Preclinics and clinics of neuroendocrine tumors

Roxanne C.S. van Adrichem

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Prekliniek en kliniek van neuroendocriene tumoren

Roxanne Caresse Samantha van Adrichem

Colofon

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General introduction and aims of the thesis

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GENERAL INTRODUCTION

Epidemiology

Neuroendocrine tumors (NETs) are relatively rare tumors with an incidence of approximately 5 cases per 100,000 population per year (1-3). The incidence of NETs has been increasing over the decades. This might be related to an aging population, since these tumors tend to present at a relatively higher age. Also, increased awareness of physicians for NETs and more sophisticated diagnostic imaging techniques for the detection of these tumors might have further contributed to this increase (1,4,5). Still, up to 70% of all NET patients present with metastases at diagnosis (4,6). These patients are usually treated in specialized centers, like European Neuroendocrine Tumor Society (ENETS) Centers of Excellence (CoE) (6,7).

Tumor origin

NETs originate from the diffuse neuroendocrine (NE) cell system (8). Although in many organs the physiological function of NE cells is yet unclear, in the gastrointestinal (GI) tract these cells are known to exert a regulatory function (8).

NETs can arise throughout the whole body (1). About 70% of all NETs will develop in the gastroenteropancreatic (GEP) system and are named GEP NETs (1,3,9,10). In some patients with metastatic NETs, the primary tumor site is unknown. However, these occult primary tumors are relatively uncommon and account for 13% of all NETs (1).

Tumor characteristics

GEP NETs originating from the various parts of the GI tract or the pancreas can manifest as different phenotypes and, therefore, can be considered as separate tumor entities (11,12). This is reflected by differences in tumor growth rates, metastatic patterns and secretory capacities (13). There is growing evidence that these heterogeneously presenting endocrine tumor types differ in their clinical behavior as well (14-16).

Depending on their primary localization, GEP and lung NETs can be subdivided according to their embryologic origin into: foregut, midgut, or hindgut NETs, as shown in **Figure 1** (2,3). Foregut NETs originate from the: bronchus, stomach, duodenum, and pancreas. NETs from the midgut are localized in the lower jejunum, ileum, appendix and ascending colon. Hindgut NETs are derived from the transverse and descending colon and rectum (2,3).

Nomenclature

GEP NETs are characterized by overproduction of metabolically active substances (hormones and amines) causing clinical syndromes. Pancreatic NETs (panNETs) are generally named after the hormone which is secreted in excess and which causes the clinical

signs and symptoms. For example, a 'PTHrP-oma' is a NET which secretes parathyroid hormone-related peptide (PTHrP) in excess (17).

In the past decades, NETs arising from the small intestine were called 'carcinoids'. More than a century ago, the German pathologist Siegfried Oberndorfer named these tumors 'karzinoide', or cancer-like, since these NETs generally have a more indolent growing pattern as compared to the more common GI adenocarcinomas (1,18).

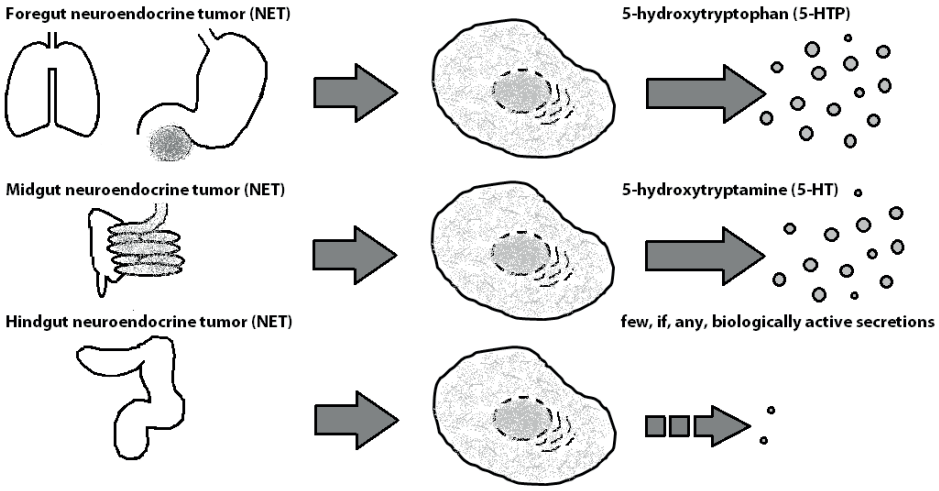

Figure 1 Origin and secretory capacity of primary neuroendocrine tumors
Neuroendocrine cells are found in several organs derived from the embryologic primitive gut. Foregut structures include: bronchus, stomach, first part of the duodenum and pancreas. Midgut organs include the second portion of the duodenum and the right hemicolon. Hindgut derivatives stretch from transverse colon to rectum. Adapted from: *https//www.netterimages.com.*

