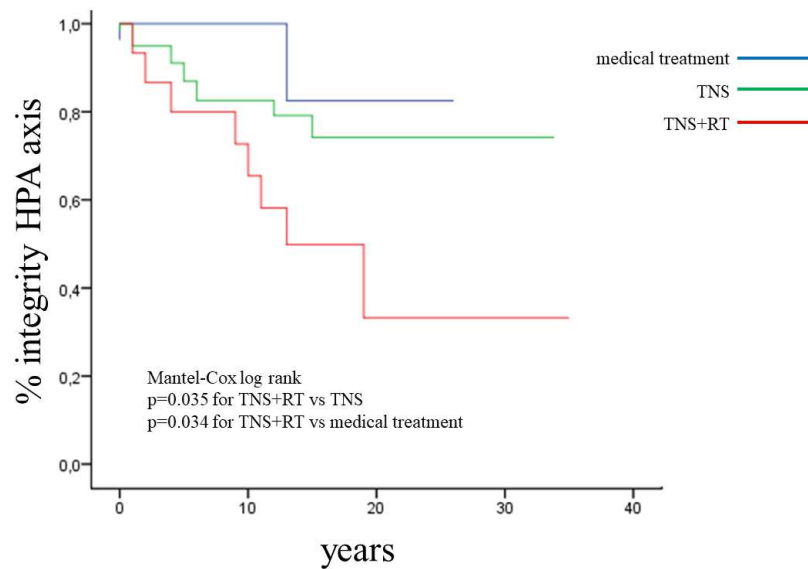
Considering treatments, in our cohort 23 acromegalic patients were treated with primary medical therapy (21 with SSA, 2 with DA; median 8 years; IQR 3.5-12). The other 74 patients underwent TNS (51 once), and among surgical patients 24 assumed medical treatment before TNS (for 3-6 months). Surgical failure was observed in 39% of patients, who then assumed medical therapy (SSA, DA or pegvisomant, alone or combined), 6 repeated TNS and 17 were irradiated (treatment strategies are summarized in figure 8).

Figure 8: Study design and CAI distribution among the various treatments



As regards medication, the use of SSA, DA or pegvisomant was similar for patients with and without CAI, for both primary and post-TNS therapy: the CAI onset rate was lower after primary medical treatment than after repeat TNS ($p=0.002$) or RT ($p=0.003$). Patients who underwent TNS without any subsequent RT revealed no HPA axis damage ($p=0.3$), whereas 50% of patients who repeated TNS developed CAI ($p=0.015$ versus patients who had TNS only once). In our cohort, 9 out of 17 of irradiated patients (after TNS) developed CAI: after conventional RT in 8/13 cases, and after radiosurgery in 1/4 ($p=0.2$). Pituitary irradiation was associated with a higher risk of developing CAI than TNS plus medical therapy, as shown in figure 9 (Mantel-Cox log rank $p=0.035$ for TNS+RT vs TNS, and $p=0.034$ for TNS+RT vs medical treatment). Overall, acromegaly was controlled in 80% of our patients (>90% after ≥ 5 years of follow-up), irrespective of adenoma size (73% of macro-adenomas and 90% of micro-adenomas, $p=0.778$), use of TNS (81% after surgery versus 75% without TNS, $p=0.585$), medical treatment (75% with versus 87% without medical treatment, $p=0.207$), or RT (94% with versus 77% without RT, $p=0.082$). The acromegaly control rate was similar for patients given RT after TNS and those who underwent surgery alone (90% vs 78%, $p=0.118$). Acromegaly control was also similar in patients with and without CAI (86% vs 78%, $p=0.423$), also taking any use of TNS or RT into account.

Figure 9: Life-table analysis indicating probabilities of initially normal HPA axis remaining normal in our cohort by type of treatment.



In a binomial logistic regression analysis performed to calculate the HR of each treatment inducing CAI, RT and repeat TNS carried the highest risk (Table 6), whereas primary or secondary medical treatment were unrelated to the onset of CAI. Among the 17 patients irradiated after TNS, the CAI rate was statistically similar among the 13 patients who had only one TNS (6 developed CAI, 46%) and among the 4 subjects who had repeat TNS before receiving RT (3 developed CAI, 75%, $p=0.312$). The rate of other pituitary deficiencies was higher for the acromegalic patients with CAI than for those judged to have a normal HPA axis ($p=0.001$), especially among the irradiated patients ($p=0.001$).

Table 6: Binomial logistic regression analyses to calculate the role of several variables in inducing CAI. HR: hazard ratio; CI: confidence interval for HR.

	p	HR (95% CI)
Gender	0.404	1.71 (0.48-6.05)
Cavernous sinus invasion	0.452	2.41 (0.24-23.93)
Medical treatment	0.368	1.81 (0.49-6.59)
TNS	1.00	0.00 (0.00-0.02)
Repeat TNS	0.037	5.84 (1.11-30.62)
Radiotherapy	0.044	4.01 (1.04-15.46)

4.4 Metyrapone in ACTH-secreting PitNET (Cushing's Syndrome)

(publication II. Ceccato F, Endocrine 2018)

31 patients with CS were enrolled: 20 with CD, 6 with ectopic CS (EAS) and 5 with adrenal CS (ACS). Median MET treatment duration was 9 months (IQR 3-12 months). All 31 patients were treated for at least 1 month (as indicated in inclusion criteria); 25 patients completed 3 months and 18 patients 6 months of treatment. Considering long-term follow-up, 13 and 6 patients completed 12 and 24 months of consecutive MET monotherapy, respectively. Median MET dose at last visit was 1000 mg (IQR 500-1500).

At baseline all 31 patients presented increased mLNSC; normal mUFC levels were observed in 5 cases (3 of them presented one out of 3 UFC measurement >ULN). mUFC and mLNSC levels decreased after the first month of treatment: median -67% (IQR 55-82) and -57% (IQR 26-80) from baseline, respectively. Data of CD are depicted in table 7. The cortisol reduction continued also in the third month of treatment: median -70% (IQR 54-91) and -63% (IQR 46-83) from baseline (median -43% and -28% from month 1), for mUFC and mLNSC respectively, as depicted in figure 1. Considering all patients, 3 months of treatment were able to normalize mUFC in 68% of patients (64% considering those with mUFC >ULN at baseline, n=14/22); mUFC levels dropped after one and 3 months, achieving a sustained normalization that continued up to 12 and 24 months. MET was able to normalize mUFC levels in the first month of treatment in patients with mild, moderate and severe hypercortisolism, otherwise 3 to 6 months of treatment were needed to control cortisol secretion in patients with very severe hypercortisolism. Considering patients with severe hypercortisolism at baseline (n=10), median mUFC and mLNSC reduction was -86% (IQR 80-92) and -80% (IQR 70-88) after 1 month of treatment, respectively. Overall 71% of patients normalized mUFC levels at the last visit (62% considering those with mUFC >ULN at baseline), independently from the severity of hypercortisolism at baseline.

The MET efficacy to recover cortisol rhythm (37% of patients presented mLNSC <ULN at last visit) was lower than that to normalize cortisol excretion (70% of patients presented mUFC <ULN at last visit). In the long term-follow-up mean mLNSC levels were still >ULN, albeit reduced from baseline. None of the CS patients presented with normal cortisol rhythm at baseline: the complete recovery of cortisol rhythm at the last

available visit was obtained especially in patients with normal mUFC or mild hypercortisolism at baseline. Patients with severe or very severe hypercortisolism required a longer treatment (from 3 to 6 months) to reduce mLNSC levels. Short-term MET therapy (1 month) was able to reduce mUFC and mLNSC; contrariwise, after at least 3-6 months, mUFC normalized in 70% of patients, and half patients showed both mUFC and mLNSC <ULN after long-term treatment (12-24 months, as depicted in figure 2). MET treatment was effective before surgery: mUFC and mLNSC median reduction was -80% (IQR 63-94) and -75% (IQR 52-92) respectively from baseline (both $p < 0.01$, median preoperative therapy 3 months).

Regarding different type of CS, we found that EAS patients were older than CD and ACS, and presented with higher levels of mUFC and mLNSC than CD. MET doses and cortisol levels at last visit were similar among subtypes of CS, nevertheless MET was used as a long-term therapy especially in CD. MET was used before surgery especially in EAS (5 out of 6) and ACS (5 out of 5) compared to CD (6 out of 20, both $p < 0.01$). The outcome of MET therapy in CD was similar when used before or after surgery, despite different follow-up. Clinical and hormonal data were similar considering baseline and last visit in CD patients (table 2), but mUFC normalization rate was higher in CD patients treated with MET after surgical failure (12 out of 14 patients, 86%) than those treated with MET before surgery (1 out of 6 cases, 17%, $p = 0.007$).

An “escape” from MET treatment was observed in 3 patients. Their mUFC levels were normalized after 6 months of therapy (and in 2 cases also mLNSC were <ULN), and the escape was observed before the next scheduled visit (9 months after MET therapy), associated with a worsening of cortisol-related signs and symptoms. We decided not to increase MET in these 3 patients with CD: one performed the first pituitary surgery, the second repeated pituitary intervention (achieving remission), and the third performed stereotactic radiotherapy (her cortisol levels are now controlled with cabergoline and ketoconazole).

On the whole, MET was well tolerated, and none of the patients reported severe side-effects (As reported in the CTCAE version 4.0). Two patients discontinued MET after the first month of therapy (despite both achieving normal UFC levels) for peripheral edema, nausea, asthenia (one patient) and allergic dermatitis with arthralgia (the other one). None of the patients developed adrenal insufficiency during MET treatment.

Table 7: biochemical and clinical results in patients with ACTH-secreting PitNET (CD). Metyrapone (MET) therapy was used as primary medical therapy (n=6) or after surgical failure (n=14, 9 with persistent and 5 with recurrent hypercortisolism after neurosurgery); data are expressed as mean and standard error. Number of patients is represented in brackets. MET dose and months of MET treatment are indicated with median and interquartile range. mUFC: mean of 3 UFC collections; mLNSC: mean of 2 LNSC collections; BMI: Body Mass Index; BP: blood pressure.

		MET before surgery (n=6)	MET after surgical failure (n=14)	p
age (years)		40 (4)	47 (4)	0.207
months of MET treatment		3 (3-6)	12 (12-24)	0.045
MET dose (mg)	baseline	750 (500-750)	500 (500-750)	0.659
	last visit	1000 (500-1250)	1000 (500-1250)	0.779
mUFC (nmol/24h)	baseline	1769 (1248)	558 (228)	0.026
	last visit	204 (36)	165 (69)	0.091
mLNSC (nmol/L)	baseline	29.6 (9.3)	10.9 (2.5)	0.072
	last visit	5.5 (2.6)	4.2 (0.8)	0.981
weight (kg)	baseline	80.5 (7.8)	73.9 (3.8)	0.444
	last visit	78 (6.7)	76 (3.7)	0.718
BMI (kg/m ²)	baseline	26.9 (1.7)	28.9 (1.7)	0.547
	last visit	26.5 (1.9)	29.5 (1.6)	0.391
waist (cm)	baseline	102 (4)	103 (4)	0.904
	last visit	101 (5)	103 (5)	0.893
systolic BP (mmHg)	baseline	136 (3)	135 (5)	0.968
	last visit	142 (6)	140 (5)	0.878
diastolic BP (mmHg)	baseline	90 (3)	82 (2)	0.718
	last visit	92 (5)	90 (3)	0.721

As depicted in figure 10, MET treatment was effective to reduce quickly UFC and LNSC levels in the first month of treatment ($p < 0.005$ with baseline for all visits), achieving UFC normalization after 3 months of therapy. Moreover, extended MET treatment (up to 12-24 months) was able to maintain the achieved results on cortisol excretion. All patients are summarized in figure 11.

Table 8: UFC and LNSC levels indicated in figure 10 in baseline and subsequent visits.

