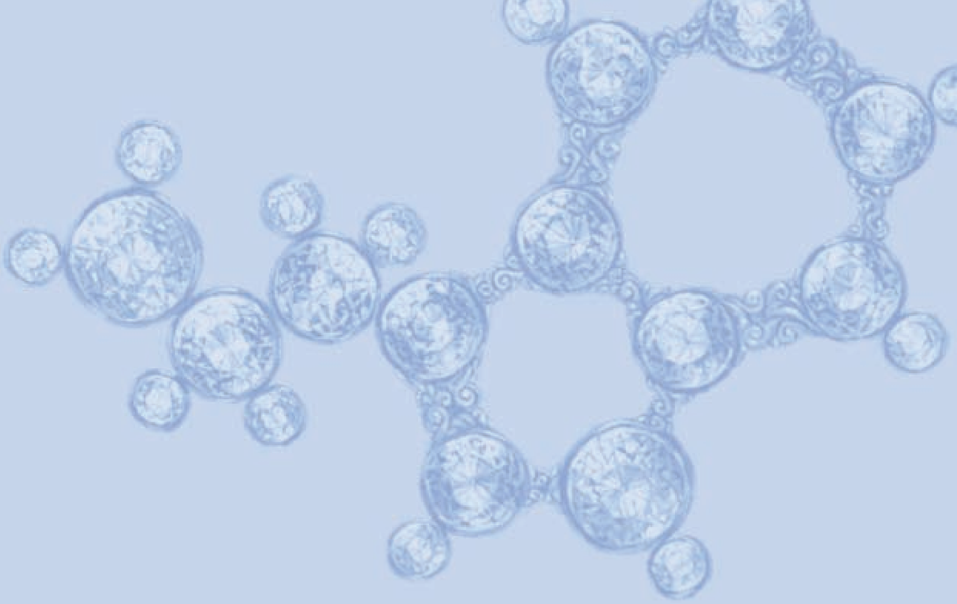
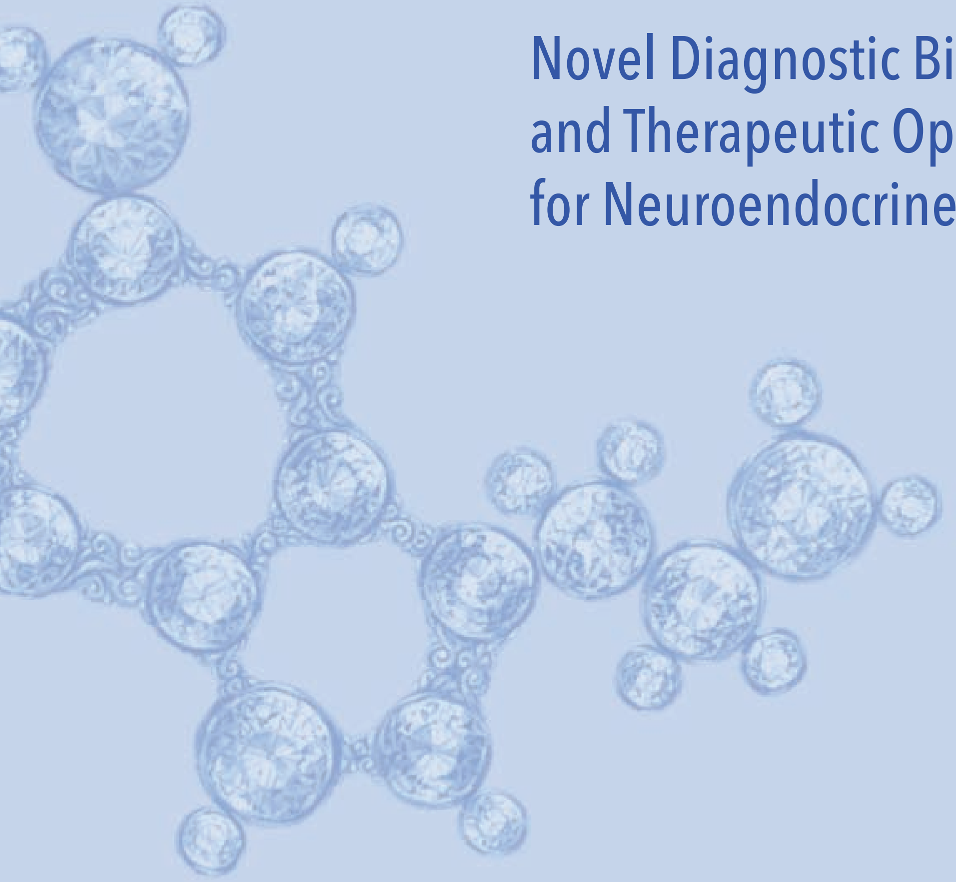


Aura Dulcínea Herrera Martínez

Novel Diagnostic Biomarkers and Therapeutic Options for Neuroendocrine Tumors



NOVEL DIAGNOSTIC BIOMARKERS AND THERAPEUTIC OPTIONS FOR NEUROENDOCRINE TUMORS

Nieuwe diagnostische biomarkers en therapeutische opties voor neuroendocriene tumoren

Nuevos marcadores diagnósticos y opciones terapéuticas en tumores neuroendocrinos

Aura Dulcínea Herrera Martínez

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Nieuwe diagnostische biomarkers en therapeutische opties voor neuroendocriene tumoren

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by

Aura Dulcínea Herrera Martínez
born in Barinas, Venezuela

Erasmus University Rotterdam

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María A. Gálvez Moreno[2], Justo P. Castaño[2] and Richard A. Feelders[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus
MC, University Medical Center Rotterdam, the Netherlands
[2] Maimonides Institute for Biomedical Research of Cordoba (IMIBIC);
University of Córdoba; Reina Sofía University Hospital; Córdoba, Spain.

Invited Review: Endocrine Related Cancer. Submitted for publication

Medical Treatment for neuroendocrine tumors: current options and future perspectives

Aura D. Herrera-Martínez[1,2], Johannes Hofland[1], Leo J. Hofland[1], Tessa Brabander[3], Ferry A.L.M Eskens[4], María A. Gálvez Moreno[2], Justo P. Castaño[2], Wouter W. de Herder[1], Richard A. Feelders[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus
MC, University Medical Center Rotterdam, the Netherlands
[2] Maimonides Institute for Biomedical Research of Cordoba (IMIBIC);
University of Córdoba; Reina Sofía University Hospital; Córdoba, Spain.
[3] Department of Radiology & Nuclear Medicine, Erasmus
Medical Center, Rotterdam, the Netherlands
[4] Department of Medical Oncology, Erasmus Medical Center, Rotterdam, the Netherlands

Invited Review: Drugs. Submitted for publication

INTRODUCTION

The diffuse endocrine system is composed of neuroendocrine cells dispersed throughout the whole body [1]. These cells, which may be found in isolation or in small aggregates, can give rise to neuroendocrine tumors (NETs) [1, 2]. NETs represent a heterogeneous group of rare, slow-growing neoplasms [3, 4], and comprise 1-2% of all gastrointestinal and pulmonary malignancies [5, 6].

According to the last National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER), the incidence of NETs has increased substantially (1.09/100,000 persons in 1973 to 6.98/100,000 in 2012) [7]. It is not known whether this is a true increase in NET incidence, the result of increased use of (improved) diagnostic procedures, or a combination of both [4, 8]. According to the SEER database, 27.4% of NETs have distant metastasis at diagnosis and 20% have regional infiltration [7]. Other series describe increased metastasis rates (localized and distant metastasis) when NETs are diagnosed (60–80%) [9]. Five to ten percent of metastasized tumors have an unknown primary tumor [10]. Despite the increase in incidence, survival in NET patients has improved, especially for patients with advanced gastroenteropancreatic- (GEP-) NETs [7].

This group of neoplasms displays a wide range of biological behavior ranging from benign to highly malignant growth [11]. In NETs, the overall 5-year survival rate ranges between 57-65% [12, 13], but depends on several parameters including the localization of the primary tumor (75.0% for jejunoileal, 42.9% for pancreatic NETs), the presence of metastasis (51.7% in presence vs 80% in absence of metastasis at initial diagnosis), tumor size, grade and stage of disease [12, 14].

1. Clinical features

NETs may produce specific clinical syndromes due to overproduction of hormones and bioactive peptides. The incidence of functioning NETs ranges from 0.01-8.4 cases per 100,000 habitants per year, depending on the secreted hormone. Carcinoid syndrome (CS) is the most frequent hormone-related syndrome (2-8.4 new cases/100,000 habitants/year) and is predominantly encountered in patients with metastasized midgut NETs [15]. The syndrome is mediated by several active hormones, especially serotonin, and comprises several symptoms, including flushing (94%), diarrhea (78%) and abdominal pain (51%), which is usually related to mesenteric fibrosis [16, 17]. Additionally, patients may present with carcinoid heart disease (CHD), which consists in the deposition of plaques on the endocardial surfaces of valve leaflets, subvalvular apparatus and cardiac chambers. CHD affects especially the right side of the heart and is observed in about 60% of patients with CS [18].

Pancreatic NETs (PNETs) are able to produce pancreatic peptides which can lead to specific hormone syndromes. Among these, the most common is the endogenous hyperinsulinism-related hypoglycemia caused by insulinomas [19]. The glucagonoma syndrome should be also mentioned and is characterized by necrolytic migratory erythema (NME), diabetes mellitus and weight loss [20]. Furthermore, multiple peptide ulcers may be related to gastric hypersecretion, specifically the Zollinger-Ellison syndrome in patients with gastrinomas [21]. Watery diarrhea may be related to functioning NETs that release vasoactive intestinal polypeptide (VIP) or calcitonin, while somatostatinomas may be asymptomatic or present with diabetes mellitus, cholelithiasis, weight loss, steatorrhea and diarrhea [89-91]. In addition, ectopic hormone production (EHP) may also be observed in NETs. In EHP, adrenocorticotropic hormone (ACTH) producing tumors are most commonly observed, but ectopic release of peptides including corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH), antidiuretic hormone, parathyroid hormone-related peptide (PTHrP) and gonadotropins by NETs has also been described [96-98].

The proportion of non-functional NETs is larger than functioning tumors. Non-functional NETs may be discovered incidentally during diagnostic procedures, or present with mechanical symptoms (e.g. bowel obstruction, cough, hemoptisis)[22, 23]. Due to their silent clinical presentation, patients with non-functioning NETs generally present late with large primary tumors and advanced disease [24].

2. Diagnosis

NETs may be diagnosed following symptoms-directed evaluation (in case of functioning tumors), due to non-specific symptoms or incidentally during endoscopic/cross-sectional imaging procedures [23]. Diagnosis should be ideally confirmed by histological evaluation, in which immunohistochemical markers are key points, particularly synaptophysin and chromogranin A [25]. Tumor grade is defined using the Ki67 index and the mitotic index according to the World Health Organization (WHO) grading system. For lung NETs, necrosis is also considered [26, 27].

2.1. Currently available markers for NETs

Currently used biochemical markers in NETs are usually hormones or amines secreted by the enterochromaffin cells, which can be influenced by several factors, including co-existent disease(s) and drugs. These biomarkers add to diagnosis, but are insufficient to accurately diagnose, to identify the primary tumor site or to differentiate tumor grading, especially due to limited sensitivity and specificity [28]. Despite this, some of them are considered for the diagnosis and follow-up of NETs according to several clinical guidelines [29-31]. In Table 1, a summary of the sensitivity and specificity of currently used and novel biomarkers in NETs is depicted.

Table 1: Sensitivity and specificity of current and novel neuroendocrine biomarkers

Tumor marker	Primary tumor location	Sensitivity	Specificity
Chromogranin A [32-38]	Non-specific	60-83%	72-85%
Urinary5-HIAA [32, 38, 39]	Midgut	35-68%	90-100%
Pancreatic polypeptide [28, 40, 41]	Pancreas, midgut	31–63%	~67%
Neuron-specific enolase [28, 32, 42]	Non-specific	33%	73%
N-terminal brain natriuretic peptide [28, 43]	Midgut (non-specific for CHD)	87%	80%
Progastrin-releasing peptide [44]	Lung	43%	99%
Paraneoplastic Ma antigen 2 [45]	SB-NETs	46-50%	
DCR [46]	SB-NETs	AUC: 0.74	
TFF3 [46]	SB-NETS	AUC: 0.72	
Midkine [46]	SB-NETS	AUC: 0.71	
Multritranscript genes [47-49]	GEP-NETs	75-98%	

Legend: CHD: carcinoid heart disease; SB: small-bowel, AUC area under the curve.

Chromogranin A (CgA): is a protein present in the secretory granules of normal and neoplastic neuroendocrine cell types,which is released with peptide hormones and biogenic amines, and is also the precursor for functional neuroendocrine peptides [50, 51]. Several guidelines recommend plasma CgA measurement during diagnosis, treatment and follow up in GEP-NETs. Baseline and serial CgA may predict clinical outcome, prognosis and tumor response [52], and may be indicative for local progression in patients with liver involvement [32]. Additionally, a progressive decrease in CgA levels may be observed in patients with extensive metastatic spread and loss of neuroendocrine differentiation [53]. However, CgA is elevated in only 60–80% of patients with NETs, has a limited sensitivity of 60-83% and also specificity is relatively low, i.e. 72-85% (Table 1) [32-36, 38, 54-56]. Moreover, proton pump inhibitors, atrophic gastritis and impaired kidney function can induce a rise in CgA levels [38, 57]. The combination of CgA with other diagnostic methods, e.g. somatostatin receptor scintigraphy, may increase its sensitivity (93%) and specificity (81%) [58-60]. Importantly, the sensitivity of CgA depends further on the threshold cut-off [37, 38, 53], NET primary location [37, 61, 62], endocrine associated syndrome [9], disease spread, liver metastases [37, 53, 56, 63] and the used assay [64]. Importantly, different analytical properties of the CgA kits give different performances, a fact that must be taken into consideration when comparing results from different clinical studies.

Neuro-specific enolase (NSE): is a soluble cerebral protein which provides information on neural, neuroendocrine and paraneuronal cells [65]. An increase in NSE levels is thought to be related to a high death rate of cells with neuroendocrine differentiation [32]. NSE is probably the most reliable tumor marker in diagnosis, prognosis and follow-up of small cell lung cancer (SCLC) [66]. This marker may be elevated in 38-40% of GEP-NETs patients, in particular in those with high grade tumors [42, 67]. The specificity of NSE is similar to CgA but with lower sensitivity (Table 1) [42, 68, 69]. NSE levels have been directly associated with tumor differentiation, aggressiveness and size [42, 67]. Despite its limited sensitivity, NSE is inversely correlated to overall survival (OS) in ENETS TNM stage IV [67] and with shorter progression-free survival (PFS), even if CgA levels are normal [70].

N-terminal brain natriuretic peptide (NT-BNP): is a peptide produced by myocardial cells in response to electrolyte and fluid balance; its serum concentration is usually elevated in mid-gut NETs with a sensitivity of 87% and a specificity of 80% [28, 43]. NT-BNP is in particular used for evaluating CHD and it has been reported that a cut-off value of 260 pg/ml has a sensitivity of 92% and specificity of 91% [71]. Interestingly, it has been suggested that patients with elevated NT-BNP levels combined with increased CgA levels have worse OS when compared to CgA alone [28, 72]. Importantly, NT-BNP is not disease specific, thus further studies for evaluating its applicability in the progression of CHD are still required [71].

5-hydroxyindoleacetic acid (5HIAA): Serotonin, produced by (midgut) NETs, is the most prominent hormone associated with diarrhea and flushes in carcinoid syndrome. Its metabolite, 5-hydroxyindoleacetic acid (5HIAA), measured in 24 h urine is used as a diagnostic and follow up marker [73]. Urinary 5HIAA levels are not directly related to the severity of symptoms and large fluctuations within an individual patient have been described [74]. The specificity of 5HIAA is around 90%, but the reported sensitivity is 35-68% in patients with NETs [32, 38, 39]. 5HIAA is mainly used as an indicator of hypersecretory activity in patients with NETs, especially in midgut NETs [32]. Its prognostic value, however, is limited, some studies have related higher urinary 5HIAA levels with mortality [75], but these results were not reproduced by other studies [39, 73]. Its combination with other markers also failed to predict OS, for this reason, 5HIAA determination is only recommended to assess carcinoid syndrome [71].

Pancreatic Peptide (PP): is a non-specific marker in NETs [76]. Around 63% of PNETs and 18-53% of primary gastrointestinal NETs show increased PP levels [41]. Its determination does not seem to increase the diagnostic performance of other markers like CgA, but changes above 50% in PP serum levels seem to correlate with tumor increase on imaging [56].

Despite the above-mentioned limitations, current biomarkers are regularly used in clinical practice and their accuracy increases when combined. Importantly, specific comparisons between markers are difficult since several publications are based on short heterogeneous cohorts and retrospective analysis. Additionally, the differences between assays limit comparisons and solid conclusions.

2.2 Potential novel diagnostic biomarkers

To improve early diagnosis and follow-up of NETs, several new prognostic and treatment-related biomarkers have been developed in the last years (Figure 1).

Most of these biomarkers are still under study and not available yet for use in clinical practice. It is aimed to develop high-specific and sensitive circulating biomarkers using DNA, RNA and a metabolomic approach. Combination markers and multianalyte analysis seem to be more effective than the current use of monoanalytes because of a higher sensitivity [28, 49, 77]. A summary of potential novel circulating and tissue biomarkers for diagnosis, prognosis, and therapy response prediction, as well as their relation with tumor localization, is shown in Figure 2.

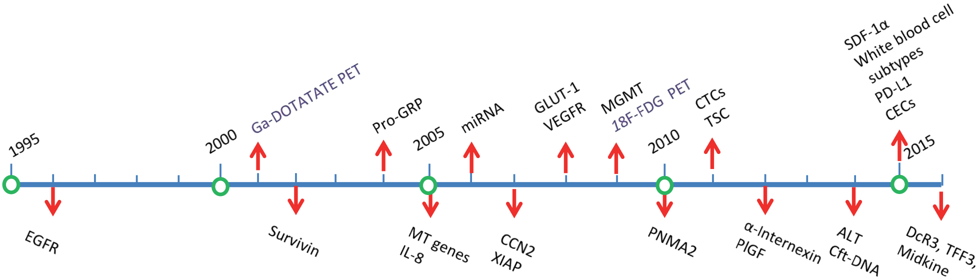

Figure 1: Timeline of the publication of potential novel biochemical and therapeutic markers in neuroendocrine tumors. Monoanalytes, transcripts, DNA-, RNA- , immune- markers are shown. They are still mostly under study and not available for use in clinical practice. Image-based modalities are represented in purple.

Legend: EGFR: epidermal growth factor receptor; proGRP: progastrin-releasing peptide; MT: multitranscript; CCN2: connective tissue growth factor for carcinoid heart disease; IL-8: interleukin 8; miRNA: microRNA; XIAP: X-linked Inhibitor of Apoptosis; GLUT-1: glucose transporters type 1; VEGFR: vascular endothelial growth factor receptor; MGMT: O-6-methylguanine-DNA methyltransferase; 18F—FDG PET: 18-fluorodeoxyglucose positron emission tomography; PNMA2: paraneoplastic Ma antigen 2; CTCs: circulating tumor cells; TSC: Tuberous sclerosis complex; PlGF: Placental growth factor; ALT: alternative lengthening of telomeres; cftDNA: circulating cell free tumor DNA; SDF-1α: stromal cell-derived factor 1α; PD-L1: programmed death ligand-1; CECs: circulating endothelial cells. Imaging techniques, as reference, are presented in purple.

a. ***Peptides and growth factors:***

Several peptides and growth factors (Table 2) have been studied for a (potential) role as biomarkers in NETs and may: (1) help to localize primary tumors (e.g. progastrin-releasing peptide in lung NETs, connective tissue growth factor (CCN2), paraneoplastic Ma antigen 2 , DcR3, TFF3, and midkine in small intestine NETs [28, 44, 46, 78]); (2) predict the outcome in functioning NETS (e.g. α-Internexin in insulinomas [79, 80]) or predict early

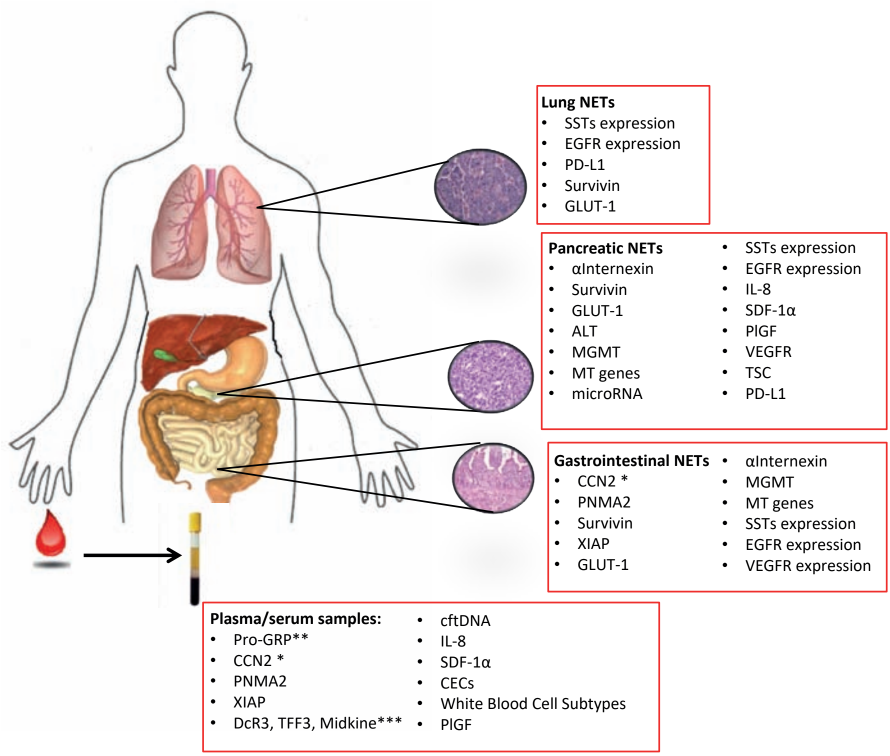

Figure 2: Summary of potential novel diagnostic and therapeutic markers in neuroendocrine tumors

Several tumor or plasma/serum biomarkers seem to play a role in the diagnosis or follow-up in lung and GEP-NETs. Its presence may be determined in serum or tissue samples.

Legend: * specific for carcinoid heart disease; ** only for lung-NETs; *** only for small intestine NETs; proGRP: progastrin-releasing peptide; CCN2: connective tissue growth factor for carcinoid heart disease; PNMA2: paraneoplastic Ma antigen 2; XIAP: X-linked Inhibitor of Apoptosis; GLUT-1: glucose transporters type 1; ALT: alternative lengthening of telomeres; MGMT: O-6-methylguanine-DNA methyltransferase; MT: multitranscript; cftDNA: circulating cell free tumor DNA; EGFR: epidermal growth factor receptor; SDF-1α: stromal cell-derived factor 1α; CECs: circulating endothelial cells; PlGF: Placental growth factor; VEGFR: vascular endothelial growth factor receptor; TSC: tuberous sclerosis complex; SSTs: somatostatin receptor; PD-L: programmed death ligand-1.

complications in patients with carcinoid heart disease [78]; and (3) add information to that provided by other circulating/tissue markers for treatment response evaluation and outcome prediction (e.g. pro-GRP and CgA for predicting outcome/therapeutic response in lung carcinoids; α-Internexin and Ki67 in insulinomas [44, 81-83] or as part of multianalyte tests [46]). Additionally, some peptides may be useful to correlate with imaging techniques. For instance, glucose transporter 1 (GLUT1) expression in NETs is associated with the Ki67 index and 18-fluorodeoxyglucose (FDG) uptake at FDG-positron emission tomography

Table 2: Peptides and growth factors as novel markers in NETs

Peptide/growth factor	Function	Potential role as marker in NETs
Progastrin-releasing peptide (proGRP)	Precursor of gastrin-releasing peptide, a neuropeptide hormone widely distributed throughout the gastrointestinal and pulmonary tract [85]	Primary tumor localization in patients with a metastatic NET of unknown origin. Complementary marker to CgA in lung NET for treatment response evaluation and survival [44, 86]
Connective tissue growth factor for carcinoid heart disease (CCN2)	CCN2 is an early gene product of the CCN family of matricellular proteins, which are involved in cell proliferation, angiogenesis, tumourigenesis and wound healing. It may be involved in the pathogenesis of carcinoid heart disease [78, 87]	Independent predictor of both reduced right ventricular function and right-sided valve regurgitation (its plasma levels are inversely related to right ventricular function levels) Early predictor of cardiac fibrosis [78]
Paraneoplastic Ma antigen 2 (PNMA2)	Antineuronal antibodies identified as markers of neurological paraneoplastic syndromes [88]	Allows the identification of almost 50% of small bowel NETs at the primary stage of the disease Correlation with disease progression and recurrence free survival [45]
α-Internexin	Cytoskeleton protein involved in tumorigenesis and disease progression [89]	Association with proliferation, ki67 index and malignancy [79]
X-linked Inhibitor of Apoptosis (XIAP)	Inhibitor of apoptotic cell death in cancer cells [90, 91]	Potential target therapies [92-94]
Glucose transporters type 1 (GLUT-1)	Mediate the transport of glucose across the cellular membrane and are commonly overexpressed in tumors, probably related with higher metabolism and cell growth [95]	Predictor of risk of death in neuroendocrine lung carcinomas and lung carcinoids [96] Relation with Ki67 index in GEP- and lung NETs [84, 97] Correlation with the uptake in 18-FDG-PET [84]
DcR3	Regulates cytokines that influence tumor growth and reduce apoptotic stimuli [98]	DcR3 correlates to liver metastasis and worse survival Predictor of treatment resistant tumors [46]
TFF3	Protects and repairs epithelial surfaces Enhances migration, angiogenesis, and inhibits apoptosis [99-101]	Higher concentrations have been correlated to reduced survival [46]
Midkine	Promotes tumor cells migration, angiogenesis and reduces apoptosis [102]	Predictive marker to chemotherapy response[103, 104]

(PET) scans [84]. GLUT-1 expression may serve as an additional marker for aggressiveness of NETs and may add to a more accurate grading [84]. Although some of these peptides have been suggested as promising biomarkers, most of them are non-specific. In addition, their applicability is limited, due to their sensitivity and specificity (Table 1) and the absence of cut-off levels. In addition, some of them have been described only in single retrospective studies, thus further validation in larger and longitudinal cohorts is still required.

b. *DNA markers:*

These markers are expected to improve diagnosis in NETs, especially when the primary tumor is unknown, and to predict drug response. They include the determination of mutations which are associated with alternative lengthening of telomeres [105, 106], the expression of the DNA repair enzyme O-6-methylguanine-DNA methyltransferase which may predict the clinical responses to alkylating agents including temozolamide [107, 108] and the evaluation of cell-free DNA in liquid biopsy which contains identical genetic defects as the primary tumor [109]. A schematic overview of DNA markers is depicted in Figure 3.

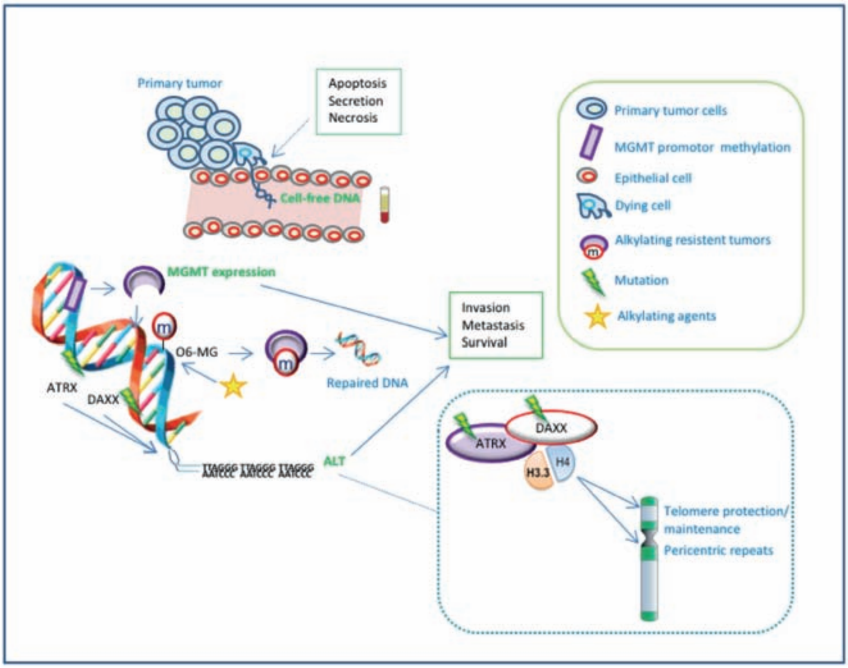

Figure 3: Summary of DNA markers in neuroendocrine tumors: DNA markers are presented in green. Tumor cells release small fragments of cftDNA into circulation by multiple mechanisms, cftDNA contains identical genetic defects compared to the primary tumor. DNA methylation at the O6 position of guanine results in apoptosis and tumor cell death; in GEP-NETs, the methylation *of MGMT* promoter and loss of MGMT protein expression have been reported. Inactivating mutations in ATRX and DAXX genes are associated with ALT.

Legend: cftDNA: cell free tumor DNA; O-6-MGMT: O-6-methylguanidine-DNA methyltransferase; O6MG: DNA methylation at the O6 position of guanine; ALT: alternative lengthening of telomeres; ATRX X linked transcriptional regulator; DAXX: death domain-associated protein 6

c. *RNA markers:*

These are novel and potentially promising minimally invasive markers used for diagnostic purpose and/or to identify therapeutic targets for NETs. Specifically, the identification of circulating target genes using PCR-amplification has been used for determining stage, prognosis, recurrence or new metastasis in several cancers [110-112]. Modlin and co-workers have developed a PCR-based molecular test using 51 genes for identifying GEP-NETs [49]. For this so-called NETtest, a score, based on tissue and peripheral blood transcriptomes, was developed [49] as a diagnostic and follow-up tool for NETs [48, 49, 77, 113]. NETtest results were shown to differentiate progressive disease [48] and to predict tumor response to somatostatin analogs (SSAs) [114] and peptide receptor radionuclide therapy (PRRT) [47]. Despite these promising results, prospective independent validation is desirable in order to establish the reproducibility of the results and their interpretation.

Additionally, dysregulated microRNAs (miRNA) have been correlated with diagnosis, staging, progression, prognosis and therapeutic response in several tumors, including NETs [115]. Their up- and down-regulation has been described and associated with histological characteristics (e.g. Ki-67, degree of malignancy) and prognosis characteristics, including OS [116, 117]. The therapeutic strategy for miRNAs includes the oncogenic miRNA inhibition or the introduction of a tumor suppressor miRNA [118]. However, the currently available technology is not robust enough to support its clinical use yet [119]. Furthermore, dysregulation of miRNAs is not tumor specific and the absence of cut-off levels for differentiating tissue and tumor subtypes, the lack of reproducibility in other NET cohorts and the difficulties in their interpretation, limit the clinical application of miRNAs. Further studies are required to evaluate the application of miRNAs as clinical and therapeutic markers in NETs.

d. *Therapeutic/prognostic biomarkers:*

Somatostatin/cortistatin system components (ligands and receptors) are widely expressed in tissues, including the gastrointestinal tract, where they inhibit endocrine secretions, motility and absorption, in a paracrine and endocrine manner [120, 121]. Somatostatin acts through the binding and activation of a family of five G-protein-coupled somatostatin receptor subtypes (SST_{1-5}), which are widely distributed in the organism [122-124]. SSTs are a family of 5 G-protein-coupled, 7 transmembrane domains receptors that trigger different intracellular signaling pathways. Through their activation, in addition to secretion processes, proliferation, differentiation and angiogenesis are regulated [124]. The complexity of somatostatin/cortistatin system has increased in recent years after the identification of SSTs splicing variants of the SST_5 gene (SST_5TMD_4 and SST_5TMD_5) [125-131], which may be dysregulated in tumor pathologies where they may be associated with aggressive features [128, 132].

SSAs are considered to be the preferred first-line treatment option in functionally active NETs, including those associated with the carcinoid syndrome and functional PNETs [133, 134]. Additionally, monthly administered long-acting preparations of octreotide and lanreotide are usually used for disease stabilization in NETs [135, 136]. The effects of SSAs depends on the presence of SSTs in the tumor (octreotide and lanreotide bind preferably to SST_2 and pasireotide has high binding affinity to multiple SSTs, particularly SST_5; see also figure 5) [137].

Tightly related with the somatostatin system, the ghrelin system is involved in the regulation of multiple (patho)-physiological functions, including hormonal secretion, β-cell survival, as well as appetite and gastric motility [138-141]. The acylation of the third serine residue in ghrelin molecule is necessary for its activation, which is catalyzed by the ghrelin-O-acyl-transferase (GOAT) enzyme [141, 142]. Acylated ghrelin binds and activates its canonical ghrelin receptor, GHSR1a. Additionally, some ghrelin system variants resulting from post-transcriptional modifications or alternative splicing have been identified, including the In1-ghrelin [138, 143] and a truncated receptor GHSR1b, with unknown ligand and function [138, 143, 144].

In recent years, there is increasing interest in somatostatin/cortistatin and ghrelin systems, since alterations in some of their components seems associated with the development/progression of various cancers [143, 145-148]. Both, ghrelin [132, 149] and somatostatin systems [22, 122, 150] have been described in NETs, but the clinical-molecular correlations have not been fully elucidated [149, 151]. Their use as tissue markers may provide information about clinical evolution and outcome [132, 149]. The molecular expression and clinical relations of both systems are described in chapter 2, 3 and 4 of this thesis. In particular, SSTs expression in NETs is considered to have therapeutic implications for treatment with SSAs and for PRRT.

In recent years, the use of molecular targeted therapies has been approved in NETs. The possibility to peripherally measure monoanalytes directly related to the drug mechanism of action represents an important approach to predict treatment response. In this sense, some molecular biomarkers could play a role as prognostic markers for treatment response to tyrosine kinase- and mTOR inhibitors. For sunitinib, such biomarkers include: the epidermal growth factor receptor [152-155], vascular endothelial growth factor (VEGF) and its transmembrane receptors (VEFGR-1, VEFGR-2, VEFGR-3) [156], interleukin-8 [157], stromal cell-derived factor-1α [157] and circulating tumor, endothelial and white cells [158, 159]]. For everolimus, circulating levels of placental growth factor [160] and tuberous sclerosis complex mutations [161] have been described. Additionally, some relations were shown between these markers, tumor response, PFS and OS [158, 159].

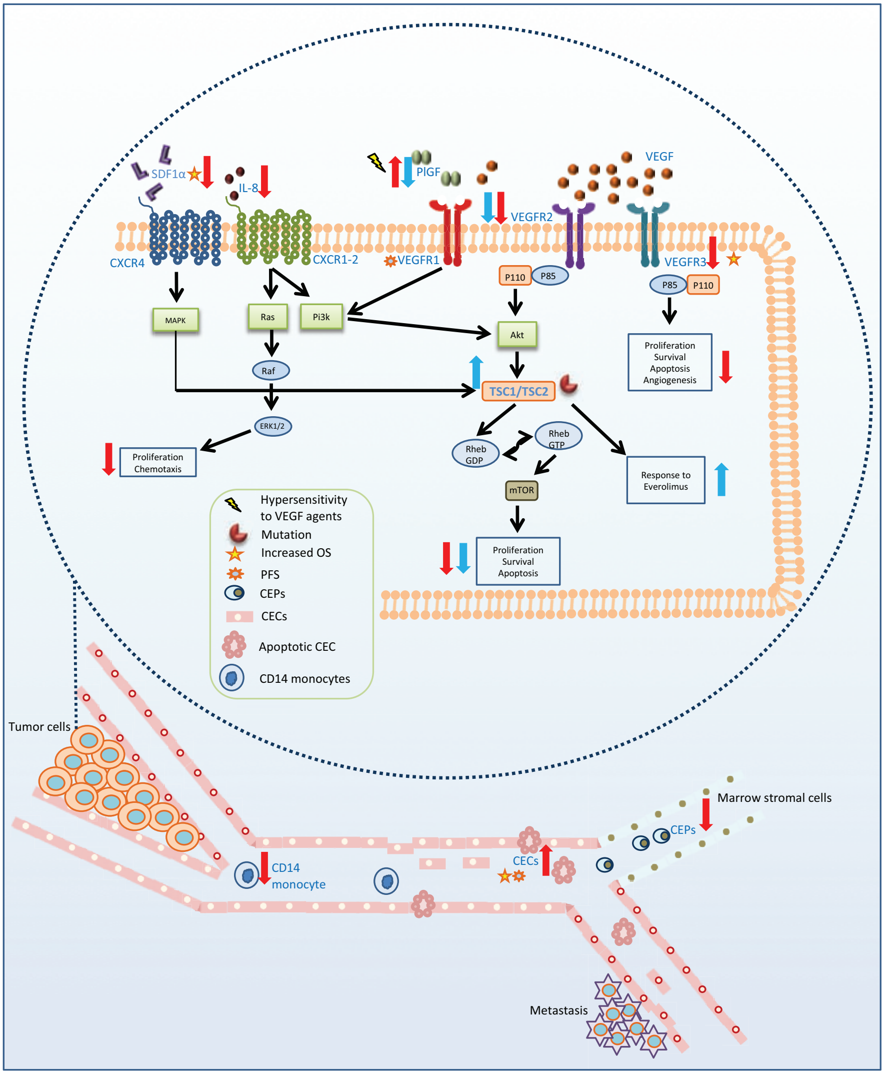

Figure 4: Therapeutic markers: molecular biomarkers for tyrosine kinase and mTOR inhibitors. Blue arrows represent the effect of the mTOR inhibitor everolimus and red arrows the effect of the tyrosine kinase inhibitor sunitinib. Molecular markers are presented in blue. Sunitinib has been related to decreased SDF1, IL-8, VEGFR 2-3, CD14 monocytes expressing VEGFR, decreased circulating endothelial precursors (CEPs), increased circulating endothelial cells (CECs) and probably decreased PlGF. Everolimus has been related to decreased PlGF and VEGFR2; additionally, mTOR directed therapies may be more effective in tumors with tuberous sclerosis complex 1 (TSC1) somatic mutation. Factors related to PFS and OS are also shown.

Legend: SDF-1α: Stromal cell-derived factor 1α; IL-8: interleukin-8; PlGF: placental growth factor; CXCR 1,2,4: chemokine family receptor 1,2,4; VEGF: vascular endothelial growth factor; VEGFR 1-3: vascular endothelial growth factor receptor 1-3; TSC 1-2: Tuberous sclerosis complex 1-2; CECs: circulating endothelial cells; CPECs: circulating endothelial precursors derived from the bone marrow.

Their use may identify patients with increased drug sensitivity and higher possibilities of drug responsiveness [161]. However, currently there is no consensus for supporting their use. Further multicenter, longitudinal studies in this field are required. A summary of the current potential tumor biomarkers for tyrosine kinase and mTOR inhibitors is shown in Figure 4.

2.3 Imaging

Tumor staging using imaging techniques should be performed in all NETs. Specifically, endoscopic evaluation in small (<1 cm) low-grade gastric- or rectal NETs is recommended [23, 162]. Most cases require evaluation with conventional imaging techniques, usually computed tomography (CT) or magnetic resonance imaging [30, 163]. SSTs-based imaging techniques help to localize primary tumor and metastasis for disease staging [164, 165]. Additionally, they help to make therapeutic decisions and are used to evaluate prognosis in NETs [165, 166]. Initially ^{111}In-DTPA-octreotide (Octreoscan®) was used. In recent years, the positron emitter ^{68}Gallium, which labels several somatostatin analogs, combined with positron emission tomography CT (^{68}Ga PET/CT), probably represents the gold standard for SSTs imaging of NETs [163, 167]. Other functional imaging techniques such as 18-fluorodeoxiglucose PET/CT (18-FDG PET/CT) have higher accuracy for poorly differentiated NETs and may be useful for evaluating atypical carcinoids. Importantly, images of 18-FDG PET/CT should be analyzed in combination with ^{68}Ga-PET/CT [163, 166, 168]. Additionally, a radiolabelled glucagon-related peptide 1 receptor agonist [Lys^{40}(Ahx-HYNIC-^{99m}Tc/EDDA)NH2]-exendin-4, has been reported as a promising imaging technique for the localization of insulinomas [169].

3. Treatment

3.1 Surgery

Surgery with margin-negative resection and, in some cases, adequate lymphadenectomy is the only curative treatment for localized NETs [170]. Unfortunately, patients often present with extended or metastatic disease at diagnosis [7]. Despite this, surgical management may still be an option with tumor debulking and resection of limited liver/lymph node metastasis with the subsequent use of liver-directed therapies [171-173]. Surgery is also useful for symptom control, specifically primary tumor resection/enucleation in localized functioning NETs is indicated. In some metastasized functioning NETs, decreased tumor load after surgery improves the associated symptoms. Additionally, in patients with recurrent/severe abdominal pain due to mesenteric fibrosis, surgery may improve clinical symptoms and obstruction [171-173]. Finally, bilateral adrenalectomy in patients with ectopic ACTH syndrome should be considered in cases of uncontrollable hypercortisolism, unknown primary tumor or extended disease [174].

3.2 Medical treatment

Hormone excess symptom control

SSAs are first-line therapy in functionally active NETs, including those associated with the carcinoid syndrome and functional PNETs [133]. The mechanisms of action of current medical options for functioning NETs are depicted in Figure 5.

Medical options for functioning NETs are summarized in Table 3. Chemotherapy may also be used in some aggressive cases [133, 175]; this therapeutic option is not discussed in this thesis. Since in Part II of this thesis novel therapeutic options for ACTH and serotonin overproduction are evaluated, only carcinoid syndrome and ectopic ACTH syndrome are described in more detail.

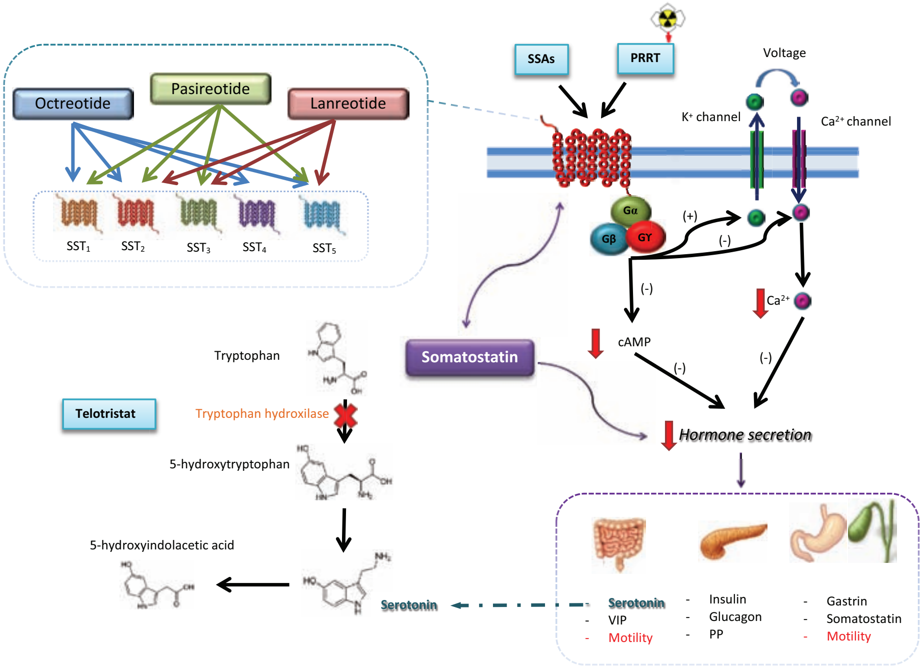

Figure 5: Current medical treatment for symptoms control in neuroendocrine tumors. Short-, long-acting and radiolabeled- somatostatin analogs bind to G-protein linked receptors on the cell surface with variable affinity. The inhibition of the cAMP and the decrease in intracellular calcium levels inhibit hormone release. Somatostatin influences hormone secretion and motility in the whole gastrointestinal tract. Serotonin production may be also decreased by telotristat, which inhibits the rate limiting step in the serotonin secretion (the enzyme tryptophan hydroxylase).