Paraneoplastic clinical syndromes

Patients with midgut NETs may also suffer from the carcinoid syndrome (CS), especially when liver metastases are present. This is the most common and well known paraneoplastic clinical syndrome and includes symptoms of flushing, dyspnoea, abdominal pain as well as, diarrhea caused by tumoral production of hormones and peptides. Among these peptides, serotonin (5-HT) hypersecretion plays an important role (19,20) (**Figure 2**). Approximately 50% of patients with CS also suffers from shortness of breath on exertion, peripheral edema, and fatigue as a result of right-sided heart failure caused by carcinoid heart disease (CHD) (19,21-24).

Patients with CHD have characteristic endocardial fibrotic plaques on both mural and valvular endocardium with a localization mainly on the right side of the heart (25,26). Most affected is the tricuspid valve followed by the pulmonary valve (27). CHD heart valves are microscopically characterized by a proliferation of (myo)fibroblasts with

matrix-rich fibrous stroma, neovascularization and inflammation (28,29). These changes can result into thickening and stiffening and, consequently tricuspid valve regurgitation and, pulmonary valve stenosis (19,22,24,30). The exact underlying pathophysiological mechanism resulting into CHD is largely unknown.

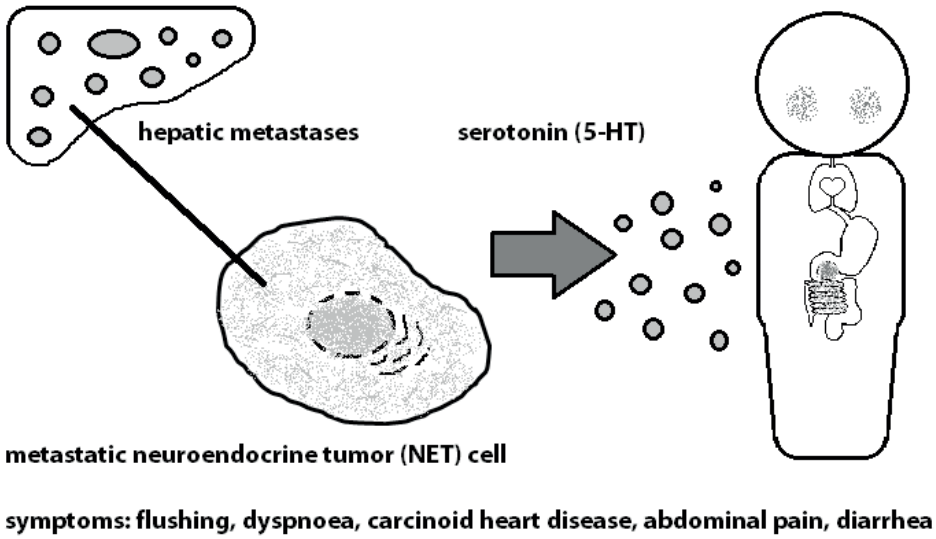

Figure 2 Carcinoid syndrome and symptoms
When neuroendocrine tumors metastasize to the liver, the biologically active 5-HT can pass directly and undegraded into the systemic circulation. The biological activity results in various symptoms in several organ systems. Most common symptoms include flushing, dyspnoea, abdominal pain and diarrhea. Carcinoid heart disease can also develop. Adapted from: *https//www.netterimages.com.*

About 60-90% of all panNETs and 20% of the small intestinal NETs (siNETs) are non-functioning, or non-syndromic (31-33). Non-functioning NETs are not associated with clinical syndromes and generally present late with locally advanced disease leading to tumor site-specific symptoms, or with distant metastases mainly to the lymph nodes, liver, or bone (13).