	UFC baseline	UFC 1_month	UFC 3_months	UFC 6_months	UFC 12_months	UFC 24_months
Mean nmol/L	1393	344	167	180	167	104
Standard deviation	1951	500	206	239	93	89
Standard error	461	110	43	60	26	40
	LNSC	LNSC	LNSC	LNSC	LNSC	LNSC
Mean nmol/L	19.8	7.5	5.4	2.8	4.5	4.2
Standard deviation	17.0	6.6	6.8	2.0	4.5	3.7
Standard error	3.9	1.7	1.7	0.6	1.4	1.9

Figure 10: mean UFC levels at each scheduled visit. Bars indicate standard error.

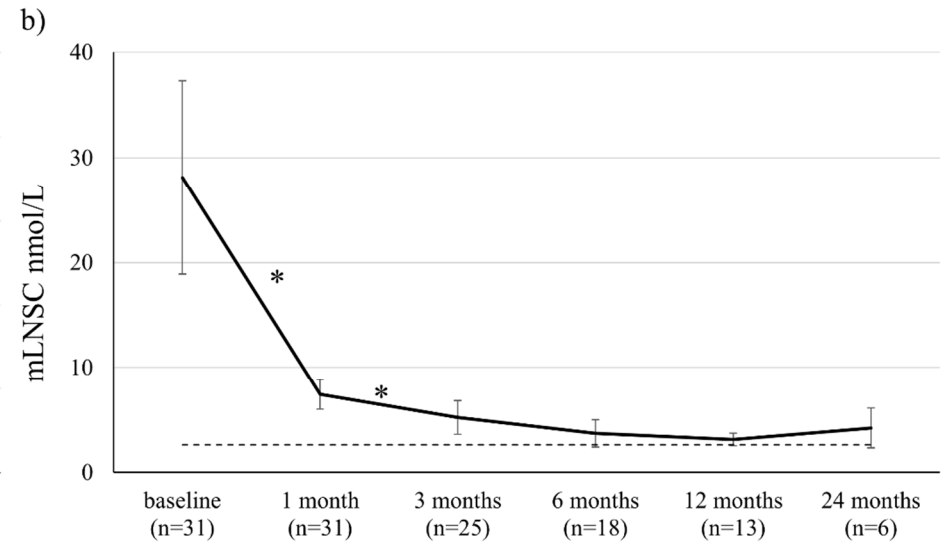
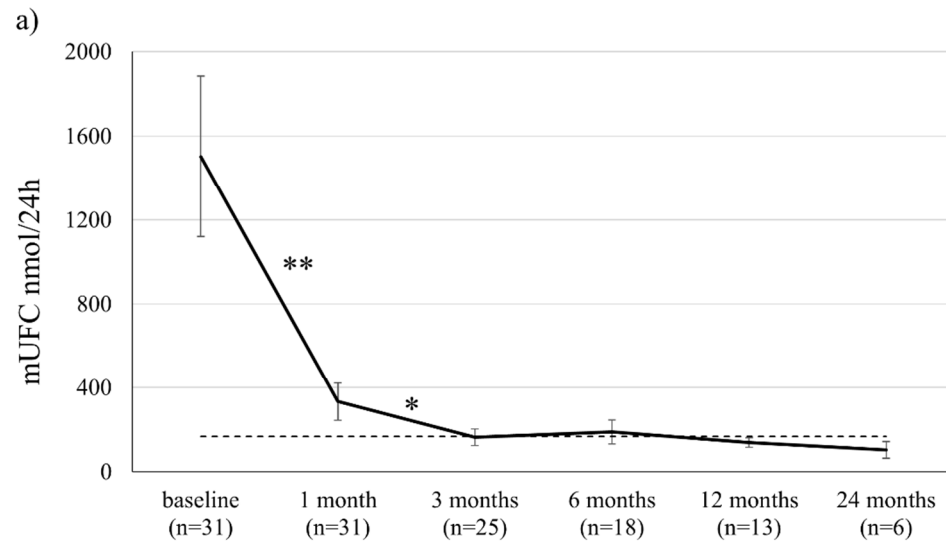
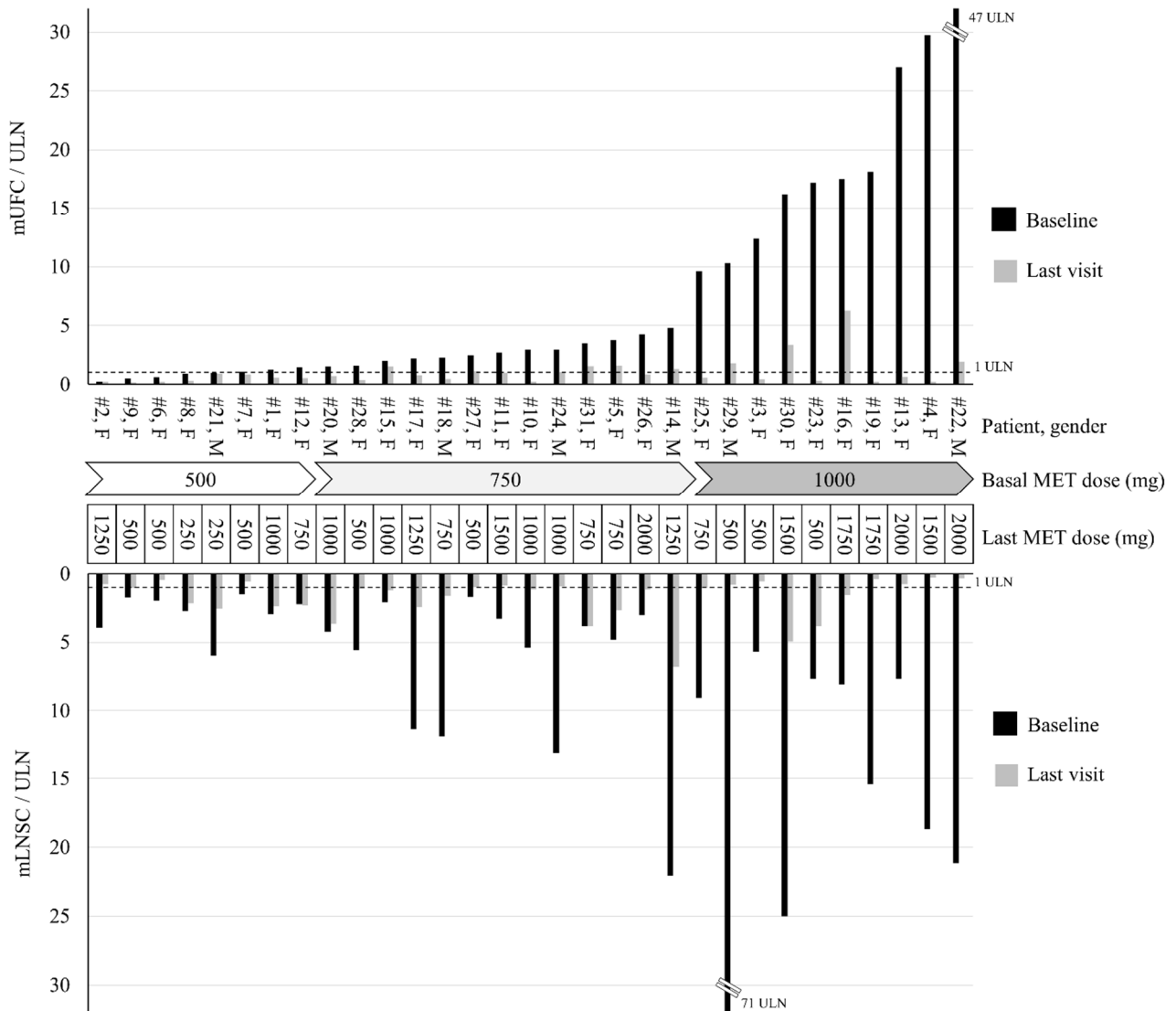
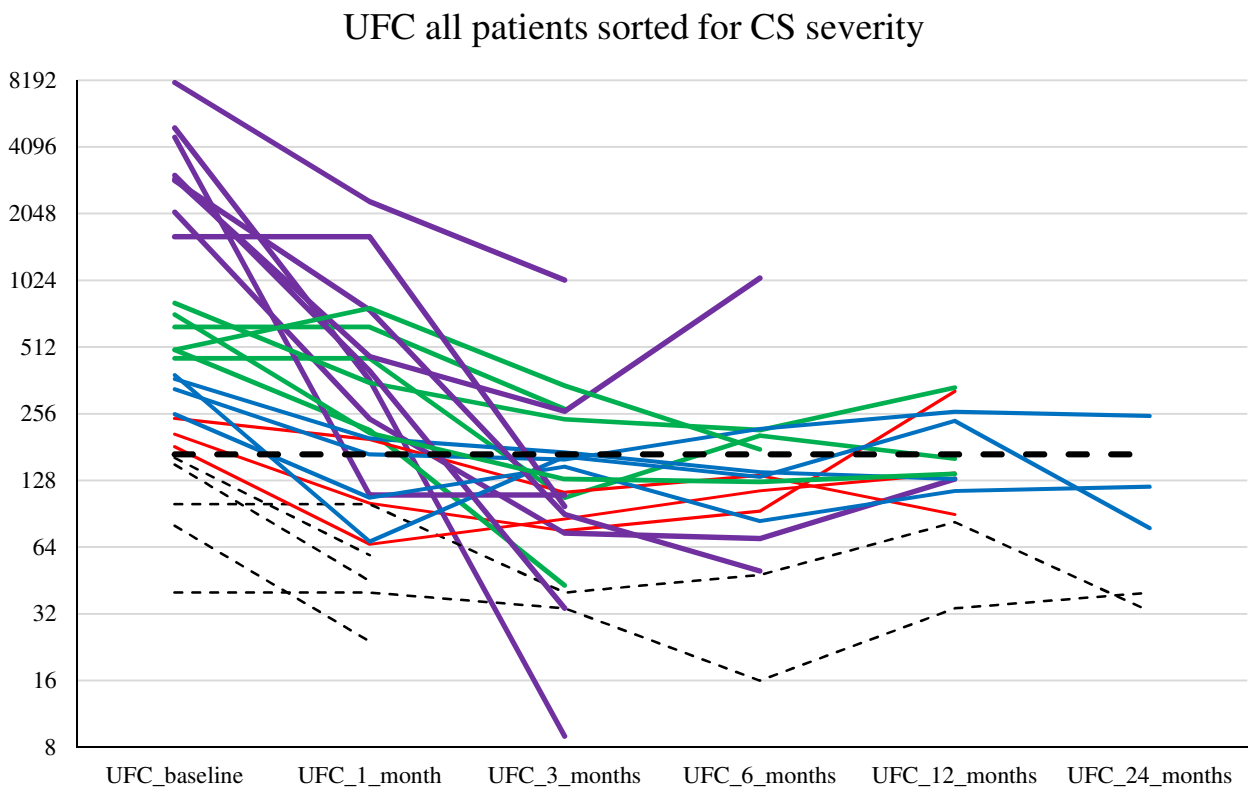


Figure 11: Individual response to MET therapy in each patient, considering baseline levels of mUFC and LNSC (related to ULN). Individual MET dose at baseline and at last visit is indicated between the graphs.