Legend: SSTs: somatostatin receptor; SSAs: somatostatin analog; PRRT: peptide receptor radionuclide therapy; cAMP: cyclic adenosine monophosphate; VIP: vasoactive intestinal peptide; PP: pancreatic polypeptide.

Table 3: Medical Treatment for functioning NETs

Functioning NET	Medical treatment	Mechanism of action/clinical relevance
Carcinoid syndrome	-SSAs, pasireotide, telotristat.	Please refer to the text for explanation of the medical options.
Insulinoma:	- Diazoxide (benzothiadiazine derivative)	- Inhibits insulin secretion, increases the hepatic glucose production and inhibits tissue glucose uptake [206-210].
	- Octreotide LAR and lanreotide autogel	- Decrease insulin release if SSTRs are expressed [209], otherwise, paradoxical decrease in blood glucose by suppressing glucagon release [208].
	- Pasireotide	- Mediates insulin secretion by binding SSTR5 [211]
	- Everolimus	- Decreases insulin release (AMP-activated protein kinase (AMPK)/c-Jun N-terminal kinase (JNK)/FoxO pathway); induce peripheral insulin resistance (glucose transporter 1 downregulation) [212-215].
	- PRRT	- Decreases insulin secretion, antitumor effect [208].
Glucagonoma:	- Octreotide LAR and lanreotide autogel	- The necrolytic migratory erythema improves despite the persistence of elevated serum glucagon levels [216]
	- Pasireotide	- Clinical response in octreotide-resistant tumors [20, 217].
	- Everolimus, sunitinib	- Clinical response after SSAs failure [218, 219].
	- PRRT with ^{90}Yttrium-DOTATOC or ^{177}Lu-DOTATATE	- Disease stabilization or tumor regression with subsequent symptoms control [220].
Gastrinoma:	- H$^+$-K$^+$-ATPase proton-pump inhibitors (PPIs)	-Control gastric hypersecretion [21]
	- SSAs	- Suppress gastrin secretion and normalizes gastric acid secretion [26–34]. Prevent the enterochromaffin-like cell hyperplasia or the development of gastric type 2 NETs [221].
	- IFN-α	- Improves clinical symptoms caused by hypergastrinemia only in stabilized tumors (41).
VIPoma, somatostatinoma:	- SSAs (Octreotide LAR and lanreotide autogel)	- Control symptoms in the majority of patients [29, 222, 223]
	- Glucocorticoids	- In SSAs refractory cases [223].
	- Molecular targeted therapy and PRRT	-In metastasized cases [218-220, 224-226].
Ectopic hormone producing syndromes:	• ***ACTH:***	- Please refer to the text for explanation of the medical options.
	• ***GHRH***:	
	-SSAs (octreotide and lanreotide)	- Low ectopic tumoral production of GHRH, with a subsequent decrease in circulating GH and insulin-like growth factor-1 (IGF-1) levels [227, 228].
	• ***PTH related protein:*** Includes hypercalcemia control	
	- Intravenous isotonic saline	- Corrects volume depletion
	- Bisphosphonates	- Interfere with the osteoclast-mediated bone resorption
	- Denosumab	- Reduces the formation, function, and survival of osteoclasts via the nuclear factor κB (RANK) pathway [190, 229, 230]
	- SSAs	- Improve symptoms control but might be insufficient in patients with tumor progression [190, 231].
	- PRRT with ^{177}Lu-DOTATATE.	- Tumor stabilization with parallel calcium control [190].

Carcinoid syndrome (CS)

CS is mediated by several active hormones, especially serotonin [16, 17]. The role of SSAs for improving secretory diarrhea and flushes in NETs was initially described in 1978 [176, 177]. Since then, short-acting octreotide was considered as a treatment option for carcinoid syndrome. The efficacy of short- and long-acting octreotide is similar once circulating octreotide steady-state concentrations are achieved [178, 179]. Long-acting preparations of SSAs are widely used, as these improve flushes and diarrhea in 53-75% and 45-80% of cases, respectively [180, 181]. Octreotide and long-acting lanreotide similarly reduce u5-HIAA acid and improve quality of life in NET patients [180]. Both octreotide and lanreotide are well tolerated and side effects are observed only in 14-29% of patients [180]. In addition, a favorable clinical response of carcinoid syndrome-related symptoms has also been reported in patients after treatment with PRRT [182, 183].

Pasireotide, a SSA with affinity to multiple somatostatin receptors, has been also tested in patients with octreotide-LAR resistant tumors. Here, pasireotide showed efficacy in 33% of patients when administered 150 µg twice daily, escalated to a maximum dose of 1200 µg per day [184]. α-interferon in combination with octreotide was suggested as an effective treatment for symptom control, but unfortunately the use of this combination is limited due to the high rate of adverse effects (attributed to α-interferon in 5-76% of cases) [185].

Recently, telotristat etiprate, a novel inhibitor of tryptophan hydroxylase, the rate-limiting enzyme in the biosynthesis of serotonin, has been developed. Telotristat etiprate decreases u5-HIAA and improves CS symptoms [186]. Remarkable, published data of its *in vitro* effects is lacking. This novel drug will be extensively described in chapter 7 of this thesis.

Importantly, SSAs and/or tumor debulking techniques may improve the hemodynamic impact of tumor vasoactive agents on CHD [178, 187, 188] but there is no concluding evidence suggesting that these treatment options can stop the progression of CHD [189].

Ectopic hormone producing syndromes

Ectopic hormone production is rare in NETs. The treatment aims in these patients include symptomatic long-term control, tumor stabilization or reduction, and prolongation of (progression-free) survival [190].

Ectopic ACTH syndrome

The ectopic ACTH syndrome (EAS) causes approximately 10% of all cases of Cushing syndrome [191, 192]. Clinical evolution is usually faster and characterized by mineralocorticoid effects (hypertension, hypokalemia, and edema), thromboembolic disease and opportunistic infections [193]. Curative surgery is the primary treatment option but is often not possible

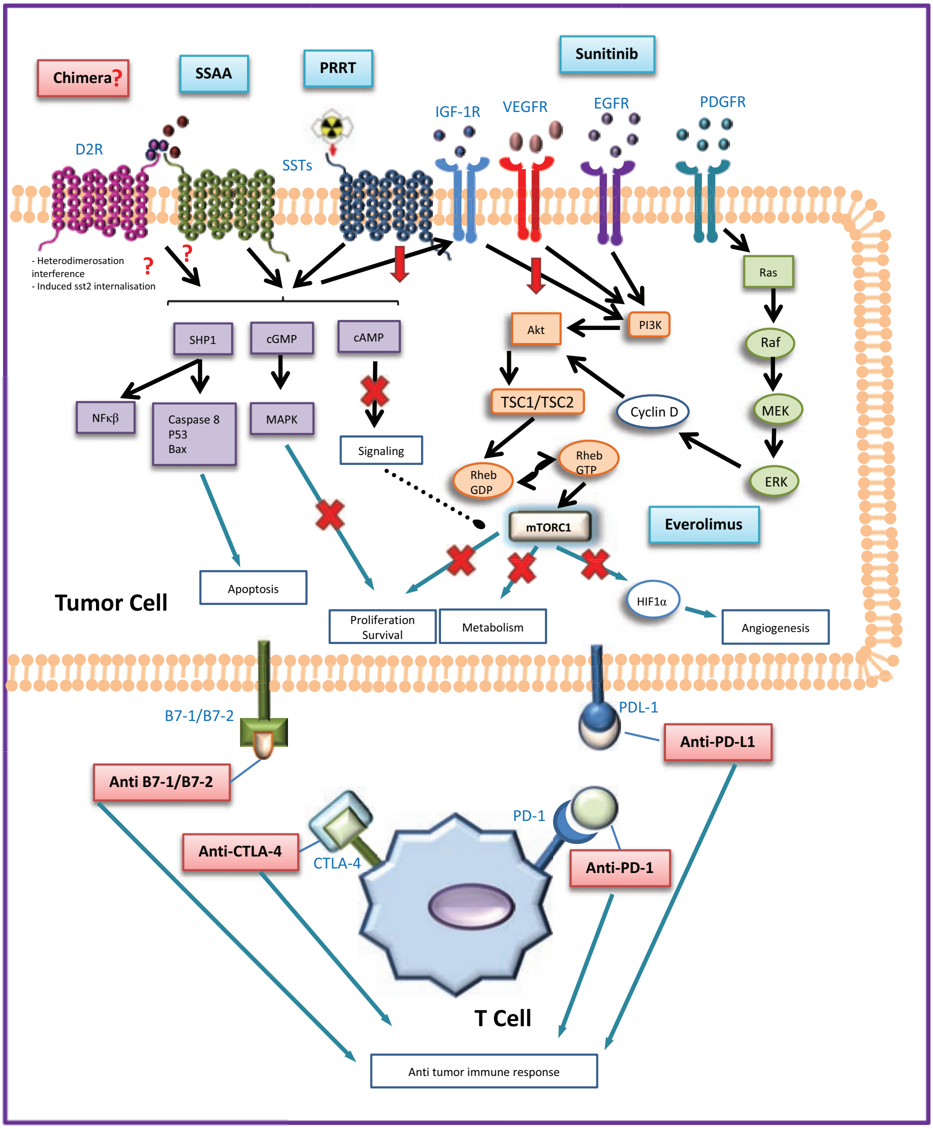

Figure 6: Current and future medical options for tumor control in neuroendocrine tumors. Current therapeutic options are presented in blue, possible novel therapeutic options are presented in red. SSAs and PRRT: increase apoptosis by activating the protein tyrosine phosphatase SHP1; decrease cell proliferation and survival through the mitogen-activated protein kinase and cyclic adenosine monophosphate; and inhibit the signaling of the insulin-like growth factor receptor type 1; additionally, PRRT produces DNA double stand breaks induced by β-irradiation, leading consequently to apoptosis. Sunitinib is a multikinase inhibitor that modulates the phosphoinositate-3-kinase/Akt pathway (it blocks the vascular endothelial growth factor receptors 1-3, the platelet-derived growth factor receptors α and β, and the epidermal growth factor receptor). Everolimus decreases tumor cell proliferation, metabolism, survival and angiogenesis through the mammalian target of rapamycin complex-1. The

indirect inhibition of mTOR through the phosphoinositate-3-kinase/Akt produced by the SSAs seems to increase sensitivity to mTOR inhibition. Multi-receptor chimeras may bind SSTs and D2R, and may enhance the signaling of the cAMP and JNK pathways; induced SST_2 internalization and SST_2 heterodimerisation interference have been also hypothesized. The interaction between some receptors expressed on the surface of cytotoxic T-cells (PD-1, CTLA-4) with ligands expressed on the tumor cells (PD-L1, B7-1/B7-2) downregulates the immune response to tumor cells; novel drugs that target these specific immune checkpoints inhibit this interaction allowing the immune system to maximize an efficient antitumor response.
Legend: SSAs: somatostatin analogs, PRRT: peptide receptor radionuclide therapy; IGF-1R: insulin-growth factor receptor type 1; VEGFR: vascular endothelial growth factor; EGFR: epidermal growth factor receptor; PDGFR: platelet-derived growth factor receptors; TSC: tuberous sclerosis complex; mTOR: mammalian Target of Rapamycin; CTL4: cytotoxic T-lymphocyte antigen-4; PD-L1: Programmed death-ligand 1.

[191, 194]. EAS can result in a critical condition for which aggressive medical therapy or life-saving bilateral adrenalectomy is necessary [193]. Medical treatment options for EAS include: (1) tumor-directed drugs including somatostatin analogs (octreotide, pasireotide) and dopamine agonists that decrease tumoral ACTH secretion [193, 195-198]. The identification of SSTs expression in the tumor using radiolabeled somatostatin analogs, may also help to identify suitable patients that might benefit from PRRT [195, 199, 200]. In addition, the tyrosine kinase inhibitors vandetanib and sorafenib may have antisecretory effects in selected cases with EAS [201, 202]; (2) steroid synthesis inhibitors which directly suppress adrenal cortisol production. In this sense, a combination of ketoconazole, metyrapone and mitotane was shown to be effective in critically ill patients with EAS [203]. Additionally, the anaesthetic drug etomidate can also rapidly suppress cortisol levels in an ICU setting [204]; and (3) glucocorticoid receptor antagonists. Mifepristone has a short onset of action and was shown to reverse morbidity of EAS in several cases [205].

Tumor growth control

According to the latest analysis of the SEER database, 27.4% of NETs have distant metastasis at diagnosis and 20% have regional infiltration [7]. Survival in NETs is related to tumor localization, tumor load and grading and these factors should be considered when selecting the appropriate medical treatment that would allow tumor stabilization and/or shrinkage. Current and promising novel medical options for tumor growth control in NETs (excluding chemotherapy) are described in this chapter and presented in Figure 6.

The current registered clinical trials of novel and combination therapies in NETs are depicted in Table 4.

Table 4: Registered clinical trials for tumor growth control medical therapies in NETs

Drug	Study Characteristics	Primary outcome/ ClinicalTrials.gov Identifier
Lanreotide (CLARINET FORTE)	Open label single group clinical trial for evaluating the efficacy and safety of lanreotide 120 mg every 14 days in well differentiated, metastatic or locally advanced, unresectable pancreatic or midgut NETs with radiological progression under lanreotide 120 mg every 28 days	PFS (102 weeks)/ NCT02651987
^{177}Lu-DOTA0-Tyr3-Octreotate (NETTER-1)	Multi-center, randomized, phase III study comparing ^{177}Lu-DOTA0-Tyr3-Octreotate to Octreotide LAR in patients with inoperable, progressive, somatostatin receptor positive midgut carcinoid tumors	PFS, OS data is pending/ NCT02651987
Sulfatinib	Randomized, multi-center phase III study to evaluate the efficacy and safety of sulfatinib (angio-immuno kinase inhibitor targeting VEGFR, FGFR1 and CSF-1R kinases) vs placebo in advanced PNETs	PFS (7 months after the last patient enrolled)/ NCT02589821
Sulfatinib	Randomized, double blind, multi-center phase III study to evaluate the efficacy and safety of sulfatinib vs placebo in advanced PNETs	PFS (9 months after the last patient enrolled)/ NCT02588170
177Lu-PRRT vs 177Lu-PRRT plus capecitabine	Open label phase II study to compare the efficacy of 177Lu-PRRT vs 177Lu-PRRT plus capecitabine in SSTR and 18-FDG PET/CT positive, G1-G2-G3 GEP-NETs	PFS (72 months)/NCT02736448
177 Lu-Octreotate -CAPTEM vs (i) CAPTEM and (ii) 177 Lu-Octreotate	Two parallel phase II randomized open label trials of PRRT with 177 Lu-Octreotate and CAPTEM (i) versus CAPTEM alone in the treatment of low to intermediate grade PNETs (ii) versus 177 Lu-Octreotate alone in the treatment of low to intermediate grade midgut NETs	PFS (12 months in PNETs and 24 months in midgut NETs)/ NCT02358356
177Lu-edotreotide vs Everolimus	Prospective, randomised, controlled, open-label, multicentre phase III study to evaluate efficacy and safety of PRRT with 177Lu-edotreotide compared to everolimus in GEP-NETs	PFS assessed up to 24 months/ NCT03049189
177Lu-DOTA0-Tyr3-Octreotate vs sunitinib	Open label randomized phase II antitumor efficacy of PRRT with 177Lu-DOTA0-Tyr3-Octreotate vs sunitinib in unresectable progressive well-differentiated PNETs	PFS (12 months)/NCT02230176
Everolimus and LEE011 (Recociclib)	Open label study to evaluate the efficacy and safety of the combination LEE011 (inhibitor of cyclin D1/ CDK4 and CDK6 pathway) 300 mg once daily for 3 weeks (4th week off) and everolimus 2.5 mg daily in foregut WDNETs	PFS (2 years)/NCT03070301
Everolimus and TMZ	Open label study to evaluate everolimus and temozolamide as first line treatment in advanced NEC with a Ki67 of 20-55%	Disease control rate/NCT02248012
Everolimus and bevacizumab	Randomized phase II study of everolimus alone versus combined with bevacizumab in patients with PNETs (currently active, not recruiting)	PFS (up to 3 years)/NCT01229943

Table 4 (*continued*)

Drug	Study Characteristics	Primary outcome/ ClinicalTrials.gov Identifier
Everolimus and cisplatinum	Open label phase II study of cisplatinum and everolimus in metastatic or unresectable NEC of extrapulmonary origin	Disease control rate/NCT02695459
Pembrolizumab	Open label phase 2 study of monotherapy with pembrolizumab (humanized anti-PD-1 monoclonal antibody) in patients with metastatic high-grade NETs who have failed platinum-based chemotherapy	Objective response rate/ NCT02939651
PDR001	Open label phase II study to evaluate PDR001 (high-affinity, ligand-blocking, humanized IgG4 antibody directed against PD-1) in advanced or metastatic, well-differentiated, non-functional, thoracic and GEP-NETs or GEP-NECs	Overall response rate/NCT02955069
Durvalumab and tremelimumab	Multi-center open label phase II study to evaluate the combination therapy between durvalumab (MEDI4736; humanized antibody against PD-1) and tremelimumab (CTLA-4 inhibitor) in advanced/ metastatic, grade 1/2 (G1/G2) lung and GEP-NETs, and grade 3 (G3) GEP- tumors or of unknown primary site after progression to previous therapies.	Clinical benefit rate/NCT03095274

Legend: PRRT: peptide receptor radionuclide therapy; CAPTEM: Capecitabine/temozolamide; PFS: progression free survival; OS: overall survival; VEGFR: vascular endothelial growth factor; FGFR : fibroblast growth factor receptor; CSF-1R: colony stimulating factor 1 receptor; PNETs: pancreatic neuroendocrine tumors; PRRT: peptide receptor radionuclide therapy; 18-FDG PET/CT:18-fluorodeoxiglucose positron emission tomography – computed tomography; GEP-NET: gastroenteropancreatic neuroendocrine tumors; CDK: cyclin-dependent kinases; WDNETs: well differentiated neuroendocrine tumors; TMZ: Temozolamide; NEC: neuroendocrine carcinoma; PDGFR: platelet-derived growth factor receptor; PD1: programmed death-1; NECs: neuroendocrine carcinomas; CTLA-4 :cytotoxic T-lymphocyte-associated protein 4.

Somatostatin analogs

The antiproliferative effect of SSAs depends on the level of expression of SSTs in the tumor, although indirect antitumor effects have been described as well [232]. SSAs may inhibit the cell cycle and increase apoptosis, and indirect effects may include immuno-modulation, antiangiogenic effects, and growth factor inhibition [135, 222, 233]. Long-acting preparations of octreotide and lanreotide are usually used for disease stabilization in NETs [135, 136]. The anti-proliferative effect of SSAs in NETs was initially evaluated in the PROMID study [234]. In this phase III B study, 85 well-differentiated metastatic midgut NETs were included. Patients randomly received placebo or octreotide-LAR 30 mg every four weeks. A difference of 8.3 months in tumor progression was observed after comparing the octreotide and the placebo group. Stable disease after six months was observed in 66.7% of patients treated with octreotide-LAR, compared to 37.2% in the placebo group [234]. Despite the initial good response to octreotide LAR, the results from the long-term survival analysis revealed that the overall survival (OS) was not significantly different in the placebo and in the octreotide group [235]. Similar to the PROMID study, the CLARINET study revealed that lanreotide Autogel (120 mg every 28 days) increased progression free survival (PFS) of patients with metastatic well- and moderate-differentiated gastroenteropancreatic NETs when compared to placebo [(PFS rate of 65.1% in the lanreotide group and 33% in the placebo group) [236]]. Usually SSAs induce tumor stabilization but in selected cases SSAs can cause tumor shrinkage, possibly due to their effects on the perfusion of liver metastases [237]. Currently, the CLARINET FORTE study is evaluating the safety and anti-tumor efficacy of lanreotide autogel 120 mg every 14 days in patients with pancreatic- or midgut NETs with progressive disease under regular dose of long-acting SSAs (NCT02651987). Pasireotide has been also studied in NETs, in which pasireotide concentrations correlated with tumor shrinkage in a non-significant manner [238]. Other studies have reported predominantly disease stabilization (60%) in treatment-naïve patients with grade 1-2 NETs, but also partial response (4%) and disease progression (36%) have been reported [239]. Additionally, pasireotide-LAR has been compared to octreotide-LAR in patients with metastatic NETs and carcinoid symptoms. In these patients, pasireotide tended to increase the tumor control rate after six months and was associated with a longer PFS [240]. In the phase 2 prospective LUNA study in advanced (unresectable or metastatic), progressive, well differentiated carcinoid tumors of the lung or thymus, pasireotide LAR treatment resulted in an objective tumor response in 39% of patients [241]. In a randomized, open-label, phase 2 study of everolimus in combination with pasireotide LAR or everolimus alone in advanced, well-differentiated, progressive pancreatic neuroendocrine tumors (COOPERATE-2 trial), the addition of pasireotide to everolimus was not associated with the improvement in PFS compared with everolimus alone [242]. Further investigation to evaluate the applicability of pasireotide alone or in combination with other therapies is required.

Interpheron-alpha

Interferon-alpha has antiproliferative, pro-apoptotic, cytotoxic/cytostatic and immunomodulatory effects in NETs [243, 244]. It has been considered as a second-line therapeutic option in progressive NETs under SSAs [133, 245]. Tumor response rates of about 10% have been reported [246] and its efficacy is similar to other agents, including bevacizumab, when combined with SSAs [247]. Unfortunately, several adverse effects have been described; a pegylated formulation seems to be associated with fewer side effects, and its combination with octreotide seems to be better tolerated [246, 248]. Despite this, the availability of novel therapeutic options with higher efficacy and lower side effects, limits the applicability of this drug for tumor control [249].

Peptide receptor radionuclide therapy

PRRT with SSAs allows targeted delivery of radionuclides to tumor cells expressing high levels of SSTs. Treatment response is directly related to the expression of SSTs in the tumor, making it a predictive marker of response [165]. Tumor response may also differ according to the primary tumor localization and tumor load [250]; OS is also different in NETs of different localization [pancreas: 71 months, CI 56-86), midgut: 60 months (95% CI52-68) [251]]. In contrast, response rates are decreased in patients with larger tumor load and higher liver infiltration [252]. The pivotal phase III NETTER-1 trial for the first time evaluated the efficacy of PRRT with ^{177}Lu-DOTATATE in a multi-center, randomized clinical trial. This study included 229 patients with well-differentiated, metastatic midgut NETs that were progressive on a standard dose of long-acting SSA. Patients were randomized to receive 4 cycles of PRRT with ^{177}Lu-DOTATATE or a double dose of octreotide LAR. The primary outcome was an increase in PFS (median not reached vs 8.4 months) in favor of patients treated PRRT. This study also reported a 79% reduction in risk of progression or death compared to octreotide and an increased overall response ratio (ORR) in the PRRT group (18%) compared to 3% in the control group [253]. In those cases with tumor progression after an initial good response, retreatment with PRRT represents an alternative. In this sense, disease control rates of 70-85% have been reported, but tumor response is limited [254, 255]. ^{177}Lu-DOTATATE has been also evaluated with radiosensitizing agents; its use in combination with 5-fluorouracil, capecitabine or temozolamide may increase the response rate (ORR 24-38%), but toxicity should still be evaluated [256-258]. Similar ORR has been reported when combined to everolimus [259]. Some case reports and series have suggested the use of pre-operative PRRT for downstaging NETs [260-262], but further investigation is still required on the efficacy of neoadjuvant PRRT in patients with initially unresectable NETs. Soon the results of the COMPETE study will be available. The aim of this multi-centre phase III study is to evaluate the efficacy and safety of PRRT (^{177}Lu-Edotreotide) compared to everolimus in progressive gastroenteropancreatic- (GEP-) NETs with positive expression of SSTs (NCT03049189). Hopefully this comparison will provide information

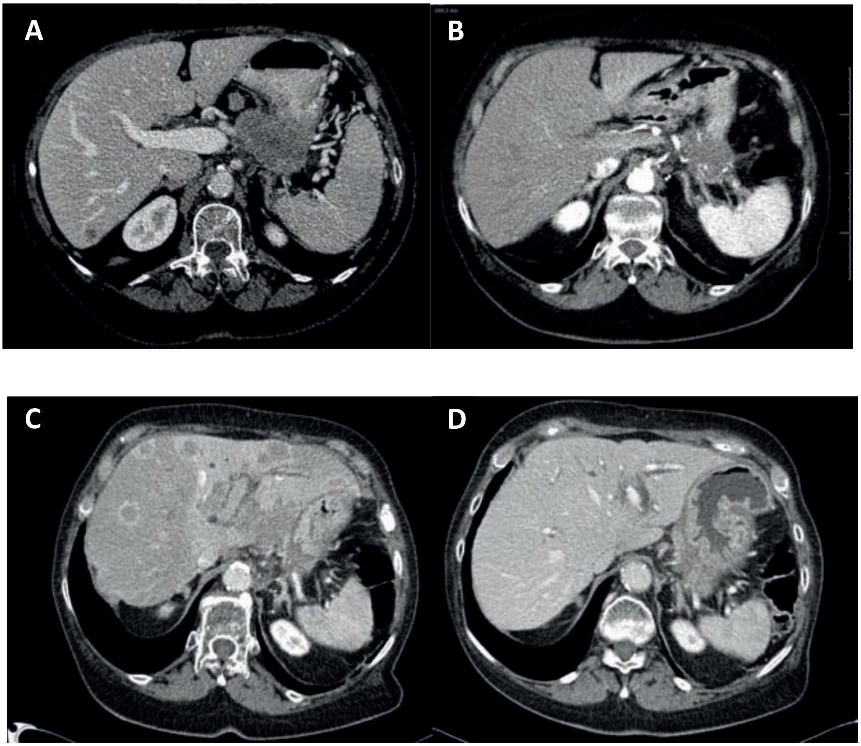

Figure 7: Peptide receptor radionuclide therapy in NETs. (A) CT imaging of a pancreas neuroendocrine tumor grade 2 with lymphatic and liver metastasis (segment 6); in this case, 4 cycles of peptide receptor radionuclide therapy (cumulative dosis 30 Gbq) was administered resulting in decreased size of the primary tumor (**B**). After 6 years, of partial response and stable disease, the primary tumor increased in size accompanied by new liver and mesenteric metastasis (**C**). Due to a first good treatment response, 2 cycles of PRRT (14.9 GBq) were administered, decreased size of primary tumor and liver metastasis were observed (**D**).

about the treatment sequence that should be followed in progressive NETs under SSAs. A representative example of tumor response to PRRT is depicted in Figure 7.

Molecular Targeted Therapy

- ***Everolimus:*** The mammalian target of rapamycin (mTOR1) pathway plays an important role in the regulation of cell proliferation in NETs [263]. The efficacy of the PI3K/AKT/mTOR inhibitor everolimus in well-differentiated NETs has been shown in several clinical trials [133]. In the phase III RADIANT 3 trial PFS was longer in the everolimus group compared to placebo [264], and its effect on PFS and OS was independent of the prior use of chemotherapy or SSAs [264-266]. Increased PFS and higher disease control rate were also reported in the RADIANT 4 study [267]. However, although everolimus is considered a safe drug, treatment can be accompanied by grade 3 and 4 drug-related adverse events (diarrhea, infections, anemia, fatigue, hyperglycemia) [267], which may limit the treatment tolerance and consequently the patient adherence. Importantly, the RADIANT 4 study, as the previous ones, failed to demonstrate statistically significant improvements in OS [267], which should be considered especially in those patients with poor treatment tolerance. The combination of everolimus and octreotide LAR improved PFS in lung [268] and colorectal NETs [269]. Everolimus is considered as first-line therapy in progressive atypical lung carcinoids, SSTs-negative lung NETs, and in well-differentiated midgut SSTs-negative NETs [133]. Currently, several studies are evaluating the combination of everolimus with other therapies including, chemotherapeutic agents, SSAs, molecular targeted therapies, radiotherapy and PRRT.

-***Sunitinib:*** Sunitinib is as an oral multi-targeted tyrosine kinase inhibitor (TKI) that inhibits multiple angiogenic factors, including the vascular endothelial growth factor receptors 1-3 (VEGFR), the stem-cell factor receptor, and the platelet-derived growth factor receptors [270]. Increased PFS in progressive PNETs compared to placebo has been reported in those patients treated with sunitinib (SUN 1111 trial) [218], but as for everolimus, significant improvement in OS has not been reported yet.

Other therapeutic targets

The comprehensive evaluation of signaling pathways regulating cell proliferation involved in NET development and progression has opened new perspectives for the medical treatment of these tumors. mTOR inhibitors and TKIs are the most representative examples, but other novel pathway-directed therapeutic compounds are also currently evaluated in (pre-)clinical studies [271]. Other examples of potential new treatment options include immunotherapy and somatostatin-dopamine multi-receptor chimeras. The mechanisms of action of immune checkpoint inhibitors and multi-receptor chimeras are depicted in Figure 6.

Immune checkpoint inhibitors have rapidly advanced and improved the management of several tumors in the last years [272, 273]. Programmed death-ligand 1 (PD-L1) is expressed on several cancer cells and interacts with PD1, which is expressed on T cells. This ligand-receptor interaction inhibits T cells and blocks the antitumor immune response [274, 275]. The expression of PD-L1 was demonstrated in GEP- and lung (large cell neuroendocrine carcinoma) NETs [274, 276], and has been associated with clinical variables including, histological type, tumor grade, and survival [274, 277]. The expression of PD-1 and PD-L1 has also been suggested as an independent survival prognostic factor in NETs [277]. Despite immunotherapy has an important role in the management of other types of cancer, the effect on well-differentiated NETs, according to preliminary data, seems to be limited although it may represent an option for G3NETs/NECs which needs further investigation [278].

Furthermore, multi-receptor interaction has been suggested as an efficacious and selective therapeutic strategy for enhancing the effects of somatostatin [279]. The presence of hetero-dimers has been described among SSTs and between SSTs and other receptor families, including dopamine receptors, especially the dopamine receptor subtype 2 (D2R) [280, 281]. Based on this, some structural chimeric molecules that combine elements of SSAs and dopamine analogs (DA) were developed [279]. *In vitro* studies using GEP-NET primary cultures, revealed inhibitory properties of chimeras on hormone secretion [282]. Importantly, BIM-23A760, a chimeric compound that activates SST_2 and D2R, acutely decreased growth hormone and prolactin secretion in pituitary tumors, but long-term effects disappeared due to a dopaminergic metabolite that may interfere with the activity of the parent molecule [279]. Multi-receptor targeting drugs are described in chapter 6 of this thesis.

Finally, other therapeutic options may have additional effects on cell proliferation and secretion in NETs. In this sense, ketoconazole is a steroidogenesis inhibitor which is widely used for medical treatment of Cushing Syndrome, since it improves clinical signs, symptoms and comorbidities [205]. Ketoconazole impairs adrenal and gonadal steroidogenesis by inhibiting side-chain cleavage, 17,20-lyase, and 11-β hydroxylase enzymes [283]. This drug could exert additive effects in the control of patients with severe hypercortisolemia [283]. Specifically, a direct effect ketoconazole on tumoral ACTH secretion has been suggested [284, 285] due to prolonged remission of hypercortisolemia in EAS patients [284-286] and reduced ACTH *in vitro* secretion [287]. Additionally, cytotoxic effects [288], ketoconazole-induced apoptosis [289] and changes in cell cycle phases have been described [290].

A putative association between treatment with metformin and cancer prevention/treatment is suggested [291]. Epidemiological studies have suggested a decreased risk for pancreas, liver, colon, lung, and breast cancer in patients with diabetes treated with metformin [292-295]. This protective effect of metformin has been also described in several meta-analysis [295-297]. Moreover, biguanides can inhibit cell proliferation *in vitro* in several cancer cell lines, including pancreatic and neuroendocrine tumor cells [298, 299]. Metformin stimulates the AMP-activated protein kinase (AMPK), which reduces hepatic gluconeogenesis/glycogenolysis and increases glucose uptake in the muscle [300, 301]. Additionally, it suppresses the mTOR1 pathway, reduces the insulin/insulin like growth factor 1 (IGF-1) signaling [302, 303] and mediates cell cycle arrest and apoptosis [304, 305]. Some of these actions may be also exerted in an AMPK-independent manner [306]. Closely related to metformin, statins are also commonly used in patients with metabolic syndrome or T2DM. Statins not only affect the rate limiting step in cholesterol synthesis, they also exert other clinical effects related with immunomodulatory mechanisms [307]. Additionally, some antitumor effects have been described in several tumor types, including melanoma, colon and breast cancer [308-311]. The antitumor mechanisms of statins may include induced cell-cycle arrest, apoptosis induction, decreased invasion/metastasis capacity and decreased *Ki67* expression [310-314]. In this context, these drugs are described in chapters 5 (ketoconazole) and 8 (metformin and statins).

3.3 Liver directed therapies

Non-surgical liver directed therapies may represent a primary treatment option, especially in functioning metastasized NETs. Radiofrequency ablation, cryoablation and microwave ablation are some options for small liver lesions [315]. Ablation is useful in cases of intrahepatic disease recurrence with limited liver surgical options, and as an adjuvant therapy to surgical resection in metastatic disease [316].

Since metastasis in NETs are highly vascular, and the hepatic artery supplies the majority of their blood, endovascular procedures are useful in several NETs with liver metastasis [317]. Bland embolization, chemoembolization, or radioembolization are recognized as a palliative treatment in hepatic-predominant metastatic NET patients who are not candidates for surgical resection [318]. Arterial directed interventions produce local effects and could deliver high chemotherapy doses or selective internal radiotherapy for symptomatic control of hormone release and tumor size [319]. Currently no modality has demonstrated to be superior to the others, but unfortunately, prospective, randomized, placebo-controlled studies are not available yet [319].

AIMS AND OUTLINE OF THIS THESIS

The general aims of the studies presented in this thesis are:

1. To identify potential novel tissue biomarkers for lung carcinoids and GEP-NETs
2. To evaluate the antitumor effect of registered drugs (for other medical purposes) in NETs
3. To evaluate the effects of novel drugs on NET hormone release and cell proliferation

Specifically, we studied the potential applicability of ghrelin and somatostatin systems as biomarkers in tissue samples of lung carcinoids and GEP-NETs. As registered drugs for other medical purposes, we evaluated the antitumor effects of ketoconazole, metformin and statins. Finally, somatostatin-dopamine receptor chimeras and telotristat were studied as novel drugs for hormone release and cell growth control.

Chapter 1 gives an overview of the current literature on epidemiological and clinical characteristics of NETs, diagnostic strategies and therapeutic options. Diagnosis is especially focused on novel circulating and (some) tissue biomarkers. **Part I** is focused on potential novel tissue biomarkers in NETs. **Chapter 2** describes the molecular and immunohistological presence of somatostatin/cortistatin, and ghrelin system components in human lung carcinoids, as well as their clinical and histological relations. In **chapter 3** the mRNA expression of somatostatin/cortistatin, system components in GEP-NETs is described and correlated with clinical features, histology and immunohistochemistry. Additionally, the *in vitro* evaluation of the observed clinical relation is also included. Finally, **chapter 4** describes the potential role of ghrelin O-acyltransferase (GOAT) and ghrelin receptor (GHSR1a) as tissue markers in GEP-NETs. **Part II** is focused on novel therapeutic options in NETs. To this aim, in **chapter 5** the *in vitro* direct and indirect effects of ketoconazole in ACTH- and non-ACTH producing tumor cells are studied. Additionally, an *in vitro* pancreas model of NETs using two-dimensional and three-dimensional culture systems is extensively evaluated in **chapter 6**, and in the same chapter the *in vitro* effect of somatostatin/dopamine agonists and somatostatin-dopamine multi-receptor targeting drugs is described. Furthermore, the first report of the *in vitro* effects of the novel serotonin syntesis inhibitor telotristat is described in **chapter** 7. The clinical relation between metabolic syndrome and NETs is described in **chapter 8**, as well as some clinical data and *in vitro* effects of biguanides and statins in NETs. Finally, **chapter 9** and **10** provide a general discussion and summary of the presented data.

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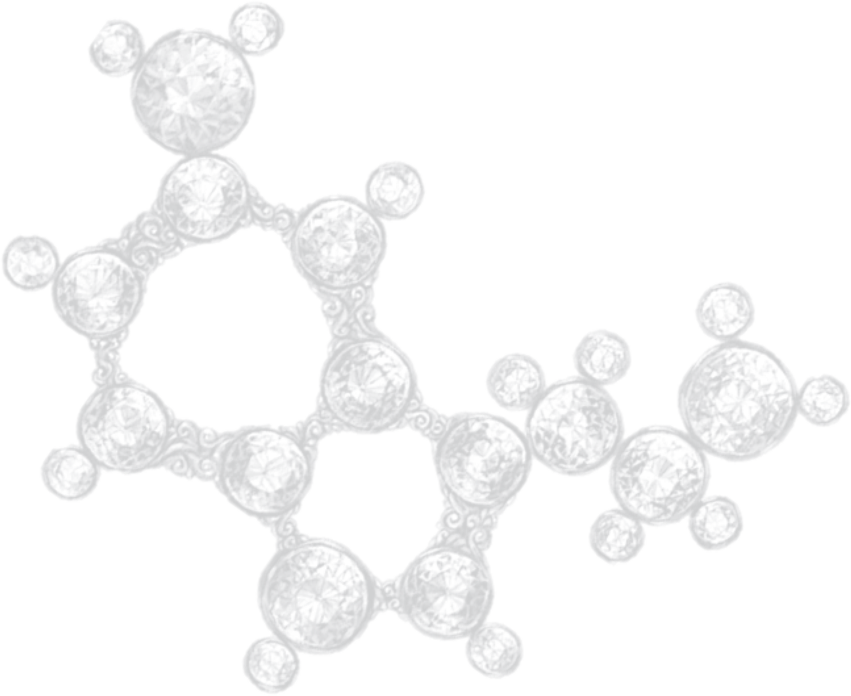
PART I

Novel diagnostic markers for neuroendocrine tumors



Chapter 2

The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features

Herrera-Martínez AD[1,2], Gahete MD[1,3], Sánchez-Sánchez R[1,2], Salas RO[1,2], Serrano-Blanch R[1,2], Salvatierra Á[1,2], Hofland LJ[4], Luque RM[1,3], Gálvez-Moreno MA[1,2], Castaño JP[1,3]

[1] Maimonides Institute for Biomedical Research of Córdoba
[2] Reina Sofi University Hospital
[3] Department of Cell Biology, Physiology, and Immunology, University of Córdoba, Spain
[4] Department of Internal Medicine, division of Endocrinology, Erasmus
MC, University Medical Center Rotterdam, the Netherlands

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ABSTRACT

Background: Lung carcinoids (LCs) are rare tumors that comprise 1-5% of lung malignancies but represent 20-30% of neuroendocrine tumors. Their incidence is progressively increasing and a better characterization of these tumors is required. Alterations in somatostatin (SST)/cortistatin (CORT) and ghrelin systems have been associated to development/progression of various endocrine-related cancers, wherein they may become useful diagnostic, prognostic and therapeutic biomarkers.

Objectives: We aimed to evaluate the expression levels of ghrelin and SST/CORT system components in LCs, as well as to explore their putative relationship with histological/clinical characteristics.

Patients and methods: An observational retrospective study was performed; 75 LC patients with clinical/histological characteristics were included. Samples from 46 patients were processed to isolate mRNA from tumoral and adjacent non-tumoral region, and the expression levels of SST/CORT and ghrelin systems components, determined by quantitative-PCR, were compared to those of 7 normal lung tissues.

Results: Patient cohort was characterized by mean age 53 ±15 years, 48% males, 34% with tobacco exposure; 71.4/28.6% typical/atypical carcinoids, 21.7% incidental tumors, 4.3% functioning tumors, 17.7% with metastasis. SST/CORT and ghrelin system components were expressed at variable levels in a high proportion of tumors, as well as in adjacent non-tumor tissues, while a lower proportion of normal lung samples also expressed these molecules. A gradation was observed from normal non-neoplastic lung tissues, non-tumoral adjacent tissue and LCs, being SST, sst4, sst5, GHS-R1a and GHS-R1b overexpressed in tumor tissue compared to normal tissue. Importantly, several SST/CORT and ghrelin system components displayed significant correlations with relevant clinical parameters, such as necrosis, peritumoral and vascular invasion, or metastasis.

Conclusion: Altogether, these data reveal a prominent, widespread expression of key SST/CORT/ghrelin system components in LCs, where they display clinical-histological correlations, which could provide novel, valuable markers for NET patient management.

Key Words: lung carcinoids; somatostatin system; ghrelin system; clinical-histological features

INTRODUCTION

Lung neuroendocrine tumors (LNETs) represent 20-30% of all neuroendocrine tumors [1-3]. The 2015 World Health Organization (WHO) classifies lung neuroendocrine tumors in low-grade typical carcinoid (TC), intermediate-grade atypical carcinoid (AC) and high-grade large cell neuroendocrine carcinomas (LCNEC) and small cell carcinoma (SCLC)[4]. Although these neoplasms share morphological, immunohistochemical and ultrastructural features, there are significant clinical, prognostic and therapeutic differences between subtypes. Indeed, high-grade tumors are very aggressive and display poor prognosis, while lung carcinoids (LCs, including TCs and ACs) have been less characterized, with a less standardized clinical management, apart from surgical resection or chemoradiotherapy [5, 6].

LCs display an incidence of 5-10 cases per million population/year [7, 8], and 5-15% of multiple neuroendocrine neoplasia 1 (MEN1) patients [9, 10]. Compared to other NETs, a lower proportion of LCs exhibits hormone hypersecretion, such as vasoactive intestinal peptide, adrenocorticotropic hormone (ACTH), and diuretic hormone, wherein the most common hormonal syndrome is the ectopic ACTH syndrome [11]. Paraneoplastic syndromes, including inappropriate antidiuretic hormone secretion, are even less frequent [9]. Importantly, LCs prognosis is tightly correlated to histotype, as TCs have a 5-year survival rate of 87%, presenting regional lymph node metastasis in 10-15% and distant metastases in 3-5% of cases. In contrast, ACs are more aggressive, with frequent nodal and distant metastases (20–50% respectively) and a 5-year survival of 60% [3, 12, 13]. Anyway, the only curative treatment for LCs is radical surgery [14].

The heterogeneity of these neoplasms, their different clinical behavior, and the possibility of recurrence or long-term metastasis, emphasize the importance of identifying new diagnostic and therapeutic markers, which could improve the diagnosis, prognosis and/or treatment of these patients. Accordingly, alterations in the regulatory neuroendocrine systems comprised by somatostatin (SST)/cortistatin (CORT), ghrelin and their receptors (ssts and GHSRs, respectively) have been associated to the development/ progression of various endocrine-related cancers. However, their expression has not been systematically characterized in LCs.

SST and CORT are two highly related neuropeptides that exert a plethora of physiological, often inhibitory, functions, by acting through their so-called SST receptors (sst1-5) [15-18], which regulate, among other activities, cell proliferation, differentiation, and angiogenesis [19]. They are widely distributed in normal and tumoral tissues, playing a useful role in tumor imaging (sst-scintigraphy or octreotide scan) [20]. More importantly, synthetic SST analogues (SSAs) represent a valuable therapeutic tool to treat ssts-positive tumors, to control hormone hypersecretion and tumor growth [21-24]. Additionally, ghrelin is a peptide hormone with multiple, and generally stimulatory functions, which span from hormone release to regulation of tumor cell proliferation [25, 26]. Ghrelin needs to be acylated by the enzyme ghrelin-O-acyl transferase (GOAT) [27, 28] to bind its receptor GHSR-1a [29]. The complexity of SST/CORT and ghrelin systems has been lately expanded by the identification of additional splicing variants of ghrelin (In1-ghrelin) [30-35], ghrelin receptor (GHSR-1b) [36, 37] and sst5 (sst5TMD4 and sst5TMD5) [38-44] genes, which are overexpressed in tumoral pathologies, including gastroenteropancreatic NETs (GEP-NETs 34, 44), where they are associated with aggressive features.