PATHOPHYSIOLOGY

Receptor expression

NETs can express a variety of receptor (sub)types on their cell surfaces. Two specific receptor types, namely somatostatin receptors (sst) and dopamine (DA) receptors, are abundantly expressed in normal, non-pathologic, human NE cells as well as in NETs (34). Sst are important therapeutic targets for the inhibition of hormonal secretion and cell

proliferation in GEP NETs. Sst and DA receptors share some structural and functional characteristics (35-37).

Somatostatin receptors (sst)

Somatostatin (SS) is a small peptide hormone that exerts a variety of inhibitory functions on GI motility, exocrine secretion, hormone secretion, neurotransmission, immunomodulation and cell proliferation in normal tissues and tumors (38,39).

Two biologically active forms of SS exist: somatostatin-14 (SS-14), consisting of 14 amino acids, and somatostatin-28 (SS-28), consisting of 28 amino acids (39-41).

There are five different G protein-coupled somatostatin receptor subtypes, named sst_{1-5} (39). Sst_2 exists in two alternative splicing forms, sst_{2a} and sst_{2b} (42-44). All sst subtypes can be expressed in variable amounts in NETs (45-55). Sst_2 is predominantly expressed in about 90% of all siNETs and 80% of all panNETs, followed by sst_1 and sst_5, while sst_3 is less often expressed and sst_4 is almost absent (55-58). Undifferentiated grade 3 GEP NETs and NECs express sst_2 less frequently and in lower density as compared to well-differentiated grade 1-2 GEP NETs (59).

After binding, SS can activate its receptors and activate guanine nucleotide-binding (G) proteins in downstream pathways resulting in the activation of potassium channels and inhibition of calcium channels and adenylate cyclase activity. Ultimately, this leads to inhibition of hormonal secretion and potentially also to control of cell proliferation (60-64). Sst signaling is presented in **Figure 3**.

All five sst subtypes bind somatostatin-14 and somatostatin-28 with high affinity (38). However, only sst_{2a}, sst_3 and sst_5 display a high, low, and moderate affinity, respectively, towards the currently available synthetic octapeptide somatostatin analogs (SSAs) lanreotide and octreotide (38).

Sst expression, especially of sst_2, is obligatory for successful treatment with SSAs, as well as radiolabeled SSAs.

Dopamine (DA) receptors

Dopamine is a neurotransmitter in the central nervous system. It is a regulator of GI motility, cardiovascular and renal function, and in the endocrine system (65). Activities can be executed after binding of DA to its receptors on the cell surface, as demonstrated in **Figure 4** (65).

There are five different G protein-coupled DA receptors, e.g. dopamine receptor type 1 (D_1)–D_5 (34). The presence of mainly D_2 has been studied in GEP NET cells (55,66-68). After dopamine binding, D_2 activates G proteins. These proteins can subsequently inhibit adenylyl cyclase activity, cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA). In addition, G proteins can modulate actions of phospholipase C (PLC) and mitogen-activated protein kinases (MAPKs). Ultimately, these signal transduction

pathways can, *in vitro,* result in decreased gene expression and inhibition of growth and hormonal secretion in NET cells (34,42).