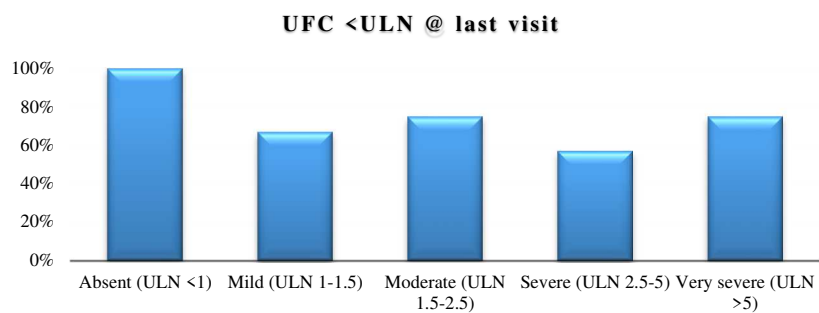
MET is able to normalize UFC levels quickly (in the first month, see figure 12) in the most part of patients with mild and moderate severe, otherwise in patients with severe or very severe hypercortisolism 3 to 6 months are needed to control cortisol secretion.

Figure 12: spaghetti plot of response to MET therapy (each line is a patient) according to severity of hypercortisolism at baseline. Dotted black line = normal UFC; red line = mild hypercortisolism; blu line = moderate hypercortisolism; red line = severe hypercortisolism; purple line = very severe hypercortisolism. UFC is depicted in a logarithmic base 2 scale.



Finally, we observed that disease control (in those patients with increased UFC) at the last available visit was similar considering baseline severity of hypercortisolism, as depicted in figure 13.

Figure 13: Patients that normalize UFC levels at last visit after MET therapy, according to severity of hypercortisolism at baseline visit



MET treatment was effective to reduce quickly LNSC levels (as reported in figure 14), nevertheless it was not able to restore completely circadian cortisol rhythm in all patients, as depicted in figure 15.

Figure 14: response to MET therapy (each line is a patient) according to severity of hypercortisolism at baseline. Blue line = moderate hypercortisolism; red line = severe hypercortisolism; purple line = very severe hypercortisolism.

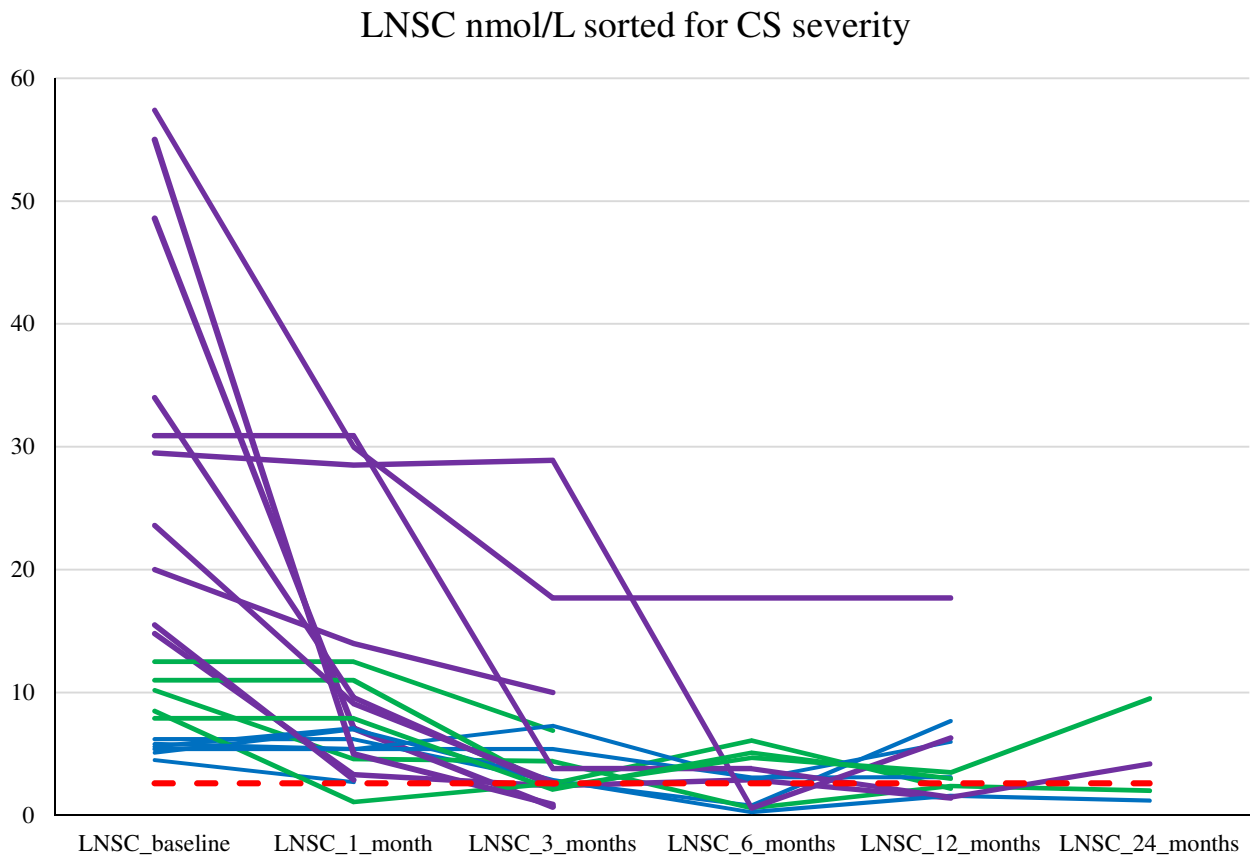
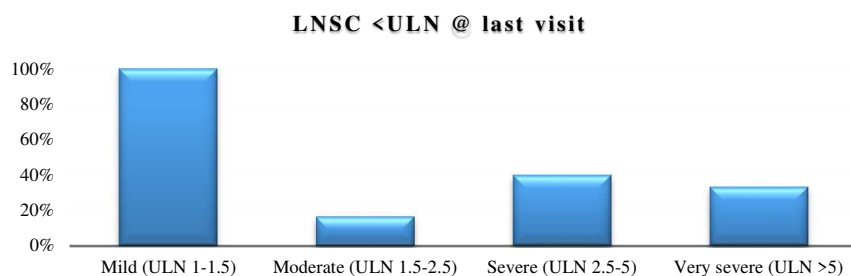
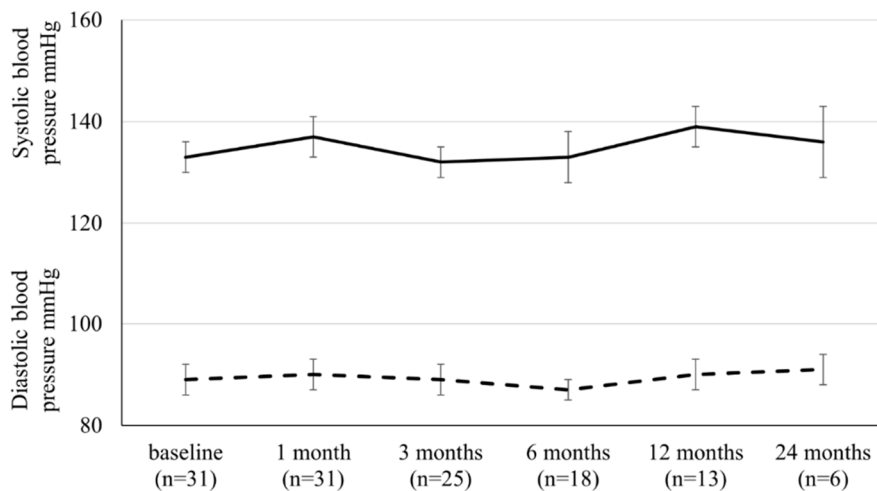


Figure 15: Patients that normalize LNSC levels at last visit after MET therapy, according to severity of hypercortisolism at baseline visit



Before enrolment, 24/31 patients presented hypertension (normalization of blood pressure levels was achieved in 10 subjects). During MET therapy, 2 normo-tensive patients presented an increase of blood pressure levels, controlled with medical monotherapy (ramipril 10 mg in one case and potassium canrenoate 100 mg in the other); 5/24 hypertensive patients needed an up-titration of their treatment and 4/24 hypertensive patients stopped their anti-hypertension drug during the study. Overall, we did not observe any increase of systolic or diastolic blood pressure (see figure 16). Potassium levels were lower at baseline in EAS than in CD (4 EAS and 3 CD patients presented with potassium <3.5 mEq/L). At last visit, most of patients (26/31) presented with normal potassium levels, and none with severe hypokalemia (potassium <2.5 mEq/L). Before enrollment 11 patients presented with impaired fasting glucose or over diabetes, and after MET 7 patients reduced the dose or the number of anti-diabetic drugs.

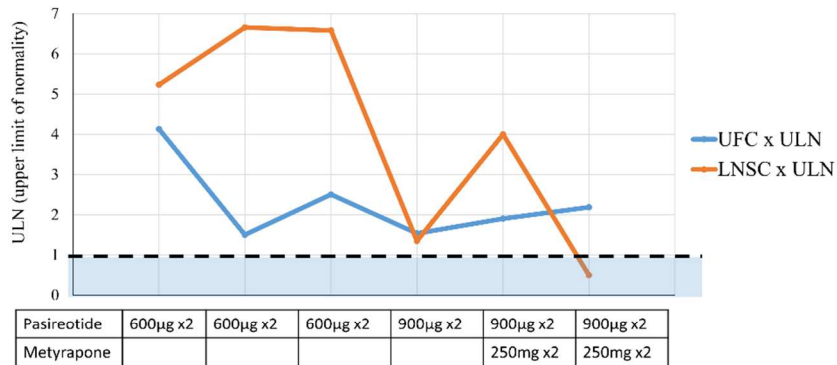
Figure 16: mean systolic blood pressure (solid line) and diastolic blood pressure (dotted line) after MET therapy. Bars indicate standard error.



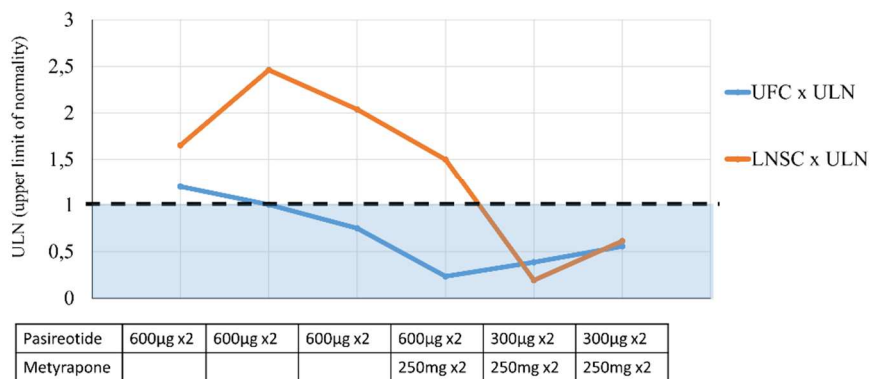
We studied, in 3 patients, the effect of combined PAS and MET treatment.