The data collected in a limited number of studies on the presence and clinical implications of certain components of these systems [34, 45-54] suggest that a more detailed knowledge of the expression pattern of their components could unveil relevant implications in the diagnosis, prognosis and medical treatment of LCs. Accordingly, we implemented an exhaustive characterization of the presence/expression of the components of SST/CORT and ghrelin systems in well-characterized LCs, compared to non-tumoral adjacent tissues and normal non-neoplastic samples, and explored their putative relationship with clinical/histological characteristics.

MATERIALS AND METHODS:

Patients

The Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain) approved the study, which was conducted in accordance with the Declaration of Helsinki and with national and international guidelines. A written informed consent was signed by every individual. Seventy-five patients with LCs who underwent surgery from 2005 to 2015 were included. Clinical records were used to collect full medical history. Endocrine-associated syndromes such as MEN or von Hippel-Lindau syndromes were excluded. LCs were evaluated and classified according to histopathology features in TC and AC. To confirm the neuroendocrine nature of all tumors, different neuroendocrine markers (including chromogranin A, synaptophysin, cytokeratin 7, cytokeratin 20, CD56, neuronal specific enolase, AE1AE3, cytokeratin, p53, glucagon, insulin, gastrin, SST, intestinal vasoactive peptide, pancreatic polypeptide and/or serotonin) were determined by immunohistochemistry following standardized protocols and evaluated by two experienced pathologists to confirm that all the included neuroendocrine tumors expressed a minimum of two different neuroendocrine markers. In addition, tissue samples were obtained from 46 of those patients and from 7 normal tissues from anonymous body organ donors. In particular, we obtained 89 formalin-fixed paraffin-embedded (FFPE) samples (46 primary tumors and 43 non-tumoral adjacent tissues) and 7 normal tissues. To ensure the appropriate identification of tumor and non-tumor adjacent areas for further RNA isolation, a comprehensive analysis of hematoxylin/eosin and immunohistochemistry sections was performed by two different experienced pathologists using conventional microscopy. Each sample was evaluated twice in order to identify, delineate and manually dissect the corresponding tissues, and when tumor and adjacent tissue were appropriately identified, 5um slides from each paraffin-embedded tissue were cut and tumor and non-tumor adjacent regions subsequently separated for further evaluations.

RNA isolation and reverse-transcription

Total RNA from FFPE samples was isolated using the RNeasy FFPE Kit (Qiagen, Limburg, Netherlands) according to manufacturer's instructions. Quantification of the recovered RNA was assessed using NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). One microgram of total RNA was retrotranscribed to cDNA with the First Strand Synthesis kit using random hexamer primers (Thermo Scientific) as previously reported [39, 55].

Quantitative real time PCR (qPCR)

cDNAs were amplified with the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) using the Stratagene Mx3000p system and specific primers for each transcript of interest, as previously reported. Expression levels (absolute mRNA copy number/50ng of sample) of native-ghrelin, In1-ghrelin, GOAT, GHSR-1a, GHSR-1b, SST, CORT, sst1, sst2, sst3, sst4, sst5 and sst5TMD4 were measured using previously validated primers [31, 33, 56, 57]. Samples were run, in the same plate, against a standard curve to estimate mRNA copy number and a No-RT sample as negative control. Thermal profile consisted of an initial step at 95°C for 30s, followed by 50 cycles of denaturation (95°C for 20s) and annealing/elongation (60°C for 20s), and finally, a dissociation cycle (melting curve: 55°C to 95°C, increasing 0.5°C/30s) to verify that only one product was amplified. RNA expression was adjusted by the expression of the housekeeping gen beta-actin (ACTB), whose levels were not significantly different among groups.

Immunohistochemistry (IHC) analysis

IHC analysis of GHSR-1a and sst4 was implemented in formalin-fixed, paraffin-embedded (FFPE) lung tissue samples (n=19), which included tumor and non-tumor regions from patients diagnosed with LCs, using standard procedures. The optimum antibody concentration to perform GHSR-1a and sst4 IHC analyses (1:300) using a commercially available human GHSR-1a and sst4 antibodies (Santa Cruz and AVIVA, respectively) was selected by performing a series of antibody dilution tests (1:100; 1:200; 1:300 and 1:400) in brain samples (a tissue that has been previously reported to express high levels of GHSR-1a and sst4). Two independent pathologists performed the IHC analysis of the samples following a blinded protocol. In the analysis, 1+, 2+, 3+ stand for low, moderate, and high intensities of the tumoral region staining compared to the adjacent region with non-tumor lung tissue.

Statistical analysis

Paired t-test analysis was used to compare the expression levels between LC samples and adjacent non-tumoral tissue. Non-paired t-test analysis was used to compare the expression levels between normal lung tissue and tumor or adjacent non-tumoral tissue. U-Mann Whitney tests were used to evaluate clinical-molecular relations. Chi-squared test compared categorical data. Statistical analyses were performed using SPSS statistical software v20 and GraphPad Prism v6. Data are expressed as mean±SEM. p-values<0.05 were considered statistically significant.

RESULTS

Patient population and clinical correlations

A total of 75 LCs patients were included. Demographic and clinical features are summarized in Table 1, while characteristics of the 49 tumor tissues are summarized in Table 2. Demographic and clinical characteristics were similar between TC and AC subjects. When all LC patients were considered together, age was positively correlated to second neoplasm presence

Table 1: Demographic and clinical characteristics of the patient population

General characteristic	Total n=75 (100%)	Typical n=34 (69.4%)	Atypical n=15 (30.6%)	p*
Gender				>0.05
Male	48% (36)	44.1% (15)	66.7% (10)	
Female	52% (39)	55.9% (19)	33,3% (5)	
Age	53.13±15.18 years	49.36±3.45	51.66±4.62	>0.05
Personal history of other tumors	17.4% (12)	22.6% (7)	20% (3)	>0.05
Smoke habit				>0.05
Active	34% (18)	33.3% (8)	28.6%(4)	
Ex-smoker	28.3% (15)	33.3% (8)	28.6% (4)	
No habit	37.7% (20)	33.3% (8)	28.6% (4)	
Family history of neoplasms	55.6 % (5)	66.7% (2)	33.3 % (1)	>0.05
Incidental tumor	21.7% (10)	29.4% (5)	36.4% (4)	>0.05
Pre-surgical treatment	6.1% (4)	3.2% (1)	14.3% (2)	>0.05
Clinical symptoms**				>0.05
Hemoptysis	17.9% (5)	23.5% (4)	9.1% (1)	
Cough	10.7% (3)	11.8% (2)	9.1% (1)	
Pneumonia	35.7% (10)	35.3% (6)	36.4% (4)	

**p* value refers to the comparison between typical and atypical carcinoids

** most common clinical symptoms

Table 2: Tumor sample characteristics

Tissue samples	**Total (%) (46)**	**Typical n=22 (66.7%)**	**Atypical n=11 (33.3%)**	**p***
Primary tumor localization				
Right lung	62.2% (28)	72.7% (16)	54.5% (6)	>0.05
Left lung	37.8% (17)	27.3% (5)	45.5% (5)	>0.05
Upper lobe	25.6% (11)	18.2%(4)	36.4% (4)	>0.05
Right middle lobe	27.9% (12)	40.9% (9)	18.2% (2)	>0.05
Lower lobe	44.2% (20)	36.4% (8)	36.4% (4)	>0.05
Immunohistochemistry				
Chromogranin A	39.6% (19)	34,28% (12)	40%(6)	>0.05
Synaptophysin	31.3% (15)	28.57%(10)	28.57% (4)	>0.05
Cytokeratin 7	4.2% (2)	2.86%(1)	9.1% (1)	>0.05
Cytokeratin 20	2.1% (1)	0% (0)	7.14% (1)	>0.05
Neuronal specific enolase	22.9% (11)	17.14% (6)	21.43% (3)	>0.05
CD56	18.8% (9)	18.2% (4)	18.2% (2)	>0.05
Others**	73% (36)	62.85%(22)	71.42% (10)	>0.05
Functionality	4.3% (2)	0% (0)	8.3% (1)	>0.05
Tumor diameter (cm)	**2.72±2.05**	**2.38±0.21**	**4.81±1.36**	**0.013**
Multiple tumors	7% (5)	3% (1)	13.3% (2)	>0.05
Peri-tumoral invasion	**22.5% (9)**	**6.7% (2)**	**46.2% (6)**	**0.028**
Vascular invasion	**16.7% (4)**	**0% (0)**	**50% (4)**	**0.005**
Neural invasion	11.8% (2)	0% (0)	33.2% (2)	>0.05
Metastasis	**12.5% (5)**	**0% (0)**	**46.2% (5)**	**0,007**
Bronchial lumen localization/infiltration	80% (32)	75% (21)	66.7% (8)	>0.05
Parenchyma localization/infiltration	32.5% (13)	35.7% (10)	58.3% (7)	>0.05
Pleural localization/infiltration	7.5% (3)	10.7% (3)	0% (0)	>0.05

**p* value refers to the comparison between typical and atypical carcinoids

**Others indicate positive immunohistochemistry for (at least) one of the following neuroendocrine markers: AE1AE3, cytokeratin, p53, glucagon, insulin, gastrin, SST, intestinal vasoactive peptide, pancreatic polypeptide and/or serotonin

(p=0.006) and parenchyma localization (p=0.013), and showed a non-significant trend to correlate with vascular invasion (p=0.055). Although all tumors were positive for at least one neuroendocrine marker, these markers did not exhibit any apparent association with clinical variables. In contrast, tumor diameter was directly correlated to necrosis (p=0.016), peritumoral invasion (p=0.006) and metastasis (p=0.026). ACs exhibited significantly higher diameter (p=0.022), necrosis (p=0.013), vascular invasion (p=0.005), peritumoral tissue invasion (p=0.028), and metastasis (p<0.001) than TCs.

Histopathological characterization of LCs and non-tumoral adjacent tissue

Representative histological images of TC and ACs, adjacent non-tumor tissues and normal lungs are depicted in Figure 1. Remarkably, adjacent non-tumor tissue displayed clear signs of pathological transformation (Figure 1C) as they presented diffuse interstitial chronic inflammation characterized by lymphocytes, scattered plasma cells and occasional multinucleated giant cells, which was not observed in normal non-neoplastic lung tissues (Figure 1D).

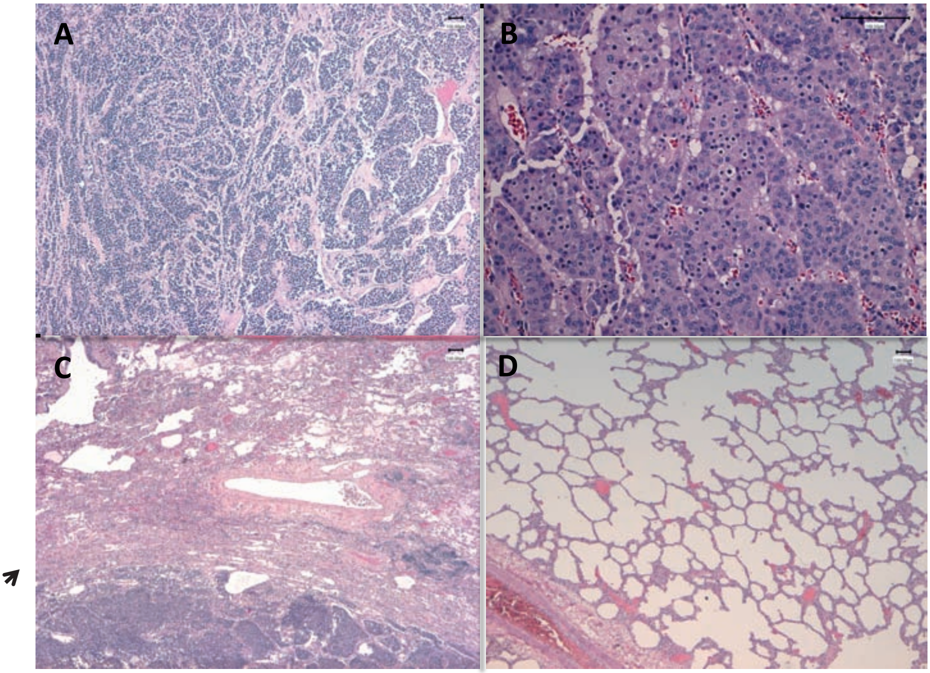

Figure 1: Histopathological evaluation of normal lung, adjacent non-tumoral tissue and LCs samples. Representative images of hematoxilin/eosin staining performed on TC (A), AC (B), adjacent non-tumoral tissue characterized by diffuse interstitial chronic inflammation with lymphocytes, scattered plasma cells and occasional multinucleated giant cells (narrows) (C) and normal lung controls (D).

Expression of SST/CORT system components in control and LC samples

SST/CORT system components were expressed in a modest proportion of normal lung samples, as determined by qPCR. Only SST and sst3 were expressed in almost 50% (3 out of 7) of normal samples, whereas other SST/CORT components were only detected in 1 or 2 samples (Figure 2A). In contrast, a high proportion of tumoral and, also, adjacent non-tumoral tissues expressed most of the SST/CORT system components (Figure 2A). Specifically, SST, sst1, sst2, sst3, sst5, and sst5TMD4 were expressed in at least 75% of adjacent non-tumoral and tumoral samples, with only sst4 being present in less than 25% of the adjacent and tumoral tissues (Figure 2A). Of note, the percentage of tumoral tissues

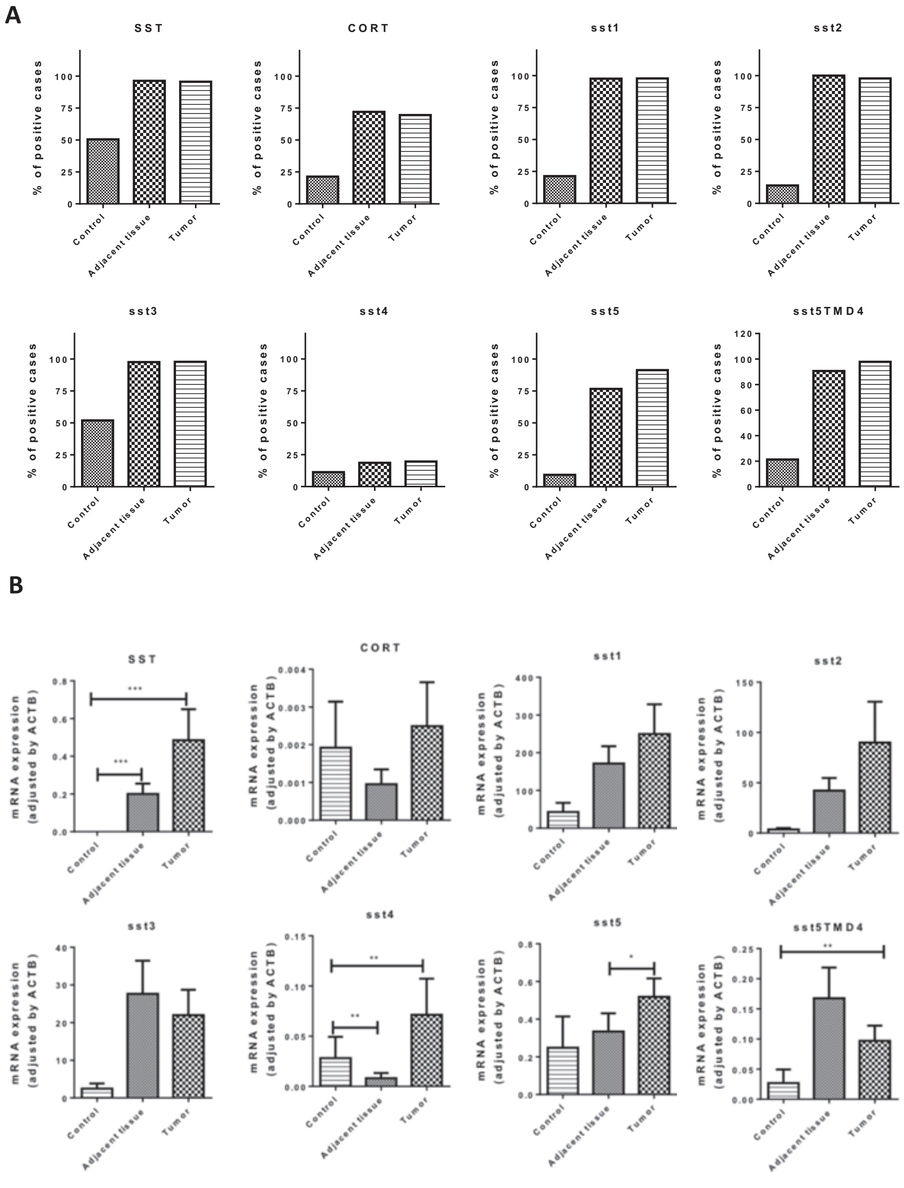

Figure 2: Presence and mRNA expression of SST/CORT system components in normal lung, adjacent non-tumoral tissue and LCs. A: The graphs indicate the percentage of samples (normal lung control, adjacent non-tumoral tissue and tumoral tissue) positive for each of the SST/CORT system components. **B:** The absolute mRNA expression of the different components of the SST/CORT system was determined by qPCR in normal lung controls, adjacent non-tumoral tissue and LC samples (values are adjusted by ACTB expression). Data represent the mean±SEM. Asterisks (*, p<0.05; **, p<0.01; ***,p<0.001) indicate significant differences by paired analysis between adjacent non-tumoral and LCs and non-paired analysis between normal lung tissue and adjacent non-tumoral or tumoral tissues.

expressing the components of SST/CORT system was similar between AC and TC (p>0,05) (Suppl. Figure 1).

Expression levels of the SST/CORT system components were largely variable. In this analysis, only cases that showed detectable expression were included. SST expression levels were 100-fold higher than those of CORT, whose mRNA levels were close to the detection limits (Figure 2B). SST levels were higher in non-tumoral adjacent and tumoral tissues compared to control tissue, being this increase more pronounced in tumoral tissue (Figure 2B). In the case of the receptors, sst1 and sst2 were highly expressed, followed by sst3, while sst5, sst5TMD4 and sst4 showed lower levels. Expression of all ssts, except sst3, displayed a similar tendency, increasing progressively from control tissues to non-tumoral adjacent tissue and being apparently higher in the tumoral regions (Figure 2B). Interestingly, in tumor samples, SST expression was correlated to sst1, sst2, sst3 and sst5 expression; CORT levels were correlated to sst5 expression and, finally, sst1, sst2 and sst3 expression levels showed significant correlations (Suppl. table 1).

Expression of ghrelin system components in control and LC samples.

Ghrelin system components were also expressed in <25% of normal lung samples, as determined by qPCR (Figure 3A). In contrast, ghrelin, In1-ghrelin, GHSR-1a and GHSR-1b were expressed in at least 75% of tumoral and adjacent non-tumor tissues; while GOAT was present in less than 50% of the adjacent non-tumoral samples but in more than 75% of tumoral samples (Figure 3A). The proportion of tumoral tissues expressing ghrelin system components was not statistically significant different between AC and TC (Suppl. Figure 2). In contrast, GHSR-1a and GHSR-1b were overexpressed in tumor tissue and adjacent-non tumoral tissue compared to normal lung tissue (Figure 3B). No significant correlation was observed among the expression levels of the ghrelin system components in tumor samples (data not shown).

Immunohistochemistry (IHC) analysis

Although qPCR is a sensitive method of assaying for gene expression, we subsequently performed IHC analysis in a set of selected samples in order to validate the observed changes at the protein level, and to determine which particular cells are expressing those markers. To this end, we selected sst4 and GHSR-1a due to their clear overexpression in tumor samples. Specifically, GHSR-1a and sst4 IHC was performed on FFPE-lung carcinoids, which revealed stronger staining in tumor samples compared to non-tumor adjacent tissue (Suppl. Figure 3B, 4B). In general, IHC analysis of non-tumol adjacent tissue revealed that only few cells from pulmonary parenchyma and glandular tissue were specifically stained (Suppl. Figure 3A, 4A). However, it is worth noting that infiltrated immune cells and especially alveolar macrophages presented an intense staining in these samples. In contrast, IHC

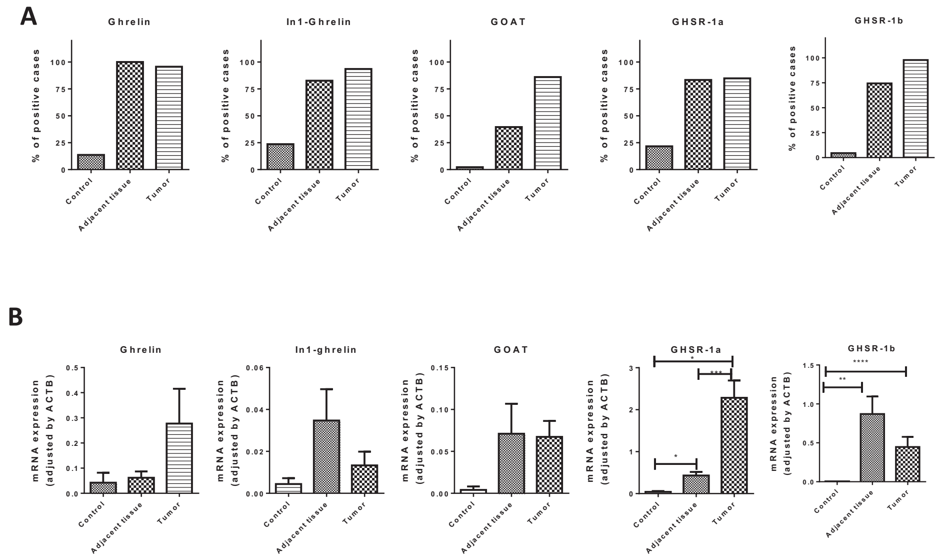

Figure 3: Presence and mRNA expression of ghrelin system components in normal lung, adjacent non-tumoral tissue and LCs. 3A: The graphs indicate the percentage of samples (normal lung control, adjacent non-tumoral tissue and tumoral tissue) positive for each of the ghrelin system components. **3B:** The absolute mRNA expression of the different components of the ghrelin system was determined by qPCR in normal lung controls, adjacent non-tumoral tissue and LC samples (values are adjusted by ACTB expression). Data represent the mean±SEM. Asterisks (*, $p<0.05$; **,$p<0.01$; ***,$p<0.001$) indicate significant changes by paired analysis between adjacent non-tumoral and LCs and non-paired analysis between normal lung tissue and adjacent non-tumoral or tumoral tissues.

analysis of tumor tissue revealed that GHSR-1a and sst4 were present in the vast majority of tumor cells. Interestingly, tumor samples presented variable levels of both molecules, although most samples were classified as having an intensity of 2+ or 3+ by the pathologists. Therefore, these data confirm the contention that the expression of certain components of the SST/CORT/ssts and ghrelin/GHSRs systems, and especially GHSR-1a and sst4, is clearly dysregulated in LC samples compared to non-tumor adjacent tissue.

Expression of SST/CORT and ghrelin systems components and clinical-histological characteristics in tumoral LC samples.

Correlation analyses revealed that sst3 was overexpressed in LCs of patients with tobacco smoke exposure ($p<0.05$), that sst5 was higher in incidental tumors, and that disease-free patients exhibited higher sst5TMD4 expression (Figure 4). Regarding the ghrelin system, ghrelin expression was correlated to vascular invasion ($p=0.042$) and tended to associate with bronchial localization ($p=0.057$) (Figure 4). Interestingly, necrotic tumors overexpressed GOAT ($p<0.05$) and GHSR-1a was overexpressed in tumors with parenchyma localization and in non-functional and metastatic tumors ($p<0.05$) (Figure 4).

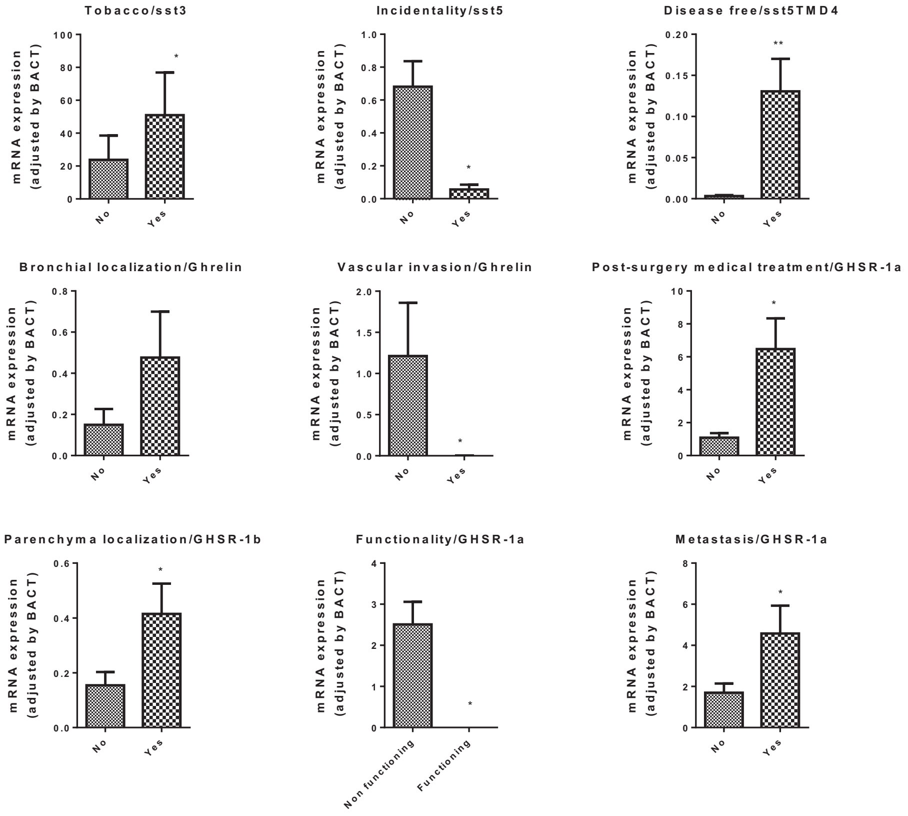

Figure 4: Correlations between epidemiological, clinical, histological and molecular parameters in LCs. The putative correlations between epidemiological, clinical, histological and molecular parameters within LC samples were assessed by U-Mann Whitney tests and asterisks (*, p<0.05; **, p<0.01) indicate significant associations.

DISCUSSION

In this study, we have comprehensively evaluated by qPCR the expression of SST/CORT and ghrelin system components in a large series of well-characterized TCs and ACs, and compared with the expression in adjacent non-tumoral and normal lung tissues. To the best of our knowledge, this study represents the first systematic characterization of the components of these regulatory systems in samples from LCs, in comparison with adjacent non-tumoral regions and normal lungs, and may therefore provide a useful overall picture of the landscape of changes associated to LCs pathology. Although several studies have explored the presence of certain SST/CORT and ghrelin systems components in LCs [45-52], their presence had never been compared to that found in adjacent non-tumoral regions or normal lungs. In addition, specific SST/CORT and ghrelin system components displayed clinical-histological correlations in tumoral tissues, suggesting that they could provide novel, valuable markers for LC-patient management.

Consistent with previous reports showing that 75% of LCs are central (bronchial) and 25% are peripheral tumors [3], our tumor series presented a similar distribution. In addition, previous studies have reported that LCs diameter is not correlated to survival or recurrence [13]. Consistently, in our series, no direct correlation with clinical outcome was observed, although tumors >2.4cm showed higher rate of peritumoral invasion, vascular invasion and distant metastasis. In fact, no correlations between clinical, histological or immunohistochemical characteristics and survival or mortality were found herein, which is in contrast with previous reports describing some independent predictors of survival [mitotic rate, tumor size, sex [58], typical histology and lymphatic invasion [59]]. Nevertheless, our analysis revealed that age correlated directly to the presence of parenchyma localization, a second neoplasm and vascular invasion, which have not been previously reported and could suggest age as a risk factor for more aggressive tumors.

Remarkably, tumoral characteristics of TCs and ACs were markedly different. ACs exhibited significantly higher diameter, necrosis, peri-tumoral and vascular invasion and metastasis, which is consistent with higher ACs malignancy compared to TCs [60]. Similar to other reports, adjacent non-tumoral tissues exhibited signs of pathological alteration compared to normal lung samples [61-64]. Consequently, to comprehensively characterize SST/CORT and ghrelin system components expression in a large series of well-characterized TCs and ACs, and compare with their expression in adjacent non-tumoral tissues and normal lungs, we applied a qPCR-based approach as previous studies have demonstrated that mRNA levels of the components of these systems correlate well with their respective protein levels [46, 48, 49, 65, 66]. Moreover, qPCR is a more sensitive detecting method than IHC [67].

NETs are known to overexpress stss [46, 53, 54], which is important in their diagnosis and management [20, 46, 68, 69]. However, to date, only a limited number of studies have reported the expression of ssts other than sst2, with variable results, likely due to the application of different experimental approaches [19, 70, 71]. Particularly, just few studies have explored a small number of cases or only single receptor subtypes [50, 65, 67, 70, 72]. Recently, additional studies have more comprehensively characterized ssts presence on LCs [46, 48, 73]. Interestingly, the data presented in the current study reveal the prominent and widespread expression of ssts in LCs, being sst1 the most abundant, followed by sst2, sst3 and sst5, with sst4 and the truncated sst5TMD4 being the least expressed. These data agree with the majority of the previous studies [48-52, 65, 67]. Moreover, this is the first study reporting the presence of sst5TMD4 in LCs and sst5 in symptomatic patients. Although initial studies suggested that ssts presence in LNETs could exhibit a progressive decrease from low- to high-grade forms [45]; however, our work and other studies [46] indicate that there are no major differences, suggesting that ssts could be a common hallmark of low and intermediate grade LNETs. Moreover, our results revealed, for first time, a prominent expression of SST in LCs, whereas CORT expression was comparatively negligible. Of note, expression of SST directly correlated with that of sst1, sst2, sst3 and sst5, which suggests an autocrine/paracrine SST/ssts loop capable to modulate *in situ* the progression of LCs. In addition, SST and ssts expressions displayed herein a gradation in normal lungs, non-tumoral adjacent tissue, and LCs. Of special interest is our observation that non-tumoral adjacent tissues also present a notable expression of SST and ssts, and in fact, a similar proportion of non-tumoral adjacent tissues presented ssts compared to LCs, although, in general, at lower expression levels. To further explore this notion, we selected sst4 (due to its differential distribution), to perform an IHC analysis, as it has been previously reported that specific antibodies against SST receptors allow an appropriate immunolocalization of receptor subtypes in tumor tissue with a comparable, although not quantitatively superior quality than that of qPCR [65]. Results from this analysis enabled visualization of sst4 in specific cells of tumor tissue and demonstrated that non-tumor adjacent cell type (in airway epithelium and associated neuroendocrine cells, as well as in pulmonary parenchyma and associated glandular tissue) are less immunopositive that other tumor cells.

Our results also revealed a differential expression of ghrelin system components in normal lung tissues, non-tumoral adjacent tissue, and LCs. In particular, ghrelin system components were expressed at low levels in a reduced proportion of normal lungs, which is consistent with previous reports showing ghrelin expression in normal and fetal lungs [30, 47, 66, 74]; while GHSR1a was undetectable [74]. In contrast, our analysis revealed a prominent and widespread expression of ghrelin system components in LCs and adjacent non-tumoral samples. Interestingly, a higher expression levels of the canonical variants (native ghrelin and GHSR1a) is consistent with previous reports showing that ghrelin is expressed in lung tumors, regardless of their neuroendocrine phenotype, and that GHSR1a is present in well differentiated functioning and non-functioning lung NETs [29, 75]. In contrast, expression of the alternative splicing variants (In1-ghrelin/GHSR1b) is lower, and had not been reported previously. This is also the first study reporting the expression of the GOAT enzyme in a high proportion of LCs, wherein the concomitant presence of ghrelin, GOAT and GHSR1a on most LCs suggests the existence of a functional regulatory association that could be modulating the development and/or progression of this pathology. Unfortunately, no studies have yet investigated the direct effect of this in LCs, and the only report in SCLC suggests that ghrelin could inhibits cell proliferation and increases apoptosis [29], in agreement with the negative association between ghrelin and vascular invasion in our cohort. However, GOAT levels were higher in tumors with necrosis, which were the ones with a larger size and higher capacity of peritumoral invasion and distant metastasis; this, together with the direct relationship between metastasis, requirements of post-surgical treatments, and GHSR1a expression, reinforces the idea that this system could be associated to the pathogenesis of the disease and might therefore provide novel potential diagnostic, prognostic and/or therapeutic tools in LCs.

In summary, this study provides a comprehensive primary mapping of the expression of SST/CORT and ghrelin system components (including their most relevant splicing variants), in LCs, as compared with their respective adjacent non-tumoral tissues, and with normal, non-neoplastic tissues. Our results indicate a prominent and widespread overexpression of SST/CORT and ghrelin system components in LCs and in non-tumoral adjacent tissues, wherein they could exert relevant regulatory roles, for they display changes in expression tightly linked to the degree of disease, and exhibit associations to fundamental clinical parameters. Hitherto, there has been a paucity of studies reporting clinical, biochemical, histological, immunohistochemical or molecular tumor markers that could help to accurately predict the efficacy of the medical treatment, as well as the cure or relapse rates in NETS. This goal is specially difficult and necessary in LCs, due to their rarity, high diversity and heterogeneity in terms of malignant capacity, localization, and growth pattern. In this context, our present findings may help to identify new potential diagnostic and prognostic factors, which could help to devise and implement improved therapeutic strategies, aimed at attaining a better quality of life and survival for this patients. Hence, our data provide novel information on the presence of both SST/CORT and ghrelin systems in LCs, and invite to suggest that their role in this pathology as putative molecular biomarkers and therapeutic targets for LC patients deserves further investigation.

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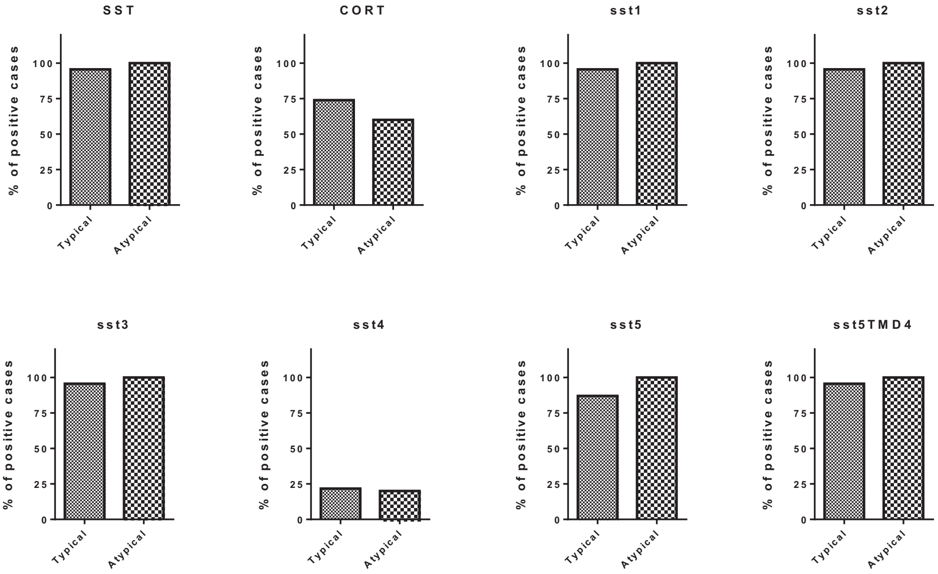
SUPPLEMENTAL DATA

Supplemental Table 1: SST/CORT system components correlations in tumor tissue

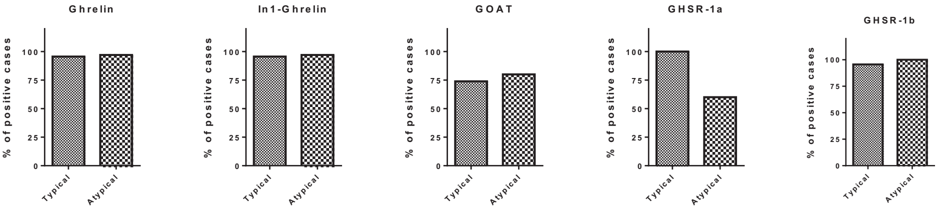
	sst1	sst2	sst3	sst4	sst5	sst5TMD4
SST	0.522***	0.613***	0.364*		0.392*	
CORT					0.430**	
sst1		0.838***	0.784***			
sst2			0.701***			

*$p<0.05$; **$p<0.001$; ***$p<0.0001$

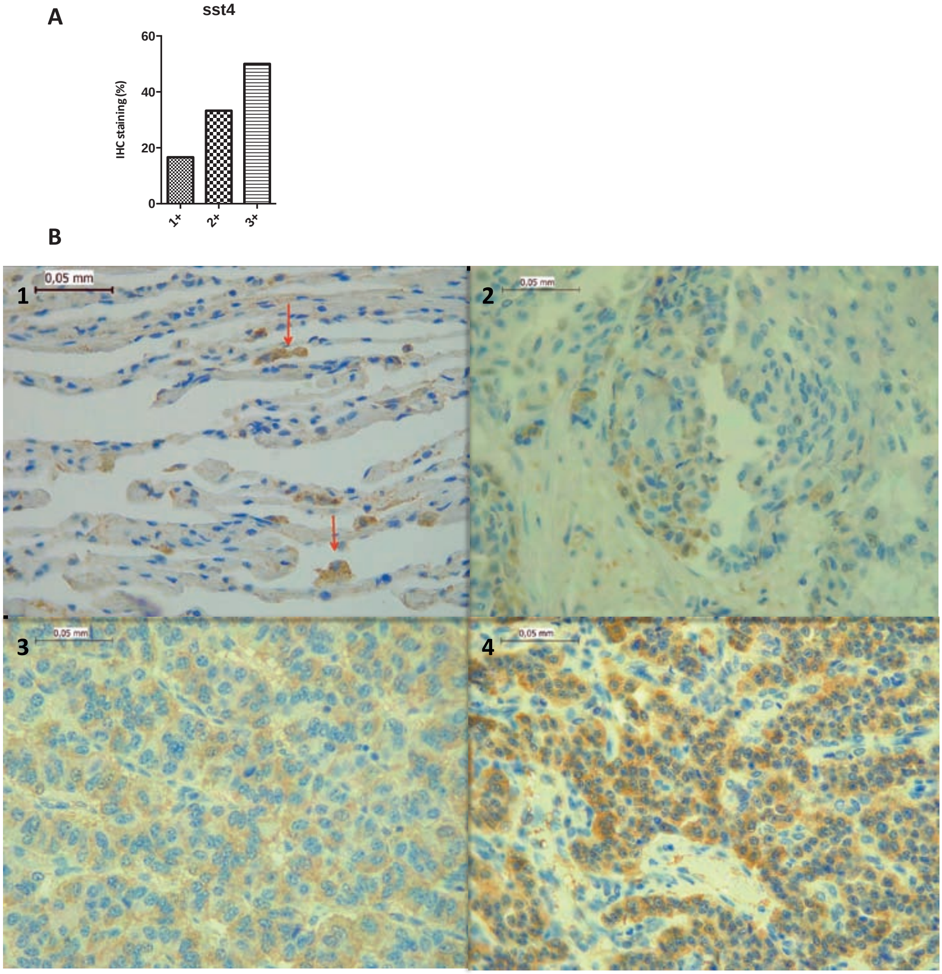
p value refers to the comparison between SST system components



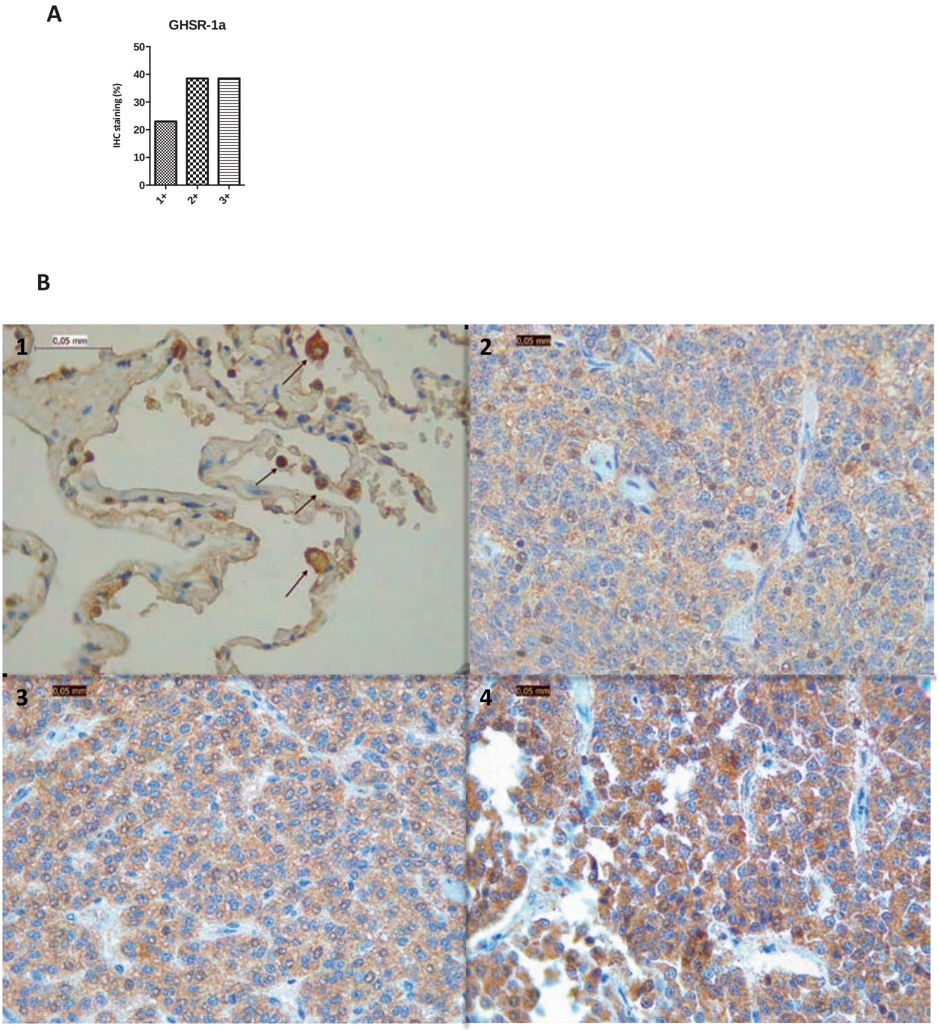
Supplemental Figure 1: Presence of SST/CORT system components in TC and AC. The graphs indicate the percentage of positive samples for each of the SST/CORT system components.



Supplemental Figure 2: Presence of ghrelin system components in TC and AC. The graphs indicate the percentage of positive samples for each of the ghrelin system components.



Supplemental Figure 3: Presence of sst4 by IHC in TC and AC. The expression of sst4 by immnunohistochemistry was determined in adjacent non-tumor tissue and LCs samples. **A:** Percentage of cases with 1+, 2+, 3+ staining, which represent low, moderate, and high intensities of the tumor region compared to the adjacent non-tumor tissue. **B**: Representative images of sst4 staining performed on non-tumor adjacent tissue (1), 1+ (2), 2+ (3) and 3+ (4) tumor LCs samples. The arrows represent isolated alveolar macrophages with positive IHC staining.



Supplemental Figure 4: Presence of GHSR1a by IHC in TC and AC. The expression of sst4 by immnunohistochemistry was determined in adjacent non-tumor tissue and LCs samples. **A:** Percentage of cases with 1+, 2+, 3+ staining, which represent low, moderate, and high intensities of the tumor region compared to the adjacent non-tumor tissue. **B**: Representative images of sst4 staining performed on non-tumor adjacent tissue (1), 1+ (2), 2+ (3) and 3+ (4) tumor LCs samples. The arrows represent isolated pneumocytes and alveolar macrophages with positive IHC staining.

Chapter 3

Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors

Herrera-Martínez AD[1,2], Gahete MD[1,3]Pedraza-Arevalo S[1,3] Sánchez-Sánchez R[1,2], Ortega-Salas R[1,2], Serrano-Blanch R[1,2], Luque RM[1,3], Gálvez-Moreno MA[1,2], Castaño JP[1,3]

[1] Maimonides Institute for Biomedical Research of Córdoba
[2] Reina Sofi University Hospital
[3] Department of Cell Biology, Physiology, and Immunology, University of Córdoba, Spain

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ABSTRACT

Purpose: Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) comprise a heterogeneous group of malignancies often presenting with metastasis at diagnosis and whose clinical outcome is difficult to predict. Somatostatin (SST) analogs (SSAs) provide a valuable pharmacological tool to palliate hormonal symptoms, and control progression in some NETs. However, many patients do not respond to SSAs or develop resistance, and there are many uncertainties regarding pathophysiology of SST and its receptors (sst1-sst5) in GEP-NETs.