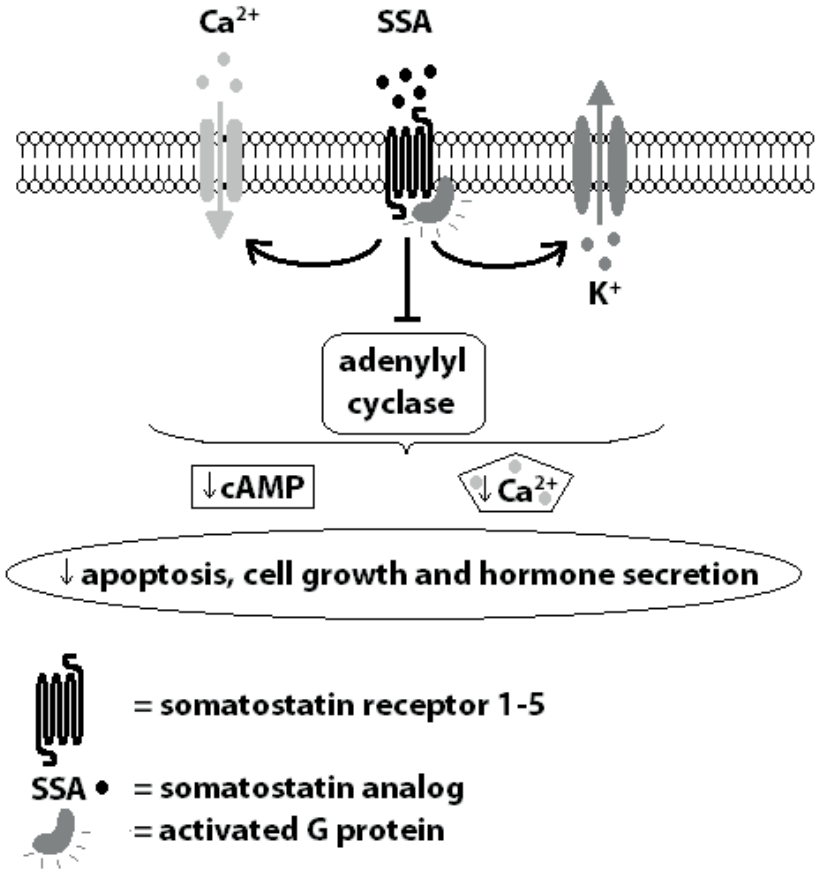

Figure 3 Somatostatin receptor signaling pathway
Somatostatin binds to sst receptor types and activates G proteins resulting in the inhibition of adenylyl cyclase activity, activation of potassium (K^+) channels and inhibition of calcium (Ca^{2+}) channels. This in turn results in effects on hormone secretion, apoptosis and cell growth. Adapted from: *International Journal of Peptides 2013 2013 926295.*

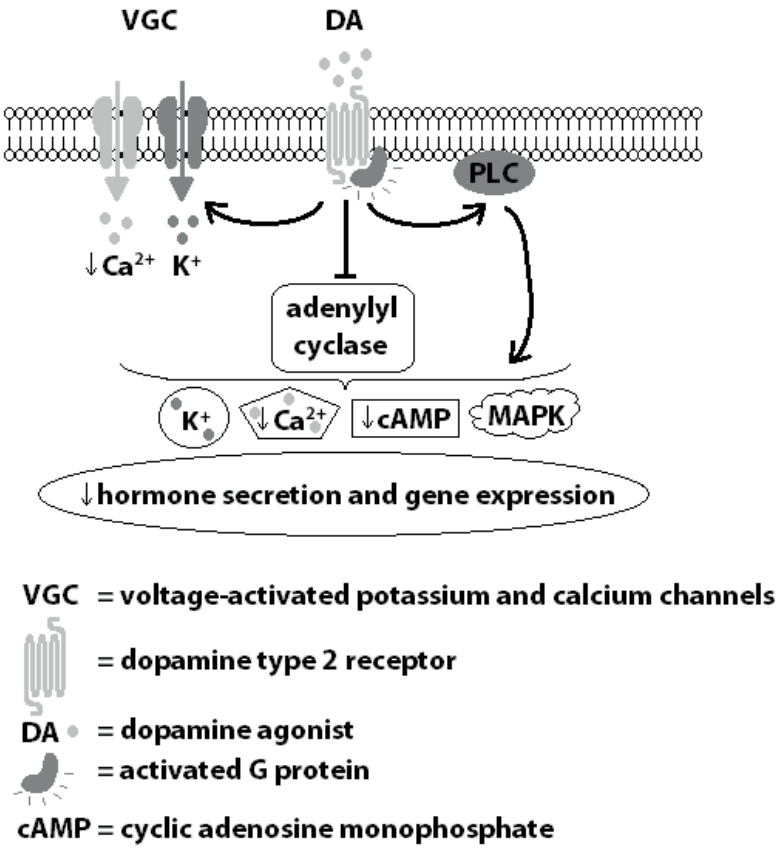

Figure 4 Dopamine receptor signaling pathway
D_2 signaling after binding of dopamine. Via stimulation of G proteins, dopamine inhibits adenylyl cyclase activity and phosphatidylinositol metabolism, activates voltage-activated potassium (K^+) and calcium (Ca^{2+}) channels, and interferes with activities of phospholipase C (PLC) and the mitogen activated protein kinases (MAPKs). Subsequently, hormone secretion and gene expression are decreased. Adapted from: *Neuropsychopharmacology 2014 39 156-168.*

Hybrid receptors

Co-expression of sst and D_2 has also been demonstrated in NETs (34,37,69). These cell surface receptors seem capable of heterodimerization, thereby generating so-called chimeric receptors or hybrid receptors with altered functional properties (35,70-72).