- Patient 1: female, 32 years, failure of combination therapy: nowadays treated with an experimental compound (LCI699). Figure 17, A
- Patient 2: female, 51 years, efficacy of combination therapy. Figure 17, B
- Patient 3: female, 46 years, efficacy of combination therapy. Figure 17, C

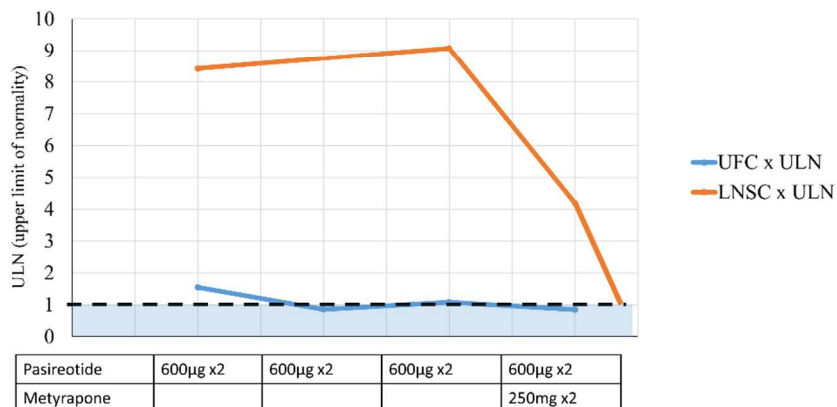
a)



b)



c)



4.5 Differential effects of medical treatment in Cushing's Syndrome

(publication V. Ceccato F, Horm Metab Res. 2017, III. Barbot M, Endocrine 2018)

Currently few papers studied body composition changes in adult patients with CS after achieving remission. Therefore, it is not yet clear whether body composition is normalized after remission of hypercortisolism. Moreover, there are no published data regarding the effects of different treatments on body composition, as surgery or medical therapy. Our study aimed to prospectively evaluate body composition changes with DXA in patients with active hypercortisolism and during remission phase, considering different therapeutic plan. We collected data at baseline (before treatment) and at last available follow-up during remission, mean observation time was 32 months (range 13-86). Overall, as presented in Table 8, patients reported a decrease of BMI and waist circumference after achieving remission (respectively mean -7.6% and -4.5%), coinciding with an improvement in blood pressure and cholesterol levels. In total body DXA scans we found a decrease in total mass (-7.5%), due to a reduction of both lean and fat mass (respectively -3.3% and -12.8%). Lean mass levels (also considering bone mineral content) decreased in total body and R1 box, whereas they did not change at trunk levels. Decrease in fat tissue (both mass and percentage) was observed in total body, trunk and R1 box (respectively -14.4% and -17%). Body composition of CS after remission was similar to matched controls, especially fat mass in all the considered DXA scans (total body, trunk and R1 box).

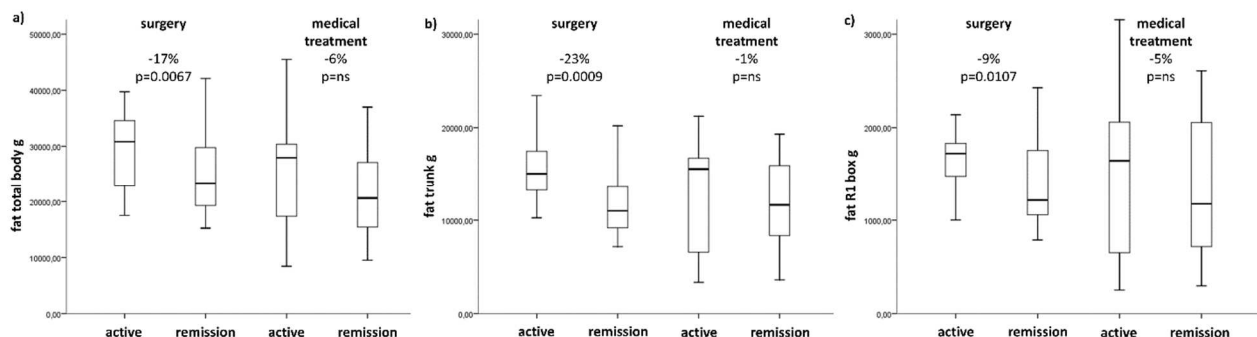
Table 8: Anthropometric and body composition measurements in the whole cohort of 23 CS patients. ^a = p < 0.05 active CS vs controls; ^b = p < 0.05 remission CS vs controls; BMC = bone mineral content

	<i>active CS</i>	<i>remission CS</i>	P (active vs remission)	controls
Anthropometry				
age (years)	46.6 ± 12.2	51 ± 13.4	0.0003	46.7 ± 8.9
BMI (kg/m ²)	27.6 ± 5.3	25.3 ± 3.9	0.0003	26.4 ± 4.6
waist (cm)	102 ± 14	97 ± 12	0.0201	-
hypertension (n)	17 out of 23 (74%)	9 out of 23 (39%)	0.0047	-
diabetes (n)	8 out of 23 (35%)	10 out of 23 (43%)	0.3173	-
glucose (mg/dL)	123.3 ± 58	106.2 ± 31.7	0.5953	-
total cholesterol (mg/dL)	233.4 ± 44.3	209.3 ± 54.2	0.0132	-
metabolic syndrome (n)	8 out of 23 (35%)	3 out of 23 (13%)	0.0588	-
DXA total body				
fat (g)	28855 ± 11198	24148 ± 8620	0.0052	23498 ± 8179 ^a
fat (%)	37.5 ± 8.9	34.6 ± 8.2	0.0249	33.8 ± 7.9 ^a
lean (g)	44060 ± 7221	42660 ± 7678	0.0207	43363 ± 8857
lean + BMC (g)	46047 ± 7479	44584 ± 7948	0.0171	45439 ± 9154
total mass (g)	74902 ± 15508	68731 ± 12537	0.0002	68856 ± 13128
DXA trunk				
fat (g)	15384 ± 6390	12398 ± 4948	0.0041	11520 ± 4979 ^a
fat (%)	38.8 ± 9.6	34.9 ± 8.4	0.0155	32.9 ± 10.7 ^a
lean (g)	22401 ± 3703	21766 ± 4109	0.618	21405 ± 3986
lean + BMC (g)	22922 ± 3724	22289 ± 4123	0.385	21934 ± 4056
total mass (g)	38262 ± 8920	34687 ± 7447	<0.0001	33454 ± 7385 ^a
DXA R1 box				
fat (g)	1747 ± 796	1375 ± 658	0.0046	1182 ± 566 ^a
fat (%)	42.8 ± 10.6	38.8 ± 10.3	0.0289	35.5 ± 12.4 ^a
lean (g)	2109 ± 363	1974 ± 387	0.0046	1760 ± 327 ^{a,b}
lean + BMC (g)	2131 ± 365	1999 ± 949	0.0046	1852 ± 345 ^a
total mass (g)	3878 ± 1097	3374 ± 949	0.0003	3038 ± 812 ^a

At baseline, the 2 groups of active CS patients divided for remission (surgical or pharmacological) presented similar UFC and LNSC levels (respectively p=0.557 and p=0.607) and DXA data (described in table 9, for fat mass p=0.305, p=0.643 and p=0.734 respectively in total body, trunk and R1 box scan). Mean remission follow-up was 39 months in the surgical and 20 months in the pharmacological group (p=0.039). Considering last available follow-up, UFC levels were similar during remission of CS (respectively after surgery and pharmacological treatment: mean 46 vs 80 nmol/24, p=0.27), otherwise LNSC levels were higher during medical treatment (mean 1±0.2 vs 1.8±0.4 nmol/L, p=0.043), despite normal in every patients, since it was an

inclusion criteria. UFC and LNSC levels were similar at baseline, as well as at last available follow up after pharmacological treatment, considering pituitary-directed drugs (n=6) and steroidogenesis inhibitors (n=3). After surgical remission of CS we confirmed the reduction of BMI (-8.3%), waist circumference (-7.4%), glucose levels and the decrease of hypertension and metabolic syndrome, as detailed in Table 2 and figure 18. The decrease of fat mass and percentage after surgery (-17.3%) was confirmed as well as in the overall remission group, considering total body, trunk and R1 box (depicted in figure 18): at the end of the study body composition parameters were similar to healthy matched controls. On the other hand, the reduction of fat mass achieved with pharmacological treatment was inferior, although body composition was similar to controls. Glucose levels increased after pharmacological remission of hypercortisolism, especially in the 6 patients treated with pasireotide; among them, three developed overt diabetes and were treated with insulin (in one patient) and with the combination of metformin+sitagliptin (in two subjects).

Figure 18: DXA results of fat mass in total body (panel A), trunk (panel B) and R1 (panel C) box during active hypercortisolism and remission of CS, divided by treatment.



To the best of our knowledge, there is only one study evaluating the effect of short-term medical therapy in clotting factors¹¹¹, but no data are available on the effects of long-term treatment with cortisol lowering medication on this aspect. Therefore, we evaluated the effectiveness of long-term therapy with PAS 600 µg twice daily in hemostatic alterations in patients with active CD. Despite significant reduction in UFC levels (up to half patients, more than the III-phase trial⁶⁸) none of the coagulative parameters explored showed any significant changes during the therapy, neither at 6 nor 12 months of therapy. No significant differences in coagulative profile were observed between patients with normal and elevated UFC at 6 and 12 months.

Table 9: Anthropometric and body composition measurements in CS patients, considering treatment strategy. p^{CS} : active CS vs remission CS; $p^{controls}$: remission CS vs controls.

	<i>SURGICAL TREATMENT (n =14)</i>				<i>PHARMACOLOGICAL TREATMENT (n =9)</i>			
	<i>active CS</i>	<i>remission CS</i>	p^{CS}	$p^{controls}$	<i>active CS</i>	<i>remission CS</i>	p^{CS}	$p^{controls}$
Anthropometry								
BMI (kg/m ²)	28.1 ± 5.8	25.5 ± 3.9	0.0085	0.353	26.9 ± 4.8	25 ± 4.2	0.0547	0.730
waist (cm)	106 ± 12	98 ± 12	0.004	-	96 ± 15	96 ± 13	0.9453	-
hypertension (n)	11 out of 14 (79%)	5 out of 14 (36%)	0.0143	-	6 out of 9 (67%)	4 out of 9 (44%)	0.1573	-
diabetes (n)	5 out of 14 (36%)	4 out of 14 (26%)	0.3173	-	3 out of 9 (33%)	6 out of 9 (67%)	0.0833	-
glucose (mg/dL)	138 ± 69	94 ± 30	0.001	-	100 ± 24	125 ± 68	0.0391	-
total cholesterol (mg/dL)	233 ± 30	210 ± 45	0.0681	-	234 ± 62	208 ± 46	0.1641	-
metabolic syndrome (n)	6 out of 14 (43%)	2 out of 14 (14%)	0.0455	-	2 out of 9 (22%)	1 out of 9 (11%)	0.5637	-
DXA total body								
fat (g)	30844 ± 10484	24937 ± 8010	0.0067	0.598	25762 ± 12188	22920 ± 9863	0.4258	0.818
fat (%)	40 ± 6.1	35.9 ± 6.8	0.0107	0.385	33.6 ± 11.4	32.6 ± 10	0.8203	1
lean (g)	43005 ± 7025	41853 ± 7303	0.1937	0.571	45700 ± 7632	43917 ± 8518	0.0195	0.645
lean + BMC (g)	44937 ± 7161	43730 ± 7492	0.2412	0.533	47772 ± 8061	45912 ± 8903	0.0195	0.618
total mass (g)	75781 ± 16030	68666 ± 12503	0.0067	0.965	73534 ± 15503	68833 ± 13349	0.0391	0.969
DXA trunk								
fat (g)	16767 ± 5990	12774 ± 4985	0.0009	0.457	13232 ± 6739	11812 ± 5130	0.7344	0.818
fat (%)	41.8 ± 5.3	36.1 ± 6.9	0.0031	0.270	34.2 ± 13.1	33.1 ± 10.4	0.7344	0.788
lean (g)	22200 ± 3780	21345 ± 4022	0.104	0.965	22719 ± 3781	22422 ± 4398	0.4961	0.489
lean + BMC (g)	22736 ± 3769	21892 ± 3999	0.0785	0.975	23211 ± 3861	22907 ± 4477	0.4258	0.465
total mass (g)	39432 ± 9289	34666 ± 7904	0.0002	0.642	36443 ± 8514	34718 ± 7141	0.25	0.645
DXA R1 box								
fat (g)	1845 ± 663	1401 ± 486	0.0107	0.214	1594 ± 992	1336 ± 897	0.25	0.848
fat (%)	45.7 ± 6.3	41.3 ± 7.4	0.0479	0.115	38.3 ± 14.5	34.9 ± 13.3	0.4258	0.939
lean (g)	2102 ± 353	1908 ± 368	0.0031	0.223	2120 ± 390	2077 ± 415	0.5703	0.041
lean + BMC (g)	2123 ± 353	1933 ± 370	0.0023	0.511	2145 ± 404	2102 ± 421	0.5703	0.151
total mass (g)	3968 ± 938	3333 ± 711	0.0012	0.247	3739 ± 1357	3437 ± 1285	0.2031	0.539

4.7 *Early recognition in aggressive PitNET*

(Publication IV. Ceccato F, Acta Neurochir, 2017)

Considering a data lock in middle 2017, we collected only patients with at least 2 years of follow-up after presentation and intervention (in order to analyse solid and reliable data). We considered 102 patients with aggressive PitNET using the combined clinical-radiological-pathological definition proposed in table 1 in the introduction. This consists in 18% of the whole cohort, 57 male and 47 female, median age was 49 years (IQR 42-61), median age at diagnosis was 43 years (IQR 35-54) and median presumed onset of disease was 42 years (IQR 30-53). We identified ACTH secretion in 14 patients (17%), GH in 18 cases (20%), PRL in 23 patients (13%) and 47 cases resulted non-functioning PitNET (31%). As reassumed in table 10, patients with NFPA were older at diagnosis and disease onset than those with ACTH- (respectively $p=0.008$ and $p=0.001$), GH- ($p=0.005$ and $p=0.001$) and PRL-secreting adenomas (both $p=0.001$). Median follow-up (5 years, IQR 2-9) was similar among all patients. Furthermore, patients with PRL- were diagnosed earlier than those with GH-secreting adenoma ($p=0.038$) and the percentage of males was greater among PRL- than ACTH- ($p=0.038$) and GH-secreting adenomas ($p=0.031$).