Methods: The expression of SST system components in GEP-NETs was determined, compared with that of non-tumor adjacent and normal tissues and correlated with clinical and histological characteristics. Specifically, 58 patients with GEP-NETs and 14 normal samples were included. Cell viability in NET cell lines was determined in response to specific SSAs.

Results: Normal samples and non-tumor adjacent tissues presented a similar expression profile, with appreciable expression of sst2 and sst3, and a lower expression of the other receptors. In contrast, cortistatin, sst1, sst4 and sst5 were overexpressed in tumors, while sst3 and sst4 seemed overexpressed in less differentiated tumors. Some SST system components were related to vascular/nerve invasion and metastasis. *In vitro*, sst1 and sst3 agonists reduced viability in BON-1 cells, while they, similar to octreotide and pasireotide, increased viability in QGP-1 cells.

Conclusions: These results provide novel information on SST system pathophysiology in GEP-NETs, including relevant associations with clinical-histological parameters, which might help to better understand the intrinsic heterogeneity of NETs and to identify novel biomarkers and/or targets with potential prognostic and/or therapeutic value for GEP-NETs patients.

Key words: Carcinoids, somatostatin analogs (SSAs), sst1, sst3, prognosis, invasion, metastasis.

INTRODUCTION

Neuroendocrine tumors (NETs) are a heterogeneous group of malignancies arising from neuroendocrine cells of the diffuse endocrine system, which present a unique rising incidence in the last decades: from 1.09 (in 1973) to 6.98 (in 2017) new cases per 100,000 inhabitants annually [1]. Indeed, the Surveillance, Epidemiology and End Results (SEER) further support this contention by showing an increase in the incidence of GEP-NETs of all origins except to appendix NETs [2]. Gastroenteropancreatic NETs (GEP-NETs) originate from the pancreas or the intestinal tract and represent approximately 65% of all NETs and 2% of all gastroenteropancreatic malignancies [3, 4]. These neoplasms include functioning and non-functioning tumors, depending on their capacity to secrete peptide hormones, and can occur sporadically or as a result of hereditary predisposition syndromes such as multiple endocrine neoplasia type 1 (MEN-1) or Von Hippel–Lindau's disease (VHL), with clinical onset occurring earlier in patients with genetic predisposition [5]. Although some prognostic factors include primary tumor localization, histological differentiation, Ki67 index, platelets, and lactate dehydrogenase (LDH) levels [6], their clinical outcome is mostly unpredictable, in that even well-differentiated low-grade tumors can display an aggressive behavior [3]. Furthermore, in about 30% of NETs the primary tumor cannot be identified [6], and metastatic disease is frequently found at diagnosis, especially in non-functioning tumors, ranging from 27 to 73% depending on the series, with an overall survival rates varying from 30 to 90% at 5 years [5, 7, 8]. As a result, most tumors are diagnosed at an advanced stage of disease, wherein the only curative therapeutic option is surgery. For this reason, identification of novel, early diagnostic biomarkers, and development of new targeted medical treatments has gained scientific and clinical interest over the past recent years [9].

Somatostatin (SST), cortistatin (CORT), and their 5 receptors ss1-sst5 (i.e. the SST system) are widely expressed in multiple tissues, including the gastrointestinal tract (GIT) [10, 11]. In fact, based on their capacity to inhibit endocrine secretions from the GIT, synthetic SST analogs (SSAs) have long been used to palliate hormonal symptoms in NETs. Moreover, SSAs can also control disease progression in well-differentiated, metastatic midgut NETs and in moderately differentiated, metastatic nonfunctioning GEP-NETs [12]. Thus, the PROMID study showed that SSAs can delay tumor progression in both functionally active and inactive NETs [13], while the CLARINET study reported an increase in median progression-free survival in SSA-treated patients [14]. Nevertheless, many tumors, despite bearing sst, do not respond or eventually develop resistance to SSA treatments.

The antisecretory and antitumor actions of SSAs are mediated through the SST receptors (sst1-5), a family of five G-protein-coupled receptors that are widely distributed throughout the body and are present in most GEP-NETs [15-17]. Some studies suggest that the antiproliferative effects of SSAs are associated to their affinity for sst2 [18]. SSA treatment commonly exhibit a good safety profile [3] but after long-term tachyphylaxis may occur, which has been associated to a possible loss of sst2 availability following receptor internalization and degradation [19]. In addition, presence of sst5TMD4, a truncated splice variant of the sst5 subtype that interacts with sst2 and disrupts its signaling, has been associated with increased aggressiveness in pancreatic NETs and other hormone-dependent tumors [20, 21]. However, the exact mechanisms by which SST and SSAs exert their effects on NET cells are complex and not fully understood [3]. In fact, although various studies have examined the presence of SST system components in NETs, the clinical implications of all their members are not completely elucidated [22-24].

The intrinsic heterogeneity of NETs coupled to the their unpredictable behavior and prognosis complicates their clinical management, particularly owing to the lack of sufficient and reliable biomarkers to predict medical treatment response and patient prognosis. Accordingly, in this study we aimed to systematically and comprehensively determine the precise expression of SST system components in GEP-NETs, compare to non-tumor adjacent tissue and normal control tissue, and correlate them with clinical, functional and histological characteristics.

MATERIALS AND METHODS

Patients and samples

The Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain) approved the study, which was conducted in accordance with the Declaration of Helsinki and according to national and international guidelines. Every individual or family member signed a written informed consent before inclusion into the study. Data from 58 patients with GEP-NETs who underwent surgery at the Hospital from 2005 to 2015 were collected (demographic and clinical characteristics of the included patients are summarized in Table 1). As well, 14 normal control tissues were also included (four whole pancreas, three stomach, four midgut and three foregut samples). Patients with neurofibromatosis, multiple endocrine neoplasia type 1, Von Hippel-Lindau or any endocrine syndrome were excluded from the present study. Clinical records were used to collect full medical history of all patients. GEP-NETs were classified according to histopathological features as: well-differentiated NETs (G1), well-differentiated neuroendocrine carcinomas (G2), and poorly differentiated neuroendocrine carcinomas (G3). Cell survival was determined by counting Ki67 positive cells by experienced pathologists [25]. After surgery, if residual disease or relapse was observed, adjuvant treatment with SSAs or chemotherapy was prescribed; however, only two cases received pre-surgery treatment with SSAs. From all these subjects, we obtained 130 formalin-fixed

Table 1: General characteristics of GEP-NETs patients

General characteristic		% (n)
Gender		
	Male	48,3% (28)
	Female	51,7% (30)
Age		56,41±15,6 years
Personal history of other tumors		11,7%
Smoke habit		
	Active	57,7% (15)
	Ex-smoker	15,4% (4)
	No habit	26,9% (7)
Family history of neoplasms		45,5% (10)
Incidental tumor		35% (14)
Functionality		43,9% (18)
Pre-surgical treatment		14% (7)
SSA pre-surgical treatment		4% (2)

paraffin-embedded samples (58 tumor samples, 58 non-tumor adjacent tissue samples and 14 normal control tissues). In order to ensure the appropriate identification of relevant areas of tumor and non-tumor tissues to carry out the RNA isolation, a comprehensive analysis of hematoxylin and eosin (H&E) sections was performed by experienced pathologists.

RNA isolation and reverse-transcription

Total RNA from formalin fixed paraffin-embedded (FFPE) samples was isolated using the RNeasy FFPE Kit (Qiagen, Limburg, Netherlands) according to the manufacturer's instructions. Quantification of the recovered RNA was assessed using NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). One microgram of total RNA was retrotranscribed to cDNA with the First Strand Synthesis kit using random hexamer primers (Thermo Scientific) as previously reported [26, 27].

Quantitative real time PCR (qPCR)

cDNAs were amplified with the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) using the Stratagene Mx3000p system and specific primers for each transcript of interest. Specifically, expression levels (absolute mRNA copy number/50 ng of sample) of SST, cortistatin (CORT), sst1, sst2, sst3, sst4, sst5 and sst5TMD4 were measured using previously validated primers [28, 29]. Briefly, samples derived from human GEP-NET tissues were run, in the same plate, against a standard curve to estimate mRNA copy number and a No-RT sample as a negative control. Thermal profile consisted of an initial step at 95°C for 30 seconds, followed by 50 cycles of denaturation (95°C for 20s) and annealing/

elongation (60°C for 20s), and finally, a dissociation cycle (melting curve; 55°C to 95°C, increasing 0, 5°C/30 s) to verify that only one product was amplified. RNA expression was adjusted by the expression of 18S.

Immunohistochemistry (IHC) analysis

IHC analysis of sst1, sst2 and sst5 was implemented in formalin-fixed, paraffin-embedded (FFPE) tissue samples, which included tumor and non-tumor regions from patients diagnosed with GEP-NETs, using standard procedures. The optimum antibody concentrations to perform the IHC analyses (1:300) using commercially available antibodies against human sst1, sst2 and sst5 (respectively, UMB7, UMB1 and UMB4 from Abcam, Cambridge, UK) were selected by performing a series of antibody dilution tests (1:100; 1:200; 1:300 and 1:400) in brain samples (a tissue that has been previously reported to express high levels of somatostatin receptors [30, 31]). Independent pathologists performed the IHC analysis of the samples following a blinded protocol. In the analysis, negative, 1+, 2+, 3+ stand for absent, low, moderate, and high intensities of the tumor region staining compared to the adjacent region with non-tumor lung tissue.

Cell cultures

The human NET model cell lines BON-1 and QGP-1 were used in this study [20, 32]. Particularly, the human pancreatic neuroendocrine tumor BON-1 cell line was established from a lymph node metastasis of a human functional pancreatic NET BON-1 (The University of Texas Medical Branch, Galveston, Tex., USA), while the cell line QGP-1 was initially obtained from primary pancreas NET tissue obtained from a 61-year-old male [33]. Both cell lines were checked for mycoplasma contamination by PCR as previously reported [34]. BON-1 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM-F12; Life Technologies, Barcelona, Spain) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Madrid, Spain), 1% glutamine (Sigma-Aldrich) and 0.2% antibiotic (Gentamicin/Amphotericin B; Life Technologies). QGP-1 cells were cultured in RPMI 1640 (Lonza, Basel, Switzerland), supplemented with 10% FBS, 1% glutamine and 0.2% antibiotic. Cells were harvested with trypsin (0.05%)–EDTA (0.53 mM) and resuspended in culture medium; cell viability always exceeded 85%. Both cell lines were cultured at 37°C in a 5% CO_2 incubator. All cell line experiments were performed at least four times.

Drugs and reagents

The sst1 agonist (IPSEN Bioscience) was diluted in medium 5% FBS prior to use until obtaining final concentration of 10^{-6} M; sst3 agonist (IPSEN Bioscience), octreotide (Novartis Pharmaceuticals Corporation), and pasireotide (Novartis Pharmaceuticals Corporation), were diluted in medium 5% FBS prior to use until obtaining final concentration of 10^{-7} M. Paclitaxel 10^{-7} M (Sigma-Aldrich) was used as positive control. The tested concentrations were chosen based on the reported dose-dependent effects and sst1-5 subtype-binding affinities of somatostatin analogs, according to previously published data [35-41].

Cell viability assay

Cells were plated in 96-well plates at the density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment (5.000 cells/well). Twenty-four h later, serum-free medium was added during 24 h. Basal and 24h cell viability was determined using Alamar-Blue reagent. Specifically, the day of measurement, cells were incubated for 3 h in 10% Alamar-Blue/serum-free medium and then, Alamar-Blue reduction was measured in a FlexStation3 system (Molecular Devices) plate reader, exciting at 560 nm and reading at 590 nm. After this, the appropriate treatments (sst1 and sst3 agonists, octreotide and pasireotide) were added to wells in 5% FBS medium. In all instances, cells were seeded per quadruplicate and all assays were repeated a minimum of four times.

Statistical analysis

Paired t-test analysis was used to compare the expression levels between GEP-NETs samples and adjacent non-tumor tissue. Non-paired t-test analysis was used to compare the expression levels between normal lung tissue and GEP-NETs samples or adjacent non-tumor tissue. Mann-Whitney U test was used to evaluate clinical-molecular relations within GEP-NETs samples. Chi-squared test was used to compare categorical data. In functional experiments, results are expressed as percentage of control (non-treated cells). Cell survival rate compared to control was assessed by multiple comparison tests (One-way ANOVA followed by Dunnet post-hoc test). All statistical analyses were performed using SPSS statistical software version 20 and Graph Pad Prism version 6. Data are expressed as mean ± SEM. P-values < 0.05 were considered statistically significant.

RESULTS

A total of 58 patients with GEP-NETs and 14 normal control samples were included in the study. Clinical features of patients are summarized in Table 1, and tumor characteristics in Table 2. Twenty-eight patients (48.3%) had pancreatic NETs and 51.7% had gastrointestinal NETs; 43.9% were functioning tumors. Quantification of Ki-67 and mitotic index was available at diagnosis in only 75% of samples, 71% of samples were low or intermediate grade tumors. Interestingly, 51.7% of patients had metastasis at diagnosis, relapsed disease was observed in 41.2% of patients, and the mortality rate reached 24.5%.

Table 2: Tumor sample characteristics

Characteristic		n (%)
Tissue samples		
	Primary tumor	58
	Non-tumor adjacent tissue	58
	Normal tissue	14
Primary tumor localization		
	Pancreas	48,3% (28)
	Stomach	5,2% (3)
	Small bowel	22,4% (13)
	Colon and rectum	24,1% (14)
Maximal tumor diameter		3,16±2,09 cm
Necrosis	<10%	14,3% (1)
	10-20%	28,6% (2)
	21-30%	42,9% (3)
	>30%	14,3% (1)
Depth of infiltration (gastrointestinal NETs)		
	Submucosa	4% (1)
	Mucosa	4% (1)
	Muscular	44%(11)
	Serosa	48%(12)
Multiple tumors		7,1% (2)
Peri-tumor tissue invasion		66,7% (36)
Vascular invasion		36% (18)
Neural invasion		32,7% (16)
Metastasis		51,7% (30)
Metastasis localization		
	Liver	10% (3)
	Spleen	3,3% (1)
	Lymphatic nodules	27,6% (16)
	Multiple	29,9% (10)
Grading (WHO 2010 criteria)		
	Low	32,8% (19)
	Intermediate	37,9% (22)
	High	5,2% (3)
	Unknown	24,1% (14)
Post-surgical treatment		40,4% (19)
Relapsed disease		41,2% (21)
Disease free		61,4% (27)
New surgery requirements		23,1% (9)

Histopathological characterization of GEP-NETs and non-tumor adjacent tissue

Primary tumor samples were delimited from the non-tumor adjacent tissues after the evaluation of two experienced pathologists using histology and immunohistochemistry (Figure 1).

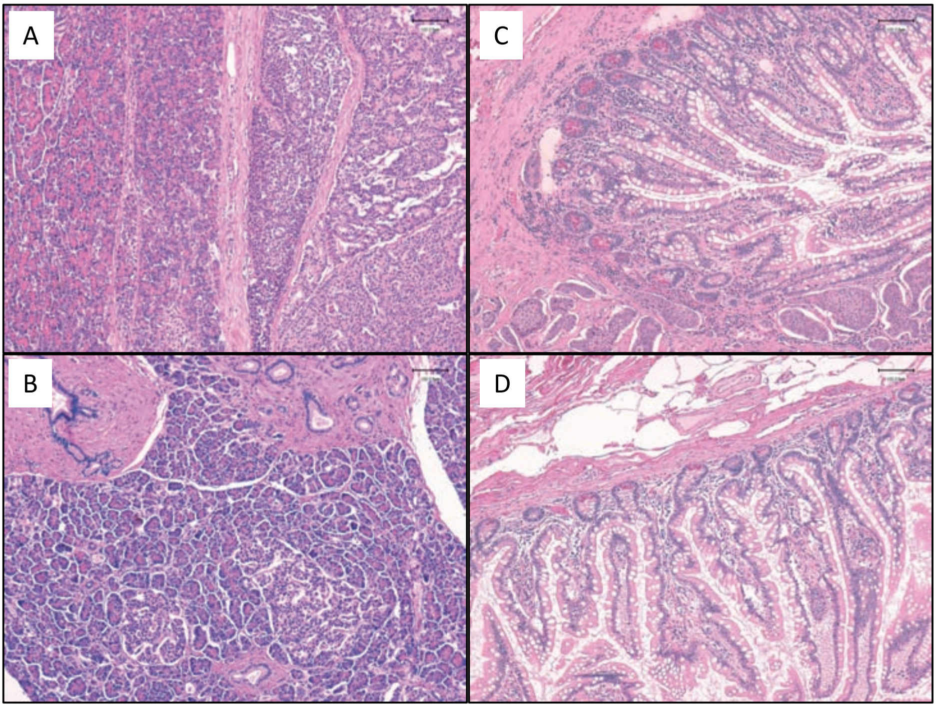

Figure 1: Representative images of pancreatic and intestinal NETs and control tissues. All images were taken from hematoxylin-eosin stained specimens at 100X. A) Pancreatic NET and adjacent tissue. B) Normal pancreas. C) Small intestine NET and adjacent tissue. D) Normal intestine. Scale bar is included in each image.

Expression of SST system components in control and GEP-NETs samples

Using a yes/no scale, SST system components were expressed in a high percentage of normal (control) GEP samples as determined by qPCR, especially SST, sst2, sst3 and sst5TMD4, which were expressed in more than 80% of the samples; while CORT was expressed in less than 30% of samples (Supplemental Figure 1). Consistently, all system components, except sst4, were expressed in at least 60% of adjacent non-tumor and tumor tissues. Instead CORT, sst1, sst4 and sst5 were present in a higher proportion of GEP-NETs samples compared to adjacent tissues, although the differences were not statistically significant (Supplemental Figure 1).

Normal tissue (control) samples and non-tumor adjacent tissues presented a similar profile of mRNA expression levels, with a considerable expression of sst2 and sst3 and a lower

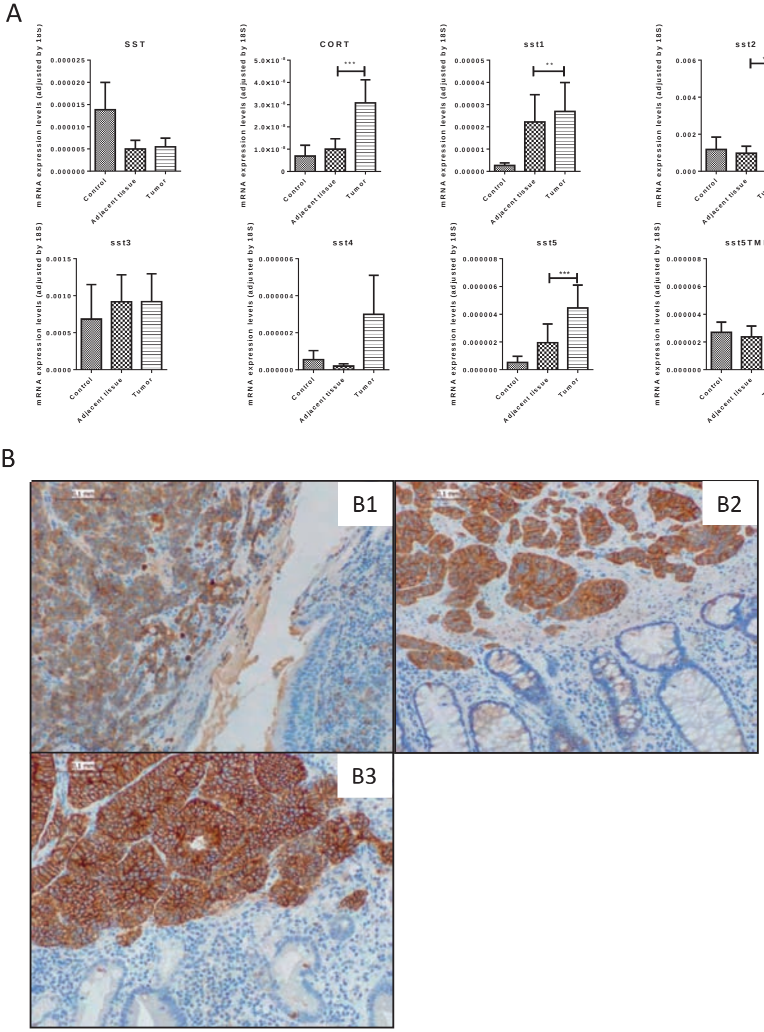

Figure 2: Presence and expression of SST/CORT system components in normal samples, adjacent non-tumor tissue and GEP-NETs. A) The absolute mRNA expression of the different components of the SST/CORT system was determined by qPCR in normal control samples, adjacent non-tumor tissue and GEP-NETs samples (values are adjusted by 18S expression). Data represent the mean±SEM. Asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) indicate significant changes by paired analysis between adjacent non-tumor and GEP-NETs and non-paired analysis between normal tissue and adjacent non-tumor or tumor tissues. B) **Immunohistochemical analysis of somatostatin** receptors sst1, sst2 and sst5 **in adjacent non-tumor tissue and GEP-NETs.** The presence of sst1, sst2 and sst5 by immunohistochemistry using specific antibodies was determined in a subset of samples, which included tumor and non-tumor regions from patients diagnosed with GEP-NETs. Representative images from pieces stained with sst1, sst2 and sst5 are depicted in B1, B2 and B3, respectively. This analysis revealed that somatostatin receptors were present in the vast majority of tumor cells compared with non-tumor adjacent tissue, with different grades of staining.

expression of the rest of receptors (sst1, sst4 and sst5) and the ligands (SST and CORT). Interestingly, CORT and the receptors sst1, sst2, and sst5 were clearly overexpressed in tumor tissues compared with adjacent non-tumor tissue samples (Figure 2). Expression of sst3 and sst4 tended to increase in GEP-NETs according to their grade, while the opposite trend (decreasing expression levels with increasing tumor grade) was observed for SST, sst1, sst2, sst5, and sst5TMD4 (Supplemental Figure 2). When tumor samples were analyzed separately, in pancreatic NETS, sst1, sst2 and sst5 were overexpressed when compared with control and adjacent tissue; at the same time, a decreased expression of sst3 and sst5TMD4 in tumor samples was observed. In contrast, small bowel and colon tumors tended to progressively increase the expression SST, sst2, sst3, sst5 and sst5TMD4 in adjacent tissue when compared to controls and also in tumors when compared to their respective adjacent tissue and/or normal controls (Supp. Figure 3). Intriguingly, in tumor samples, SST expression was correlated to sst1, sst4 and sst5 and sst5TMD4; in turn, sst5TMD4 was positively correlated to the molecular expression of all evaluated SST system components, while CORT was correlated to sst1. In addition, sst1 expression was correlated with sst4 (Table 3).

Table 3: SST/CORT system components correlations in tumor tissue

	sst1	sst2	sst3	sst4	sst5	sst5TMD4
SST	0.306*			0.324*	0.288*	0.389***
CORT	0.344***					0.268*
sst1				0.288*		0.377***
sst2			0.902***			0.455***
sst3						0.460***

*p<0.05; **p<0.01; ***p<0.001
p value refers to the comparison between SST/CORT system components

Immunohistochemistry (IHC) analysis

Although qPCR is a sensitive method to assess gene expression, we subsequently performed an IHC analysis in a set of selected samples, in order to validate the observed changes at the protein level, and to determine which particular cells are expressing those markers. To this end, we selected sst1, sst2, and sst5 due to their clear overexpression in tumor samples. Specifically, assays performed on FFPE-samples revealed stronger staining in tumor tissue compared to non-tumor adjacent tissue (Fig. 2B). In general, IHC analysis of tumor tissue revealed that somatostatin receptors were present in the vast majority of tumor cells compared with non-tumor adjacent tissue, with different grades of staining (Supp. Figure 4). These data further suggest a dysregulated presence of somatostatin receptors in GEP-NETs samples compared to non-tumor adjacent tissue.

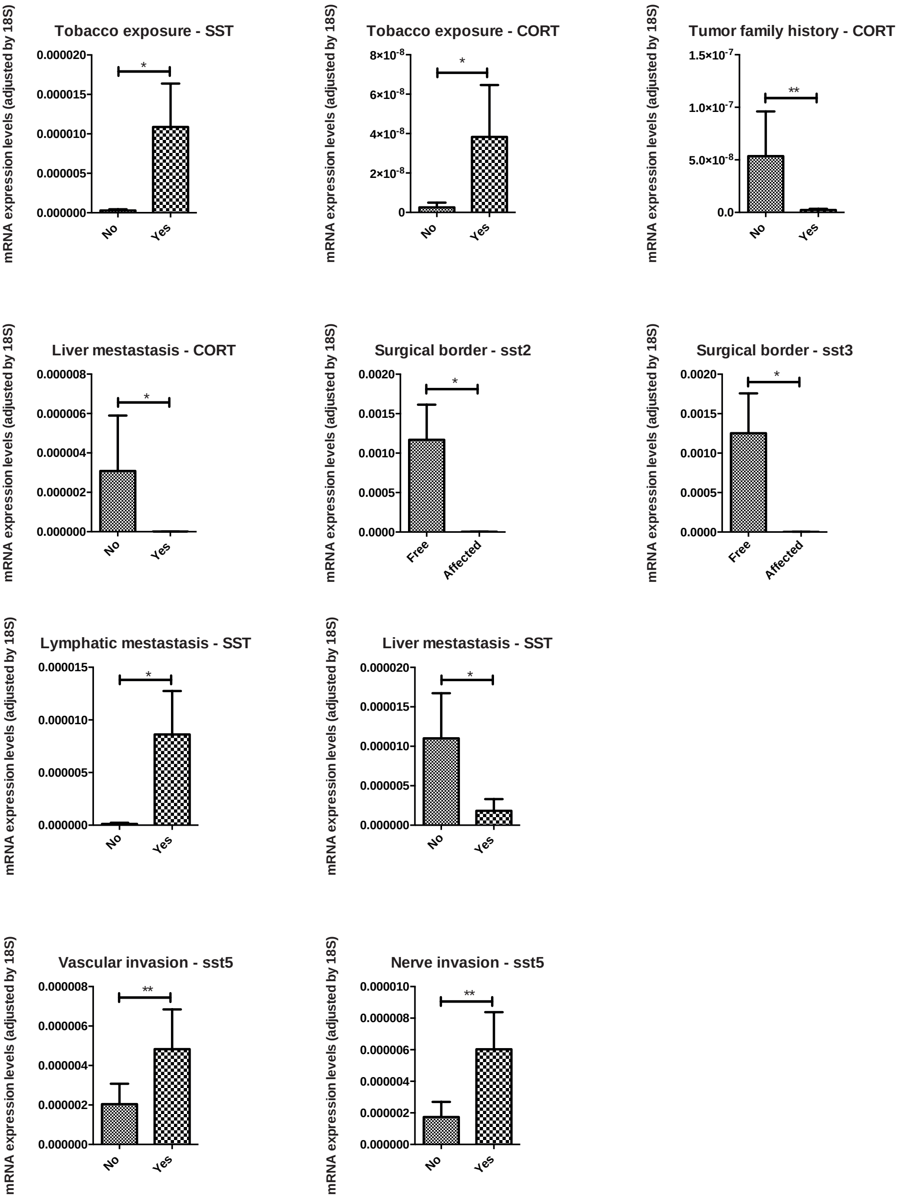

Figure 3: Correlations between epidemiological, clinical, histological and molecular parameters in GEP-NETs. The correlations between epidemiological, clinical, histological and molecular parameters within GEP-NETs samples were assessed by U-Mann Whitney tests. Data represent the mean±SEM. Asterisks (*, $p<0.05$; **, $p<0.01$) indicate significant associations.

Associations between expression levels of SST system components and clinical-histological characteristics in GEP-NETS patients

Analysis of the demographic, epidemiological and clinical data revealed that patients with tobacco exposure presented GEP-NETs with higher expression of SST and CORT (Figure 3), while in those cases with family history of tumor disease, a lower expression of CORT was observed (Figure 3). Conversely, no correlation was observed with sex, personal history, previous neoplasm history, clinical symptoms or diagnostic techniques.

Importantly, the expression of some SST system components was related to aggressiveness and prognosis of GEP-NETs. Indeed, tumors with lymph node metastasis overexpressed SST while a lower SST and CORT expression was observed in those with liver metastasis. In addition, tumors with free surgical borders overexpressed sst2 and sst3, but tumors with vascular or nerve invasion overexpressed sst5 (Figure 3).

Effect of SSAs and specific sst agonists on NETs cells survival

To further explore the possible role and potential clinical utility in NET pathophysiology of the two less explored SST receptors, sst1 and sst3, we used two commonly accepted NET model cell lines, BON-1 and QGP-1 cells. Cells were treated with specific sst1 and sst3 agonists and compared with the classic multi-receptor compounds octreotide and pasireotide (Figure 4). This revealed that BON-1 cells decrease their viability rate after an acute treatment (24-h incubation) with sst1 and sst3 analogs (p=0.054 and p<0.05, respectively), whereas no such effect was observed in response to octreotide and pasireotide (Figure 4). In

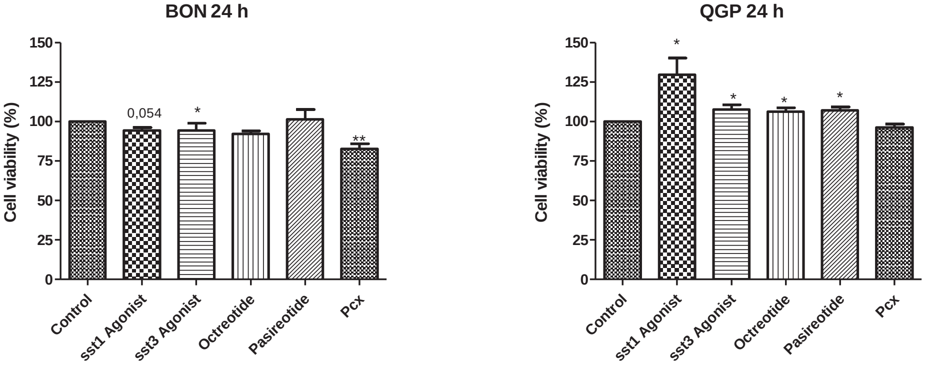

Figure 4: Cell survival rate in BON-1 and QGP-1 cell lines in response to sst stimulators. Cell survival rate was determined by Alamar-blue technique in BON-1 and QGP-1 cells in response to 24h incubation with sst1 agonist, sst3 agonist, octreotide and pasireotide compared to vehicle-treated control. Paclitaxel (Pcx) was used as inhibitory control. Data represent the mean±SEM from at least n=3 independent experiments. Differences were assessed by multiple comparison tests. Asterisks (*, p<0.05; **, p<0.01) indicate significant differences with control.

contrast, QGP-1 cell line exhibited an increased survival rate in response to sst1 and sst3 agonists, as well as to octreotide and pasireotide after 24-h of treatment.

DISCUSSION

In the present study, we have systematically evaluated the expression of SST system components (ligands and receptors) in a large series of clinically well-characterized GEP-NETs, including primary tumors of the whole GEP system. Gene expression in tumor tissues was compared with that in the corresponding adjacent non-tumor tissues and in normal control tissues. Some studies [42, 43], including a recent one from our group [20], have explored the presence of certain components of the SST systems in GEP-NETs, but, to the best of our knowledge, this study represents the first comprehensive characterization of the components of this regulatory system in tumor samples, in comparison not only with their corresponding adjacent non-tumor regions but also with normal tissue samples for each analyzed organ. In addition, we thoroughly characterized the demographic, epidemiological, and clinical characteristics of the patients, and the disease progression and prognosis after 2-10 years, and assessed their putative relationship with the expression levels of the components of this SST system. Our results revealed a distinctive expression of most SST system components in tumor and peritumoral tissues compared to normal samples. In addition, specific components of the SST system displayed clinical-histological correlations in tumor tissues, suggesting their potential value as novel markers for GEP-NETs patient management.

The intrinsic variability and heterogeneity that characterize GEP-NETs [44] is further confirmed by the results obtained in this particular cohort of samples, which, first of all, revealed a marked overexpression of CORT, sst1, sst2 and sst5 in tumor tissues compared to non-tumor adjacent samples. The presence of the different ssts in NETs has been analyzed previously by various groups [15, 24, 42], including ours, in a different cohort of patients [20]. Interestingly, SST system components are expressed not only in tumor tissue but also in non-adjacent tissue, although the expression in tumors is consistently increased compared to the surrounding tissue [20, 24, 45]. These findings, which have been demonstrated by RT-qPCR and IHC, probably relate to their biological regulatory role in secretion and, also, cell proliferation [15], suggesting a dysregulation of these system that could be related with tumor progression. Although the majority of the alterations found in the expression patterns analyzed herein are in agreement with those reported previously (e.g. of sst1 and sst2 overexpression in tumor vs. control tissues) [15, 20, 24, 42], certain changes do not seem to be completely consistent among the different studies, likely owing to obvious differences among the distinct cohorts, but also, to the intrinsic heterogeneity of NETs mentioned above, even when comparable experimental approaches are implemented to characterize tumor expression profiles. In this regard, it is remarkable the difference in the expression of somatostatin receptors in pancreatic NETs when compared to other localizations, and despite the reduced number of samples, our findings underscore the heterogeneity of these tumors, and could be related to lower progression free-survival [46, 47] when compared with other tumors, for example, small bowel NETs [48]. In spite of these differences, and given the emerging relevance of SSAs therapy and peptide receptor radionuclide therapy in the management of well-differentiated GEP-NETs [15, 49, 50], it seems worth emphasizing the high levels of sst2 expression in these tumors observed in our work, which is consistent with previous studies [15, 20, 24, 42], for most currently available SSAs preferentially target this receptor subtype. For this same reason, the sst5 overexpression in tumor tissue observed in our set of samples would also be a relevant marker supporting the potential benefit of SSAs (such as octreotide) as an appropriate treatment for controlling symptoms and tumor growth in well-differentiated GEP-NETs. Furthermore, inasmuch as some tumors are (or become) resistant to SSAs, and some proposed mechanisms suggest defects in sst2 presence, activation, or downstream signaling events [51], it is not unreasonable to propose that the decreased expression of sst2 in less differentiated GEP-NETs observed herein could be related to this phenomenon.

It is also important to note the increased expression of sst2 and sst3 observed in completely resected tumors, which may suggest that the presence of this receptor is associated with the development of well-defined and localized tumors. In fact, it is well known that sst2 can mediates the antiproliferative effects of SST and, as mentioned above, the currently available SSAs have high affinity for this receptor, even its presence and uptake imaging techniques using labeled somatostatin analogues with positron emitting isotopes, especially Gallium-68 being necessary for considering the use of peptide receptor radionuclide therapy in these patients [35, 52-54]. Around 10% of all hepatic metastases are related to a primary NET [55], liver metastatic disease affects between 25 and 90% of NET-patients, and its presence decreases the 5-year survival about 35-60% [56-58]. Interestingly, in our cohort, liver metastases were not observed in tumors overexpressing SST despite the presence of lymphatic invasion, suggesting a relevant role of SST in tumor behavior. Regarding the expression of other receptors, previous reports have described the presence of the truncated variant of sst5 (sst5TMD4) in breast cancer and poorly differentiated thyroid carcinomas [59, 60]; apparently the balance between this receptor, sst2 and sst5 (full-length) may influence the stage of disease [60]. Similarly, sst5TMD4 has been associated with increased tumor aggressiveness in somatotropinomas and NETS [20, 21] and its dysregulation may also influence the response to SSAs [21, 60]. In the present group of patients, we did not find associations between the truncated isoform and tumor aggressiveness, whereas tumors with vascular and nerve invasion overexpressed sst5, suggesting that a dysregulation in this receptor could be related to aggressive features and the patient outcome.

Indeed, when there is tumor progression or treatment intolerance in response to SSAs, patients require alternative clinical strategies [54, 61, 62], for which novel target treatments [e.g. vascular endothelial growth factor (VEGF) inhibitors and inhibitors of mammalian target of rapamycin (mTOR)] have shown to improve progression-free survival with a low rate of severe adverse events compared with placebo among patients with advanced pancreatic NETs [46, 63]. Unfortunately, despite these new therapeutic options, treatment strategies are still limited and unsatisfactory and, until now, we lack clinical, histological or molecular makers that can satisfactorily predict the evolution of these patients. In this scenario, alternative SST receptors other than sst2, such as sst1 and sst3, could represent suitable options for the development of novel therapeutic strategies. However, expression and functionality of sst1 and sst3 in NETs are hitherto largely unexplored. In our cohort, a significant overexpression of sst1 in tumor tissue was observed, which is consistent with previous reports [20, 64]. On the other hand, although sst3 was not overexpressed in all tumor samples in our cohort, it tended to be overexpressed in less differentiated, more aggressive tumors, and, importantly, it has been found to be overexpressed in other cohorts [20]. These findings suggest the interest of exploring the potential utility of sst1 and sst3 selective agonists in the treatment of NETs. In support of this contention, our present *in vitro* studies demonstrated that treatment with specific sst1 or sst3 agonists was capable to reduce cell survival in BON-1 cells, while, in contrast, SSAs mainly targeting sst2 and/or sst5 (octreotide and pasireotide) were ineffective in this regard. In contrast, QGP-1 cells exhibited an opposite response, wherein treatment with sst1 and sst3 agonists increased cell survival. The reason for these unexpected differences is unknown, but it could be related to the distinct sst1-sst5 expression in tumor cell lines; according to published results, QGP-1 cells have lower mRNA expression levels of all somatostatin receptors, specially sst1 and sst3 [20, 65]. Moreover, similar stimulatory effects on cell survival were observed with other somatostatin analogs (octreotide and pasireotide), in QGP-1 cells but not in BON-1, which suggests that it is a cell type-dependent effect, which could be related to a dysregulation in the expression, activation or signaling of sst1 and/or sst3, or to their particular interaction with other ssts, in this tumor cell line. These findings further emphasize the potential relevance of NET heterogeneity in the functional response of these tumors to the SSA treatment.

Earlier studies suggested that sst5 could mediate antiproliferative effects when transfected in heterologous cell models [66]. In our cohort, sst5 was overexpressed in tumor tissue compared with adjacent non-tumoral tissue, an abundance that appeared to be associated to aggressive features, for sst5 expression was higher in tumors with vascular and nerve invasion. This suggests that sst5 presence could be related to aggressiveness in NETs, similar to that found in other pathologies [29, 67, 68]. The possible mechanisms underlying this potential association are still to be elucidated. On the other hand, and at variance with previous studies from our group reporting an overexpression of the splicing variant sst5TMD4 in various tumor pathologies, including NETs, in the present set of samples sst5TMD4 was not overexpressed in tumor samples, [20], an observation that could be explained by intrinsic differences in the cohort and would reinforce the idea of the variability and heterogeneity of GEP-NETs [69, 70].

In contrast to the SST receptors, the presence of the ligands SST and CORT in NETs has received less attention in recent years, and given their potential autocrine actions we sought to explore their expression in our cohort. Interestingly, despite its relatively low expression levels, we observed that CORT was clearly overexpressed in tumor samples, and that its levels were higher in patients without family tumor history, suggesting a potential, distinct involvement of this peptide, and its regulation, in NET development and/or progression, which has not been described previously and deserves further investigation. On the other hand, SST was expressed at higher levels but its expression was not altered in tumor vs. not-tumor tissues; however, a closer view to key clinical parameters of GEP-NETs (e.g. invasion capacity) revealed interesting associations, in that tumors with lymph node metastasis over-expressed SST, whereas expression of both SST and CORT was decreased in tumors with liver metastasis, suggesting that a differential expression of SST and CORT could be related to the behavior and aggressiveness in GEP-NETs. In this sense, it has been proposed that CORT synthetic analogs may provide potentially valuable tools as diagnostic/therapeutic tools in SST and ghrelin expressing tumors [10], although their development is still lacking, and should be considered in the future in the field of GEP-NETs.

In summary, this study provides a primary comprehensive mapping of the expression of SST/CORT system component in GEP-NETs, as compared with their respective adjacent non-tumor tissues, and with normal, non-neoplastic tissues. Our results indicate a prominent and widespread overexpression of SST/CORT system components in tumor tissues, wherein they could exert relevant regulatory roles in that the expression levels of some components are tightly related to the degree of disease, and exhibit associations to key clinical parameters, including invasion and metastasis. Thus, our data provide novel information that might help to better understand NETs pathophysiology and its intrinsic heterogeneity and to identify novel molecular targets with potential prognostic and/or therapeutic value for GEP-NETs patients.