Overall 75% of patients with aggressive pituitary adenoma presented a radiological diagnosis of invasion; as depicted in table 3, we observed a higher incidence of invasiveness in patients with GH-, PRL- and non-secreting adenoma (respectively $p=0.017$, $p=0.039$ and $p=0.014$). Most of them (69%) presented cavernous sinus invasion (CSI), especially patients with GH-, PRL- and NFPA than ACTH-secreting adenoma (respectively $p<0.001$, $p=0.010$ and $p=0.002$). Remission rate was similar in patients with an invasive adenoma, irrespective of hormonal secretion type.

Table 10: clinical characteristic of disease onset and follow up of patients with aggressive PitNET sorted by secretion; data are reported as median and interquartile range (IQR). M: male; F: female.

	Gender (M/F)	Age (years)	Age of diagnosis (years)	Age of presumed onset (years)	follow-up (years)
ACTH (n=14)	5/9	48 (38-53)	41.5 (33-51.3)	34.5 (23-50)	5 (2-7.3)
GH (n=18)	7/11	46 (42-53)	40.5 (34.5-51)	39 (30.2-45.8)	5 (2.8-9)
PRL (n=23)	17/6	42 (32-47)	34 (24-41)	30 (23-38)	6 (4-10)
Non secreting (n=47)	27/20	61 (50-69)	52 (43-64)	51 (40-64)	5 (2-10)
Total (n=102)	44/46	49 (42-61)	44 (35-53)	42 (30-52)	5 (3-9)

We observed a pathological report consistent with atypical adenoma in 24% of patients, as reassumed in table 11. Considering only those patients with an atypical pituitary adenoma (n=23), remission rate ranged from 30% to 80%, and was higher in patients with PRL- than in those with NFPA (83% vs 37%, $p=0.036$), irrespective of CSI or size of the adenoma. In our series 33% of surgical specimens revealed MIB-1 >3%, with higher prevalence in ACTH- and PRL- than in NFPA (both $p=0.041$); MIB-1 was higher in ACTH- and PRL- compared with GH-secreting adenoma (near significance, respectively $p=0.086$ and $p=0.079$). The rate of radiological invasion was similar among typical and atypical adenomas.

Among 44 patients with NFPA, 21 patients (48%) presented a silent adenoma with positive immunostaining for ACTH (n=6), GH (n=1), PRL (n=2) and FSH/LH (n=12). The rate of atypical adenomas, their radiological invasion and their remission rates (after therapy) were similar among patients with silent compared to those with null-cell adenomas. Atypical adenomas, invasiveness and remission rates were also similar comparing patients with ACTH-silent or FSH/LH-silent and null-cell adenomas.

Table 11: radiological and pathological criteria of aggressiveness in patients with aggressive PitNET. RR: remission rate; CSI: cavernous sinus invasion. Atypical adenoma is considered as that proposed by WHO 2004 (increased or atypical mitoses, Ki67 >3%, positive p53)

	Invasive Adenoma n (%)	RR in invasive adenoma (%)	CSI (%)	RR in CSI (%)	Atypical Adenoma (%)	RR in atypical Adenoma (%)
ACTH (n=14)	6/14 (43)	2/6 (33)	3/6 (50)	1/3 (33)	5/14 (36)	4/5 (80)
GH (n=18)	15/18 (83)	7/15 (47)	12/15 (80)	5/12 (42)	4/18 (22)	2/4 (50)
PRL (n=23)	18/23 (78)	9/18 (50)	12/18 (67)	7/12 (58)	6/20 (30)	5/6 (83)
Non secreting (n=47)	38/47 (81)	14/38 (37)	26/38 (68)	10/26 (38)	8/44 (18)	3/8 (37)
Total	77/102 (75)	32/77 (42)	53/77 (69)	23/53 (43)	23/96 (24)	14/23 (61)

Surgery was the most performed treatment among all patients, especially in those secreting ACTH or GH (see table 12). Considering all cases, surgical remission rate was 24% (23 out of 96), higher in ACTH- than in GH-

and PRL-secreting adenoma (respectively $p=0.049$ and $p=0.026$). Overall 22% of patients were submitted to radiotherapy (in 18 subjects conventional and 4 radiosurgery with cyberknife), with a remission rate of 43% after median 3 years. Radiotherapy was more applied to patients with ACTH- than GH-secreting adenoma ($p=0.03$), the obtained remission rate was similar among the different types of pituitary adenoma evaluated. We calculated also the efficacy of medical-therapy (MT) with pituitary-directed drugs (at least two consecutive years): overall 41% patients presented a controlled disease; MT was used more often in patients with GH and PRL- rather than in those with ACTH-secreting adenoma (respectively $p=0.001$ and $p=0.029$), with similar results on disease control considering the different adenomas. Comparing various treatments, disease control was better achieved with surgery than with MT in patients with ACTH-, as opposed to those with GH- and PRL-secreting adenoma (because in the latter MT was more effective, respectively $p=0.008$ and $p=0.03$). Disease control was similar between MT and radiotherapy.

Table 12: description of treatment in aggressive PitNET; data are reported as percentage or mean and standard deviation (when specified). TNS: Trans-Nasal Surgery, MT: Medical Therapy; na: not available. NFPA were not considered computing total data for MT.

	TNS (%)	TNS per patient	TNS Remission (%)	RT (%)	RT Remission (%)	MT (%)	MT Control (%)
ACTH (n=14)	14/14 (100)	1.6±1.1	6/14 (43)	5/14 (36)	1/5 (20)	6/14 (43)	2/6 (33)
GH (n=18)	18/18 (100)	1.3±0.7	2/18 (11)	2/18 (11)	1/2 (50)	17/18 (94)	8/17 (47)
PRL (n=23)	20/23 (87)	1±0	2/20 (10)	3/23 (13)	2/3 (67)	18/23 (78)	7/18 (39)
Non functioning (n=47)	44/47 (94)	1.5±0.6	13/44 (30)	12/47 (26)	6/12 (50)	na	na
Total	96/102 (94)	1.4±0.7	23/96 (24)	22/102 (22)	10/22 (45)	41/55 (75)	17/41 (41)

4.8 *Temozolomide and Cabergoline in PitNET*

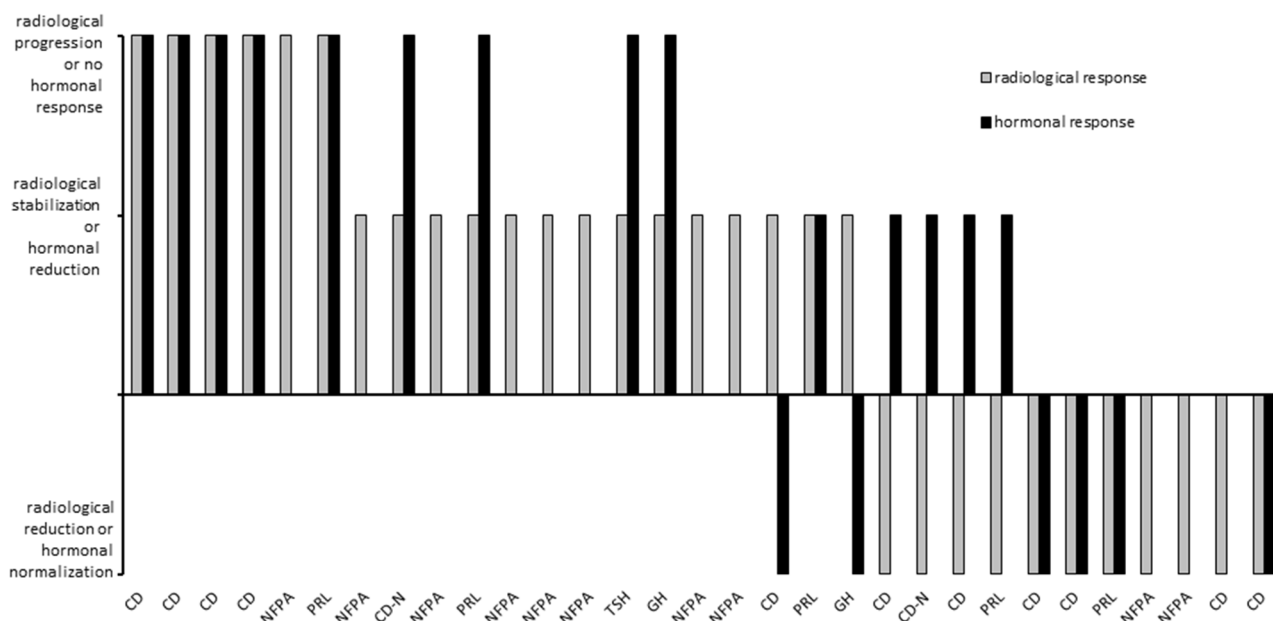
(Publication [IX.Losa M, J Neurooncol 2016](#); New data are unpublished)

In 2015 we performed a national web-based survey study of patients with aggressive pituitary adenomas or carcinomas treated with TMZ, on behalf of the Italian Society of Endocrinology. During the study period, 31 patients received TMZ as a salvage therapy. Mean age at start of TMZ treatment was 58 ± 2 years, ranging from 39 to 78 years. The mean time from diagnosis of the pituitary adenoma and TMZ treatment was 10 ± 2 years (range 1-35 years). 6 out of 31 patients had pituitary carcinoma (metastatic disease) at the time of TMZ treatment. Among the 31 patients, there were 13 cases of ACTH-secreting PitNET (42%; 11 patients had Cushing's disease and the remaining two Nelson's syndrome), 10 cases of non-functioning PitNET (32%), 5 cases of prolactin (PRL)-secreting PitNET (16%), 2 cases of acromegaly (6%), and 1 case of thyrotropin (TSH)-secreting adenoma (3%). Despite previous multiple therapeutic attempts, all patients had evidence of progressive disease at the time of TMZ treatment. In particular, all patients had been subjected to pituitary surgery. Most of patients undergone more than one surgical intervention: 5 patients received one procedure, 13 patients received two procedures, 6 patients received three procedures, 5 patients received four procedures, one patient received five procedures and the remaining patient received ten surgical procedures. Twenty-seven patients (87%) had also been treated with radiotherapy, either fractionated or radiosurgery, before TMZ treatment. Of these, 19 patients received one radiation treatment, 5 patients received two radiation treatments, and the remaining 3 patients received three radiation treatments. The median interval between the last radiation treatment and start of TMZ treatment was 39 months, range 0–396 months. In all cases, except three patients who received TMZ concomitantly with radiotherapy or one month thereafter, the tumor had recurred after the last radiation treatment. Moreover, all growth hormone (GH)- and TSH-secreting adenomas were resistant to somatostatin analogues (SSA) and all PRL-secreting adenomas were resistant to DA.

All patients had at least one MRI three months after beginning TMZ treatment and were, therefore, included in the analysis of efficacy. Figure 19 summarizes the results of TMZ treatment according to the type of pituitary tumor. Overall, 11 patients (35%) had reduction of the tumor, while 6 patients (19%) had progressive disease during TMZ treatment. The remaining 14 patients (45%) had stable disease. Reduction of tumor size occurred

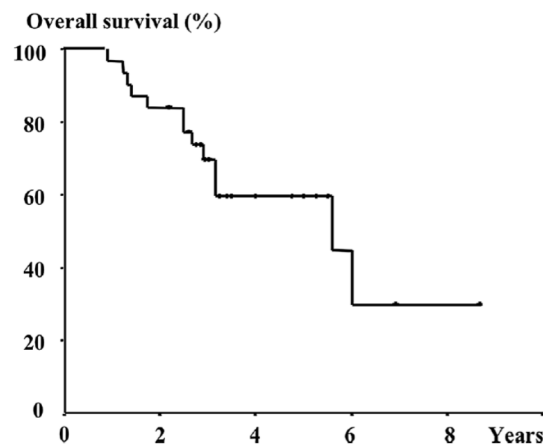
within 3 months from start of TMZ therapy in all cases. Combining patients with tumor reduction and those with stable disease, 25 patients (80%) had disease control during TMZ treatment.

Figure 16: individual responses to TMZ



The median follow-up after start of TMZ treatment was 43 months (IQR, 24–72 months). PFS at 2 years in the entire cohort of patients was 47.7 % (95 % CI 29.5–65.9 %). A more specific analysis on tumor recurrence was done in the 25 patients who had disease control during treatment and was calculated as disease control duration (DCD). At the end of the follow-up period, the tumor regrowth in thirteen patients (52%). The 2-year DCD was 59% (95 % CI 39.1–79.1%). Further treatments in the 19 patients with treatment failure or tumor regrowth were very heterogeneous. At the end of the follow-up period, 13 patients (42%) had died. Cause of death was disease progression in all cases, except two cases. As detailed before, among the 13 deceased patients, 4 cases did not respond to TMZ treatment while the other 7 had recurrence of disease after an initial response to TMZ (3 had a partial response and the other 4 stable disease). The 2-year and 4-year Overall Survival rates were 84% (95 % CI 70.7–97.1%) and 60% (95 % CI 40.0–79.2 %; figure 20).

Figure 20: Kaplan–Meier analysis showing overall survival in 31 Italian patients that received TMZ treatment for a PitNET



On the basis of our data above-reported regarding aggressive PRL-secreting PitNET (medical treatment is more effective than surgery), recently we treated a patient with an aggressive giant PRL-secreting PitNET with a primary combined treatment with CAB and TMZ. A 47-year-old man presented to Emergency Department for visual defect and transient global amnesia. Cerebral MRI revealed a giant polylobate mass extending in the middle skull base, with irregular margins and median localization, occupying the sellar region. The neoplasm caused the stretching of the pituitary stalk and compression of the optic chiasm up to the third ventricle. Hormonal evaluation confirmed a PRL-secreting PitNET with secondary hypogonadotropic hypogonadism: basal PRL 142500 ng/mL (normal value n.v. 4.6-21.4), testosterone 3.1 nmol/L (n.v. 9.7-38.2), TSH 1.91 with FT4 1.67 (n.v. 0.9-1.7), morning serum cortisol 350 nmol/L (676 nmol/L after 1 μ short synacthen test). Menin gene was wild-type. Medical treatment with dopamine-agonist (cabergoline 1.5 mg/week) was started, after 6 weeks PRL levels were normalized (11.4 ng/mL). Despite the recovery of visual field in 2 weeks, a pituitary MRI after 3 months of cabergoline treatment revealed a not-significant size reduction of the PitNET. Therefore, the drug was increased up to 3.5 mg/week; after 6 months of treatment the PRL levels were suppressed (1.3 ng/mL) with recovery of testosterone secretion, while MRI revealed a stable pituitary mass. The case has been discussed in the Pituitary Multidisciplinary Team: a gross-total resection was not feasible, and rejected by the patient. Therefore, we proposed a primary neoadjuvant cytoreductive TMZ treatment.

After obtaining patients' written consent, first cycle of TMZ was administered at 150 mg/m² for 5 days every 28 days, then 200 mg/m² for 5 days every 28 days were scheduled for 13 cycles (combined with cabergoline 1.5 mg/week). Pituitary MRI was scheduled every 3 months. The patient did not report TMZ-related adverse

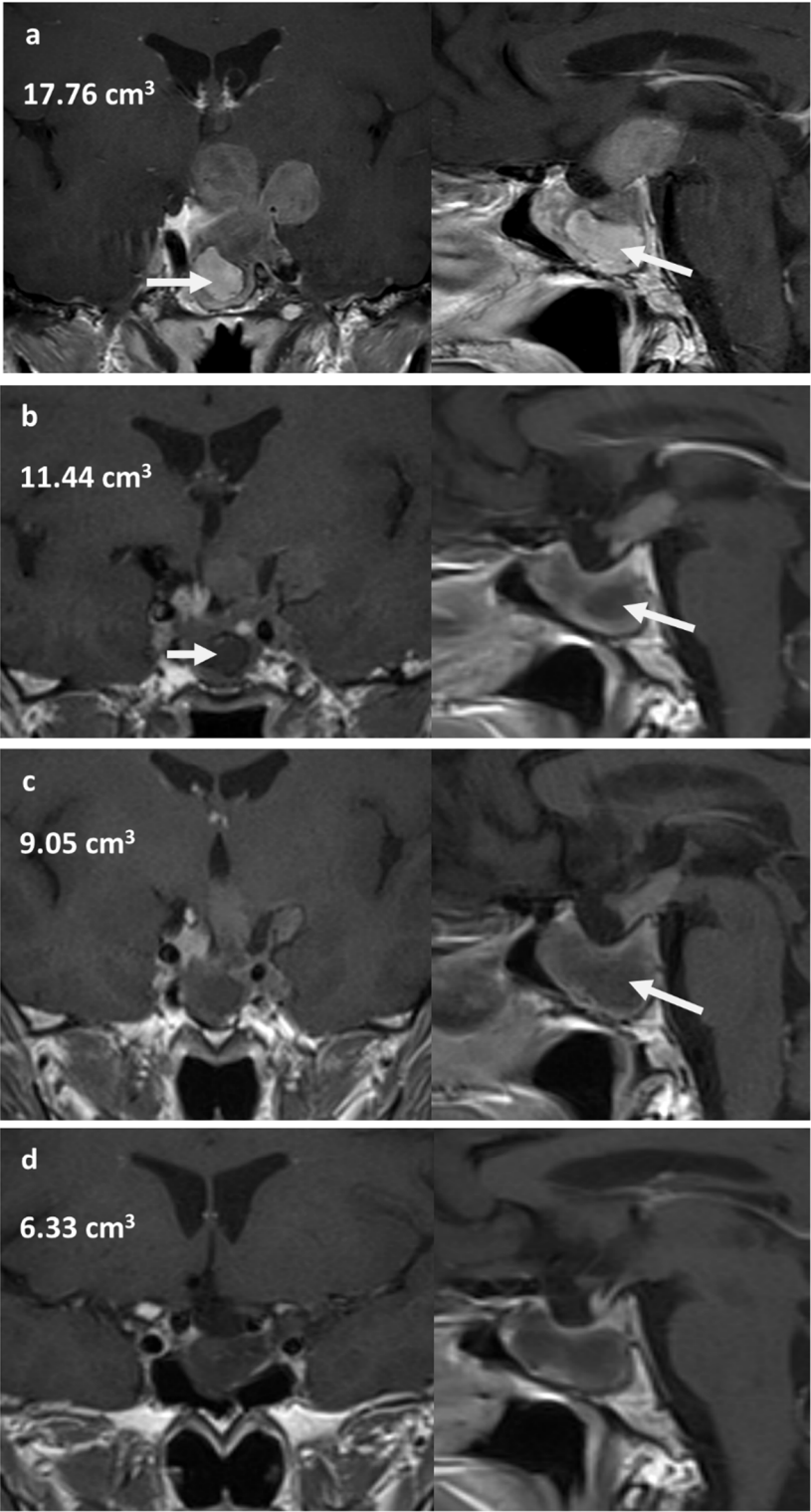
levents and no new-onset pituitary deficiencies were observed during and after treatment. During the combined treatment (TMZ and cabergoline), PRL levels remained suppressed, and a dramatic reduction in the size and changes in the radiological features of the PitNET were observed.

The volume of the mass was measured before, during and after TMZ discontinuation. As reported in figure 21a, after 6 months of cabergoline and before TMZ treatment the PitNET revealed a maximum extension of 6cm and a small hemorrhagic area (the compression of the optic chiasm and third ventricle and the stretching of the pituitary stalk were similar to baseline MRI). After 3 cycles of TMZ we observed a reduction of the overall sizes of the pituitary adenoma with regular evolution of the methaemoglobinic components and enlargement of the necrotic area, leading to an overall reduced compression of the third ventricle (fig 21b). After 6 cycles of TMZ the shrinkage of the adenoma persists, with disappearing of the methaemoglobin, and increase of the necrotic area (figure 1c). In figure 1d we show that after 9 cycles of TMZ a large necrotic area appeared, with a little remaining of tissue's enhancement in the carotid sinus. MRI performed during follow-up (4 and 10 months after TMZ discontinuation) revealed a stable necrotic tissue (5.9cm³) with a minimal area of tissue enhancement (0.5cm³, table 13).

Table 13: Prolactin-secreting PitNET volume before and after TMZ

date MRI	TMZ treatment	Total Volume (cm ³)	Total Volume without necrosis (cm ³)
28/01/2016	Before TMZ	17.76	15.1
05/08/2016	After 3 months (3 cycles)	11.44	9.18
04/11/2016	After 6 months (6 cycles)	9.05	3.15
10/02/2017	After 9 months (9 cycles)	6.33	0.7
21/06/2017	After 12 months (13 cycles)	5.99	0.5
03/11/2017	4 months after TMZ discontinuation	5.94	0.5
03/06/2018	12 months after TMZ discontinuation	5.9	0.5

Figure 21: Pituitary MRI and adenoma's volume before TMZ (panel a), and after 3, 6 and 9 cycles of TMZ (respectively panel b, c and d).



5. Discussion

Considering our results, a significant proportion of patients with PitNET could benefit from a complete clinical and molecular characterization, in order to personalize their therapeutic plan.

Everolimus in PitNET

As far as we know, we first attempt to evaluate by a molecular approach the involvement of a TSC-related gene variant in the pathogenesis of the PitNET. Considering the c.4006-71C>T variant causative for TSC – that is reasonable given the frequency of intronic mutations in TSC (i.e. 5%)¹⁰³ and its absence in the normal population – we could exclude the involvement of pituitary gland alterations in the pathological process of TSC. On the other hand, however, until functional data on this variant has not been gathered, we cannot exclude possible alternative genetic mechanisms causative of TSC including mosaic mutations in *TSC1* regulatory regions – the mosaicism would easily explain the mild phenotype of our patient¹⁰³ – or an additional TSC causative gene. Nearly 10-15% of TSC patients lack, indeed, a conclusive molecular diagnosis^{101,102}. Although relevant, our data are thus not conclusive for establishing unequivocally the causal nature of the association between TSC and PitNET that might only be a coincidence due to the relative high prevalence of pituitary tumors in the general population¹.