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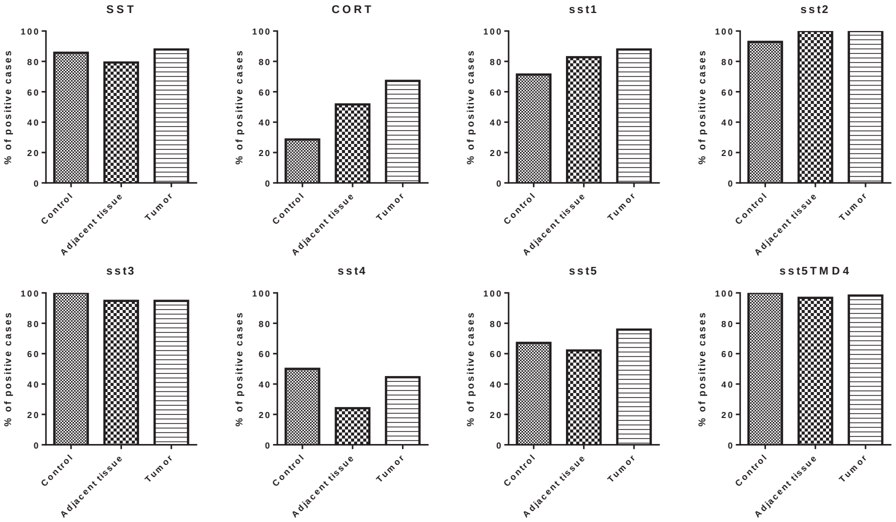
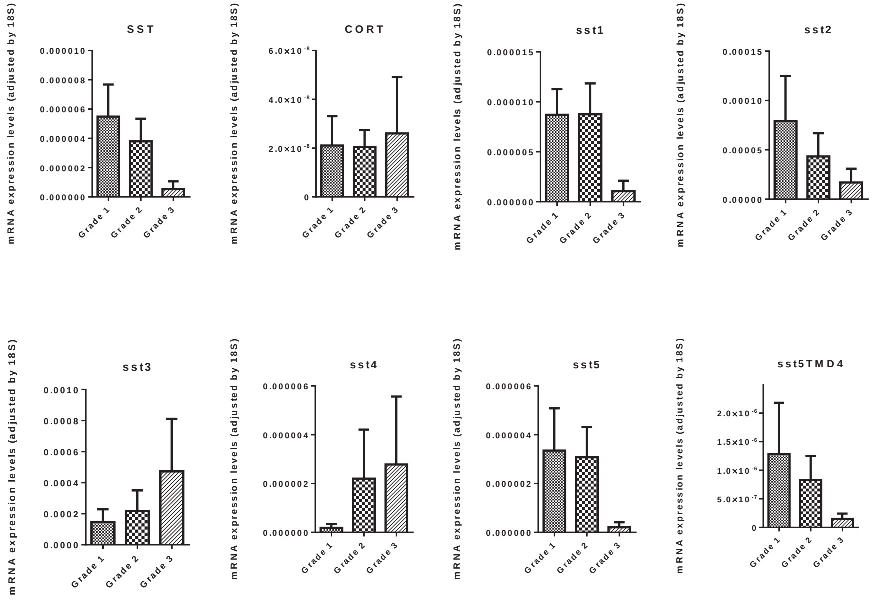
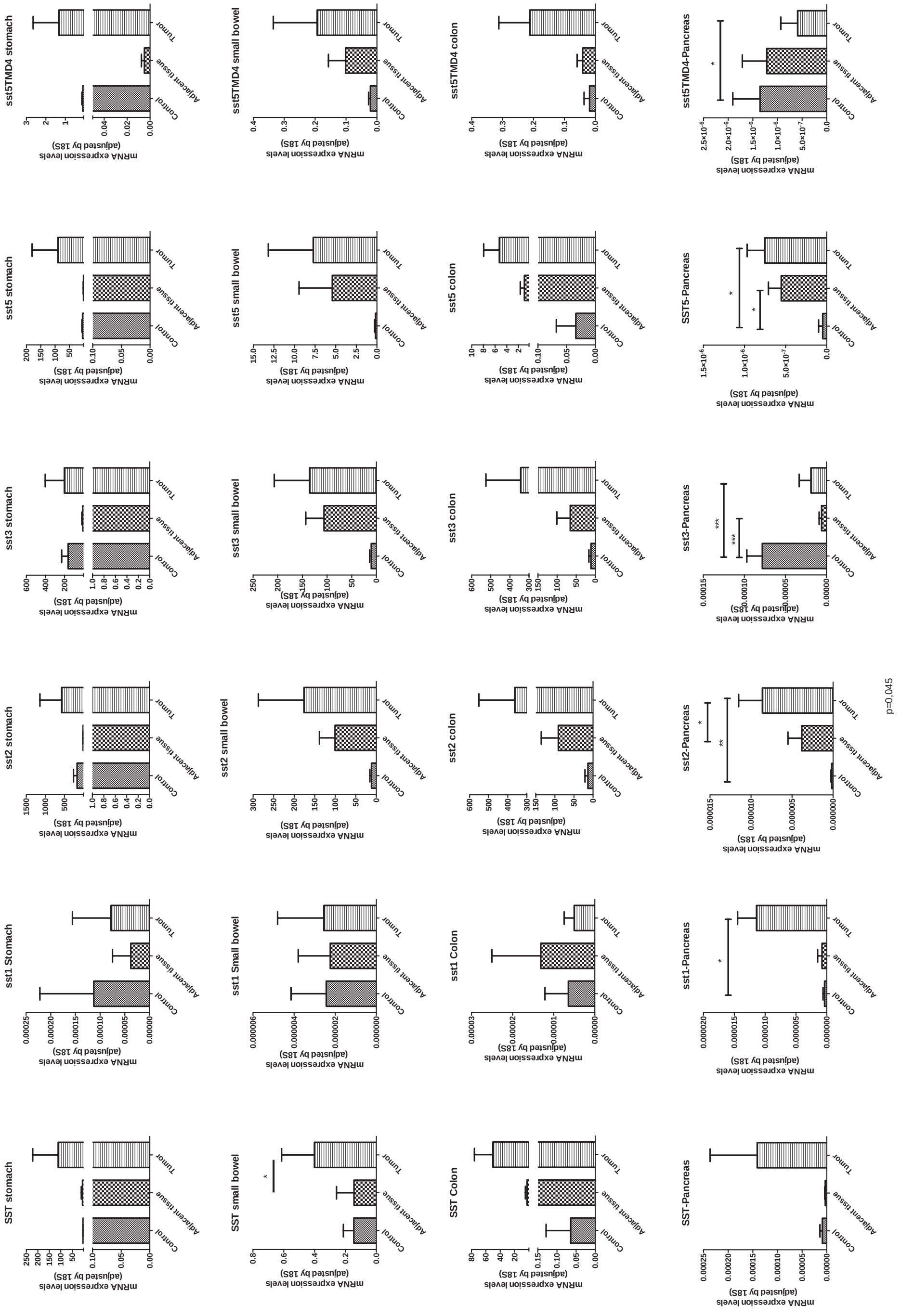
SUPPLEMENTAL DATA



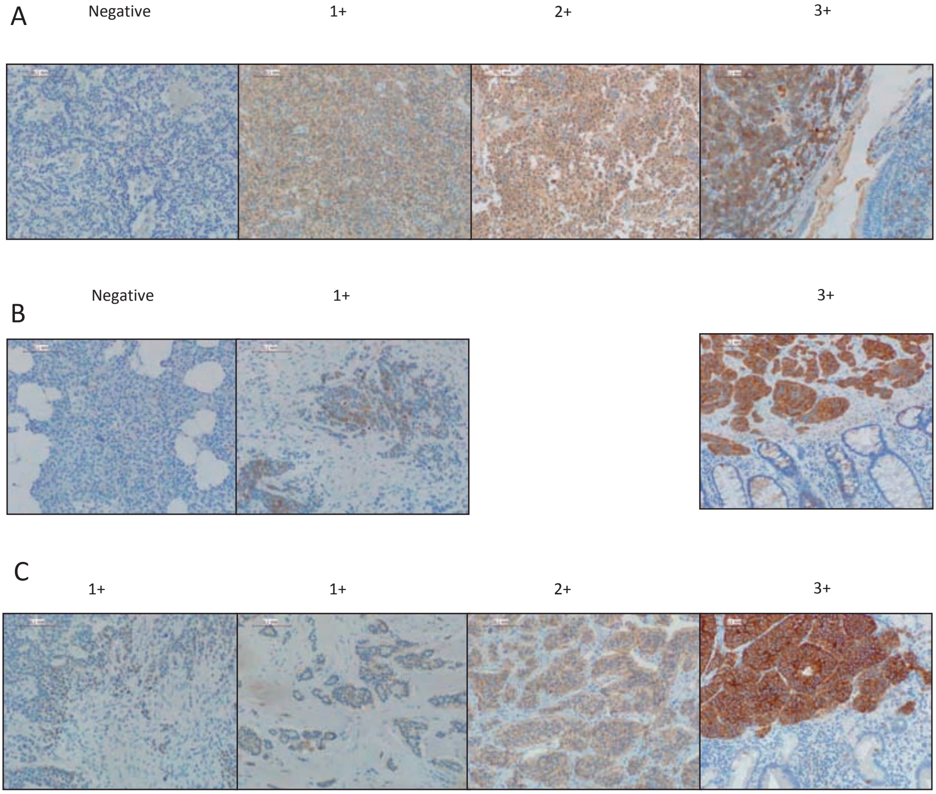
Supplemental Figure 1: Presence of SST/CORT system components in normal tissues, adjacent non-tumor tissue and GEP-NETs. The graphs indicate the percentage of samples (normal control tissue, adjacent non-tumor tissue and tumor tissue) positive for each of the SST/CORT system components.



Supplemental Figure 2: Expression of SST/CORT system components in GEP-NETs of different grade. The absolute mRNA expression of the different components of the SST/CORT system was determined by qPCR in grade 1, 2 and 3 GEP-NETs samples (values are adjusted by 18S expression). Data represent the mean±SEM.



Supplemental Figure 3: Expression of SST system components in normal samples, adjacent non-tumor and tumor tissue of stomach, small bowel, colon and pancreatic NETs. The absolute mRNA expression of the different components of the SST/CORT system was determined by qPCR in normal control samples, adjacent non-tumor tissue and GEP-NETs samples (values are adjusted by 18S expression). Data represent the mean±SEM. Asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) indicate significant changes by paired analysis between adjacent non-tumor and GEP-NETs and non-paired analysis between normal tissue and adjacent non-tumor or tumor tissues.



Supplemental Figure 4: Presence of sst1, sst2, sst5 by IHC in GEP-NETs. The expression of sst1 **(A)**, sst2 **(B)** and sst5 **(C)** by immunohistochemistry was determined in adjacent non-tumor tissue and NET samples. Samples are classified as negative, 1+, 2+, 3+ staining, which represent absent, low, moderate, and high intensities of the tumor region compared to the adjacent non-tumor tissue.

Chapter 4

Ghrelin O-acyltransferase (GOAT) enzyme as a novel potential biomarker in gastroenteropancreatic neuroendocrine tumors.

Aura D. Herrera-Martínez[1,2], Manuel D. Gahete[1,3], Rafael Sánchez-Sánchez[1,2], Emilia Alors-Perez[1,3], Sergio Pedraza-Arevalo[1,3], Raquel Serrano-Blanch[1,2], Antonio J. Martínez-Fuentes[1,3], Maria A. Gálvez-Moreno[1,2], Justo P. Castaño[1,3], Raúl M. Luque [1,3]

[1] Maimonides Institute for Biomedical Research of Córdoba
[2] Reina Sofi University Hospital
[3] Department of Cell Biology, Physiology, and Immunology, University of Córdoba, Spain

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ABSTRACT

The association between the presence and alterations of the components of the ghrelin system and the development and progression of neuroendocrine tumors (NETs) is still controversial and remains unclear. Here, we systematically evaluated the expression levels (by quantitative-PCR) of key ghrelin system components of in gastroenteropancreatic (GEP)-NETs, as compared to non-tumor adjacent (NTA; n=42) and normal tissues (NT; n=14). Then, we analyzed their putative associations with clinical-histological characteristics. The results indicate that ghrelin and its receptor GHSR1a are present in a high proportion of normal tissues, while the enzyme ghrelin-O-acyl transferase (GOAT) and the splicing variants In1-ghrelin and GHSR1b were present in a lower proportion of normal tissues. In contrast, all ghrelin system components were present in a high proportion of tumor and NTA tissues. GOAT was significantly overexpressed (by qPCR) in tumor samples compared to NTA, while a trend was found for ghrelin, In1-ghrelin and GHSR1a. In addition, expression of these components displayed significant correlations with key clinical parameters. The marked overexpression of GOAT in tumor samples compared to NTA regions was confirmed by IHC, revealing that this enzyme is particularly overexpressed in gastrointestinal NETs, where it is directly correlated with tumor diameter. These results provide novel information on the presence and potential pathophysiological implications of the ghrelin system components in GEP-NETs, wherein GOAT might represent a novel diagnostic biomarker.

Keywords: GEP-NETs, ghrelin, GOAT, GHSRs, In1-ghrelin, prognosis, invasion

Highlights:

- Key components of ghrelin system are markedly dysregulated in GEP-NETs and associated to key clinical parameters
- Changes in the expression of ghrelin system components are associated with the development and/or progression of GEP-NETs
- These molecular targets, especially GOAT, may represent putative diagnosis and/or prognostic markers in GEP-NETs.

INTRODUCTION

Neuroendocrine tumors (NETs) comprise a heterogeneous family of malignancies with complex clinical behavior and increasing incidence [1-3]. Primary tumor is identified only in 70% of patients [4], while distant metastases are frequently found at diagnosis (27-73%), influencing the overall survival [5-7]. Despite that histological differentiation and Ki67 index are some prognosis factors [4], well-differentiated low-grade tumors may behave aggressively [8]. Unfortunately, surgery is often not applicable since most tumors are diagnosed at advanced stage. For these reasons, the development of novel diagnostic markers has gained scientific and clinical interest [9, 10].

The ghrelin system is involved in the regulation of multiple (patho)-physiological functions, including hormonal secretion, β-cell survival or appetite and gastric motility [11-14]. Ghrelin must undergo a unique modification, consisting of the acylation of the third serine residue, which is catalyzed by the ghrelin-O-acyl-transferase (GOAT) enzyme [14, 15]. Acylated ghrelin (AG) represents the peptide binding and activating its canonical ghrelin receptor, GHSR1a. Interestingly, several ghrelin system variants, resulting from post-transcriptional modifications or alternative splicing, have been identified, including the In1-ghrelin [11, 16] and a truncated receptor GHSR1b, with unknown ligand and function [11, 16, 17].

Alterations in the expression of specific components of this system have been associated with the development/progression of various neoplasms [16, 18-21], including NETs, but the clinical-molecular correlations have not been elucidated [22, 23]. Accordingly, in this study we aimed to: 1) analyze systematically the expression of different components of ghrelin system in gastroenteropancreatic-(GEP-)NETs compared to non-tumor adjacent (NTA) tissue and, most importantly, to normal control tissues by quantitative real-time PCR (qPCR); 2) correlate the expression of these components with clinical/histological characteristics; and 3) perform *in vitro* experiments to elucidate the potential pathophysiological role of GOAT enzyme as a key component particularly altered in our cohort of NET samples, using BON-1 and QGP-1 cell lines.

MATERIALS AND METHODS

Patients and samples

This study was approved by the Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain), was performed according to the Declaration of Helsinki, and patients were treated following national and international clinical practice guidelines. A written informed consent was required before inclusion. Data from 42 patients with GEP-NETs were collected (demographic and clinical characteristics of the cohort are summarized in Table 1). Additionally, 14 normal control tissues from healthy donors were also included. Patients with hereditary endocrine syndrome were excluded. Clinical records were used to collect full medical history. GEP-NETs were classified according to histopathology features as well-differentiated NETs (G1), moderately differentiated (G2) and poorly differentiated NETs (G3) [24]. Formalin-fixed paraffin-embedded (FFPE) samples were also collected (42 tumor samples, 42 NTA and 14 normal tissues).

Table 1: General characteristics of the patient population

General characteristic		% (n)
Sex		
	Male	52.4% (22)
	Female	47.6% (20)
Age at diagnosis		55.66±17 years
Personal history of other tumors		15.0% (6)
Smoke habit		
	Active	45.0% (9)
	Ex-smoker	20.0% (4)
	No habit	35.0% (7)
Family history of neoplasms		52.9% (9)
Incidental tumor		37.9% (11)
Functionality		43.3% (13)
Mortality rate		18.9% (7)

RNA isolation and reverse-transcription

Total RNA from FFPE samples (n=98) was isolated using the RNeasy-FFPE Kit (Qiagen, Limburg, Netherlands) according to manufacturer's instructions. Quantification of the recovered RNA was assessed using NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, NC). Total RNA was retrotranscribed to cDNA with the First-Strand Synthesis kit using random hexamer primers (Thermo Scientific) as previously reported [25-28]

Quantitative real time PCR (qPCR)

cDNAs were amplified with the Brilliant III SYBR-Green Master Mix (Thermo Scientific) using the Stratagene Mx3000p system and specific primers for each transcript of interest. Specifically, expression levels (absolute mRNA copy number/50ng of sample) of ghrelin, In1-ghrelin, GOAT-enzyme, GHSR1a and GHSR1b, were measured using previously validated primers [21, 29, 30]. RNA expression was adjusted by *18S* gene expression[28, 31].

Immunohistochemistry (IHC) analysis

IHC analysis of GOAT was implemented in all 42 FFPE samples (tumor and NTA regions) using standard procedures [32]. Optimum antibody concentration (1:300) using a commercially available antibody against human GOAT (AA257-287, Acris-antibodies, Herford, Germany) was selected by performing a series of antibody dilution tests in normal pancreas [33]. Two independent pathologists performed the IHC analysis following a blinded protocol. In the analysis, 0, 1+, 2+, 3+ stand for absent, low, moderate, and high staining intensities of GOAT enzyme in the tumor compared to the NTA region.

Cell culture

In vitro experiments were performed using human NET cell lines BON-1 [34] and QGP-1 [35]. BON-1 cells were cultured in DMEMF12 (Life Technologies, Barcelona, Spain) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Madrid, Spain), 1% glutamine (Sigma-Aldrich) and 0.2% antibiotic (Gentamicin/Amphotericin-B; Life Technologies). QGP-1 cells were cultured in RPMI-1640 (Lonza, Basel, Switzerland), supplemented with 10% FBS, 1% glutamine and 0.2% antibiotic. Both cells lines were cultured at 37°C in a 5% CO_2 incubator and monthly checked for mycoplasma contamination by PCR [36].

Cell proliferation assay in response to GOAT inhibitor

The only commercially available GOAT inhibitor (GOATi; GO-CoA-Tat; Ref: 032-37) was purchased from Phoenix Pharmaceuticals (Burlingame, CA). The final concentration (10^{-5}M) was selected based on dose-response experiments performed in prostate cell-lines and on previous reports [37]. Cell proliferation was determined by using Alamar-blue assay (basal, 24h, 48h and 72h) as previously reported [21, 22, 32]. Cells were seeded per quadruplicate and assays were repeated four times. Paclitaxel (PAX; Sigma-Aldrich) was used as control for the inhibition of proliferation [27, 30].

Migration capacity assay

The ability of BON-1 cells to migrate after 24h of treatment was evaluated by wound healing technique [22, 38-40]. Briefly, stable cells were plated at sub-confluence in 6 well plates. The wound was made on confluent cells using a 100 µl sterile pipette tip. Wells were rinsed in PBS and treated for 24 h in FBS-free medium. Wound healing was calculated as the area of a rectangle centered in the picture 24 h after the wound vs. the area of the rectangle just after doing the wound. Three experiments were performed in independent days, in which three random pictures per well along the wound were acquired and, the mean area of these pictures was used for analysis. Images were analyzed using the ImageJ software [41].

Statistical analysis

Paired t-test analysis was used to compare the expression levels between GEP-NETs samples and NTA tissue. Non-paired t-test analysis was used to compare the expression levels between normal tissue and GEP-NETs samples or NTA tissue. U-Mann Whitney tests were used to evaluate clinical-molecular relations within GEP-NETs samples. Chi-squared test was used to compare categorical data. All statistical analyses were performed using SPSS and GraphPad Prism. Data are expressed as mean ± SEM. *p*-values<0.05 were considered statistically significant. In functional experiments, results were expressed as percentage vs. control (vehicle-treated cells). Cell proliferation rate compared to control was assessed by multiple comparison test (Two-way ANOVA followed by Newman-Keuls post-hoc test).

RESULTS

Forty-two patients with GEP-NETs were included. Demographic/clinical features are summarized in Table 1. Specifically, 15 patients presented PNETs and 27 patients presented gastrointestinal (GI)-NETs [52.3% males (22/42); mean age 55.6±17 years]. Tumor characteristics are summarized in Table 2. In our cohort, 43.3% (13/30) were functioning tumors; 63.2% (24/38) had peritumoral invasion [34.3% (12/35) vascular and 35.3% (12/34) neural invasion], 52.4% (22/42) had metastasis at diagnosis [multiple localization in 36.3% (8/22)], 63.2% (24/38) were invasive tumors and the mortality rate reached 18.9% (7/37). Relapsed disease was observed in 36% of patients (13/36). Finally, almost 70% of samples (29/42) were considered as low/intermediate tumors [38.1% (16/42) grade 1 and 31% (13/42) grade 2]. PNETs were statistically larger in size compared to those GI-NETs (4.0±0.47 vs 2.36±0.34 cm respectively; p<0.01).

Histopathological characterization of GEP-NETs and NTA tissue

Primary tumor samples were delimited from the NTA tissues after the evaluation of two experienced pathologists using histology and immunohistochemistry, as previously reported [32].

Expression of components of the ghrelin system in control and GEP-NETs samples

Ghrelin system components were present at variable proportions in normal GEP samples, as determined by qPCR. Ghrelin and its native receptor GHSR1a were expressed in more than 80% of healthy controls (34/42 and 39/42, respectively), while their splicing variants In1-ghrelin and GHSR1b were expressed in about 40% of the samples (17/42 and 19/42, respectively). In contrast, expression of GOAT enzyme was only detected in less than 20% (7/42) of normal samples (Supp. Figure 1). Ghrelin and GHSR1a were also present in a high proportion (more than 60%) of the NTA and tumor samples (32/42 and 29/42,

Table 2: Tumor sample characteristics

Characteristic		% (n)
Tissue samples		
	Primary tumor	42
	Non-tumor adjacent tissue	42
	Normal tissue	14
Primary tumor localization		
	Pancreas	35.7% (15)
	Stomach	7.1% (3)
	Small bowel	31.0% (13)
	Colon and rectum	26.2% (11)
Maximal tumor diameter		2.98±1.86 cm
	Pancreas NETs	4.0±0.47 cm
	Gastrointestinal NETs	2.36±0.34 cm
Necrosis		
	<10%	16.7% (1)
	10-20%	16.7% (1)
	21-30%	50% (3)
	>30%	16.7% (1)
Depth of infiltration (gastrointestinal NETs)		
	Submucosa	4.5% (1)
	Mucosa	4.5% (1)
	Muscular	40.9%(9)
	Serosa	50.0%(11)
Multiple tumors		8.0% (2)
Peri-tumoral tissue invasion		63.2% (24)
Vascular invasion		34.3% (12)
Neural invasion		35.3% (12)
Metastasis		52.4% (22)
Metastasis localization	Liver	9.1% (2)
	Lymphatic nodules	54.5% (12)
	Multiple	36.3% (8)
Grading (WHO 2010 criteria)	Low	38.1% (16)
	Intermediate	31.0% (13)
	High	4.8% (2)
	Unknown	26.2% (11)
Post-surgical treatment		43.2% (16)
Relapsed disease		36.1% (13)
Disease free		55.9% (19)
New surgery requirements		18.2% (6)

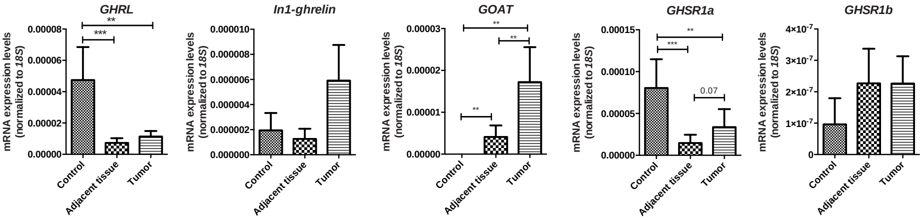


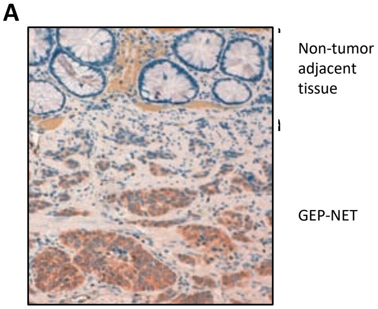
Figure 1: Expression of ghrelin system components in normal GEP, adjacent non-tumor tissue and GEP-NETs. The absolute mRNA expression of the different components of the ghrelin system was determined by qPCR in normal GEP controls, adjacent non-tumor tissue and GEP-NETs samples (values are adjusted by 18S expression). Data represent the mean ± SEM. Asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) indicate significant changes by paired analysis between adjacent non-tumor and GEP-NETs and non-paired analysis between normal tissue and adjacent non-tumor or tumor tissues.

respectively); while GOAT enzyme and the splicing variants In1-ghrelin and GHSR1b were present in more than 40% of the samples ((25/42, 21/42 and 17/42, respectively; Supp. Figure 1). Of note, ghrelin expression levels were decreased in NTA and tumor tissue compared with normal samples, with a slightly but not significantly increased expression in tumor compared with NTA tissue (Figure 1). A similar observation was found for GHSR1a expression, while GOAT enzyme was clearly overexpressed in tumor tissues compared with NTA regions and normal tissues, wherein it was virtually absent (Figure 1). Finally, In1-ghrelin was more expressed in tumor tissues than in control samples but these differences were not statistically significant, while no significant changes were found in the case of the splicing variant GHSR1b (Figure 1).

In terms of tumor grade, no significant differences in the expression of any of the ghrelin system components analyzed were found between differentiated (G1/G2) and non-differentiated (G3) GEP-NET (Supp. Figure 2). However, we found that the expression of GOAT enzyme and GHSR1a in GI-NETs was markedly higher than in PNETs, while the expression of ghrelin was lower in GI-NETs compared to PNETs (Supp. Figure 3). Additionally, ghrelin expression levels correlated with those of In1-ghrelin ($R^2=0.532$; $p<0.01$) and GOAT ($R^2=0.422$; $p<0.05$) in tumor samples, while the expression of GHSR1a was correlated with GHSR1b ($R^2=0.444$; $p<0.05$).

Immunohistochemistry analysis of the presence of GOAT enzyme

Based on the marked overexpression of GOAT enzyme, as well as on previous reports [16, 18], we also sought to analyze its presence at the protein level. IHC analysis of tumor tissue revealed that GOAT enzyme was present in the vast majority of tumor cells compared with NTA tissue (Figure 2A), with different grades of staining. Indeed, in our cohort, 86% of the tumor samples (36/42) evaluated were positive for the presence of GOAT enzyme by IHC



B

	GOAT IHC analysis				Total
	0+	1+	2+	3+	
Number of cases	6	19	9	8	42

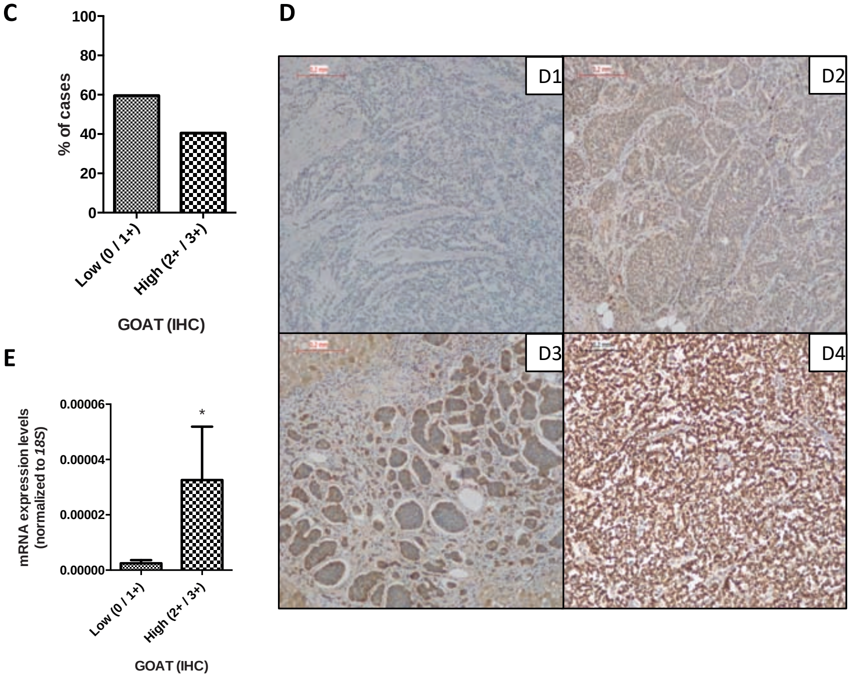


Figure 2: Immunohistochemical analysis of GOAT enzyme in adjacent non-tumor tissue and GEP-NETs. The presence of GOAT by immunohistochemistry using a specific antibody was determined in a subset of samples, which included tumor and non-tumor regions from patients diagnosed with GEP-NETs. **A)** Representative images of the IHC analysis of GOAT enzyme in a GEP-NET sample compared with the non-tumor adjacent tissue. **B)** Absolute number of cases according to the intensity of GOAT IHC staining (0, 1+, 2+, 3+). **C)** The graph indicates the percentage of tumor samples according to the intensity of GOAT expression by IHC, 0 and 1+ have been grouped as low expression while 2+ and 3+ have been grouped as high intensity by IHC. **D)** Representative images of different GOAT staining in GEP-NETs. In the analysis, 0, 1+, 2+, 3+ stand for absent, low, moderate, and high intensities of the tumor region staining compared to the adjacent region with non-tumor tissue (3C1, 3C2, 3C3, 3C4 respectively). This analysis revealed that GOAT was present in the vast majority of tumor cells compared with non-tumor adjacent tissue, with different grades of staining. **E)** Correlation between the absolute mRNA expression of GOAT determined by qPCR in GEP-NETs samples (values are adjusted by 18S expression) and the intensity of GOAT staining.

(Figure 2B), wherein 40% of the tumor cases (17/42) presented a strong staining (2+ or 3+) for GOAT compared to NTA tissue (Figure 2C and 2D). Of note, mRNA expression levels in tumors samples correlated with GOAT expression by IHC (Figure 2E). Additionally, strong staining (2+ or 3+) for GOAT was correlated to increased age at diagnosis (62.5±4 years) compared to those tumors with absent or lower staining (1+; 51±2 years; $p>0.05$).

Correlations between the expression levels of ghrelin system components and clinical-histological characteristics in GEP-NETs

Epidemiological data revealed that patients with tobacco exposure exhibited higher expression of ghrelin and In1-ghrelin (Figure 3A). Moreover, patients with family history of tumor disease had a lower expression of ghrelin (Figure 3B). Conversely, sex, personal history, previous neoplasm history, clinical symptoms, or other histological parameters (vascular/peritumoral invasion, lymph node metastasis) were not associated with the expression of any of the components of the ghrelin system.

Expression of some ghrelin system components was also associated to tumor characteristics, invasion capacity and prognosis in GEP-NETs. Specifically, functioning tumors presented higher levels of GHSR1a (Figure 3C), while lower expression levels of this receptor were associated to the presence of affected surgical borders and mortality (Figure 3C). Tumors with necrosis had lower GOAT mRNA levels and those with liver metastasis had decreased expression levels of In1-ghrelin (Figure 3D). Interestingly, functionality was also associated with increased expression of GHSR1b (Figure 3E). Finally, tumor diameter was directly correlated to GOAT expression ($R=0.33$; $p<0.05$). Remarkably, no further associations were found between expression levels of ghrelin system components and clinical/histological characteristics when considering separately PNETs and GI-NETs (data not shown)

In vitro analysis of the role of GOAT in PNETs cell lines

We decided to further investigate the pathophysiological role of GOAT enzyme using the only available GOATi in PNETs cell lines. However, GOATi did not affect cell proliferation in BON-1 and QGP-1 cells (Figure 4A) or the migration capacity of BON-1 cells (Figure 4B).

DISCUSSION

This study aimed at evaluating systematically the expression of various components of the ghrelin system in an ample series of clinically well-characterized GEP-NETs, and to compare these expression levels with those in the corresponding adjacent non-tumor tissues and in normal control tissues. Previous studies have reported certain components of the ghrelin

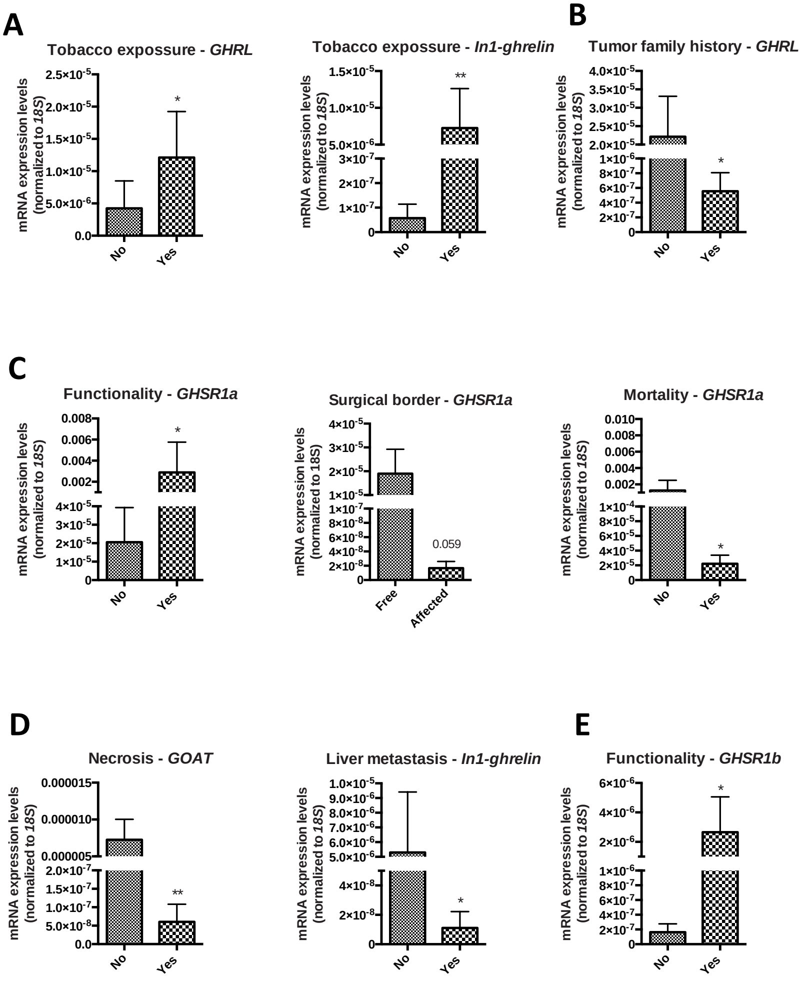


Figure 3: Correlations between epidemiological, clinical, histological and molecular parameters in GEP-NETs. The correlations between epidemiological, clinical, histological and molecular parameters within GEP-NET samples were assessed by U-Mann Whitney tests. Asterisks indicate significant associations (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

system in GEP-NETs [22, 42-44]; however, to our knowledge, this is the first study that comprehensively characterizes these components in tumor samples compared to their corresponding adjacent non-tumor regions, as well as with normal tissue samples. Moreover, we analyzed the demographic, epidemiological, and clinical characteristics as well as the disease progression and prognosis after 2–10 years of the patients with GEP-NETs. Overall, our results revealed that most of the components of the ghrelin system exhibit a distinctive expression in tumor and peritumoral tissues compared to normal tissue samples. Indeed, specific components of the ghrelin system, and especially GOAT, displayed remarkable

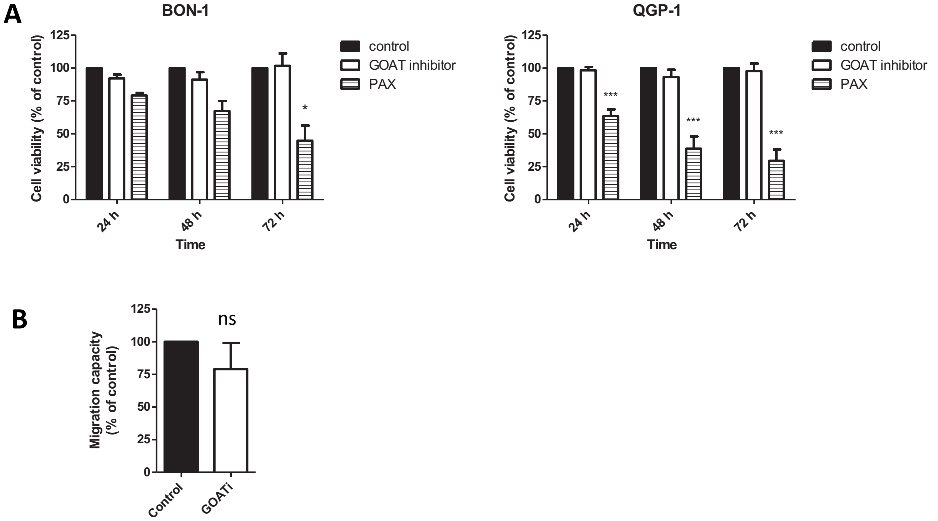


Figure 4: ***In vitro*** **analysis of the consequences of GOAT inhibitor (GOATi) treatment in NET cell lines.** A) Cell proliferation rate in BON-1 and QGP-1 cell lines after 24h, 48h and 72h of GOATi treatment determined by Alamar-blue assay. Paclitaxel (PAX) was used as inhibitory control in proliferation assays. B) Cell migration rate in BON-1 after 24h of treatment with GOAT inhibitor by wound-healing assay. Cell proliferation rate compared to control was assessed by multiple comparison tests while migration was assessed by U-Mann Whitney test. Values represent the mean ± SEM of at least three individual experiments. Asterisks indicate significant differences (*,$p<0.05$; ***,$p<0.001$) compared with control (set at a 100%). Legend: ns means non-significant.

alterations and clinical-histological correlations in tumor tissues, suggesting their potential value as novel biomarkers in GEP-NETs.

Also in our cohort, GEP-NETs exhibited a substantial molecular heterogeneity and variability [22, 32, 45]. Our results are consistent with previous reports showing that different components of the ghrelin system are present in tumor and non-adjacent tissues, and that, some of these components can be overexpressed in tumor samples compared to the surrounding tissue [22, 42-44]. Differences among these studies may be related to the differences among patient cohorts.

Ghrelin system regulates key bodily functions, such as hormonal secretion and cell proliferation, in both normal and tumor cells [11-13, 46, 47]. In this context, our and other studies support the notion that the dysregulation of ghrelin system components observed in NETs could be pathologically relevant and may participate in tumor progression. The diverse localization and morphology of ghrelin-producing cells in the GI tract, and their implications on metabolic/endocrine functions, might suggest a role of this component in the regulation of GEP-NETs patho-physiology [12, 13, 42], and could also explain the ample molecular heterogeneity found herein in the expression of ghrelin in different normal control tissues. Moreover, it could also be related to the overall overexpression of ghrelin in normal tissues compared to tumor samples. Although expression of canonical ghrelin has been described in various tumor types, its potential role in cancer is still controversial [48, 49]. Ghrelin has been described in NETs using immunohistochemistry and qPCR [22, 25, 42, 43], in our cohort ghrelin was expressed in NET samples, albeit in substantially lower amounts than in normal tissues. In addition, in our cohort ghrelin expression levels were higher in PNETs than in GI-NETs, which is consistent with previous evidence [43] but differed from other reports [44]. At variance with previous studies that did not find any clinical correlation between ghrelin expression and clinical features [22, 42], we observed here that ghrelin expression was higher in patients without tumor family history. Similarly, the *in vitro* effects of ghrelin on cell proliferation are also controversial [48, 50-56] and some studies have reported an association between ghrelin and poor survival in renal cell carcinoma patients [57, 58]. Altogether, these data reinforce the notion that NETs are highly heterogeneous tumors, wherein the particular ghrelin expression profile and its clinical implications may depend on the type of tumor and the particular cohort of patients analyzed.

Expression of the canonical ghrelin receptor GHSR1a has been described in tumors including NETs [22, 25, 48]. Here, GHSR1a expression was highly variable in normal control samples, but tended to be overexpressed in tumor samples compared to adjacent non-tumor tissue, which is consistent with our previous study in a different cohort [22]. The relation between GHSR1a, functionality and mortality invites to explore further the potential relationship of this receptor with tumorigenesis, and its putative value as a molecular prognostic marker in NETs.

The pathophysiological implications of the ghrelin system have been recently expanded with the discovery of new molecular components [11, 16, 17, 29], which have been found to be overexpressed in several tumors [16, 22, 27] and associated to relevant clinical parameters [22]. Herein we found comparable tendencies in the expression of these variants; however, these differences did not reach statistical significance. Nevertheless, in the present cohort, more than 40% of tumor samples presented detectable levels of In1-ghrelin and GHSR1b, while in the previous study, more than 80% of the tumor samples exhibited detectable levels [22]. These differences could likely reduce the statistical power of the comparisons and correlations, and, again, would illustrate the elevated heterogeneity of NETs.

The most novel and relevant finding of this study is the marked overexpression of GOAT in NET samples. Whereas the expression of this enzyme was almost absent in control tissues, it was present in adjacent non-tumor tissue and notably overexpressed in tumor tissues. These, together with previous results showing a similar, remarkable overexpression of GOAT in breast and pituitary tumors [11, 21] provide suggestive evidence for a striking dysregulation of this enzyme in endocrine-related tumors. The expression levels GOAT does not always correlate with those of ghrelin, whereas they do parallel more consistently the expression levels of In1-ghrelin, suggesting the existence of additional targets for GOAT enzyme [11]. In NETs, GOAT levels have been correlated with those of In1-ghrelin, and associated with worse outcome [22], these findings were not reproducible in our cohort, which may be explained by the tumor heterogeneity and the limited number of tumor samples. Despite this, in the present study, GOAT expression is associated to larger tumors, especially in GI-NETs, reinforcing the notion of a possible association between the dysregulation of this enzyme and the pathophysiology of NETs. This is the first study that demonstrates an intense overexpression of GOAT enzyme by IHC in GEP-NETs tissues compared to non-tumor adjacent tissues; however, its functional implications should be precisely defined. Here, a GOAT inhibitor administered on two NET cell lines, BON-1 and QGP-1, did not show relevant changes in cell proliferation or migration *in vitro*. Thus, future studies should explore this further, using novel inhibitors or other inhibiting/silencing approaches.

Notwithstanding this, our current and previous [22] studies provide compelling evidence that certain components of the ghrelin system, and specially GOAT enzyme, are clearly overexpressed in NETs, suggesting their potential value as diagnostic and/or prognostic biomarkers for this pathology. In support of the present finding in NETs, GOAT has been also recently reported as non-invasive plasma biomarker in prostate cancer [18]. Additionally, the association between GHSR1a and GHSR1b with the functionally of these tumors and the mortality of these patients further supports this notion and emphasizes the importance of exploring the modulation of this receptor for improving patient outcome. Therefore, although it is difficult to predict the specific clinical impact of these findings, taken together, all these results invite to analyze in more detail the putative utility of GOAT overexpression as a diagnostic biomarker in NETs.

In summary, we present the first systematic characterization of the components of the ghrelin system, including splicing variants, in GEP-NETs tissues in comparison with their adjacent non-tumor regions, and also with normal tissue samples. Our results demonstrate that key components of this system are markedly dysregulated in GEP-NETs and associated to key clinical parameters, suggesting the interest of further studying these molecular targets, especially GOAT, as putative diagnosis and/or prognostic markers in GEP-NETs.

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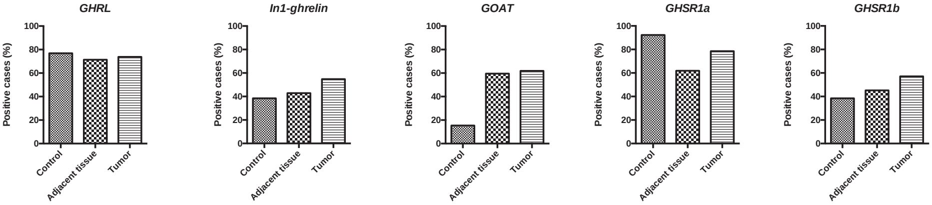
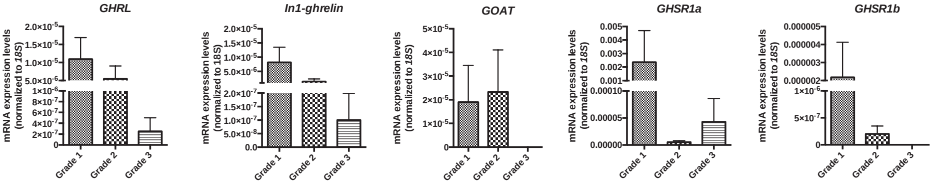
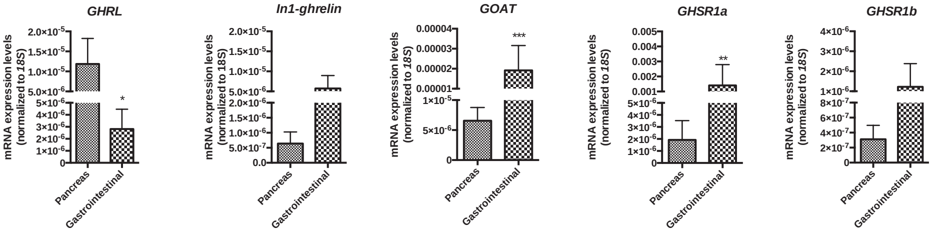
SUPPLEMENTAL DATA



Supplemental Figure 1: Presence of ghrelin system components in normal GEP, adjacent non-tumor tissue and GEP-NETs. The graphs indicate the percentage of samples (normal GEP control, adjacent non-tumor tissue and tumoral tissue) positive for the expression of each of the ghrelin system components.



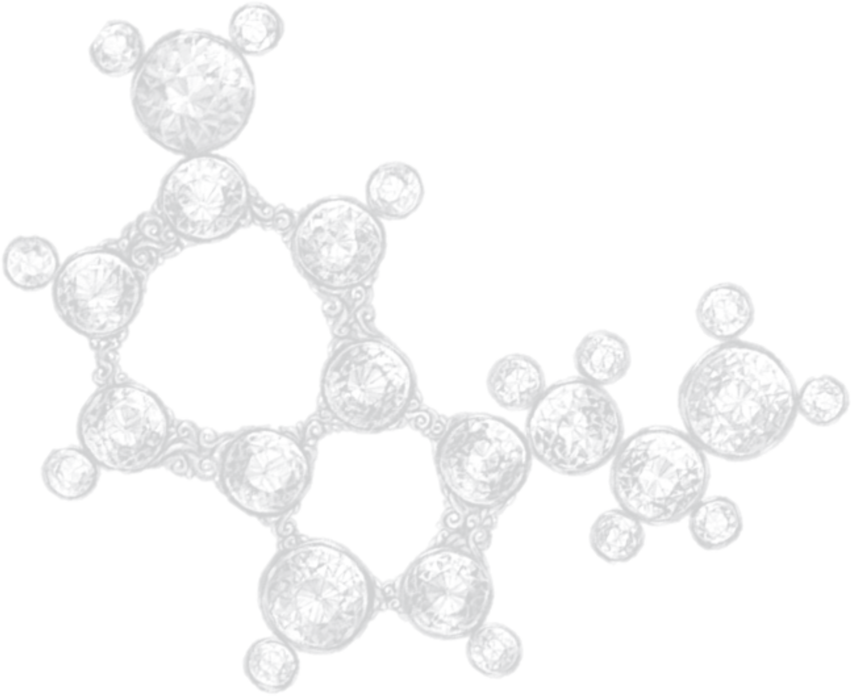
Supplemental Figure 2: Expression of ghrelin system components in GEP-NETs of different grade. The absolute mRNA expression of the different components of the ghrelin system was analyzed in grade 1, 2 and 3 GEP-NETs samples (values are adjusted by 18S expression). Data represent the mean ± SEM.