Although everolimus has been successfully tested in non-functioning and GH secreting PitNET cellular models^{110,112,113}, very few data are available on its use in PitNET patients. Everolimus has been indeed used for treating only two patients with pituitary carcinomas resistant to repeated resections, radiations and combined treatment with chemotherapeutic agents – i.e. TMZ and Capecitabine – with limited effects^{114,115}. Our data corroborate the efficacy of everolimus in reducing cell viability in non-secreting PitNET, and support the need of further clinical studies for demonstrating its efficacy and safety for treating this group of tumors.

Prediction of response to SSA in acromegaly

The current treatment algorithms for acromegaly are based upon a “trial-and-error” approach with additional treatment options provided when disease is not controlled. In many other diseases, therapeutic algorithms have been evolving towards personalized treatment with the medication that best matches individual disease characteristics, using biomarkers that identify therapeutic response⁹¹. Surgical success is affected by the size of the tumour and the skill of the surgeon, as well as the type of surgical procedure used. Patients with a microadenoma (<1 cm) may reach a 90% surgical cure rate; conversely, patients with a macroadenoma (>1 cm) and especially those with extrasellar extension have <50% chance of surgical remission^{3,7,80,91}. Most cases that are not surgically cured are treated with a SSA, and response is evaluated after a few months. Unfortunately, treatment failure to control GH and IGF-1 secretion with first-generation SSA (octreotide and lanreotide) may occur in approximately half patients⁸⁴. Currently, predictive factors of response to SSA include the following: age, SSTR phenotype, AIP expression, Ras-Raf-MEK-ERK1/2-p27 pathway, G-protein-linked receptor mutations, densely granular histological pattern, T2-weighted MRI signal of the adenoma and initial and residual (after surgery) size of tumour as previously indicated^{20,91}. Since none of the aforementioned tools is able to completely predict the response to SSA, it is crucial to define also new predictive markers. In such scenario, we studied genetic variants (as AIP) and paradoxical response of GH after OGTT.

Regarding genetic variants, we participate in a large multicentric Italian study in order to collect genetic variants and the relationship between pollution and GH-secreting PitNET. We observed that acromegaly is more biochemically severe and resistant to SSA treatment in patients from highly polluted areas, especially if they also carry specific AHR or AIP gene variants. The AHR is a transcription factor belonging to the basic helix-loop-helix/Per/ARNT/Sim family and is the only one that is activated by a ligand. It is stimulated by several natural compounds that are present in food such as indoles and flavonoids, or by tryptophan derivatives. The most potent AHR ligand known so far is dioxin (TCDD) but more than 400 exogenous compounds act as AHR ligands, most of which are environmental endocrine disruptors, including polycyclic aromatic hydrocarbons^{27,29}.

Considering response to SSA treatment, most of the proposed biomarker are studied in the adenoma's cells, therefore surgery is mandatory to predict the outcome, in a non-sense vicious circle. Therefore, it is of utmost importance to develop new biomarker that are not related to surgery, ideally able to predict the response. The

GH response after OGTT, the hallmark of acromegaly diagnosis, could be one of this. We confirmed that impaired expression of a functional GIPR could promote the GH-paradoxical response in a proportion of acromegalic patients. GIP plasma levels could be high enough to chronically trigger adenylyl cyclase/cAMP signaling³⁷. In addition, the stimulatory effect of IGF-1 on GIPR promoter activity in STC-1 may suggest the possible presence of a self-sustaining GH/IGF-I/GIP axis in these patients. Food dependency indeed might characterize the first phase of the disease and prolonged exposure to high circulating GH levels might induce persistently elevated GIP levels that continuously trigger the adenylyl cyclase/cAMP signaling cascade in the GIPR overexpressing GH-secreting PitNET.

Long-term effects of medical treatment in acromegaly

We considered the long-term effects of available treatments for acromegaly (medical therapy, surgery, radiotherapy), especially regarding their impact on the pituitary function. Hypopituitarism severe enough to require replacement therapy¹¹⁶ can occur both before and after treatment for acromegaly as a result of the adenoma damaging the pituitary gland, or secondary to the effects of the different treatments on pituitary cells^{7,117}. The impact of central adrenal insufficiency (CAI) in acromegaly is still not entirely clear because some authors reported its occurrence only in patients who had undergone TNS, with or without medical treatment, and others excluded irradiated patients from their analyses¹¹⁷⁻¹²⁰. A higher mortality rate in irradiated acromegalic patients and in those who developed CAI has also been reported¹²⁰. There is still a shortage of data on other aspects, such as the effect of long-term primary medical treatment on hypothalamic-pituitary-adrenal (HPA) axis function. We describe a large series of consecutive, unselected patients who could access all available treatments, with median follow-up of 13 years. We found a high overall prevalence of CAI (22%), similar to that previously reported^{117,120}. Other authors reported a lower prevalence of CAI, close to 10% of all patients^{118,121}. We also studied the effect of medical therapy both as primary treatment (when TNS was contraindicated⁸³) and after TNS proved unsuccessful or while awaiting the effects of RT. Two patients developed CAI after primary medical therapy with SSA: although there have been reports of somatostatin inhibiting ACTH secretion¹²², SSA treatment did not affect HPA axis integrity in our cohort. In the present study we considered octreotide or lanreotide, but not pasireotide (the latest SSA to become available for acromegaly), so it will be interesting in future to examine the effects of this last drug on corticotroph cells,

given its greater affinity for SSA receptor 5, and consequent potential effect on ACTH secretion⁵⁴. To our knowledge, there is currently no data available on the risk of cabergoline (and other DAs), or pegvisomant inducing CAI in acromegaly, nor any information on their role after unsuccessful TNS^{87,88}. Our acromegalic patients who underwent TNS only once did not have a higher rate of CAI than those in medical treatment, providing they received no RT. A repeated surgery was clearly associated with the onset of CAI, harboring a 5-fold higher risk. As expected, pituitary RT coincided with a 4-fold higher risk of CAI. This risk effect increased over time, the delay probably being related to a progressive fibrotic degeneration and/or vascular remodeling after irradiation, which can result in of HPA axis derangement. The Kaplan-Meier curve for HPA axis integrity showed that RT correlated with CAI more closely than TNS or medical therapy. The onset of CAI in irradiated patients was unrelated to the number of TNS performed before RT, whether acromegaly was controlled or not. Judging from our results, we would suggest medical therapy (wherever possible) after unsuccessful surgery, rather than RT, with a view to preserving the HPA axis, bearing in mind that both RT and ACTH deficiency are strongly related with a higher mortality rate in acromegaly¹²⁰.

Medical treatment in Cushing's Syndrome

Considering patients with CS we evaluated, in an observational and prospective study (all available studies in literature are retrospective^{58-60,123}), the efficacy of MET to control hypercortisolism (in term of both UFC and LNSC). We used LC-MS/MS to routinely measure cortisol levels since 2013, because this method is accurate (cross-reactivity with other steroids is reduced) and cost-effective (when large numbers of samples are analyzed). We reported a consistent (-67% of mUFC and -57% of mLNSC) and fast reduction of cortisol levels after the first month of MET treatment, that continued up to the third month, achieving mUFC normalization in about 70% of patients, with a median dose of 1000 mg. MET was effective also in long-term treatment, especially after surgical failure: 13 patients continued MET for one year and 6 up to two years (one primary treatment in an occult EAS), without reporting severe AEs and achieving a sustained mUFC normalization. We observed a response to MET higher than previously described: UFC normalization rate was 57% and 43% of subjects (respectively reported by Valassi *et al.* and also Daniel *et al.*), describing MET doses similar to our cohort^{59,60}. Our higher response rate to MET could be related to the design of the study: we treated patients with planned doses of MET based upon severity of baseline hypercortisolism, and up-titrated until UFC

normalization. The stratification of CS severity, based upon baseline mUFC levels, could be useful to choose the correct starting dose and to reduce AEs. As previously reported, we confirmed that patients with CD after surgical failure might present with a mild hypercortisolism^{108,124,125}. MET was able to normalize mUFC levels especially in patients with mild and moderate hypercortisolism after one month of treatment. At baseline 7 CS patients revealed mild cortisol excess (mUFC <1.5-fold ULN, being normal in 5 cases), and most of them were CD patients with recurrent or persistent hypercortisolism after surgical failure (pseudo-CS was excluded in all patients, as reported in inclusion and exclusion criteria). They were all characterized by impaired cortisol rhythm: they should be treated to reduce their cortisol-related comorbidities (as recently described in patients with normal UFC¹²⁶). In selected CS patients with severe hypercortisolism or cortisol-related critical illness, hormonal control must be attempted first, as soon as possible after CS diagnosis^{123,127}. In our series 10 patients presented with very severe hypercortisolism at baseline (3 CD, 2 ACS and 3 EAS): they started with 1000mg of MET. In patients with severe hypercortisolism median observed mUFC and mLNSC reduction after 1 month of treatment was -86% and -80%, respectively, and 7 out of 10 presented with normal UFC at last visit (after median 3 months). Therefore, in patients with severe CS a prompt treatment with MET is able to reduce hypercortisolism quickly before definitive therapy, i.e. surgery, if feasible. As expected, patients with EAS presented higher cortisol levels at baseline, as previously reported^{109,125}, nevertheless their prompt decrease of cortisol levels and their required MET dose were similar to that for CD and ACS patients. The length of MET treatment was lower in ACS and in EAS (median 3 months, vs 12 months in CD), because in our series MET treatment was used before the surgical removal of cortisol- or ACTH-secreting neoplasm, in order to reduce quickly the hypercortisolism (and to reduce the cortisol-related surgical risk, i.e. infection or thromboembolism¹²⁸). The recovery of circadian cortisol rhythm (mLNSC <ULN at last visit in about 40% of patients) was not as satisfactory as the normalization of daily cortisol excretion (mUFC) during medical treatment, as previously reported^{168,72,73,129,130}. The importance of the circadian cortisol rhythm preservation in driving physiological processes is critical: several metabolic alterations are common among subjects with increased cortisol levels in the evening¹³¹, and increased LNSC is a common marker of endogenous CS^{132,133}. In our cohort, the combined end-point (control of both cortisol secretion with UFC and rhythm with LNSC) was achieved in half patients (30% after 3 months and 50% after 12 months of therapy). Regarding AEs, MET was well tolerated, and none of the patients reported severe side-effects. Mild nausea and gastrointestinal pain

were the most reported, two patients discontinued MET after the first month of therapy (despite mUFC normalization), none of them developed adrenal insufficiency. We observed an increase in androgen levels in 10 female patients, 5 complaining hirsutism (treated with spironolactone).