Supplemental Figure 3: Expression of ghrelin system components in pancreas and gastrointestinal NETs. The absolute mRNA expression of the different components of the ghrelin system was analyzed in pancreas and gastrointestinal NETs samples. Data represent the mean±SEM. Asterisks (**,p<0.01; ***, p<0.001) indicate significant changes by non-paired analysis.

PART II

Novel therapeutic options for neuroendocrine tumors



Chapter 8

Type 2 diabetes in neuroendocrine tumors: are biguanides and statins part of the solution?

Aura D. Herrera-Martínez[1,2], Sergio Pedraza-Arevalo[1,3], Fernando López-López[1,3,4], Manuel D. Gahete[1,3,4], María A. Gálvez-Moreno[1,2], Justo P. Castaño[1,3,4], Raúl M. Luque[1,3,4]

[1]Maimonides Institute for Biomedical Research of Cordoba (IMIBIC); Córdoba, Spain
[2]Endocrinology and Nutrition Service, Reina Sofia University Hospital; Córdoba, Spain
[3]Department of Cell Biology, Physiology, and Immunology, University of Córdoba; Córdoba, Spain

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ABSTRACT

Background: Biguanides and statins have been reported to exert beneficial effects on various cancer types. However, their precise effects and underlying molecular mechanisms are still poorly understood. **Materials and Methods:** We analyzed the relation between metabolic-syndrome, i.e., presence of type-2 diabetes (T2DM), hyperlipidemia and their treatment, with histological, epidemiological, and prognosis variables in two patient cohorts with neuroendocrine-tumors [NETs: lung-carcinoids (LCs; n=81) and gastro-entero-pancreatic (GEP-NET; n=100)]. Additionally, we investigated the antitumoral effects of different biguanides and statins by evaluating proliferation/migration/secretion/gene-expression and involved molecular pathways using BON1/QGP1-cell cultures. **Results:** In T2DM patients, pleura invasion was higher (LCs group; $p<0.05$) and tumor diameter tended to be increased (GEP-NET group). mRNA levels of somatostatin and ghrelin systems were different in tumor tissue of T2DM patients with and without metformin. Biguanides (metformin/buformin/phenformin) decreased proliferation rate in BON1/QGP1-cells (24-72h). However, the effects of statins on proliferation-rate were dependent of the statin-type, cell-type, and time. Specifically, only simvastatin/atorvastatin decreased proliferation in BON1-cells (48/72h and 72h, respectively), while all statins decreased proliferation rate in QGP1-cells (48/72h). Remarkably, metformin and simvastatin decreased migration capacity in BON1-cells and biguanides decreased serotonin secretion in BON1-cells. Phenformin increased apoptosis in BON1/QGP1-cells, and simvastatin in QGP1-cells. These antitumor effects likely involved altered expression of key genes related to cancer aggressiveness (i.e. *GLUT4, INSR*). Altogether, our results reveal a clear inhibitory effect of biguanides and statins on NET-cell aggressiveness. **Conclusion:** Given the demonstrated clinical safety of these drugs, our results invite to further explore their potential therapeutic role for the treatment of NET patients.

INTRODUCTION

Biguanides comprise a class of drugs with relevant effects as insulin-sensitizing agents, which are consequently used to treat type 2 diabetes (T2DM), a severe disease with distinct co-morbidities and whose incidence is growing worldwide, along with its associated metabolic syndrome and their other concomitant diseases [1]. The inflammation and insulin resistance present in patients with T2DM or metabolic syndrome have been associated with increased incidence of neoplasms [2]; thus, some treatment options targeting related pathways, as may be the case of biguanides, could be beneficial in some types of cancer. In this context, a putative specific relationship between T2DM-metabolic syndrome and neuroendocrine tumors (NETs) has not been established yet.

Among biguanides, only metformin is commercially available for medical use, since it has a safe profile and is well tolerated. Phenformin and buformin were withdrawn in the early 1970s because of an association with lactic acidosis and increased cardiac mortality [3, 4]. Interestingly, a putative association between metformin treatment and cancer prevention/treatment was suggested in 2005 [5], and multiple investigations have been subsequently published on this topic. Specifically, some epidemiological studies have suggested a decreased risk for pancreas, liver, colon, lung, and breast cancer in patients with diabetes treated with metformin [6-9]. This protective effect of metformin for cancer has been also found in diabetic patients according to several meta-analysis [9-11]. Moreover, biguanides can inhibit cell proliferation *in vitro* in several cancer cell lines, including pancreatic and neuroendocrine tumor cells [12, 13]. In terms of signaling, biguanides stimulate AMP-activated protein kinase (AMPK), reduce hepatic gluconeogenesis/glycogenolysis and increase glucose uptake in the muscle [14, 15]. AMPK activation also suppresses the mammalian target of rapamycin (mTOR1), which is a key regulator of proliferation in cancer cells. AMPK induces cell cycle arrest and reduces the insulin/insulin like growth factor 1 (IGF-1) signaling [16, 17]. Metformin-mediated AMPK activation may also result in p53-mediated cell cycle arrest or apoptosis [18, 19]. It has been also described that metformin could inhibit cell proliferation by G0/G1, G2/M or S phase arrest [20]. However, metformin may also exert antineoplastic properties in an AMPK-independent manner [21].

Statins are also commonly used drugs in the therapeutic arsenal for patients with metabolic syndrome or T2DM. Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR, HMGCoAR), affecting the rate limiting step in cholesterol synthesis, but they also exert other clinical effects related with immunomodulatory mechanisms in vascular diseases, autoimmune diseases and organ transplantation [22]. In addition, statins also reduce bone marrow stimulation and have been shown to exert anti-proliferative effects on smooth muscle cells [23-26]. The antitumor mechanisms of statins may include: induced cell-cycle arrest, apoptosis induction and activation of the signaling of c-Jun N-terminal kinases (JNKs), decreased invasion/metastasis capacity and decreased *MKI67* expression [27-31]. These antitumor effects have been described in several tumor types, including melanoma, colon and breast cancer [30-33]. Moreover, statins have been proposed as well as an useful treatment option to induce apoptosis and decrease proliferation in pheocromocytomas and paragangliomas [34, 35]; but, to the best of our knowledge, studies with statins have not yet been reported in NETs.

Since the anti-neoplastic therapy in advanced NETs is still unsatisfactory, novel drugs for tumor growth control are required, especially in progressive and hereditary NETs, which are characterized by early onset and multiple lesions [12]. Therefore, based on the potential association between T2DM, metabolic syndrome and cancer, we explored this association in a well-characterized cohort of lung carcinoids (LCs) and gastroenteropancreatic neuroendocrine tumors (GEP-NETs). In addition, we analyzed the use of antidiabetic drugs and statins in these cohorts and explored their putative relationship with clinical/histological characteristics. Finally, we also investigated , for the first time, the potential *in vitro* antitumoral effects of different biguanides (i.e. metformin, buformin and phenformin) and statins (i.e. atorvastatin, lovastatin rosuvastatin and simvastatin) in two different NET-cell models, BON1 and QGP1 cell lines.

MATERIALS AND METHODS

Patients and tissue samples

This study was approved by the Ethics Committee of the Reina Sofia University Hospital (Córdoba, Spain), and was conducted in accordance with the Declaration of Helsinki, and according to national and international guidelines. A written informed consent was signed by every individual before inclusion into the study. A total of 181 patients [81 with lung carcinoids (LCs), and 100 with gastroenteropancreatic (GEP-NETs)] who underwent surgery at the Reina Sofia University Hospital from 2005 to 2015 were included in the study. Clinical records were used to collect full medical history. Endocrine-associated syndromes were excluded. Patients with T2DM before the diagnosis of NET (31 patients) were analyzed separately: 14 T2DM patients in the LCs group, 6 treated with metformin; and 17 T2DM patients in the GEP-NETs group, 9 treated with metformin. Similar analysis was performed in those patients treated with statins: 4 in the LCs and 6 in the GEP-NETs group. Demographic and clinical characteristics of both cohorts are summarized in Tables 1-4. Patients were managed following the available guidelines and recommendations. After surgery, if residual or relapsed disease was observed, adjuvant treatment with or without surgery was prescribed. To confirm the neuroendocrine nature of all tumors, chromogranin

A, synaptophysin, cytokeratin 7, cytokeratin 20, CD56 and neuronal specific enolase were determined by immunohistochemistry, which was performed following the standardized diagnosis protocol of our hospital and evaluated by two experienced pathologists. Formalin-fixed paraffin-embedded samples were available in 46 LCs and 55 GEP-NETs cases, in which total RNA was isolated. Tumor samples were re-evaluated by two experienced pathologists before RNA isolation. Only primary tumor samples were included. Samples were analyzed individually and mRNA expression levels were correlated with the clinical/histological characteristics of the corresponding patient.

Culture of cell lines

We used two human pancreatic NET (PNET) cell lines: BON-1 and QGP-1 [36-39]. BON-1 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM-F12; Life Technologies, Barcelona, Spain) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Madrid, Spain) and 0.2% antibiotic (Gentamicin/Amphotericin B; Life Technologies). QGP-1cells were cultured in RPMI 1640 (Lonza, Basel, Switzerland), supplemented with 10% FBS, 1% glutamine and 0.2% antibiotic. Cells were harvested with trypsin (0.05%)–EDTA (0.53 mM) and resuspended in culture medium. Cell viability always exceeded 85%. Both cell lines were cultured in 75cm^2 flasks at 37°C in a 5% CO_2 incubator. All experimental procedures in both cell lines were performed at least three times.

Drugs and reagents

Metformin, phenformin, simvastatin, atorvastatin, lovastatin and rosuvastatin were purchased from Sigma Aldrich. Buformin was purchased from Santa Cruz Biotechnology (Texas, USA). All treatments were dissolved in the respective FBS-free-medium and diluted until obtaining final concentrations prior to use (metformin: 10^{-2}M; phenformin and buformin: 5 x10^{-3}M; statins: 10^{-5}M). IGF1 and paclitaxel were purchased from Sigma Aldrich (Madrid, Spain). Drug doses were selected based on *in vitro* dose–response curves (unpublished results) or in previous studies [40, 41].

Cell viability assay

Cells were plated in 100 µL of medium in 96-well plates at the density necessary to obtain a 65–70% cell confluence in the control groups at the end of the experiment. Twenty-four hours later, serum free medium was added during 24 h. After this, biguanides and statins were added into wells in medium with 5% serum. Cell viability was measured using the alamar-blue assay at basal, 24 h, 48 h and 72 h of incubation by measuring the fluorescent signal exciting at 560 nm and reading at 590 nm (Flex Station 3; Molecular Devices). The day of each measurement, cells were incubated for 3 h in 10% alamar blue/serum free-media and then, alamar reduction was measured. After each measurement, medium was replaced immediately by fresh medium. In all cases, cells were seeded per quadruplicate and all assays were repeated a minimum of four times. IGF1 and paclitaxel treatment were used as positive and negative controls, respectively.

Migration capacity assay

The ability BON-1 cells to migrate after 24 h of treatment with biguanides and statins was evaluated by wound healing technique. Briefly, cells were plated at sub-confluence in 12 well plates. Confluent cells were serum-starved for 24 h and after synchronization the wound was made using a 100 µl sterile pipette tip. Cells were incubated for 24 h in FBS free medium. Wound healing was calculated as the area of a rectangle centered in the picture 24 h after the wound vs. the area of the rectangle just after doing the wound, as previously reported [42]. At least three experiments per cell line were performed in independent days, in which three random pictures along the wound were acquired per well.

RNA isolation and reverse-transcription

Total RNA from formalin fixed paraffin-embedded (FFPE) samples was isolated using the RNA easy FFPE Kit (Qiagen, Limburg, The Netherlands) according to the manufacturer's instructions. Quantification of the recovered RNA was assessed using NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). One microgram of total RNA was retrotranscribed to cDNA with the First Strand Synthesis kit using random hexamer primers (Thermo Scientific) as previously reported [43, 44].

Quantitative real time PCR (qPCR)

cDNA was amplified with the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) using the Stratagene Mx3000p system and specific primers for each transcript of interest. Specifically, expression levels (absolute mRNA copy number/50ng of sample) of insulin receptor (*INSR*), *GLUT4* genes were measured in the cells using previously validated primers [41], and the expression level of each transcript was adjusted by the expression of Beta Actin (*BACT*; used as housekeeping gene). Experiments were performed at least three times.

In human tumor samples, somatostatin system [i.e. somatostatin (*SST*), cortistatin (*CORT*), their receptors *SSTR1*, *SSTR2*, *SSTR3*, *SSTR4*, *SSTR5*, sst5TMD4], and ghrelin system [i.e. ghrelin (*GHRL*), In1-ghrelin variant, ghrelin-o-acyltransferase enzyme (*GOAT*), and the receptors *GHSR1a*/GHSR1b] were evaluated using previously validated primers [41, 45-48]. mRNA levels were normalized by 18S in GEP-NETs and by BACT in LCs, as previously described [45, 46]. In NET cell lines, the expression of insulin receptor (*INSR*) and glucose transporter GLUT4 was analyzed in response to biguanides and statins treatment using previously validated primers [49, 50].

All samples were run, in the same plate, against a standard curve to estimate mRNA copy number and a No-RT sample as a negative control. Thermal profile consisted of an initial step at 95°C for 30 s, followed by 50 cycles of denaturation (95°C for 20s) and annealing/elongation (60°C for 20s), and finally, a dissociation cycle (melting curve; 55°C to 95°C, increasing 0.5°C/30 s) to verify that only one product was amplified.

Serotonin assay

BON-1 and QGP-1 cells were cultured in 12-well plates. At 70% confluence, cells were serum starved and after a 24 h incubation period with specific treatments or with vehicle, media were collected and stored at −20°C until measurements. Secretion of serotonin was detected using a serotonin ELISA kit (ALPCO, Salem, NH, USA) following the instructions of the manufacturer.

Apoptosis assay

BON-1 and QGP-1 cells were cultured in 24 well plates. At 70% confluence, cells were serum starved and after 48 h incubation with specific treatments or with vehicle-treated controls in 5% FBS medium, apoptosis levels were measured using a cell death detection ELISA kit (Sigma Aldrich) following the manufacturer's instructions.

Measurement of ERK1/2 and AKT signaling pathways by western blotting

500,000 cells (BON-1 and QGP-1) were cultured in 6-well plates and incubated for 8 min with specific treatments and vehicle-treated controls. Briefly, after the corresponding treatment, medium was removed and cells were washed twice using PBS, detached using a scrapper and immediately lysed in pre-warmed SDS-DTT sample buffer at 65°C (62.5 mM Tris-HCl, 2% SDS, 20% glycerol, 100 mM DTT and 0.005% bromophenol blue) followed by sonication for 10 s and boiling for 5 min at 95 °C, as previously described [51, 52]. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Darmstadt, Germany), and then membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/0.05% Tween 20 and incubated with the primary antibodies for total-ERK1/2, p-ERK1/2, total-AKT, p-AKT (Santa Cruz, CA, USA) and then, with the appropriate secondary antibodies [anti-rabbit antibody from Cell Signaling (Danvers, MA, USA)]. Protein analyses were developed using an enhanced chemiluminescence detection system (GE Healthcare, UK) with dyed molecular weight-markers. A densitometric analysis of the bands was carried out with the ImageJ software [53]. Relative phosphorylation of ERK and AKT was obtained from normalization of p-ERK1/2 or p-AKT against the total ERK1/2 or AKT, respectively.

Statistical analysis

In functional experiments, results are expressed as percentage vs. vehicle-control (non-treated cells). mRNA levels are expressed as mean ± SEM. Cell survival rate compared to control was assessed by multiple comparison tests. U-Mann Whitney tests were used to evaluate clinical relations within LCs and GEP-NETs samples. Chi-squared test was used to compare categorical data. All statistical analyses were performed using SPSS statistical software version 20 and Graph Pad Prism version 6. *p* values smaller than 0.05 were considered statistically significant.

RESULTS

Clinical evolution in patients with LCs and GEP-NETs and correlations with T2DM

In the LC group, weight loss was more frequently observed in patients with T2DM than in non-diabetic patients (36.4% vs. 8.6%; $p<0.05$; Table 1). Likewise, pleura invasion was also higher in T2DM patients (37.5% vs. 2.2%; $p<0.05$; Table 1). Despite the increased incidence of weight loss in diabetic patients with LC, none of the metformin-treated T2DM patients exhibited this symptom ($p<0.05$; Table 2). In this cohort, the clinical outcome did not differ in those patients receiving metformin or other antidiabetic treatment (Table 2). Mortality tended to be increased in patients with T2DM ($p=0.09$; Table 1).

In the GEP-NETs group, an increased incidence of a second neoplasm was observed in the non-diabetic group (25.7% vs. 0%; $p<0.05$; Table 3). Tumor diameter tended to be higher in patients with T2DM compared to non-diabetic patients (3.4±0.5 vs 2.5±0.2 cm, $p=0.06$). In addition, the proportion of patients with complete surgical resection was lower in T2DM compared with non-diabetic patients (69.2% vs. 93.8%; $p<0.05$; Table 3). In this cohort, the clinical outcome of patients treated with metformin was also similar to those treated with other antidiabetic drugs or insulin (Table 4).

None of the rest of clinical parameters evaluated (including functionality or incidental finding), histopathological variables (including, necrosis, local invasion, presence of metastasis, vascular or nerve invasion), tumor grading or evolution parameters (including relapsed disease, disease-free survival and mortality) were associated to the presence of T2DM or the use of metformin in our cohorts of patients with LCs or GEP-NETs.

No clinical, histological or molecular variable was associated to the presence of hyperlipidemia in our cohort of LCs (Table 5) or GEP-NETs (Table 6). A higher proportion of patients treated with statins were free of disease during the follow up (X^2 7.07; $p<0.05$). None of the other clinical, histological or evolution parameters were associated with the use of statins in our cohorts of patients with LCs or GEP-NETs.

Table 1: General characteristics of the LCs patient population

General characteristic		Total (n=81)	Non-diabetic patients (n=61)	T2DM patients (n=14)	*p*
Gender					0.55
	Male	51.8% (42/81)	52.5% (32/61)	50.0% (7/14)	
	Female	48.1% (39/81)	47.5% (29/61)	50.0% (7/14)	
Age (years old)		56.4±15.6	56.1±2.7	58.3±3.7	0.75
Personal history of other tumors		18.7% (14/75)	18.3% (11/60)	21.4% (3/14)	0.52
Smoke habit (Active/ Ex-smoker)		65.5% (38/58)	68.1% (32/47)	60% (6/10)	0.44
Family history of neoplasms		55.6% (5/9)	80.0% (4/6)	20.0% (1/3)	0.40
Weight loss		**14.6% (7/48)**	**8.6% (3/35)**	**36.4% (4/11)**	**0.046**
Functionality		7.5% (4/53)	7.7% (3/39)	8.3% (1/12)	0.67
Incidental		19.2% (10/52)	21.1% (8/38)	16.7% (2/12)	0.55
Maximal tumor diameter (cm)		2.9±2.4	2.8±0.3	3.5±0.8	0.36
Multiple tumors		6.9% (5/72)	5.5% (3/55)	8.3% (1/12)	0.55
Vascular invasion		16.1% (5/50)	17.4% (4/23)	0% (0/6)	0.37
Neural invasion		11.8% (2/17)	8.3% (1/12)	25.0% (1/4)	0.45
Metastasis		25.0% (17/68)	25.0% (13/52)	25.0% (3/12)	0.65
Bronchial infiltration		75.0% (45/60)	90.0% (36/47)	50.0% (4/8)	0.13
Parenchyma infiltration		39.0% (23/59)	37.0% (17/46)	62.5% (5/8)	0.16
Pleura infiltration		**6.8% (4/59)**	**2.2% (1/46)**	**37.5% (3/8)**	**0.008**
Classification					0.17
	Typical	69.4% (34/49)	65.8% (25/38)	77.8% (7/9)	0.69
	Atypical	30.6% (15/49)	34.2% (13/38)	22.2% (2/9)	0.69
Relapsed disease		11.8% (6/51)	12.5% (5/40)	0% (0/9)	0.34
Disease free during follow-up		77.6% (45/58)	77.8% (35/45)	81.8% (9/11)	0.56
Mortality		19.4% (13/67)	15.4% (8/52)	35.7% (5/14)	0.09

p value refers to the comparison between non-diabetic patients and T2DM patients.

Table 2: Metformin in patients with T2DM and LCs

General characteristic	Metformin (n=6)	Other antidiabetic treatment (n=5)	*p*
Weight loss	**0% (0/4)**	**100% (4/4)**	**0.04**
Maximal tumor diameter (cm)	2.6±0.3 cm	6.7±2.7 cm	0.38
Metastasis	0% (0/6)	60.0% (3/5)	0.12
Bronchial infiltration	50.0% (2/4)	50.0% (1/2)	0.8
Parenchyma infiltration	7.05% (3/4)	25.0% (1/4)	0.6
Pleura infiltration	50.0% (2/4)	50.0% (1/2)	0.8
Disease free during follow-up	100% (4/4)	50.0% (2/4)	0.21
Mortality	33.3% (2/6)	60.0% (3/5)	0.39

Table 3: General characteristics of the GEP-NETs patient population

General characteristic		Total (n=100)	Non-diabetic patients (n=70)	T2DM patients (n=17)	*p*
Gender					0.28
	Male	57.0% (57/100)	58.6% (41/70)	47.1% (8/17)	
	Female	43.0% (43/100)	41.4% (29/70)	52.9 % (9/17)	
Age (years)		55.7±17.5	57.8±3.1	55.7±2.2	0.80
Personal history of other tumors		**20.7% (18/87)**	**25.7% (18/70)**	**0% (0/17)**	**0.012**
Smoke habit		67.4% (29/43)	65.7% (23/35)	71.4% (5/7)	0.57
Family history of neoplasms		46.4% (13/28)	45.0% (9/20)	50.0% (4/8)	0.56
Weight loss		38.5% (20/52)	41.5% (17/41)	25.0% (2/8)	0.32
Incidental tumor		40.3% (29/72)	42.3% (22/52)	33.3% (4/12)	0.41
Functionality		31.5% (23/73)	32.1% (17/53)	33.3% (4/12)	0.59
Primary tumor localization					
	Pancreas	36.0% (36/99)	34.3% (24/70)	50.0% (8/16)	
	Stomach	6.0% (6/99)	4.3% (3/70)	6.3% (1/16)	
	Small bowel	19.0% (22/99)	16.3% (14/70)	25.1% (4/16)	
	Colon and rectum	36.0% (36/99)	33.4% (29/70)	0% (0/16)	
Maximal tumor diameter (cm)		2.6±2.2	2.5±0.2	3.4±0.5	0.06
Free surgical border		**87.3% (62/71)**	**93.8% (45/48)**	**69.2% (9/13)**	**0.032**
Multiple tumors		7.5% (4/53)	7.5% (3/40)	0% (0/6)	0.65
Local infiltration		53.1% (43/81)	57.1% (32/56)	42.9% (6/14)	0.25
Vascular invasion		28.4% (21/74)	30.8% (16/52)	25.0% (3/12)	0.50
Neural invasion		29.6% (21/71)	32.7 % (16/49)	16.7% (2/12)	0.24
Metastasis		47.4% (45/95)	45.5% (30/66)	47.1% (8/17)	0.56
Grading (WHO 2010 criteria)					
	Low	46.4% (32/69)	52.2% (24/46)	23.1% (3/13)	0.08
	Intermediate	39.1% (27/69)	34.8% (16/46)	69.2% (9/13)	0.08
	High	14.5% (10/69)	13.0% (6/46)	7.7% (1/13)	0.27
Relapsed disease		34.2% (27/79)	36.8% (21/57)	27.3% (3/11)	0.41
Disease free during follow-up		61.4% (43/70)	60.8% (31/51)	70.0% (7/10)	0.43

p value refers to the comparison between non-diabetic patients and T2DM patients.

mRNA expression of SST and ghrelin system components in LCs and GEP-NETs and their correlations with T2DM

The mRNA levels of several genes of interest were measured in the tumor tissue obtained from LCs (Figure 1A) and GEP-NET (Figure 1B) patients. In the LCs group, mRNA levels of SST and several receptor subtypes (*SSTR1*, *SSTR2*, *SSTR4, SSTR5* and sst5TMD4), but not of *CORT* or *SSTR3*, were numerically, albeit non-significantly decreased in patients with T2DM compared to the non-diabetic patients (Figure 1A). A similar pattern of expression

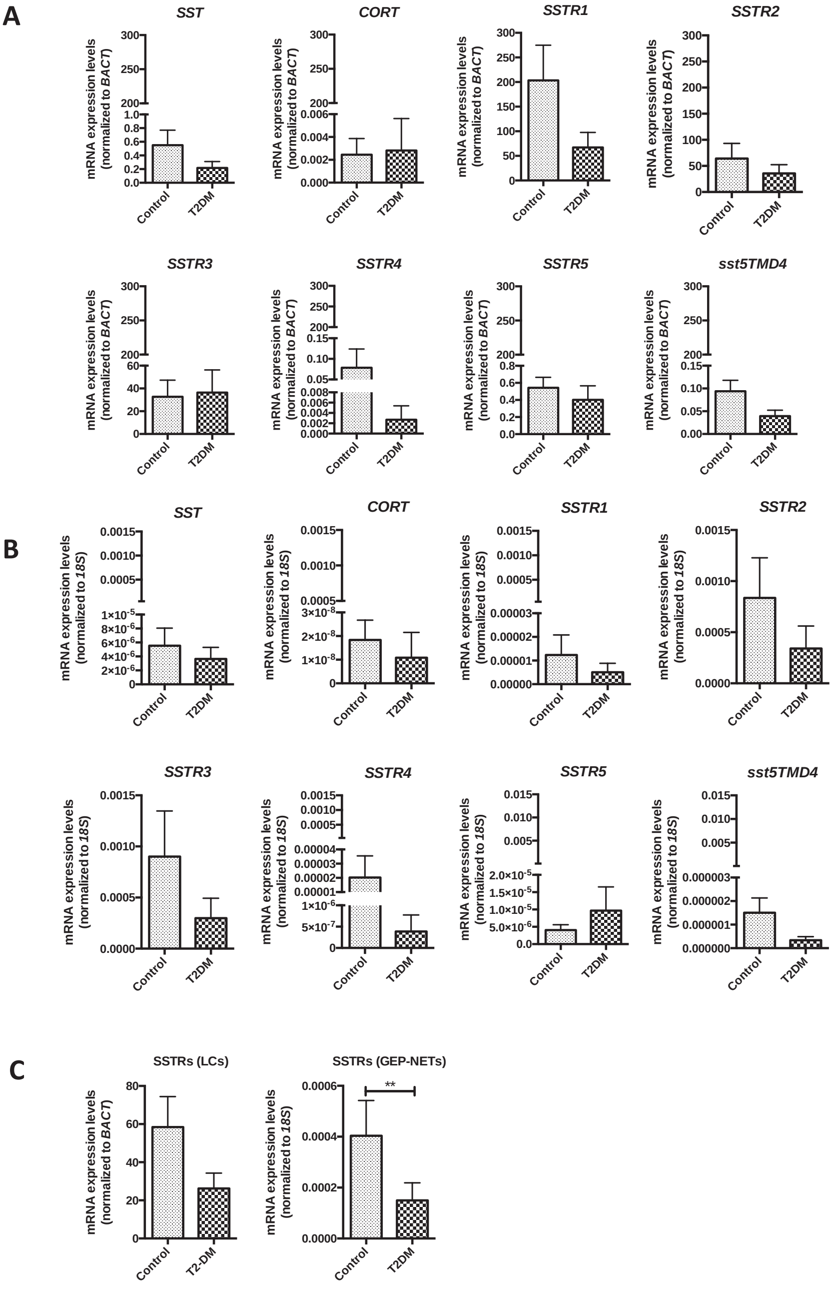

Figure 1: mRNA expression of somatostatin system components in diabetic and non-diabetic patients with LCs (A) and GEP-NETs (B). (C) Total mRNA expression of somatostatin receptors in LCs and GEP-NET patients. The absolute mRNA expression of the different components of the SST system was determined by qPCR in tumor samples (values were normalized to *BACT* in LCs and *18S* in GEP-NETs). Data represent the mean±SEM (**, p<0.01).

Table 4: Metformin in patients with T2DM and GEP-NETs

General characteristic	Metformin (n=9)	Other antidiabetic treatment (n=7)	*p*
Weight loss	25.0% (1/4	33.3% (1/3)	0.71
Maximal tumor diameter (cm)	3.5±0.8 cm	3.5±0.9 cm	0.95
Necrosis	25.0% (1/4)	0% (0/4)	0.50
Vascular invasion	40.0% (2/5)	16.7% (1/6)	0.42
Neural invasion	0% (0/5)	33.3% (2/6)	0. 27
Metastasis	44.4% (4/9)	57.1% (4/7)	0.50
Mortality	55.6% (5/9)	28.6% (2/7)	0.29

Table 5: Statins in patients with LCs

General characteristic	No statins use (n=71)	Statins (n=4)	*p*
Previous other tumor	16.9% (12/71)	50.0% (2/4)	0.16
Weight loss	13.0% (6/46)	50.0% (1/2)	0.27
Maximal tumor diameter (cm)	2.8±0.3	4.6±2.3	0.42
Necrosis	33.3% (8/24)	0% (0/1)	0.68
Metastasis	25.0% (16/64)	25.0% (1/4)	0.74
Bronchial infiltration	74.1% (43/58)	100% (2/2)	0.55
Parenchyma infiltration	40.4% (23/57)	0% (0/2)	0.37
Pleura infiltration	7.0% (4/57)	0% (0/2)	0.87
Disease free during follow-up	78.6% (44/56)	50% (1/2)	0.40
Mortality	17.5% (11/63)	50% (2/4)	0.167

Table 6: Statins in patients with GEP-NETs

General characteristic	No statins use (n=80)	Statins (n=6)	*p*
Weight loss	36.4% (16/44)	60.0% (3/5)	0.29
Maximal tumor diameter (cm)	2.6±0.2 cm	4.3±1.2 cm	0.13
Necrosis	32.0% (8/25)	0% (0/1)	0.69
Peritumoral invasion	52.3% (34/65)	75.0% (3/4)	0.36
Vascular invasion	28.3% (17/60)	33.3% (1/3)	0.64
Neural invasion	28.1% (16/57)	33.3% (1/3)	0.64
Metastasis	44.7%(34/76)	66.7% (4/6)	0.27
Mortality	28% (21/75)	66.7% (4/6)	0.07

was observed in the GEP-NETs group, except for the mRNA levels of *SSTR5* (Figure 1B). Interestingly, an overall decreased in the mRNA levels of all SSTRs was found in both, the LC and GEP-NET groups, but this difference only reached statistical significance in the GEP-NET group (Figure 1C).

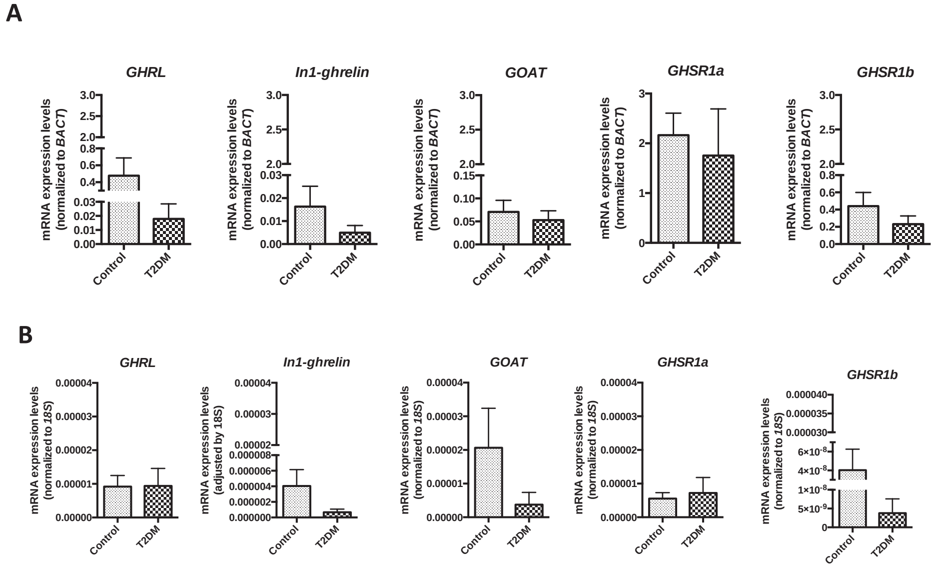

Figure 2: mRNA expression of ghrelin system components in diabetic and non-diabetic patients with LCs (A) and GEP-NETs (B). The absolute mRNA expression of the different components of the ghrelin system was determined by qPCR in tumor samples. Data represent the mean±SEM.

Similarly, mRNA levels of all the components of the ghrelin system (*GHRL*, In1-ghrelin, *GOAT* and the receptors *GHSR1a* and GHSR1b) displayed non-significant lower levels in diabetic LCs patients compared with non-diabetic LCs patients (Figure 2A). In GEP-NETs, the mRNA levels of In1-ghrelin, *GOAT* and GHSR1b, but not *GHRL* or *GHSR1a*, also tended to be lower in patients with T2DM (Figure 2B).

Interestingly, a sub-analysis showed that despite the overall expression of SSTRs was significantly lower in the GEP-NETs group with T2DM compared to non-diabetic patients, these levels were not decreased in T2DM patients treated with metformin compared to non-diabetic patients (Figure 3A). Specifically, a non-significant increase in the mRNA levels of *SST*, *CORT*, *SSTR1*, *SSTR2* and *SSTR3* was observed in T2DM patients treated with metformin compared with T2DM patients without metformin (Figures 3B), as well as in the mRNA levels of *GHRL*, In1-ghrelin and *GHSR1a* (Figures 3C).

Cell survival of PNET cells after treatment with biguanides or statins

All biguanides tested clearly decreased survival rate in both BON-1 and QGP-1 cell lines, in a time-dependent manner (Figure 4A and 4B, respectively). The most remarkable effect was observed in BON-1 cells with phenformin (5x10^{-3} M), which decreased survival rate by 76.6%, 93.1% and 97.13% after 24, 48 and 72h of incubation, respectively. Metformin

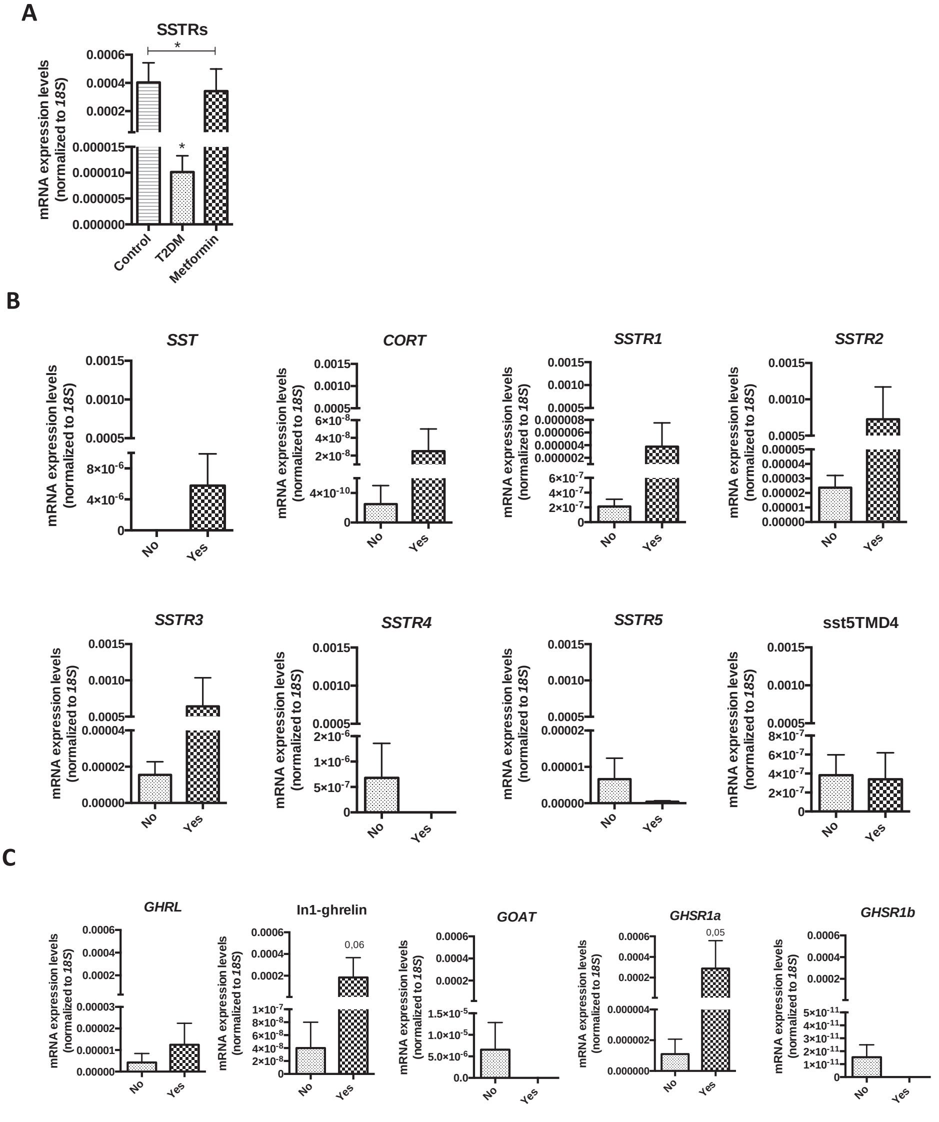

Figure 3: Effects of metformin in patients with T2DM and GEP-NETs. (A) Total mRNA expression of somatostatin receptors in GEP-NETs. Specific SST and ghrelin system components (**B, C** respectively) in GEP-NETs. The absolute mRNA expression of the different components of the SST system was determined by qPCR and normalized to *18S*. mRNA expression was assessed in GEP-NET patients with and without T2DM. Among the last ones, two subgroups were analyzed: those treated with metformin vs those treated with other antidiabetic drugs/insulin. mRNA expression was compared to control. Data represent the mean±SEM (*, $p<0.05$).

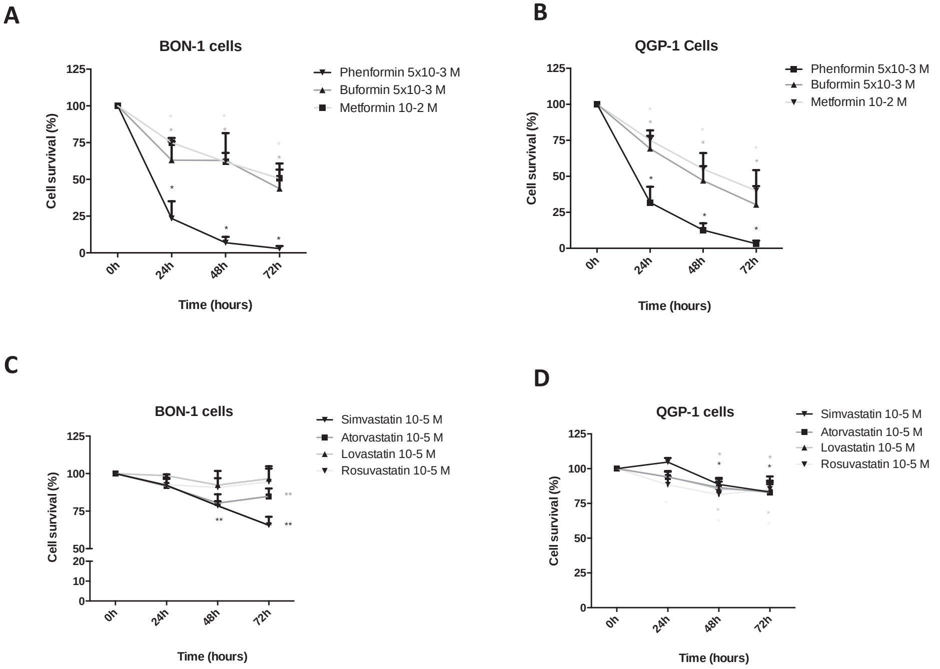

Figure 4: Time-dependent effect on cell viability of biguanides in BON-1 (A) and QGP-1 (B) cell lines; and statins in BON-1 (C) and QGP-1 (D). Cell viability is expressed as cell survival in percentage after 24h, 48 h and 72 h. Cell proliferation rate compared to control was assessed by multiple comparison tests and asterisks (*, $p<0.05$; **,$p<0.01$) indicate significant associations.

(10^{-2} M) decreased the survival rate in these cells by 25.1%, 38.1% and 49.4%, whereas buformin ($5x10^{-3}$ M) reduced it by 36.9%, 37.1% and 56.3% after 24, 48 and 72h of incubation, respectively (Figure 4A).

A similar effect was observed in QGP-1 cell line. Specifically, phenformin was also the most effective biguanide since survival rates decreased by 68.2%, 87.4% and 96.9% after 24, 48 and 72h of incubation, respectively; whereas, metformin decreased the survival rate by 24.9%, 45% and 60%, and buformin by 30.7%, 53.0% and 69.7% after 24, 48 and 72 h of incubation, respectively (Figure 4B).

We also analyzed the effect of different statins in cell survival in BON-1 and QGP-1 cell lines (Figure 4C and 4D, respectively). Specifically, a decreased survival rate was observed after 48 and 72h of treatment with simvastatin (10^{-5} M; 21.4% and 34.5%, respectively), and after 72h of treatment with atorvastatin (10^{-5} M; 15.2%) in BON-1 cells (Figure 4C), being the effect of simvastatin more pronounced than that of atorvastatin at 72h ($p<0.05$; Figure 4C). In QGP-1 cells, a reduction in the proliferation rate was observed after 48 and

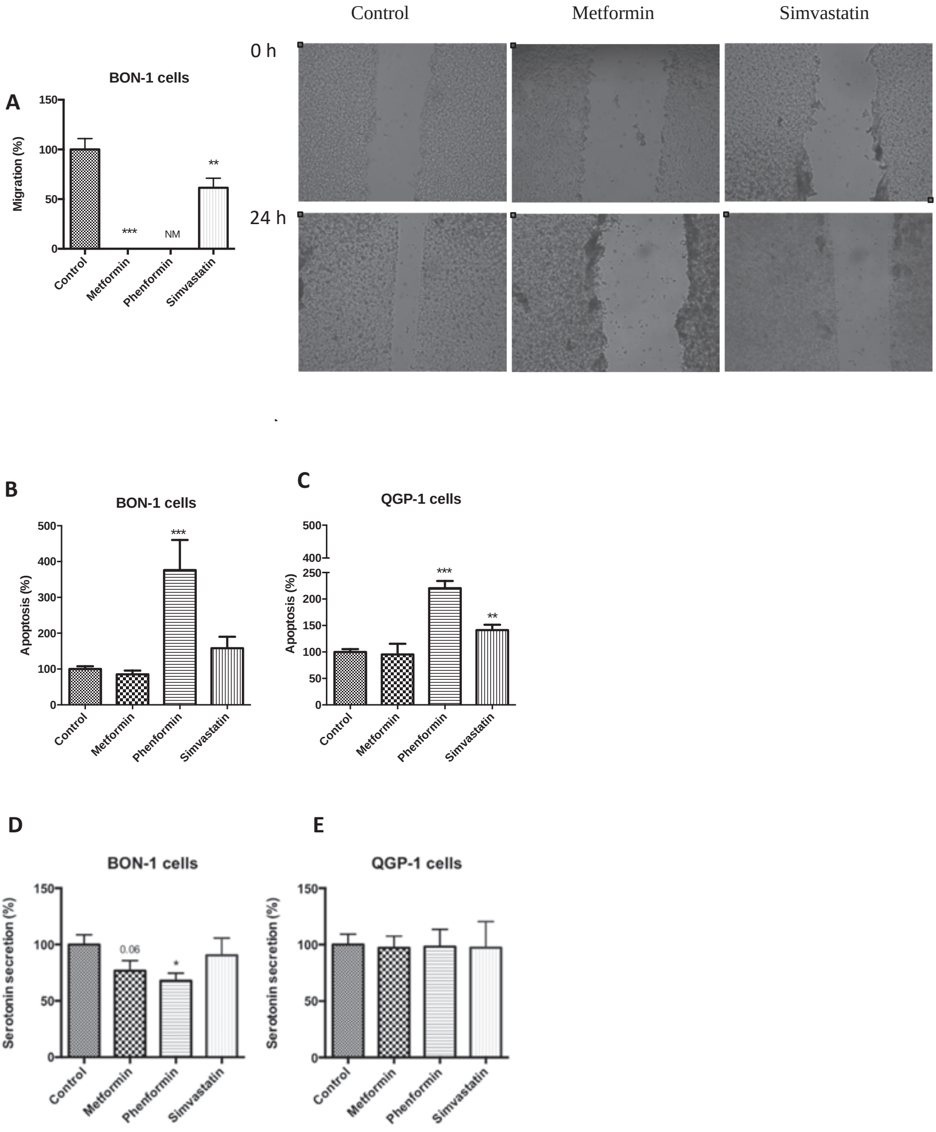

Figure 5: Effect of biguanides and statins on (A) cell migration in BON-1 cells; (B) apoptosis rate in BON-1 (B) and QGP-1 (C) cells; serotonin secretion in BON-1 (D) and QGP-1 (E) cell lines. Migration and serotonin secretion were assessed after 24 hours of incubation; apoptosis rate was evaluated after 48 hours. Representative images of wound healing after 24 hours of treatment are presented in Figure 5A, lower panels. Treatment rates were compared to control by multiple comparison tests and asterisks (*, p<0.05; **, p<0.01; ***, p<0.001) indicate significant associations. Legend NM: non-measurable.

72h of incubation with all the statins tested (Figure 4D). Thus, simvastatin, atorvastatin, lovastatin and rosuvastatin (10^{-5} M) decreased survival rate ranging from 14.7% to 17.2% after 72h.

Based on their antiproliferative effects, phenformin and simvastatin were chosen as representative compounds of these two classes of drugs in order to perform further functional experiments (i.e. migration, apoptosis and serotonin secretion). Moreover, metformin was also included in these analyses due to its relevance in the clinical practice.

Migration capacity in PNET cells in response to metformin, phenformin and simvastatin treatment

Metformin and simvastatin (24h of incubation) significantly decreased the migration capacity of BON-1 cells (100% and 38.6%, respectively; representative images are depicted in Figure 5A). In contrast, it was not possible to measure the migration capacity in response to phenformin in BON-1 cells, perhaps due to a treatment-related toxicity of this compound (see below). As previously reported [54] it was not feasible measuring this functional assay on QGP-1 cells since these cells form aggregates/clusters in culture, which do not allow to correctly measure the migration capacity under basal conditions or in response to any given treatment.

The effect of metformin, phenformin and simvastatin treatment on apoptosis

In BON-1 cells, phenformin caused a three-fold increase in apoptosis (Figure 5B). However, metformin or simvastatin treatment did not alter apoptosis in BON-1 cells. In QGP-1 cells, a two-fold increase in apoptosis was also observed in response to phenformin (Figure 5C). In addition, simvastatin increased apoptotic rate in QGP-1 cells by 58.1% (Figure 5C). Conversely, metformin treatment did not alter apoptosis in QGP-1 cells.

Effect of biguanides and statins on serotonin secretion in PNET cell lines

In BON-1 cells, phenformin, but not simvastatin decreased serotonin secretion after 24h of incubation ($p<0.05$; Figure 5D). Metformin treatment also tended to decrease serotonin release ($p=0.06$) (Figure 5D). In contrast, none of these treatments altered serotonin secretion from QGP-1 cells (Figure 5E).

Effects of metformin, phenformin and simvastatin on ERK1/2 and AKT signaling pathways

To start exploring the signaling pathways affected by biguanides (metformin and phenformin) and simvastatin to induce their functional actions in NET cells, the levels of phosphorylation of AKT and ERK were evaluated. In BON-1 cells, both biguanides and simvastatin similarly decreased phosphorylation levels of AKT and ERK compared to controls (Figure 6A). In

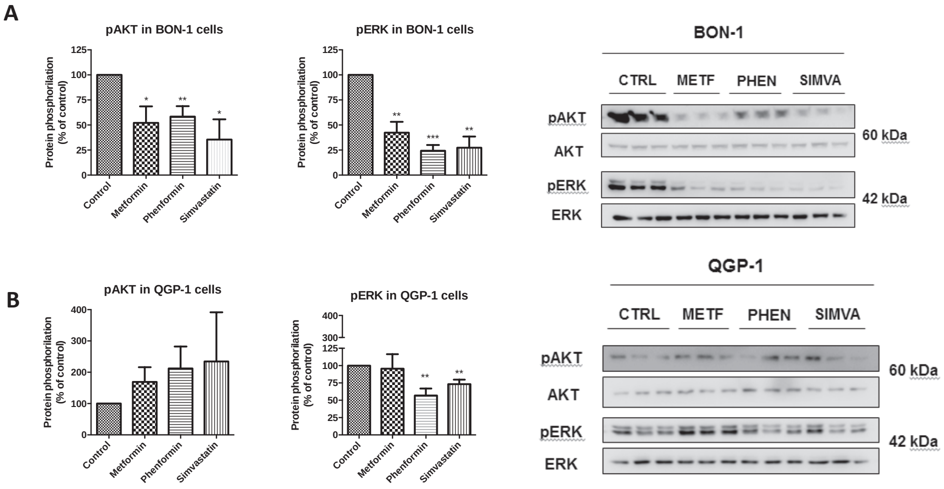

Figure 6: Effects of biguanides and statins on (A) AKT and ERK phosphorylation in BON-1 (A) and QGP-1 (B) cells. Phosphorylation levels compared to control was assessed by multiple comparison tests and asterisks (*, p<0.05; **, p<0.01; ***, p<0.001) indicate significant associations.

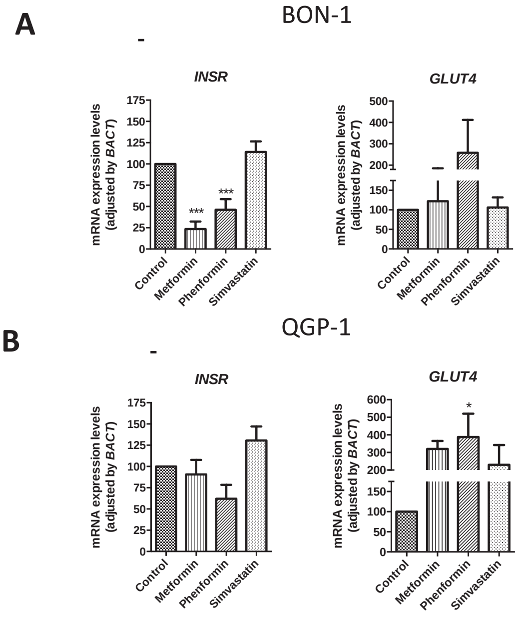

Figure 7: Effects of biguanides and statins on mRNA expression in (A) BON-1 and (B) QGP-1 cells. mRNA expression compared to control was assessed by multiple comparison tests and asterisks (*, p<0.05; **, ***, p<0.001) indicate significant associations.

marked contrast, in QGP-1 cells, only phenformin and simvastatin decreased phosphorylation levels of ERK without altering those of AKT (Figure 6B).

The effect of metformin, phenformin and simvastatin in the expression of key genes in PNET pathophysiology

In BON-1 cells, metformin and phenformin severely decreased the mRNA levels of *INSR* ($p<0.001$) (Figure 7A). Also, a trend to an increase in the expression *GLUT-4* was observed in response to phenformin (Figure 6A). In QGP-1 cells, *GLUT-4* expression was increased in response to both biguanides and simvastatin, but this difference only reached statistical significance in the case of phenformin (Figure 7B). Whereas, no significant changes were observed in the expression of *INSR* in QGP-1 cells in response to these compounds (Figure 7B). Finally, metformin treatment did not significantly alter the expression of SSTRs in BON-1 and QGP-1 cells (data not shown).

DISCUSSION

NETs are a widely heterogeneous group of neoplasms, which are frequently diagnosed at an advanced stage of disease [55]. Therapeutic options for advanced metastasized NETs include somatostatin analogs, interferon-α, chemotherapy and peptide receptor therapy [55, 56]. In the last years, target-directed therapies have increased the therapeutic spectrum for progressive NETs (e.g. sunitinib as tyrosine kinase inhibitor and everolimus for the mTOR pathway inhibition) [57-59]. However, despite the improvements in progression-free survival of these therapeutic options, their effect on the overall survival is still controversial [60]. Therefore, novel treatments are still required, especially for patients with advanced disease.

The incidence of metabolic syndrome is continuously increasing, reaching almost 35% in some countries, with a consequent increase in the prevalence of some types of cancer [61]. T2DM has been also related to an increased risk of malignancies [62, 63] and is frequently developed in patients using everolimus and some somatostatin analogs [64, 65]. In this context, metformin is one of the most widely prescribed oral hypoglycemic agents and, in the last years, it has received an increased attention because of its potential antitumorigenic effects [5, 66]. Likewise, some publications have described that statins exert an inhibitory effect on tumor-induced angiogenesis and an antitumor effect in cellular/animal models of human cancer [22, 67]; however, other studies have also suggested a potential risk of cancer when statins are used [68]. In this sense, the present study, although using a limited number of samples, is the first that: 1) assesses the association of T2DM with clinical evolution parameters in patients with different NET types (LCs and GEP-NETs); 2) evaluates the expression levels of all the components of two key regulatory systems (SST and ghrelin) in the tumor tissue of patients with these two NET types, in relation to T2DM and metformin treatment; and 3) analyzes and compares the effects of different biguanides and statins in key functional parameters in two representative models of NET cell lines, BON-1 and QGP-1 cells.

T2DM is linked to relevant defects in the INSR signaling pathway, which regulates growth and metabolic responses in insulin target cells and tissues [69]. Some epidemiological studies have described an increased risk for several types of cancer (breast, colon, rectum, liver, and pancreas) in insulin-resistant patients [70]. Remarkably, in our cohort of patients, a more aggressive pattern in LCs (increased incidence of pleura invasion) and increased tumor size in GEP-NETs were observed in patients with T2DM when compared to non-diabetic patients, suggesting a possible association between T2DM and NET pathophysiology.

Since insulin is related to increased risk of cancer, treatment options targeting this pathway could be effective in cancer prevention [71]. Indeed, a meta-analysis showed a 31% reduction in overall cancer incidence and a 34% decline in cancer mortality in patients with diabetes treated with metformin [72]. A retrospective study in patients with T2DM and NETs showed lower recurrence rate in those treated with metformin compared with non-metformin treated or non-diabetic patients [72]. Moreover, in a cohort of pancreatic NET patients receiving everolimus and octreotide LAR, progression free survival was higher in patients treated with metformin compared to other drugs [73]. To the best of our knowledge, no other specific reports in NETs have been published yet. In our cohort, metformin appeared to avoid weight loss in patients with LCs and T2DM. Interestingly, the numerical records assessed in patients with LCs receiving metformin suggested that tumor size were smaller, incidence of metastasis was lower and disease-free during follow-up was higher than in non-metformin treated patients; however, these results did not reach statistical significance, likely due to the limited size of the groups. In contrast, no association was observed between clinical/histological variables and the use of metformin in the GEP-NETs group. Obviously, we should underline that the main limitation of this work might be the limited number of patients with T2DM and those treated with metformin included in the analysis, although the size of the total cohort evaluated was large enough for making general comparisons. Therefore, like other studies reporting a limited cohort of samples [73], the results of this study should be interpreted with caution.

Novel mechanisms of action have been proposed for metformin in recent years. Among them, the induction of the expression of the glucagon-like peptide 1 (GLP-1) receptor on pancreas β-cells was described [74]. To the best of our knowledge, this is the first report showing that the expression of several SSTR subtypes is reduced in NETs from diabetic patients (LCs and GEP-NETs) compared with those from non-diabetic patients, and most importantly, that the overall expression of SSTR is significantly increased in LCs from diabetic patients treated with metformin compared with LCs from diabetic patients without metformin treatment. In fact, these expression levels of SSTR in LCs from diabetic patients treated with metformin achieved the levels observed in LCs from patients without T2DM. These novel results provide suggestive evidence that metformin treatment could increase SSTRs expression in NETs in diabetic patients, which might be important from a clinical point of view, in that a previous study has suggested that metformin could have potential synergistic effect when combined with somatostatin analogs via the inhibition of PI3K/AKT/mTOR axis [73]. Thus, it will be worth to elucidate in the future the mechanisms involved in the capacity of metformin to regulate the expression of SSTRs, as well as a putative synergistic effect between somatostatin analogs and metformin. In this sense, we analyzed the SSTR expression in BON1 and QGP1 cells after metformin treatment, but we did not observe changes in SSTRs mRNA expression levels, which could be in line with the idea that metformin could reverse the changes previously altered under diabetic conditions and maybe only have reduced potential to modulate basal expression of SSTRs. In addition, it has to be noted that pre- or co-treatment with biguanides and statins was not evaluated in this study, since the anti-proliferative *in vitro* response to somatostatin analogs is limited in these NET cell lines [45, 75, 76].

Biguanides increase insulin sensitivity as well as glucose use by peripheral tissues [3]. Anti-tumoral effects of metformin and phenformin have been evaluated in *in vitro* and *in vivo* studies, and metformin is also being tested as an adjuvant therapy to classic chemotherapeutic regimens [66, 77]. Specifically, an earlier study showed that metformin inhibited cell proliferation in pancreatic, bronchopulmonary and midgut neuroendocrine tumor cell lines in a dose-dependent manner, wherein this antitumoral effects appeared to be mediated via inhibition of mTORC1 signaling [12]. Metformin has been also shown to inhibit breast cancer cell growth *in vitro* in an AMPK-dependent manner, in association with a decreased mTOR activation [78]. In our study, we also observed a time-dependent antiproliferative effect of different biguanides in PNET cell lines. Similarly, our study revealed that biguanides were able to exert additional, beneficial effects on NET cell function by measuring other relevant functional endpoints (i.e. migration capacity and apoptotic rate). These results support and extend previous data showing that metformin exerted antitumoral actions *in vitro* by modulating cell proliferation and apoptosis in breast cancer cells [79]. However, we found that phenformin, but not metformin, was able to increase apoptosis in both NET cell lines, which is partially in agreement with previous data indicating that apoptosis induced by metformin would differ depending on the NET cell type [12]. We also found that metformin and phenformin were able to decrease serotonin secretion in BON-1, but not in QGP-1 cells. Although the exact mechanisms are still to be elucidated, these results could be clinically relevant in patients with carcinoid syndrome, since elevated serotonin levels are directly associated with symptoms in this pathology [79]. In this sense, we should remark that this is not the first time that different results are observed in the functional response of BON-1 and QGP-1 cells [45, 81-83], which further emphasizes their potential distinct value to study the intrinsic heterogeneity of NETs. Indeed, the reason for these differences is still unknown, but could be related to the distinct expression pattern of key regulatory systems (e.g. SST, ghrelin, IGF-I, etc.) [81-84] and/or to the different activation or signaling of these NET cells in response to the same treatment as it has been previously observed, for instance, for SST analogs (i.e. octreotide and pasireotide) [81, 82].

Statins can also exert antitumoral actions. Thus, a phase II trial has reported a statin-induced anti-proliferative effect in breast cancer [85]. As well, the antiproliferative effect of statins has also been reported in several cancer cell lines including cervical [86], leukemic natural killer [87], cholangiocarcinoma [88] and prostate [89]. In line with these previous studies, we observed here, for the first time, that different statins exerted a clear antiproliferative effect in NET cells. Additionally, we found that simvastatin was able to significantly increased apoptosis levels in QGP-1 cells, an effect that has been also previously described in cervical cancer, leukemia, natural killer and cholangiocarcinoma cell lines [85-87].

It is well known that the PI3K/AKT/mTORC1 pathway exerts important roles in NETs pathogenesis [90]. In LCs, metformin inhibited AKT, ERK and mTOR pathways, suggesting that its antiproliferative effects can be both AMPK-dependent and AMPK-independent [91]. In fact, Vlotides et al. have suggested that the functional effect of metformin is cell type-dependent, since they reported that AMPK and AKT phosphorylation was elevated in pancreatic and midgut NET-cell lines in response to metformin (48h of incubation) but this effect was not observed in bronchopulmonary neuroendocrine cells [12]. Interestingly, it was also suggested that the inhibition of the mTOR pathway was associated to the induction of GSK3 phosphorylation following the ERK or AKT pathway [12]. In our study, we observed an inhibition of phosphorylated AKT and ERK pathways after treating cells with biguanides (and also with simvastatin), which also reveals the AMPK-dependent and -independent effects of these drugs in NET cells. It should be mentioned that the differences between our results and those reported by Vlotides et al. may be related to the drug-incubation period (8min vs. 48h). However, as found in our study, cell inhibition of the ERK pathway has been also reported in non-small lung cancer and cholangiocarcinoma cell lines with concomitant induction of apoptosis [88, 92].

Nowadays, the mechanisms linking T2DM and cancer comprise a most exciting and interesting research topic. It has been proposed that chronic hyperinsulinemia may promote the development of neoplasms via abnormal stimulation of multiple cellular signaling cascades by insulin, enhancing growth factor-dependent cell proliferation and/or modifying cell metabolism [66]. In our results, we observed changes in the molecular expression of key genes involved in tumor aggressiveness (e.g. *INSR* and/or *GLUT-4*) in response to metformin or phenformin, but not simvastatin, suggesting a putative modulatory effect of biguanides in these signaling pathways. In line with this, some studies have suggested that the anti-proliferative effect of statins in cancer cell lines might be associated to a cell-cycle regulatory effects [85], epigenetic alterations [93], or to gene expression modifications of cancer signaling [94]. However, the effects of simvastatin treatment on these regulatory endpoints, as well as whether metformin and simvastatin could play synergistic effects in NETs (which has been demonstrated in different tumor pathologies [95-97]) could not be evaluated in our study, but deserve further attention in the future.

In sum, our study using a limited cohort of patients reveals a potential association between key clinical parameters of NET aggressiveness (i.e. incidence of pleura invasion or metastasis, tumor size, etc.) and the presence of diabetes and/or treatment with antidiabetic drugs in patients with different NET types (LCs and GEP-NETs). Moreover, this study provides evidence that the expression of multiple components of two key regulatory systems for the pathophysiology of NETs, the SST and ghrelin systems, are modulated in diabetic patients with LCs and GEP-NETs compared to non-diabetic patients. Finally, our results also showed that different biguanides and statins are capable to directly exert clear antitumoral actions in NET cells, probably due to their effect on cell survival, cell migration, apoptosis, gene expression and metabolic pathways modifications. Therefore, since metformin and statins are low-cost commercially available drugs, with a safe profile and large experience in their clinical use, our present results invite to further explore their potential value as adjuvant therapy for the treatment of NET patients.

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Chapter 9

General Discussion

Partially based on:
Neuroendocrine tumors: diagnostic, predictive and prognostic markers

Aura D Herrera-Martínez.[1,2], Leo J. Hofland[1], Wouter W. de Herder[1],
María A. Gálvez Moreno[2], Justo P. Castaño[2] and Richard A. Feelders[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus
MC, University Medical Center Rotterdam, the Netherlands
[2] Maimonides Institute for Biomedical Research of Cordoba (IMIBIC);
University of Córdoba; Reina Sofía University Hospital; Córdoba, Spain.

Invited Review: Endocrine Related Cancer. Submitted for publication.

Medical Treatment for neuroendocrine tumors: current options and future perspectives

Aura D. Herrera-Martínez[1,2], Johannes Hofland[1], Leo J. Hofland[1], Tessa Brabander[3], Ferry A.L.M Eskens[4], María A. Gálvez Moreno[2], Justo P. Castaño[2], Wouter W. de Herder[1], Richard A. Feelders[1]

[1] Department of Internal Medicine, division of Endocrinology, Erasmus
MC, University Medical Center Rotterdam, the Netherlands
[2] Maimonides Institute for Biomedical Research of Cordoba (IMIBIC);
University of Córdoba; Reina Sofía University Hospital; Córdoba, Spain.
[3] Department of Radiology & Nuclear Medicine, Erasmus
Medical Center, Rotterdam, the Netherlands
[4] Department of Medical Oncology, Erasmus Medical Center, Rotterdam, the Netherlands

Invited Review: Drugs. Submitted for publication

GENERAL DISCUSSION

Neuroendocrine tumors (NETs) are a very heterogeneous group of neoplasms, which can present with a wide range of clinical symptoms and behavior. The possibility of recurrence or long-term metastasis adds to the complexity to the clinical management of patients with NET. Moreover, heterogeneity of NETs, in terms of clinical behavior and response to medical treatment, emphasizes the importance of identifying new diagnostic, prognostic and therapeutic markers [1].

Some biomarkers for functioning and non-functioning NETs are currently available. However, despite the application of these biomarkers in clinical practice, their results should be interpreted with caution [2]. Considering the variable sensitivity and specificity of these parameters, there is an unmet need for novel biomarkers to improve diagnosis and predict patient outcome. Nowadays, several new biomarkers are under study, and may become future tools for the management of NETs. These biomarkers include: 1) peptides and growth factors; 2) DNA and RNA markers based on genomic and transcriptomic analysis, including the so-called NET test, which has been developed for analyzing gene transcripts in circulating blood; 3) circulating tumor/ endothelial/progenitor cells or cell free tumor DNA, which represent non-invasive methods that may provide additional information for monitoring treatment response and tumor recurrence; and 4) improved imaging techniques with novel radiolabeled somatostatin analogs or peptides. Unfortunately, nowadays there is no consensus for supporting the use of these novel biomarkers as an indicator of tumor burden [3]. Furthermore, dysregulation of several biomarkers is not tumor specific, and the absence of cut-off levels for differentiating tissue and tumor subtypes, the lack of reproducibility in other NET cohorts and the difficulties in their interpretation, limit their clinical application. Thus, it is aimed to develop highly specific and sensitive circulating/tissue biomarkers using DNA, RNA and metabolomic approaches.

Despite the rising incidence of NETs in the last years, prognosis has improved, probably due to the increased detection of early stage of disease and the availability of novel therapeutic options. Treatment of patients with unresectable NETs is complex and aims to control hormone secretion and tumor growth. The development of next-generation multireceptor targeting and radiolabeled somatostatin analogs, as well as target-directed therapies (everolimus, sunitinib) improved progression-free survival (PFS) in NET patients. However, only a proportion of patients responds to these therapies. Validated biomarkers that predict response to target-directed therapies are not yet available, whereas the expression of somatostatin receptors (SSTs) in NETs can be used to select patients that are eligible for peptide receptor radionuclide therapy (PRRT). Therefore, there is a need to develop biomarkers that can be used to achieve a more personalized treatment approach in patients with NET.

Medical treatment with somatostatin analogs (SSAs) is well-known in differentiated, locally advanced or metastatic NETs [4]. SSAs act through binding to G-protein-coupled SSTs which are broadly expressed in NETs and may modulate tumor cell proliferation and hormone secretion [5, 6]. Despite their efficacy in disease stabilization and symptom control, SSAs cannot control hormone production and tumor growth in the long term in many patients. For this reason, novel therapeutic options have been developed, such as PRRT [7], and specific pathways involved in cell proliferation, migration and/or angiogenesis in NETs have been targeted [8, 9]. In addition, chemotherapy schemes have been updated [10], new therapeutic options for functioning NETs have been developed, and several novel treatments are currently under study.

Based on the above-mentioned considerations, this thesis evaluated, on the one hand, the putative role of somatostatin and ghrelin system components as tissue (bio-)markers in NETs, and on the other hand, the antitumor effect of registered drugs for other medical purposes (ketoconazole, biguanides and statins), as well as the effect of novel drugs for hormone release control (somatostatin-dopamine receptor chimeras and telotristat) in NETs. For these aims, we used clinical data and cell-based assays, which represent a valuable, simple, and cost-effective tool for drugs discovery and evaluation [11]. Specifically, we used traditional two-dimensional (2D) monolayer cell cultures, in some studies, we completed our results with three-dimensional (3D) cell culture models using spheroids. This last culture system is a novel promising method which seems to appropriately reproduce tumor cells environment including, cell-cell signaling, growth kinetics, extracellular matrix deposition, nutrients-oxygen conditions, gene expression, drug resistance, cell heterogeneity and cell-cell physical interactions [12]. It has been described that 3D models with spheroids would allow better evaluation of long-term treatment [13-16], especially in NET cell lines [17]. According the results presented in this thesis, 3D cultures allowed studies with a reliable measure of hormone secretion in serum-deprived conditions, whereas an increased apoptosis, lactate dehydrogenase levels and decreased cell proliferation rates characterized long incubation periods (7 days) in 2D monolayer cell cultures. In this context, 3D culture systems with spheroids were especially useful in serum-deprived conditions, since they permit longer incubation periods with optimal (time-dependent) cell growth.

NET and somatostatin system

It has been widely described that the somatostatin system is linked to several intracellular signaling pathways including the regulation of cell proliferation, differentiation, and angiogenesis, amongst others [18]. Despite the fact that the presence of the somatostatin system has been widely described in NETs [19, 20], a specific role in tumorigenesis has not been fully elucidated yet [20, 21]. This includes the use for tumoral scintigraphy [22] and as target for long acting SSA and peptide receptor radionuclide therapy (PRRT) [22-24]. Recent publications have suggested that somatostatin system dysregulation may be associated with specific clinical features and prognosis in NETs patients [21, 25]. As such its molecular and immunohistochemical evaluation may represent a valuable prognostic marker in NETs [25, 26].

In the studies described in **chapters 2 and 3** of this thesis, we evaluated the mRNA expression of specific somatostatin system components, including the ligands somatostatin, cortistatin, and their receptors, SST_1, SST_2, SST_3, SST_4, SST_5 and SST_5TMD_4. Comparisons were made with normal (control), non-tumor adjacent (NTA) and tumor tissues. The presence of somatostatin system components was different between normal lung and gastroenteropancreatic (GEP) tissue. Specifically, in normal lung tissue, all somatostatin system components except somatostatin and SST_3 were expressed in less than 25% of samples, whereas in GEP-tissue, all system components (except cortistatin and SST_4) were expressed in more than 60% of cases. Additionally, the expression profile was also different among both groups: in lung carcinoids the mRNA expression of somatostatin and SST_4 was higher in tumor tissues compared to normal samples, while SST_5 was overexpressed in tumor samples compared to NTA. In contrast, in GEP-NETs, cortistatin, SST_1, SST_2, SST_5 were clearly over- expressed in tumor tissues compared with NTA samples.

As described in **chapters 2 and 3** of this thesis, differences in the expression of somatostatin system components in control (normal) and NTA tissue, as well as the correlation between the molecular expression of somatostatin and its receptors suggests an autocrine/paracrine effect that may modulate *in situ* tumor progression of NETs [27]. Additionally, interactions with other family receptors (opioids, dopamine, chemokine) have been described and may be associated with pathway activations [28], and even with tumor grade and malignancy [29].

According to our results, the different molecular expression of somatostatin system components in NTA tissue compared to normal samples (in lung carcinoids and in GEP-NETs) suggest that they may exert a biologically regulatory role in proliferation/secretion of these tumors. Among them, in GEP-NETs the overexpression of somatostatin was associated with increased lymphatic invasion but not with liver metastasis, additionally, SST_5 was associated with vascular and nerve invasion. In addition, other authors have suggested an association between its truncated isoform (SST_5TMD_4) with increased tumor aggressiveness, suggesting that a dysregulation in this receptor could be related to aggressive features and patient outcome [21, 30]. In contrast, in lung carcinoids, no relationship between SST_5 and agressiveness/invasion parameters was observed, while the truncated receptor SST_5TMD_4 was positively associated with disease free status during the follow-up. These results confirm the intrinsic variability and heterogeneity of NETs, specially in those with different localization, suggesting that an appropiate characterization of the tumor may allow a personalized clinical management. In this sense, not only the routine evaluation of SST_2 provides clinical information, but probably in the future the determination of other receptors, specially SST_5, may offer additional data about clinical outcome and prognosis.

As previously mentioned, SSTs expression in NETs is fundamental for the therapeutic management of these tumors. SSAs are first-line therapy in functionally active NETs, including those associated with the carcinoid syndrome and functional pancreatic neuroendocrine tumors (PNETs) [31]. Both long-acting octreotide and lanreotide, which target the SST_2, similarly improve symptoms and quality of life in NET patients [32]. Unfortunately, some patients may be resistant to octreotide or lanreotide. For this reason, a novel SSA (pasireotide), which has high affinity to multiple SSTs, including SST_2 and SST_5, may represent a valuable therapeutic option due to its higher affinity to more SSTs [33], and is suggested to be of value in insulinomas resistant to other treatment options [34, 35]. In concordance to this, in **chapter 5 and 7** a not yet described *in vitro* inhibitory effect of pasireotide on serotonin release in PNET cell lines is shown, which may be related to the SST_5 expression in this cell line. This observation further enhances the importance of the molecular study of somatostatin system components in tumor samples of NETs. Importantly, the use of radiolabeled SSAs forms in PRRT has been shown as a successful therapeutic alternative in metastatic functioning NETs with SSTs expression. Specifically, therapy using peptide receptor radionuclide therapy (PRRT) in GEP-NETs is associated with response rates (18-44% by RECIST criteria), improvement in quality of life [36, 37], reduction in risk of progression or death (79%) and increased overall response rate (ORR; 18%) [38]. Despite PRRT is a promising treatment in GEP-NETs, the application in lung NETs may be limited due to a lower expression of SSTs, especially in some atypical lung carcinoids; notwithstanding, ORR of 15% and disease stabilization of 47% have been reported [39, 40], which is related to the findings observed in our cohort, in which SST_2 was expressed in NTA and tumor samples. Interestingly, clinical effects have been described even when tumor progression was observed after initial good response, suggesting that not only the antitumor effect of PRRT may be involved in the clinical response [41].

NETs and the Ghrelin system

Tightly associated with the somatostatin system, ghrelin system is also involved in the regulation of multiple (patho)-physiological functions, including hormonal secretion, β-cell survival or appetite and gastric motility [42-45]. Alterations in the expression of specific components of this system have been associated with the development/progression of various neoplasms, but these associations are still controversial and remain unclear [46-50].

According to our results, the differences in the expression of ghrelin system components between normal tissue, non-tumor adjacent tissue and tumor tissue, suggest a dysregulation of ghrelin system in these tumors Additionally, the concomitant expression of ghrelin, its activating enzyme ghrelin-O-acyl-transferase (GOAT) and the native receptor GHSR1a suggests that the whole machinery of this system may modulate the development or progression in this pathology, and may include some splicing variants (e.g. In1-ghrelin and GHSR1b) [27].

In this context, ghrelin system components were expressed in < 25% of normal lung samples, as determined by qPCR, but ghrelin, In1-ghrelin, GHSR1a and GHSR1b were expressed in at least 75% of lung carcinoids and NTA tissues. Specifically, GHSR1a and GHSR1b were overexpressed in tumor tissue and NTA tissue compared to normal lung tissue. Interestingly, while the mRNA expression of ghrelin was associated with decreased vascular invasion capacity, the canonical receptor GHSR1a was associated with increased proportion of metastasis, suggesting that other unknown ligands may also bind this receptor. In contrast, in GEP-NETs, ghrelin and its native receptor GHSR1a were expressed in more than 75% of healthy controls, additionally, their expression levels were decreased in NTA and tumor tissue compared with normal samples, with a slightly, but not significantly, increased expression in tumor compared with NTA tissue. In these tumors, GHSR1a was associated with functioning tumors and decreased mortality, contrasting with the clinical findings observed in lung carcinoids.

An important finding of this thesis is the potential role of GOAT enzyme as biomarker in NETs. Specifically, GOAT acylates the third serine residue of ghrelin. After this unique modification, acylated ghrelin represents the peptide binding and activating its canonical ghrelin receptor GHSR1a [45, 51]. In lung carcinoids, GOAT was virtually absent in control tissue, whereas it was present in less than 50% of the NTA samples and raised to more than 75% of tumor samples, despite this, it was not overexpressed in tumor tissue compared to NTA. In contrast, in GEP-NETs, GOAT was detected in about 20% of normal samples and was remarkably overexpressed in tumor tissues compared with NTA regions and normal tissues, especially in gastrointestinal NETs, compared to pancreatic NETs. Despite GOAT was not associated with any invasion/aggressiveness parameter, its remarkable overexpression in tumor tissue suggests a potential role in NETs, further research is required and would probably include its determination as circulating marker, or even more, the evaluation of its role as a therapeutic marker by inducing changes in its molecular expression or function.

Although clinical results presented in this thesis have been evaluated in ample, well-characterized patient cohorts, the heterogeneity of the included patients and the retrospective analysis, may limit their reproducibility. Despite this, results presented in Part I of this thesis, provide a bases for future characterization and study of these system components in NETs, including e.g. circulating markers analysis in prospective cohorts.

Novel targeted therapies

The availability of molecular-target therapies in NETs has increased the therapeutic alternatives for NETs. Sunitinib and everolimus have been described as successful treatment options in both functioning and non-functioning tumors, since they may control tumor growth, improve clinical symptoms and increase PFS [52, 53]. Unfortunately, significant prolongation of overall survival (OS) with these medical options is still controversial and novel therapeutic options, especially for hormone release control, are necessary.

Somatostatin-dopamine chimeric drugs

Multi-receptor interaction has been suggested as an efficacious and selective therapeutic strategy for enhancing the effects of somatostatin [28]. It has been hypothesized that this modulation may be achieved by the activation of different receptor systems with a common endpoint, an increased activation of a single/common pathway by multiple receptors, or the activation of multiple complimentary intracellular signal transduction systems [28]. The presence of hetero-dimers has been described among SSTs and between SSTs and other receptor families, including dopamine receptors, especially the dopamine receptor type 2 (D2R) [54, 55]. Based on this, some structural chimeric molecules that combine elements of SSAs and dopamine agonists (DA) were developed [28]. The potential effect of these chimeras depends on the affinity for each receptor subtype, which may be modified according to the therapeutic interest. An ideal chimeric compound would have potent SST_2 and D2R activity, with moderate SST_5 activity in order to inhibit hormone secretion without altering the glucose homeostasis [28, 56]. In **chapter 6**, the effect on cell proliferation and secretion of two SSTs-D2R multi receptor targeting drugs (BIM065 and BIM23A760) is described using two-dimensional (2D; monolayer) and three-dimensional (3D; spheroids) culture systems. Their inhibitory effect on serotonin and CgA secretion, which was comparable to cabergoline but not to octreotide, suggests that in this PNET model, the activity of both chimeric drugs is probably related to their affinity for D2R. *In vivo* reports have described that BIM-23A760 acutely decreases growth hormone and prolactin secretion in pituitary tumors, but long-term effects disappeared due to a dopaminergic metabolite that may interfere with the activity of the parent molecule [28]. In our study, BIM-23A760, followed by cabergoline and BIM-065, were the most potent drugs for inhibiting serotonin and CgA secretion. In NETs, an open label, multicenter clinical trial in patients with carcinoid syndrome was started for evaluating the efficacy of BIM-23A760. This study was prematurely terminated, and unfortunately, primary/secondary outcomes were not analyzed (NCT01018953). Due to their putative promising effects, current research is focused in the improvement of chimeric molecules that could keep a long-term effect. The results described in **chapter 6** also underline that it is important to recognize the effect of cabergoline in hormone release control in NETs, in addition to that of SSA. This is further strengthened with previous reports of the use of cabergoline in ectopic ACTH-producing NETs (monotherapy or in combination with lanreotide) when D2R expression is observed in the tumor [57-59].

Telotristat

Interestingly, the novel inhibitor of tryptophan hydroxylase, telotristat has been described as an effective therapeutic option for serotonin release control [60]. The use of telotristat has been recently approved by the European Commission and the Food and Drug Administration [61]. Decreased urinary 5-hydroxyindoleacetic acid and small bowel movements were reported in the TELESTAR [62] and TELECAST phase III clinical trials [63]. Surprisingly, despite its clinical use, to the best of our knowledge no pre-clinical data exist in which the *in vitro* effect of this novel drug in NET has been tested. In this sense, the first report of the *in vitro* effects of telotristat is presented in **chapter 7** of this thesis. According to our *in vitro* model, a dose-dependent decreased serotonin release was observed. Importantly, the effects were achieved with clinically relevant concentrations, without concomitant cytotoxic effects or changes in the molecular expression of SSTs. Remarkably, serotonin has been described as a potent mitogen in different cell types [64]. However, in the evaluated pancreas NET 3D culture model, no effect on cell growth was observed, whereas serotonin secretion was totally suppressed. These data suggest that these cells may not respond to a direct paracrine/autocrine effect of serotonin on cell proliferation. On the other hand, serotonin might influence tumor growth via an interaction with the tumor microenvironment as well. Finally, we unexpectedly observed a slightly decreased effectiveness of the combination therapy with octreotide in BON-1 cells. This finding requires further investigation because of its implication in the clinical practice.

Importantly, in the studies presented in chapter 6 and 7 of this thesis, we used a PNET model for studying serotonin secretion in NETs, whereas an ideal model might be a midgut cell line. Unfortunately, midgut NET cell lines are not widely available. A recent study showed that of seven established NET cell lines, only the small intestine NET lines GOT-1, P-STS and the PNET lines BON-1 and QGP-1 display a neuroendocrine phenotype, as well as disease-characteristic mutations. Other cell lines were identified as lymphoblastoid (e.g. KRJ-1) [65]. Unfortunately, the GOT-1 and P-STS cell lines were not available in our laboratory to confirm our findings in a midgut-NET model. Finally, it will be important to evaluate the effects of SSTs/D2R chimeric drugs, as well as telotristat, in NET primary cultures.

Ketoconazole

It is important to recognize that some drugs that are prescribed for other medical purposes may have some direct or indirect effects for tumor control. Their combination with SSAs would represent a valuable method for increasing the antitumor effects or even to control hormone secretion. In this sense, previous reports of the cytotoxic effect of ketoconazole in cancer [66] were confirmed in **chapter 5** using a lung- and pancreas model of adrenocorticotropin- (ACTH)-producing and non-ACTH producing NET cells, respectively. In these two NET models, ketoconazole may exert its action following different mechanisms including apoptosis induction, cell toxicity and decreased cell division. The cell growth inhibitory effects observed in our study suggest that a direct antitumor action of ketoconazole may be involved in the sustained effect achieved with this drug [67]. Despite some effects of ketoconazole were reached with clinically relevant concentrations, its use in non-ACTH producing tumors is controversial and requires further studies.

Metformin and statins

It is well-known that the inflammation and insulin resistance observed in patients with type 2 diabetes (T2-DM) or metabolic syndrome are associated with increased incidence of neoplasms [68]. Thus, targeting related pathways may represent appropriate therapeutic options in some types of cancer. Based on this, the putative antitumor effects of biguanides and statins in NET were described in **chapter 8**. Biguanides stimulate AMP-activated protein kinase (AMPK), which induces cell cycle arrest, reduces the insulin/insulin growth factor 1 (IGF-1) signaling [69, 70] and, additionally, suppresses the mammalian target of rapamycin (mTOR1) which is a key regulator of cell proliferation in cancer cells [71, 72]. For this reason, the biguanide metformin has been evaluated in multiple *in vitro* and *in vivo* studies [73] and is now being tested in registered clinical trials in NETs in combination with lanreotide (NCT02823691) in well-differentiated gastrointestinal or lung NETs. Interestingly, those clinical variables that in our cohort were related to metabolic syndrome were reversed when treated with metformin. In addition, the inverse expression of somatostatin and ghrelin systems when diabetic patients were treated with metformin, suggests that this drug may exert an additive effect for tumor control since it improves the expression of SSTs and may therefore potentiate the effects of SSAs. Although preliminary, these results are encouraging and allow to hypothesize that the effect of metformin, as an adjuvant therapy to classic therapeutic regimens, should be evaluated in appropriately designed prospective clinical studies.

Statins may also have additional anti-tumor effects, including induced cell-cycle arrest, apoptosis induction, decreased invasion/metastasis capacity and decreased Ki67 expression [74-78]. Our *in vitro* results are concordant with previous reports of the anti-proliferative effect of these drugs [79-81]. Despite this, in our cohort of patients, we did not observe a statistically significant effect of statins on clinical features of NET patients, but a higher proportion of disease-free patients was observed in this group. Importantly, the size of the cohort may have affected the significance of the results and unfortunately, there are no clinical data available for comparing the effects of statins in NETs. To the best of our knowledge, no clinical trial for evaluating the effects of statins in NETs is currently registered.

Other future directions for diagnostic markers and treatment in NETs

Current circulating biomarkers for NETs have several limitations, especially in terms of sensitivity, specificity and reproducibility. To date, several prospective trials are evaluating the effect of novel therapeutic strategies in NETs (e.g. 90Y, 177Lu, Tyr octreotate, lenvatinib, palbociclib, tremelimumab, bevacizumab, temozolamide, pasireotide, PDR001), and most of them include the evaluation of treatment-related follow-up markers. Despite the advances in NET biomarkers in recent years, it is not possible yet to accurately predict treatment response and patient outcome. Additionally, the intrinsic heterogeneity of NETs, their variable behavior and prognosis complicates the identification of specific and reliable biomarkers to predict medical treatment response and patient prognosis.

Nowadays, several biomarkers for NETs are under study. Probably multianalyte measurements based on genomics represent the most accurate system for early outcome stratification and decision making. The development of blood-based analysis and liquid biopsy may represent non-invasive methods for diagnosis and prognosis as well. Imaging techniques may also improve when combined with molecular markers, not only radiolabeled SSAs, but also peptides, including the combination of glucose transporters/positron emission tomography for *in vivo* imaging. Circulating tumor cells, miRNAs, cytokines and gene transcripts represent putative novel biomarkers that could predict clinical outcome and response to treatment. Genomics will probably become the basis for developing multitranscript biomarkers, their combination would provide multifaceted information, may offer better medical management and may improve the use of resources in order to improve diagnosis, treatment, quality of life and survival in NET patients [1].

Medical treatment options in NETs have significantly increased and improved in the last years. Despite this, some patients may not respond or develop treatment resistance. In these cases, more therapeutic options that allow a personalized management would be useful. A higher number of clinical trials and approved therapeutic agents facilitate the management of patients. As a consequence, a treatment sequence should be established taken into account response rate, improvements in progression-free survival and overall survival. To this aim, head to head studies would help to identify the best therapeutic options in specific cases. Importantly, overall survival in NETs has improved despite an increased incidence of these tumors, which may be related to the advances in tumor diagnosis and treatment. However, tumor heterogeneity as well as several aspects of the biologic, genetic and molecular background in NETs are still unknown and require further investigation. For this reason, it is needed to determine novel therapeutic targets that could improve the medical management of hormone-related syndromes and the antiproliferative treatment in NETs. Novel medical options and treatment combinations will be available in the upcoming years. Although preliminary results of clinical trials and *in vitro* models are encouraging, large longitudinal randomized studies are still required to provide accurate evidence of their effect on survival and symptom control.

Targeting signaling and immune pathways involved in NETs development and progression has provided novel therapeutic options for these tumors. Immunotherapy is probably one of the most interesting fields that will be evaluated in NETs in the upcoming years. Despite immunotherapy has an important role in the management of other types of cancer, the effect on well-differentiated NETs according to preliminary data seems to be limited, but it may have a role in neuroendocrine carcinomas. Several clinical trials in this field are ongoing and hopefully in the upcoming years, some results about their real clinical applicability in NETs will be available.