Long-term effects of medical treatment in Cushing's Syndrome

We also evaluate the effect of medical treatment on some clinical parameters of cortisol-related comorbidities. We prospectively studied body composition changes in patients with CS, evaluated from baseline (during active hypercortisolism) to remission (at least 6 consecutive months of both UFC and LNSC normal levels), considering the differential outcome of surgical or pharmacological treatment. Although it is well known that body composition is impaired in active CS (especially increased visceral fat mass)^{74-76,79,134}, contrasting data are reported about body composition's changes after long-term remission. Several imaging techniques have been proposed to assess body composition (DXA, magnetic resonance, computed tomography, bioelectrical impedance analyses); however, in routine clinical practice, DXA is an accurate and precise exam that exposes patients to a low dose of radiations. Moreover, it is easily available, worldwide used and allows the estimation in the same scan of body composition and bone mineral content (that is useful also to stratify fracture risk in CS). Finally, DXA evaluates visceral adipose tissue: we considered R1 box (as proposed by Snijder), which is as accurate as computed tomography to measure visceral fat and to predict cardiovascular risk^{135,136}. At our best knowledge, this is the first prospective study that consider body composition, evaluated with DXA, in a group of patients with hypercortisolism achieving stable remission with different treatment plan. We collected 23 patients, evaluated for about 3 years, whose remission was obtained with surgery (n=14) or with pharmacological treatment (n=9). In the whole cohort considered, remission of CS led to an improvement of metabolic syndrome's features (BMI, waist circumference, dyslipidaemia and blood pressure), confirming that successful treatment of hypercortisolism improves cardiovascular risk¹³⁷. Treatment plan of CS presented different body composition outcomes: we observed an improvement in cardiovascular risk factors (especially BMI, waist circumference, hypertension, glucose levels and metabolic syndrome) and in body composition (mostly fat mass, in all DXA scans) after surgery. We observed an increase in glucose levels during pharmacological therapy: this is an expected side effect during pasireotide treatment, despite anti-diabetic therapy^{55,68}. LNSC levels of the pharmacologically treated group were higher than after surgical remission,

despite normal. Nevertheless, in our clinical practice we measure cortisol with LC/MS-MS and we adopt selected thresholds in order to enhance sensitivity, to exclude also subtle cortisol secretion. In our paper follow-up was longer after surgery than medical treatment, and probably in the latter group the observation period after cortisol normalization was not sufficient to achieve a modification of body composition.

Another interesting aspects is the coagulation in CS patients. The recently published Endocrine Society Guidelines about hypercortisolism treatment focused the attention on prevention of possible complications of CS including thrombotic events⁶. The thrombophilic state induced by glucocorticoids excess is indeed one of the main determinants of increased morbidity and mortality in CD¹³⁸. The basis of this coagulopathy has not been fully understood; the main alteration is represented by an increase in clotting factors, especially factors VIII and von Willebrand (vWF), which causes an elevation in thrombin generation and consequent fibrin thrombus formation¹³⁹. This alteration of clotting factors involves the intrinsic pathway of coagulation, producing a shortening in activated partial thromboplastin time (aPTT). The concomitant increase in endogenous anticoagulants, reported in some papers, seems unable to balance the pro-coagulative tendency^{140,141}. Moreover, also the fibrinolytic system has been found to be impaired in patients with hypercortisolism concurring to determine the high thrombotic risk of these patients^{138,142}. Therefore, we evaluated the effectiveness of long-term therapy with PAS in hemostatic alterations in patients with active CD. Despite reduction in UFC levels (higher than that reported in previous clinical trial^{67,68}) none of the coagulative parameters explored showed any significant changes during the therapy, neither at 6 nor 12 months of therapy. No significant differences in coagulative profile were observed between patients with normal and elevated UFC at 6 and 12 months. The scarce restoration of coagulative parameters might depend on persistence of typical alterations of CD such as obesity and hypertension and, in our series, might reflect also on the worsening in glycemetic metabolism induced by PAS.

Medical treatment in aggressive PitNET

Aggressive PitNET are rarely identified at an early stage in routine clinical practice, representing therapeutic challenges for endocrinologists and neurosurgeons. Therefore, we decided to propose a combined and comprehensive definition of aggressive pituitary adenoma, in order to overcome some previously definition (ie atypical adenomas, invasive adenomas and so on), that could explain only partially the aggressiveness of

an adenoma. Other than atypical nature or local invasiveness, we considered other features of adenoma that could describe their clinical aggressiveness. As expected, patients with aggressive non-functioning PitNET were older: the diagnosis might be delayed until mass-symptoms occur in the former cases, while in case of endocrine secretion the signs or symptoms related to hormonal hypersecretion might receive a prompt recognition by physicians. Two thirds of patients revealed CSI, especially PRL-secreting adenomas; nevertheless, it is worth remembering that aggressive forms are a small part of all PRL-secreting pituitary adenomas, since they are both controlled with dopamine agonists and early diagnosed⁸. Moreover, medical treatment could be proposed to patients with aggressive PRL-secreting PitNET; in order to achieve a better result¹⁴³. In our cohort 25% of patients with aggressive adenoma presented an “atypical adenoma” as that proposed in the previous WHO definition of 2004, with an observed prevalence similar among all types. Overall the remission rate of atypical adenoma was 61% (confirming previously reported data⁴⁵). Therefore, we confirm that the definition of atypical adenomas was not predictive of worse outcome, thus it should not be longer used in clinical practice as recently underlined in the new WHO classification of pituitary tumors^{52,53}. Silent adenomas are nowadays considered as a “high risk” category for aggressiveness^{44,45,53}. Obviously, since the diagnosis of silent adenoma is based upon immunohistochemistry after surgery, this marker could not be considered as a predictive tool of outcome before surgery. All considered characteristics of aggressiveness were similar among silent adenomas and NFPA, even analyzing separately silent corticotropinomas and gonadotropinomas. We also considered the efficacy of 2 years of consecutive MT with pituitary-directed drugs in patients with secreting aggressive adenoma. Less than half of the patients presented a well-controlled disease with MT. It has to be noticed that MT was used more frequently in patients presenting aggressive GH and PRL-secreting than in those with ACTH-secreting adenoma, since target therapy with PAS has been used only in the latter years, substituting steroidogenesis inhibitors (which are not effective to control pituitary adenoma growth).

After diagnosis of aggressive PitNET, the treatment of choice is challenge and need a multidisciplinary approach in the era of PTCOE¹⁴⁴. However, after several attempts with surgery, conventional medical treatment and radiotherapy, nowadays TMZ must have to be considered as an option. TMZ is an oral alkylating chemotherapeutic agent, previously indicated for glioblastoma, and is currently recommended in the treatment of aggressive pituitary tumors, considered as radiologically invasive tumour or those with unusually rapid

tumour growth rate, or clinically relevant tumour growth despite optimal standard therapies^{5,99,145}. We conducted a national web-based survey study in 2015 in patients with aggressive pituitary adenomas or carcinomas treated with TMZ. In most neoplastic disorders, a favorable response to chemotherapy is considered as a measurable reduction in tumor size; nevertheless, stabilization of disease in some cases improves the quality of life and decreases morbidity. All our patients had tumor progression before TMZ treatment: in this setting, an arrest of tumor progression corresponds to a favorable clinical, although not radiological, response, as also suggested in previous mini-series of TMZ treated pituitary tumors. Thus, a new strategic approach to tumoral disease could be to stabilize it and to revisit the idea of treating cancer as a chronic disease. This concept may particularly apply to very aggressive pituitary tumors, for which, with the possible exception of some patients with severe forms of hypercortisolism, the leading cause of death is local growth of the tumor. Because of the aforementioned considerations, tumor reduction and stable disease were grouped together and considered as disease control. Overall, 81% of our patients were classified as having disease control. However, when we split the data of the positive response, the frequency of tumor reduction in Italian patients (36%) was clearly lower than that reported in the literature. It is likely that reporting bias plays a role, as single cases with a positive response to an innovative therapeutic treatment may have high chances to be described and published: it is important therefore to publish a large series, also with “negative results”, in order to describe the real efficacy of TMZ in clinical practice. A positive response to TMZ was always recorded within 3 months of therapy. In keeping with previous suggestions^{5,98,100}, this evidence strongly suggests that an alternative therapeutic strategy should be considered if disease progression is demonstrated within 6 months of treatment with TMZ. We could not identify baseline clinical characteristics associated with a favorable response to TMZ. However, it should be stressed that the small number of patients and events might have obscured the existence of any association, if any exists. Regrowth of the tumor after an initial positive response to TMZ occurred in 52 % of our patients. Treatment of patients who have a relapse after TMZ treatment is still a largely unmet need.

Starting from our observation previously described (medical treatment is highly effective in aggressive PitNET), we proposed a TMZ primary medical treatment, never described in a patient with aggressive prolactin-secreting PitNET. As reported in the Endocrine Society’s Guidelines regarding PRLomas, cabergoline is the first-line treatment to lower hormonal levels and to decrease tumor size⁸. Despite size and

secretion, in our case the term “insufficient response to cabergoline” may be better than “resistance to cabergoline”, because cabergoline treatment (up to 3.5 mg/week) was effective to reduce PRL levels. In such cases, indication to cytoreductive surgery should be carefully considered and balanced, because complete surgical removal of a giant adenoma is challenging and tumour persistence after surgery occurs frequently⁹³. Recently, the Pituitary Society decided to create general criteria for developing Pituitary Tumors Centers of Excellence (PTCOE), indicating that the best care for pituitary patients is provided by an interdisciplinary team composed of dedicated endocrinologists, experienced pituitary surgeons, neuroradiologists, neuropathologists, neuro-oncologists and other dedicated physicians and nurses¹⁴⁴. In our patient the transsphenoidal surgery could only achieve a partial resection of the whole adenoma. Hence, other treatments should be considered, and some authors previously suggested an early chemotherapy treatment in patients with PitNET¹⁴⁶. Therefore, after a multidisciplinary team evaluation, we decided to propose a cytoreductive TMZ treatment, which revealed its effectiveness shortly after 3 months of therapy (3 months are sufficient to evaluate an initial response to treatment). TMZ has few side effects, is available in oral form, and its ability to cross the blood-brain barrier makes it superior to other chemotherapy drugs. TMZ has been previously proposed mainly after surgical failure in patients with aggressive PRL-secreting PitNET as a salvage therapy¹⁴⁷⁻¹⁵⁰, however in this case we considered TMZ as a neoadjuvant cytoreductive treatment, after a careful balance between potential side effect (TMZ is an oral alkylating) and benefit. In the present case we describe the use of 12 months treatment with TMZ in a patient with PRL-secreting PitNET, describing a significant and prompt reduction of tumor volume, without reporting severe side effects or hypopituitarism, common after pituitary surgery or radiotherapy. Therefore, an early initiation of TMZ could be considered when the targets of pituitary surgery reported in the PTCOE (to eliminate pituitary secretory syndromes, to reduce or control the tumor mass and to preserve the normal pituitary gland function and surrounding neural structures)¹⁴⁴ could not be achieved in patients with PitNET.

6. *Conclusions and future prospects*

In this complex scenario, a multidisciplinary team for the management of patients with pituitary adenoma is fundamental, to suggest the correct treatment plan, tailored to the patient. Recently Pituitary Tumors Centers of Excellence have been proposed, in order to suggest the best treatment to each patient¹⁴⁴. The major conclusion of this study are as follows:

- Everolimus is effective in primary culture of non-secreting PitNET. Since no medical treatments are available in non-secreting PitNET, everolimus should be considered also in patients, at least in those with aggressive PitNET (not completely removed or with early re-growth) and in a prospective trial.
- Most of the factors suggested to predict responsiveness to SSA in patients with acromegaly need surgical intervention, in order to study the cellular biology of the adenoma. We suggest three novel “surgery-independent” biomarker (pollution, AIP-AR variants and GH response to OGTT) that can be considered in the therapeutic plan.
- In patients with acromegaly, a long term primary medical treatment (more than 10 years) is effective and safe, especially to reduce the incidence of hypopituitarism.
- A prospective study about medical treatment with metyrapone has never been reported. Therefore we conduct an observational planned study, confirming that metyrapone is effective and safe to reduce cortisol levels (also in patients with severe hypercortisolism) and to improve cortisol-related comorbidities.
- Regarding body composition after achieving remission from Cushing’s Syndrome, we observe an improvement in cardiovascular risk factors (BMI, waist circumference, hypertension, glucose levels and metabolic syndrome) and in body composition (mostly fat mass, in all DXA scans) especially after surgery. Further studies are needed to compare directly surgery and medical treatment with strong outcomes (morbidity and mortality).
- A combined definition of aggressive PitNET is needed, considering pathological, clinical and radiological data. In patients with aggressive PitNET temozolomide is an effective and safe option, that must be considered not only as salvage treatment but also at an early stage, after the identification of an aggressive behaviour: prospective studies are needed to explore primary TMZ treatment.

7. *References*

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