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Chapter 10

Summary

Samenvatting

Resumen

SUMMARY

Neuroendocrine tumors (NETs) represent a heterogeneous group of rare, slow-growing neoplasms, originating from enterochromaffin cells. Their annual incidence has progressively increased, although it is not known whether this is a true increase in NET incidence, the result of increased use of (improved) diagnostic procedures, or a combination of both. While metastatic disease is frequently observed at diagnosis, survival in NET patients has increased, which may be related to the advances in diagnostic and therapeutic procedures. NETs require appropriate standardized diagnostic procedures to assure early diagnosis, monitor disease progression and guide an optimal treatment. Additionally, medical treatment options in NETs have significantly increased and improved in the last years. This thesis evaluates a number of novel diagnostic and therapeutic options for patients with NET.

Chapter 1 provides a general overview about NETs, emphasizes the applicability and limitations of the current diagnostic biomarkers and summarizes some putative novel biomarkers in NETs. At the same time, this chapter describes current and novel therapeutic options for functioning a non-functioning NETs. The chapter introduces the main content of this thesis: novel diagnostic and therapeutic options for NETs. Chapter 1 ends with an overview of the main aims of this thesis.

Chapter 2 comprehensively evaluates the expression of several components of the somatostatin/cortistatin and ghrelin systems in a large series of well-characterized typical and atypical lung carcinoids. To the best of our knowledge, this study represents the first systematic characterization of the components of these regulatory systems in samples from lung carcinoids (LCs), in which a comparison is made with adjacent non-tumor regions and normal lung tissue. In this study, we observed that the expression of somatostatin, of some receptor subtypes (SSTs), and of the ghrelin receptor GHSR gradually increases from normal to non-tumoral adjacent and tumor tissue. Moreover, the presence of the truncated isoforms SST_5TMD_4, In1-ghrelin and the ghrelin receptor GHSR1b are first reported in LCs. Higher expression levels of ghrelin-O-acyltransferase (GOAT) were observed in tumors with necrosis, which were tumors with a larger size and higher capacity of peritumoral invasion and distant metastasis. These data suggest that a dysregulation of ghrelin system may be involved in the development/progression of these tumors.

Chapter 3 evaluates the expression of the somatostatin/cortistatin system components in gastroenteropancreatic- (GEP-) NETs. The observed overexpression of SST_5 in tumor tissue compared to adjacent non-tumor and normal tissue, as well as its clinical relation with vascular and nerve invasion suggests the putative role of SST_5 as a target for treating aggressive GEP-NETs. Furthermore, it emphasizes that the evaluation of SSTs profile in

GEP-NETs may provide additional information for clinical decision making, including the use of second-generation somatostatin analogs (SSAs).

The most novel and relevant finding of **chapter 4** is the marked overexpression of GOAT in GEP-NET samples. Whereas the expression of this enzyme is almost absent in the corresponding normal tissues, it is present in adjacent non-tumoral tissue and notably overexpressed tumor tissues. Additionally, its overexpression was demonstrated using immunohistochemistry. Importantly, this enzyme was associated to larger tumors, especially in gastrointestinal-NETs. These findings, in combination with previous publications, suggest a putative role of GOAT as a diagnostic biomarker in GEP-NETs.

Chapter 5 evaluates the effect of ketoconazole on proliferation, cell cycle, apoptosis and secretion of adrenocorticotropic hormone (ACTH) or serotonin and chromogranin in an ACTH-producing bronchial NET and a non-ACTH producing pancreatic NET cell line, respectively. Ketoconazole exerted a predominant pro-apoptotic or cytotoxic effect, depending on the cell line. Additionally, ketoconazole induced changes in the cell cycle progression, e.g. it increases G0/G1 phase in both cell lines and an arrest in G2/M phase in the pancreatic NET cell line. Furthermore, a non-previously reported inhibitory effect of the SSA pasireotide on serotonin secretion by pancreatic NET cells was reported, which is probably related to the predominant SST_5 expression in this cell line.

In **chapter 6,** a comprehensive characterization of two pancreatic NET models was performed using two- (2D; monolayer) and three- dimensional (3D; spheroids) culture systems in different medium conditions. According to our results, NET 3D spheroid cultures represent a promising method for evaluating cell proliferation and secretion in NET cell lines, even in serum-deprived conditions. A non-previously reported dynamic expression of SSTs and dopamine receptor (D2R) was observed during growth of both 2D and 3D cultures. The effects of the somatostatin analog octreotide, the dopamine agonist cabergoline and of two novel SSTs-D2R multi-receptor targeting drugs (BIM-065, BIM-23A760) were evaluated using both culture systems. Cabergoline and SSTs-D2R multi-receptor targeting drugs, but not octreotide, inhibited CgA and serotonin secretion, but not NET cell growth. This suggests that the effect of the SSTs-D2R multi-receptor targeting drugs on secretion is mediated by D2R, and may indicate a putative role of dopamine agonists for treating D2R expressing functioning NETs, including carcinoid syndrome.

Chapter 7 shows the *in vitro* effects of the novel tryptophan hydoxylase inhibitor telotristat on pancreatic NET cells. Telotristat potently inhibited serotonin release in a dose-dependent manner at a clinically feasible concentration. Its combination with pasireotide, but not with octreotide, had an additive effect on serotonin secretion. In 3D cultured pancreatic NET cells, serotonin secretion was completely blocked by telotristat, whereas this drug did not influence cell growth. These data suggest that serotonin has no autocrine/paracrine effect on cell growth in this 3D PNET model.

In **chapter 8** decreased expression of SSTs was observed in tumor tissue of GEP-NET patients with type 2 diabetes, compared to non-diabetic GEP-NET patients. This decreased expression was not observed in type 2 diabetic patients that were treated with metformin. The *in vitro* experiments showed an antiproliferative effect of biguanides and statins in NET cell lines, with consequent increased apoptosis and decreased cell migration. Since metformin and statins are low cost commercially available drugs, with a large experience in their clinical use, they may represent putative options for adjuvant therapy in NETs.

In **chapter 9**, the results and conclusions of the studies included in this thesis, are discussed in relation to current and possible novel future diagnostic modalities, as well as therapeutic options for NET patients.

SAMENVATTING

Neuroendocriene tumoren (NET) vormen een heterogene groep van zeldzaam voorkomende, langzaam groeiende tumoren. NET ontstaan vanuit enterochromaffine cellen. De jaarlijkse incidentie van NET is progressief toegenomen in de afgelopen jaren. Het is echter niet duidelijk of deze toename in incidentie een werkelijke toename is, veroorzaakt wordt door verbeterde diagnostische procedures, of door een combinatie van beiden. Hoewel NET vaak al gemetastaseerd blijken te zijn bij de diagnose, is de gemiddelde overleving van patiënten met NET toegenomen. Dit is waarschijnlijk toe te schrijven aan verbeterde diagnostiek en toegenomen therapeutische mogelijkheden. Er zijn goede en gestandaardiseerde diagnostische procedures nodig om patiënten met een NET in een zo vroeg mogelijk stadium te diagnosticeren, om het beloop van de ziekte nauwlettend te vervolgen en om deze patiënten een optimale behandeling te kunnen geven. In de afgelopen jaren is het aantal medicamenteuze behandelingsmogelijkheden voor patiënten met een NET sterk toegenomen. In dit proefschrift worden een aantal studies beschreven die betrekking hebben op diagnostische en therapeutische mogelijkheden voor NET patiënten.

Hoofdstuk 1 betreft een algemene inleiding over NET en benadrukt de toepassing en beperkingen van de huidige diagnostische procedures voor patiënten met NET. Er wordt een overzicht gegeven van een aantal mogelijk nieuwe diagnostische (bio)merkers. In dit hoofdstuk worden tevens huidige en nieuwe behandelingsmogelijkheden voor functionele en niet-functionele NET besproken. Dit hoofdstuk vormt de inleiding voor het onderwerp van dit proefschrift, n.l. nieuwe diagnostische en therapeutische mogelijkheden voor patiënten met NET. Hoofdstuk 1 wordt afgesloten met een overzicht van de doelstellingen van het onderzoek van het proefschrift.

In het onderzoek beschreven in **hoofdstuk 2** is de expressie van verschillende componenten van het somatostatine/cortistatine en het ghrelin systeem onderzocht in een grote serie van goed gekarakteriseerde typische en atypische long carcinoiden (LC). Voor zover bekend is dit de eerste studie waarin beide systemen tegelijkertijd worden onderzocht in LC weefsels. Het onderzoek toont aan dat de expressie van somatostatine, van enkele somatostatine receptor subtypen (SSTs), en van de ghrelin receptor GHSR, gradueel toeneemt van normaal naar niet tumor-aangrenzend weefsel en tumor weefsel. Bovendien werd voor de eerste keer in LC expressie aangetoond van de korte iso-vormen SST5TMD4, In1-ghrelin en de ghrelin receptor GHSR1b. Hogere expressie van ghrelin-O-acetyltrasferase (GOAT) werd gevonden in tumoren met necrose, grotere tumoren met meer peritumorale invasie en metastasen op afstand. Deze bevindingen suggereren dat een ontregelde regulatie van het ghrelin systeem betrokken zou kunnen zijn bij de ontwikkeling/progressie van de tumoren.

In **hoofdstuk 3** wordt onderzoek naar de expressie van componenten van het somatostatine/cortistatine systeem in gastroenteropancreatische- (GEP-) NET beschreven. De gevonden hogere expressie van SST_5 in tumor weefsel in vergelijking met tumor-aangrenzend en met normaal weefsel, en de klinische relatie van SST_5 expressie met vasculaire- en zenuw invasie, suggereert dat SST_5 een mogelijk doelwit is om agressieve GEP-NET te behandelen. Bovendien kan de evaluatie van het SSTs profiel in GEP-NET aanvullende informatie opleveren die mogelijk gebruikt kan worden bij klinische besluitvorming, waaronder het gebruik van een tweede generatie somatostatine analogen (SSA).

De meest nieuwe en relevante bevinding van het onderzoek beschreven in **hoofdstuk 4** is de duidelijke overexpressie van GOAT in GEP-NET weefsel. Terwijl de expressie van dit enzym vrijwel afwezig is in bijbehorend normaal weefsel, wordt expressie gevonden in aangrenzend niet-tumor weefsel en is expressie van GOAT met name aanwezig in tumor weefsel. De hogere expressie van GOAT in GEP-NET weefsel werd tevens aangetoond met behulp van immunohistochemie. Hoge expressie van dit enzym was geassocieerd met grotere tumoren en voornamelijk in gastro-intestinale NET. Deze bevindingen, tezamen met eerdere publicaties, suggereren dat GOAT een mogelijke diagnostische biomerker is in GEP-NET.

Hoofdstuk 5 beschrijft onderzoek naar de effecten van het geneesmiddel ketoconazol op de groei, cel cyclus, apoptose en afgifte van ACTH of serotonine en chromogranine in respectievelijk een ACTH-producerende long NET cellijn en een niet-ACTH producerende pancreas NET cellijn. Ketoconazol heeft een voornamelijk pro-apoptotisch of cytotoxisch effect, afhankelijk van het type cellijn. Bovendien zorgt behandeling met ketoconazol voor veranderingen in celcyclus, namelijk een toename in G0/G1 fase in beide cellijnen en een afname in G2/M fase in de pancreas NET cellijn. Bovendien tonen de resultaten in hoofdstuk 5 een niet eerder gerapporteerd remmend effect aan van het SSA pasireotide op serotonine afgifte van de pancreas NET cellijn. Dit effect wordt waarschijnlijk gemedieerd via de hoge SST_5 expressie in deze cellijn.

Het onderzoek in **hoofdstuk 6** beschrijft een uitgebreide karakterisering van twee pancreas NET modellen in tweedimensionale (monolayer) en driedimensionale (sferoïden) kweeksystemen, in verschillende kweekmedium condities. Op basis van de resultaten van dit onderzoek wordt geconcludeerd dat een driedimensionaal (sferoïde) kweeksysteem een veelbelovend systeem is om groei en hormoon secretie van NET cellijnen te bestuderen, zelfs onder serum arme kweekcondities. In dit onderzoek wordt tevens aangetoond dat de expressie van SSTs en de dopamine D2 receptor (D2R) verandert tijdens de groei van cellen, zowel in twee- als driedimensionale kweken. De effecten van het somatostatine analoog octreotide, de dopamine agonist cabergoline en van twee nieuwe SSTs-D2R bindende geneesmiddelen (BIM-065 en BIM-23A760) werden onderzocht in de twee kweeksystemen. Cabergoline en de twee SSTs-D2R multi-receptor bindende geneesmiddelen, en niet octreotide, hadden een remmend effect op de afgifte van chromogranine en serotonine, maar niet op de celgroei. De bevindingen suggereren dat het effect van de twee SSTs-D2R bindende geneesmiddelen op de afgifte van hormonen in dit pancreas NET model verloopt via de D2R, en een mogelijke rol voor dopamine agonisten bij de behandeling van D2R-bevattende functionele NET, waaronder het carcinoid syndroom.

Hoofdstuk 7 beschrijft onderzoek naar de *in* vitro effecten van de nieuwe tryptofaan hydroxylase remmer telotristat op pancreas NET cellen. Telotristat remt krachtig en dosis-afhankelijk de serotonine afgifte, bij klinisch relevante concentraties. De combinatie met pasireotide, maar niet met octreotide, had een aanvullend remmend effect op de serotonine afgifte. In driedimensionaal gekweekte pancreas NET cellen kon de serotonine afgifte volledig worden geremd door telotristat, maar was er geen effect op de celgroei. Deze resultaten tonen aan dat serotonine in dit pancreas NET model geen autocrien/paracrien effect heeft op de celgroei.

Het onderzoek in **hoofdstuk 8** toont dat de SSTs expressie in GEP-NET weefsel van patiënten met type 2 diabetes verlaagd is ten opzichte van GEP-NET weefsel van patiënten zonder diabetes. In type 2 diabetes patiënten die behandeld werden met metformine werd deze verlaging in SSTs expressie niet gevonden. *In vitro* experimenten toonden groei remmende effecten van biguanides en statines aan op NET cellijnen, met een toegenomen apoptose en afgenomen migratie van de cellen. Aangezien metformine en statines commercieel verkrijgbare en relatief goedkope geneesmiddelen zijn en er een langdurige ervaring is in het gebruik ervan, kunnen deze geneesmiddelen mogelijk van aanvullende waarde zijn bij de adjuvante behandeling van patiënten met NET.

In **hoofdstuk 9** worden de resultaten en conclusies van de studies uit dit proefschrift bediscussieerd in relatie tot huidige en mogelijk nieuwe toekomstige diagnostische en therapeutische mogelijkheden voor patiënten met een NET.

RESUMEN

Los tumores neuroendocrinos (NETs) son un grupo heterogéneo de neoplasias poco comunes, caracterizadas por un lento crecimiento y que se originan a partir de las células enterocromafines. Su incidencia anual viene aumentando progresivamente, aunque se desconoce si éste incremento es real, el reflejo de mayor uso de (mejores) técnicas diagnosticas o una combinación de ambas. A pesar de que frecuentemente los NETs tienen metástasis al diagnóstico, la supervivencia en estos pacientes ha aumentado, lo cual puede relacionarse con mejoras en las opciones diagnósticas y terapéuticas disponibles. Los NETs requieren métodos diagnósticos estandarizados para asegurar un diagnóstico precoz, así como para monitorizar la progresión de la enfermedad y orientar un tratamiento óptimo. Adicionalmente, el número y la calidad de las opciones terapéuticas se ha incrementado en los últimos años. En este sentido, esta tesis evalúa algunas nuevas opciones diagnósticas y terapéuticas para pacientes con NETs. El **capítulo 1** ofrece una visión general sobre los NETs, y se centra en la utilidad/limitaciones de los biomarcadores actualmente disponibles, así como en la descripción de algunos nuevos (posibles) marcadores en NETs. Además, resume opciones terapéuticas para tumores neuroendocrinos funcionantes y no funcionantes, tanto aquellas nuevas como las actualmente disponibles. Este capítulo representa la introducción al contenido fundamental de esta tesis: nuevos marcadores diagnósticos y opciones terapéuticas en NETs. El capítulo 1 termina con una visión general de los objetivos generales de esta tesis.

El **capítulo 2** evalúa detalladamente la expresión de algunos componentes de los sistemas somatostatina/cortistatina y ghrelina en una seria amplia, bien caracterizada, de carcinoides pulmonares típicos y atípicos. Hasta donde sabemos, este estudio representa la primera caracterización sistemática de los componentes de estos sistemas reguladores en muestras de carcinoides pulmonares (LCs), tejido adyacente no tumoral y pulmón sano. En este estudio, la expresión de somatostatina, de algunos de sus receptores (SSTs) y del receptor de ghrelina se incrementó gradualmente en el tejido no tumoral adyacente y en el tejido tumoral en comparación a pulmón normal. Además, la presencia de isoformas truncadas SST_5TMD_4, In1-ghrelin y el receptor de ghrelina GHSR1b fue reportada por primeras vez en LCs. Asimismo, una expresión aumentada de la enzima ghrelin-O-aciltransferasa (GOAT) se observó en tumores con necrosis, los cuales (a su vez) fueron aquellos con mayor tamaño, mayor capacidad de invasión y de producir metástasis a distancia. Estos datos sugieren que una desregulación del sistema ghrelina puede estar involucrada en el desarrollo/progresión de estos tumores.

El **capítulo 3** discute específicamente la expresión de componentes de los sistemas somatostatina/cortistatina en tumorres gastroenteropancreáticos (GEP-NETs). La sobre-expresión observada de SST_5 en el tejido tumoral comparado con el tejido adyacente no tumoral y el control sano, así como su relación clínica con la invasión vascular y nerviosa del tumor, sugieren un probable rol del SST_5 como diana terapéutica en GEP-NETs agresivos, además, enfatiza la importancia de evaluar el perfil de SSTs en GEP-NETs, el cual aportaría información adicional para la toma de decisiones terapéuticas, incluida la utilización de análogos de somatostatina (SSAs) de segunda generación.

El hallazgo más novedoso y relevante del **capítulo 4** es la marcada sobre-expresión de la enzima GOAT en muestras de GEP-NETs. Específicamente, cuando la expresión de esta enzima es prácticamente ausente en tejido normal, ésta se observó en tejido adyacente no tumoral y se sobre-expresó en tejido tumoral, una sobre-expresión que fue confirmada utilizando inmunohistoquímica. Asimismo, esta enzima se asoció con tumores de mayor tamaño, especialmente en NETs gastrointestinales. Estos hallazgos, combinados con publicaciones previas, sugieren el posible papel de la enzima GOAT como un biomarcador en NETs.

El **capítulo 5** describe los efectos de ketoconazol en la proliferación, ciclo celular, apoptosis, secreción de adrenocorticotropina (ACTH) o serotonina y cromogranina en una línea celular de NET bronquial productora de ACTH y una línea celular de NET pancreático no productora de ACTH. Específicamente, ketoconazol mostró características predominantemente pro-apoptóticas o citotóxicas dependiendo de la línea celular; estos efectos se mantuvieron a pesar de la combinación con SSAs. Además, ketoconazol fue capaz de inducir cambios en el ciclo celular, específicamente el aumento de las fases G0/G1 en ambas líneas celulares y una disminución de las fases G2/M en la línea celular pancreática. Incluso se observó un efecto inhibitorio en la secreción de serotonina del análogo de somatostatina pasireotide en la línea celular pancreática, el cual no había sido previamente descrito y que probablemente se debe a la expresión de SST_5 en esta línea celular.

En el **capítulo 6** se caracteriza de forma detallada un modelo de líneas celulares pancreáticas utilizando sistemas de cultivo en dos- (2D; monolayer) y tres- dimensiones (3D; esferoides) en diferentes condiciones de nutrientes. Al analizar los resultados, se observa que los cultivos en 3D con esferoides representan un método valioso para evaluar la secreción y proliferación celular en líneas celulares de NETs, incluso en condiciones con deprivación de suero. Se observó una expresión dinámica de los SSTs y receptor de dopamina (D2R) en los sistemas de cultivo en 2D y 3D, lo cual no se había descrito previamente. En este capítulo, los efectos del análogo de somatostatina octreotide y el agonista de dopamina cabergolina, así como de los nuevos fármacos quimera SSTs-D2R (BIM-065, BIM-23A760) se evaluaron en ambos modelos de cultivo. El tratamiento con cabergolina y ambas quimeras inhibió la secreción de cromogranina A y serotonina, pero no la proliferación celular. Esto sugiere que el efecto de las quimeras en la secreción parece estar mediado por el D2R, sugiriendo un posible papel de los agonistas de cabergolina para el tratamiento de NETs funcionantes, incluido el síndrome carcinoide, siempre y cuando expresen D2R.

El **capítulo 7** muestra los efectos *in vitro* de telotristat etiprate, un nuevo inhibidor de la enzima hidroxilasa de triptófano en líneas celulares pancreáticas. Este fármaco mostró un potente efecto en la inhibición de la secreción de serotonina, el cual fue dependiente de dosis en un modelo de células pancreáticas de NETs. Su combinación con pasireotide, pero no con octreotide, tuvo un efecto aditivo en el control de la secreción de serotonina. En el modelo 3D de NET pancreático, la secreción de serotonina fue totalmente inhibida por telotristat, sin embargo, este bloqueo no produjo cambios en el crecimiento celular. Estos datos sugieren que la serotonina no ejerce un efecto autocrino/paracrino en la proliferación celular en este modelo 3D de NET pancreático.

En el **capítulo 8** se observó una expresión disminuida de SSTs en pacientes con diabetes tipo 2 y GEP-NETs comparado con pacientes con GEP-NETs pero sin diabetes. Esta expresión disminuida no se observó cuando los pacientes diabéticos fueron tratados con metformina. Los estudios *in vitro* demostraron el efecto anti-proliferativo de las biguanidas y estatinas en líneas celulares de NETs, con el consiguiente incremento de la apoptosis y disminución de la migración celular. Puesto que la metformina y las estatinas son fármacos comercialmente disponibles, económicos y con una amplia experiencia en su uso, nuestros resultados sugieren que estos fármacos podrían representar una opción terapéutica factible como terapia adyuvante en NETs.

Finalmente, en el **capítulo 9** se discuten los resultados y conclusiones de los estudios presentados en esta tesis, especialmente en relación a las modalidades diagnósticas y opciones terapéuticas, tanto actuales como futuras, para los pacientes con NETs.

Appendices

PhD PORTFOLIO

Erasmus MC Department	Internal Medicine- Section of Endocrinology
Reina Sofia UH Department	Endocrinology and Nutrition Unit
Research School	Molmed Maimonides Institute of Biomedical research
Research Period	2014-2018
Promotors	Leo J. Hofland/Justo P. Castaño
Co-promotors	Richard A. Feelders/María Angeles Gálvez

Erasmus General Academic Courses	**Year**	**Workload (ECTS)**
The Introduction in GraphPad Prism Version 6	2018	0.3
Workshop on Microsoft Access 2010: Advanced	2018	0.3
Workshop on Microsoft Access 2010: Basic	2018	0.3
Course Bayesians statistics and JASP	2018	0.3
Biomedical English Writing Course for MSc and PhD-students	2018	2.0
Microscopic Image Analysis: From Theory to Practice	2018	0.8
Workshop Writing Successful Grant Proposals	2017	0.5
Research Integrity	2017	0.3

Clinical Courses and meetings	**Year**	**Workload (ECTS)**
Post-graduate course 15th Annual Conference for diagnosis and treatment of neuroendocrine tumor disease. *European Neuroendocrine Tumor Society*	2018	5
Course in systematic reviews and metaanalysis. *Reina Sofia University Hospital. Maimonides Institute of Biomedical Research*	2017	4.5
Problems and solutions for treating osteoporosis. *Spanish Society of the Bone and Mineral Research*	2017	
Clinical scenarios for type 2 diabetes. *Córdoba Medical College*	2017	0.4
Pre-conference course 14th Annual Conference for diagnosis and treatment of neuroendocrine tumor disease. *European Neuroendocrine Tumor Society*	2017	3
Course in Clinical Nutrition. *Andalusian Society of Endocrinology, Diabetes and Nutrition*	2017	0.6
I meeting for heart and diabetes. *Andalusian Society of Endocrinology, Diabetes and Nutrition*		
IX meeting in neuroendocrinology. *Spanish Society of Endocrinology and Nutrition*	2017	
Update course in diabetes. *Andalusian Health Board*	2016	2
Pre-conference course in neuroendocrinology. *Andalusian Society of Endocrinology, Diabetes and Nutrition*	2016	
Mellanby centre Training Course in osteoporosis. *Spanish Society of the Bone and Mineral Research*	2016	0.8

Actuality in Osteoporosis Spanish Society of the Bone and Mineral Research	2016	0.6
XXI update course in neuroendocrinology and nutrition. *Spanish Society of Endocrinology and Nutrition*	2016	2
XVII advanced course in diabetes. *Spanish Society of Endocrinology and Nutrition*	2016	
Appropriate use of antimicrobials. *Andalusian Health Board*	2015	1
Update in enteral nutrition. *Reina Sofia University Hospital*	2015	
Translational research in rare diseases. *Maimonides Institute of Biomedical Research*	2015	0.8
Pre-conference course in thyroid cancer. *Andalusian Society of Endocrinology, Diabetes and Nutrition*	2015	
XXI update in clinical nutrition. *Spanish Society of Endocrinology and Nutrition*	2015	
Diabetes Live. Attention to the patient with diabetes		0.5
Cardiovascu.ar health in diabetic patients with enteral nutrition	2015	0.8
Course of thyroid ultrasound for endocrinologists. *Reina Sofia University Hospital*	2015	0.6
Course of diagnostic and therapeutic neck ultrasound. *Reina Sofia University Hospital. Andalusian Health Board*	2015	1
Diabetes live! Patients with Diabetes. *Andalusian Health Board*	2015	6.88
III course in scientific divulgation. University of Córdoba	2015	1
Pre-conference course in thyroid ultrasound. *Andalusian Society of Endocrinology, Diabetes and Nutrition*		
Nutritional support in elderly. *Spanish Society of Enteral and Parenteral Nutrition*	2014	4.2
III course in neuroendocrinology. *Andalusian Society of Endocrinology, Diabetes and Nutrition*	2014	1.82
Management of the diabetic patient in the hospital and after the delivery. *Andalusian Health Board*	2014	0.57
8th update course in Diabetes. *Reina Sofia University Hospital*	2014	2
Update in Osteoporosis. *Reina Sofia University Hospital*	2014	0.4

Conferences oral/poster presentations	**Year**
Type 2 diabetes in neuroendocrine tumors: are biguanides and statins part of the solution? *18th Congress of the European Neuroendocrine Association.*	2018
Hyperlipidemia during gestational diabetes: maternal and offspring complications. *20th European Congress of Endocrinology.*	2018
Utility of 3D cell culture systems with spheroids in neuroendocrine tumors. *20th European Congress of Endocrinology.*	2018
Effects of multi-receptor targeting drugs in neuroendocrine tumors using 3D cell culture. *15th congress of the European Neuroendocrine Tumor Society*	2018
Potential effects of ketoconazole on ACTH-producing and non- ACTH-producing neuroendocrine tumors. *15th congress of the European Neuroendocrine Tumor Society.*	2018

Ghrelin O-acyltransferase (GOAT) enzyme and ghrelin receptor GHSR1a as putative prognosis markers and therapeutic targets in gastroenteropancreatic neuroendocrine tumors. *15th congress of the European Neuroendocrine Tumor Society.*	2018
Effects of ketoconazole on ACTH-producing and non ACTH-producing neuroendocrine tumor cells. *19th European Congress of Endocrinology*.	2017
Potential anti-tumor activity of biguanides and statins in neuroendocrine tumor cells. *14th European Neuroendocrine Tumor Society Conference.*	2017
Planning for pregnancy in type 1 diabetes *XXVIII conference of the Spanish Diabetes Society*	2017
Muscular area by computed tomography in liver transplant patients *IX National Conference for the Clinical Assistance of the Chronic Patient of the Spanish Society of Internal Medicine*	2017
Role of the computed tomography in the evaluation of the body composition in liver transplant patients *XIX conference of the Spanish Society of Musculoskeletal Radiology*	2017
Components of Splicing Machinery are Drastically Dysregulated on Neuroendocrine Tumors and Associated with Malignancy. *17th Congress of the European Neuroendocrine Association.*	2016
Early nutrition support therapy in patients with head-neck tumors. *38th Conference of the European Society of Parenteral and Enteral Nutrition*	2016
Radiation and chemotherapy side effects in patients with head-neck tumors receiving early nutrition support therapy. *38th Conference of the European Society of Parenteral and Enteral Nutrition*	2016
The role of body composition evaluation by computerized tomography in pre-liver trasplant patients. *38th Conference of the European Society of Parenteral and Enteral Nutrition*	2016
Adrenal incidentalomas: functionality study. *18th European Congress of Endocrinology*	2016
Presence and clinical-histological correlates of ghrelin and somatostatin systems components in gastroenteropancreatic neuroendocrine tumors and lung carcinoids. *18th European Congress of Endocrinology*	2016
Splicing Dysregulation Impacts on Neuroendocrine Tumors: Evidence from Altered Spliceosoma Components and Somatostatin and Ghrelin Systems. *13rd European Neuroendocrine Tumor Society Conference.*	2016
Thyroid nodule: from the finding to the diagnosis of thyroid cancer *58th conference of the Spanish Society of Endocrinology*	2016
Effects of ketoconazole on ACTH-producing and non ACTH-producing neuroendocrine tumor cells. *58th conference of the Spanish Society of Endocrinology*	2016
Percutaneous endoscopic gastrectomy: Third level Hospital Experience *58th conference of the Spanish Society of Endocrinology*	2016
Clinical evaluation of gestational diabetes *XXVII conference of the Spanish Diabetes Society*	2016

Delivery outcome in patients with type 1 diabetes *XXVII conference of the Spanish Diabetes Society*	2016
Nutritional Evaluation In Pre-Liver Transplant Patients. *37th Conference of the European Society for parenteral and Enteral Nutrition*	2015
Nutritional Evaluation In Pre-Lung Transplant Patients. *37th Conference of the European Society for parenteral and Enteral Nutrition*.	2015
Expression of ghrelin and somatostatin systems components in pancreatic neuroendocrine tumours and their relationship with clinical-histological characteristics. *17th European Congress of Endocrinology*.	2015
Bariatric surgery and reduction in cardiovascular risk. *36th Congress of the European Society of Parenteral and Enteral Nutrition*	2014
Clinical, histological and molecular characteristics of gastroenteropancreatic neuroendocrine tumors *57th conference of the Spanish Society of Endocrinology*	2015
Combination of invasive techniques and intraoperatory ultrasound for the identification of insulinomas *57th conference of the Spanish Society of Endocrinology*	2015
Differences in the diagnostic of gestational diabetes according to the oral glucose tolerance test criteria *57th conference of the Spanish Society of Endocrinology*	2015
Differences in the molecular profile of pituitary adenomas after the second surgery *57th conference of the Spanish Society of Endocrinology*	2015
In differentiated thyroid cancer, is useful a second stimulatory test with rhTSH in patients with negative post-surgery control? *57th conference of the Spanish Society of Endocrinology*	2015
Bone and mineral metabolism evolution after bariatric surgery *XXVI conference of the Spanish Diabetes Society*	2015
Determining factors in the metabolic control of type 1 diabetes *XXVI conference of the Spanish Diabetes Society*	
Efficacy of denosumab in menopausal osteoporotic patients who require to suspend bisphosphonates *XIX conference of the Spanish Society of the Bone and Mineral Research*	2014
Glutamine and total parenteral nutrition in bone narrow transplant patients. *36th Congress of the European Society of Parenteral and Enteral Nutrition*	2014
Lateral amyotrophic sclerosis: enteral nutrition using percutaneous endoscopic gastrectomy *56th conference of the Spanish Society of Endocrinology*	2014
Subclinical hypothyroidism associated with thyroid autoimmunity *56th conference of the Spanish Society of Endocrinology*	2014

Awards and prizes/Travel grants	**Year**
European Neuroendocrine Association Travel Grant. *European Neuroendocrine Association*	2018
European Conference Travel Grant. *Spanish Society of Endocrinology and Nutrition*	2018
Basic Science Travel Grant. *European Society of Endocrinology*	2018
Double doctorate grant. *University of Córdoba*	2018
Excellence Prize for Endocrinology Residents. *Spanish Society of Endocrinology and Nutrition*	2017
Rio Hortega Research Grant. *Institute of Health Carlos III*	2017
Best Oral Presentation. *Andalusian Society of Clinical Nutrition and Dietetics*	2016
Basic Science Travel Grant. *European Society of Endocrinology*	2016
Colaboration for short internships. *Spanish Society of Diabetes*	2016
European Conference Travel Grant. *Spanish Society of Endocrinology and Nutrition*	2015
Best oral presentation Basic Endocrinology. *Andalusian Society of Endocrinology, Diabetes and Nutrition*	2015

LIST OF PUBLICATIONS

Publications in peer reviewed journals

1. Aura D Herrera-Martínez, Leo J. Hofland, Wouter W. de Herder, María A. Gálvez Moreno, Justo P. Castaño and Richard A. Feelders. Neuroendocrine tumors: diagnostic, predictive and prognostic markers. Endocrine related Cancer. Submitted
2. Aura D Herrera-Martínez, Johannes Hofland, Leo J. Hofland, Tessa Brabander, Ferry Eskens, María A. Gálvez Moreno, Justo P. Castaño, Wouter W. de Herder, Richard A. Feelders. Medical Treatment for neuroendocrine tumors: current options and future perspectives. Drugs. Submitted
3. Aura D Herrera-Martínez, Richard A. Feelders, Wouter W. de Herder, Justo P. Castaño, María Ángeles Gálvez Moreno, Fadime Dogan, Rosanna van Dungen, Peter van Koetsveld, Leo J. Hofland. Effects of ketoconazole on ACTH-producing and non-ACTH-producing neuroendocrine tumor cells. Submitted
4. Aura D Herrera-Martínez, Rosanna van den Dungen, Fadime Dogan-Oruc, Peter M. van Koetsveld, Michael D Culler, Wouter W. de Herder, Raúl M. Luque , Richard A. Feelders, Leo J. Hofland. Effects of novel somatostatin-dopamine chimeric drugs in 3D spheroid cell culture models of neuroendocrine tumors. Submitted
5. Aura D Herrera-Martínez, Richard A. Feelders, Rosanna van den Dungen, Fadime Dogan-Oruc, Peter M. van Koetsveld, Justo P. Castaño, Wouter W. de Herder, Leo J. Hofland. Efficacy of the tryptophan hydroxylase inhibitor telotristat on growth and serotonin secretion in 2D and 3D cultured neuroendocrine tumor cells. Submitted
6. Johannes Hofland, Aura D. Herrera-Martínez, Wouter T. Zandee, Wouter W. de Herder. Management of carcinoid syndrome: a systematic review and meta-analysis. Submitted
7. Aura D. Herrera-Martínez, Sergio Pedraza-Arevalo, Fernando L-López, Manuel D. Gahete, María A. Gálvez-Moreno, Justo P. Castaño, Raúl M. Luque. Type 2 diabetes in neuroendocrine tumors: are biguanides and statins part of the solution?. J Clin Endocrinol Metab. 2018 Sep 27. doi: 10.1210/jc.2018-01455.
8. Aura D. Herrera-Martínez, Manuel D. Gahete, Rafael Sánchez-Sánchez, Emilia Alors-Perez, Sergio Pedraza-Arevalo, Raquel Serrano-Blanch, Antonio J. Martínez-Fuentes, Maria A. Gálvez-Moreno, Justo P. Castaño, Raúl M. Luque. Ghrelin O-acyltransferase (GOAT) enzyme as a novel potential biomarker in gastroenteropancreatic neuroendocrine tumors. Clinical and Translational Gastroenterology. Clin Transl Gastroenterol. 2018 Oct 8;9(10):196.
9. Aura D. Herrera-Martínez, Rodrigo Bahamondes, Rafael Palomares Ortega, Paloma Moreno Moreno, M. Angeles Gálvez. Hyperlipidemia during gestational diabetes: maternal and offspring complications. Nutrición Hospitalaria 2018;35:698-706.

10. María Rosa Alhambra Expósito; Aura D Herrera-Martínez; Gregorio Manzano García; María Espinosa Calvo; Carmen María Bueno Serrano; María Ángeles Gálvez Moreno. Early nutrition support in head neck cancer patients. Nutrición Hospitalaria 2018;35:505-510.
11. Aura D. Herrera-Martínez, Manuel D. Gahete, Sergio Pedraza-Arevalo, Rafael Sánchez-Sánchez, Rosa Ortega-Salas, Raquel Serrano-Blanch, María A. Gálvez-Moreno, Raúl M. Luque, Justo P. Castaño. Clinical and functional implication of the components of somatostatin system in gastroenteropancreatic neuroendocrine tumors. Endocrine. 2018 Feb;59(2):426-437. doi: 10.1007/s12020-017-1482-3.
12. Aura D Herrera-Martínez; Manuel D. Gahete; Rafael Sánchez-Sánchez; Rosa Ortega Salas; Raquel Serrano-Blanch; Ángel Salvatierra; Leo J. Hofland; Raúl M. Luque; María A. Gálvez-Moreno; Justo P. Castaño. The components of somatostatin and ghrelin systems are altered in neuroendocrine lung carcinoids and associated to clinical-histological features. Lung Cancer. 2017 Jul;109:128-136. ISSN 0169-5002
13. Aura D. Herrera-Martínez; Patricia Enes; María Martín-Frias; Belén Roldán; Rosa Yelmo; Raquel Barrio. La respuesta monofásica a la sobrecarga oral de glucosa como factor predictivo del riesgo de diabetes tipo 2 en pacientes pediátricos con obesidad. Anales de Pediatría, 2017, http://dx.doi.org/10.1016/j.anpedi.2017.01.009.
14. Aura D. Herrera-Martínez; José Carlos Padillo Cuenca; Rodrigo Bahamondes Opazo; Ana Barrera Martín; Angel Rebollo Roman; Carlos Díaz Iglesias; María A. Gálvez Moreno. ACTH Producing Pancreatic Neuroendocrine Tumors in Multiple Endocrine Neoplasia Type 1. Journal of the Pancreas. ISSN 1590-8577
15. Paloma Moreno Moreno; María Rosa Alhambra Expósito; Aura Dulcinea Herrera Martínez; Rafel Palomares Ortega; Luis Zurera Tendero; Juan José Espejo Herrero; María Angeles Gálvez Moreno. Arterial Calcium Stimulation with Hepatic Venous Sampling in the Localization Diagnosis of Endogenous Hyperinsulinism. International journal of endocrinology. 2016, pp. 4581094.
16. Aura D Herrera Martínez; Rodrigo Bahamondes Opazo; Rafael Palomares Ortega; Concepción Muñoz Jiménez; Maria A. Gálvez Moreno; José M. Quesada Gómez. Primary hyperparathyroidism in pregnancy: a two-case report and literature review. Case reports in obstetrics and gynecology. 2015, pp. 171828. 2015.
17. Aura D Herrera Martínez; José C Padillo Cuenca; Alfonso Calañas Continente; Rodrigo Bahamondes Opazo; Concepción Muñoz Jiménez; María A Gálvez Moreno. Invasive techniques and intraoperative echography in the localization of insulinomas; a case report. Nutrición Hospitalaria. 32 - 1, pp.426 - 435. 2015.
18. Aura Herrera Martínez; Montserrat Viñals Torràs; Ma Concepción Muñoz Jiménez; Antonio Pablo Arenas de Larriva; Ma José Molina Puerta; Gregorio Manzano García; Ma Ángeles Gálvez Moreno; Alfonso Calañas Continente. Metabolic encephalopathy secondary to vitamin D intoxication. Nutrición Hospitalaria. 31 - 3, pp. 1449- 1500. 2015.

19. Aura Dulcinea Herrera Martínez; María R Alhambra Expósito; Gregorio Manzano García; María J Molina Puertas; Alfonso Calañas Continente; Rodrigo Bahamondez Opazo; Concepción Muñoz Jiménez; Rafael Rojas Contreras; María A Gálvez Moreno. Use of glutamine in total parenteral nutrition of bone marrow transplant patients. Nutrición hospitalaria. 31 - 4, pp. 1620 - 1624. 2015.
20. Inmaculada Prior Sánchez; Aura Dulcinea Herrera Martínez; Carmen Tenorio Jiménez; María José Molina Puerta; Alfonso Jesús Calañas Continente; Gregorio Manzano García; María Ángeles Gálvez Moreno. Percutaneous endoscopic gastrostomy in a myotrophic lateral sclerosis. Experience in a district general hospital.Nutrición Hospitalaria. 30 - 6, pp. 1289 - 1383. 2014.
21. Aura D Herrera Martínez; Pedro Estrada Corona. Spondylo-epiphyseal dysplasia associated with craniosynostosis, cleft palate and mental retardation. A case report. Investigación clínica. 51 - 4, pp. 553 – 613. 12/2010.
22. Aura D Herrera Martínez; Alfonso J Rodriguez Morales. Trends in the seroprevalence of antibodies against Trypanosoma cruzi among blood donors in a Western Hospital of Venezuela, 2004-2008.Acta tropica. 116 - 1, pp.115 - 122. 10/2010.
23. Aura D Herrera Martinez; Alfonso J Rodríguez Morales. Potential influence of climate variability on dengue incidence registered in a western pediatric Hospital of Venezuela. Tropical biomedicine. 27 - 2, pp. 280 - 286. 08/2010.
24. Alfonso J Rodriguez Morales; Aura D Herrera Martínez; Yiraldine Herrera Martínez. Imported cases of malaria admitted to a hospital in Western Venezuela, 1998-2009. Travel medicine and infectious disease. 8 - 4, pp. 269 -340. 07/2010.
25. Aura D Herrera Martínez; Yiraldine Herrera Martínez; Antonio Maria Pineda. Neurological opportunistic diseases in HIV infected patients in a western hospital of Venezuela, 2007 -2009.Brazilian journal of infectious diseases. 14 - 6, pp. 643 - 647. 2010.

ABOUT THE AUTHOR

Aura Dulcinea Herrera Martínez was born in Barinas, Venezuela. After finishing high school, she started her medical training at the Centrooccidental Lisandro Alvarado University in Barquisimeto, Venezuela, where she graduated Magna cum laude (2011). Subsequently she worked as a medical doctor in the Rafael Rangel Hospital in Yaritagua, Venezuela. Concurrently she studied education, defended a thesis on Learning Styles in Medical Universities (awarded Honorable Mention) and received the title of Magister Scientiarum in Higher Education (2013). In 2013 she started specializing in Endocrinology and Nutrition at the Reina Sofia University Hospital in Córdoba, Spain, under the supervision of dr. Rafael Palomares Ortega and dr. María Angeles Gálvez Moreno. Simultaneously she started research in neuroendocrine tumors as part of a Master Program in Translational Research under supervision of Prof. dr. Justo Castaño and Prof. dr. Raúl Luque. In 2015 she started her PhD training in the University of Córdoba, under supervision of Prof. dr. Justo Castaño, Prof. dr. Raúl Luque and dr. María Ángeles Gálvez Moreno.

In 2016 Aura performed a clinical/research rotation in the Erasmus Medical Center in Rotterdam, the Netherlands, under the supervision of Prof. dr. Leo J. Hofland, Prof. dr. Wouter de Herder and dr. Richard Feelders, and subsequently started a combined PhD training in both the University of Córdoba and the Erasmus University Rotterdam.

She finished her clinical training in Endocrinology and Nutrition in 2017, and received the 2017 Resident Award of the Spanish Society of Endocrinology and Nutrition. Since then she worked as a researcher at the department of Internal Medicine, section Endocrinology of the Erasmus Medical Center under the supervision of Prof. dr. Leo J. Hofland and dr. Richard Feelders. Currently she works as a consultant/researcher in Endocrinology at the Reina Sofia University Hospital and the Maimonides Institute of Biomedical Research of Córdoba.

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