Pathophysiologic pathways

A variety of signaling pathways are involved in normal cell physiology and are crucial for activities like cell metabolism, proliferation, migration, differentiation and survival. However, these pathways are frequently deregulated in tumors (73-82). How exactly these different pathways are involved in causing cancer, and more specific in NETs, is generally

unknown. Specific pathways that are known to be involved in the pathophysiology of GEP NETs are the insulin-like growth factor (IGF) pathway, the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway and the RAS/RAF/mitogen-activated protein kinase kinase/extracellular-signal-regulated kinase (RAS/RAF/MEK/ERK) or RAS/RAF/MEK/MAPK pathway (**Figure 5**).

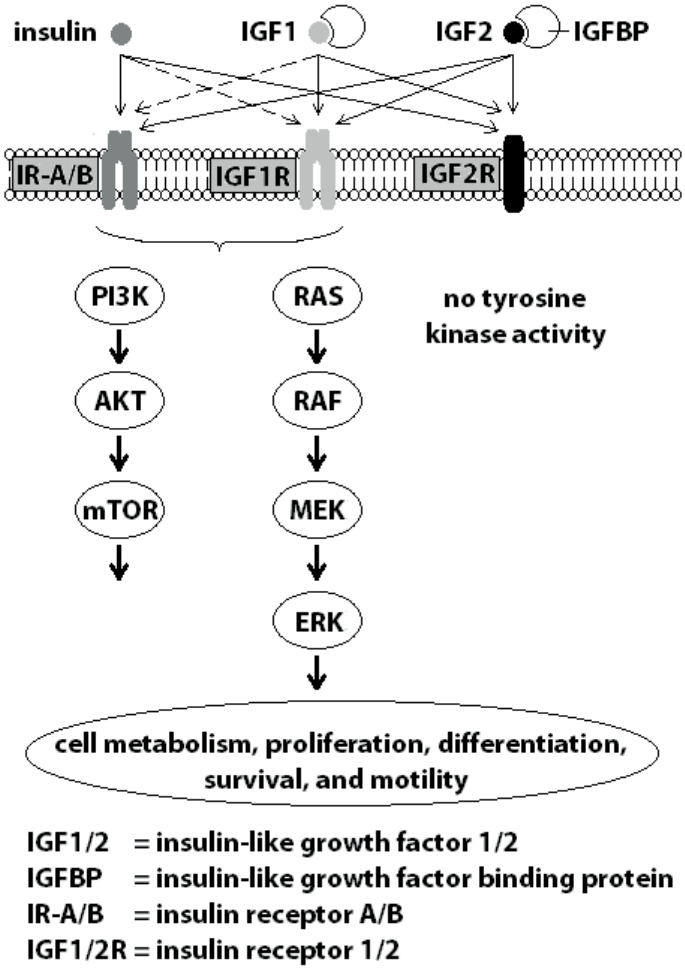

Figure 5 IGF, PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathway
A simplified representation of the IGF pathway, PI3K/AKT/mTOR pathway and the RAS/RAF/MEK/ERK pathway. Adapted from: *Biochemical Sciences 2011 36 320-328.*

Insulin-like growth factor (IGF) pathway

In the insulin-like growth factor (IGF) pathway, different polypeptide hormones or 'IGF-related factors' participate. These include the ligands, e.g. insulin-like growth factor 1 (IGF1), IGF2 and insulin, the IGF receptors, e.g. IGF1R, IGF2R, and the insulin receptors (IRs), insulin receptor isoform-A (IR-A) and -B (IR-B). The IGF-binding proteins 1-6 (IGFBP1-6) play an important role as well (73,74,83,84). The IGF1R and IR-A have predominantly mitogenic effects, IGF2R has scavenging functions, and IR-B is mainly involved in metabolic processes (73).

IGFRs and IRs have been identified *in vitro* in GEP NETs (85-87). After binding to IGF1Rs, IR-As or hybrid receptors, IGFs can exert tumor-stimulating functions in an auto- and/or paracrine way (83,84). Phosphorylation of these receptors activates downstream the insulin receptor substrate (IRS) 1-3 to the cell membrane, which subsequently activates both the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways (73).

PI3K/AKT/mTOR pathway

Upon activation of receptor tyrosine kinases (RTKs) during receptor binding (e.g. IGF1R), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) converts substrate phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3) (45,79). Furthermore, induction of AKT subsequently phosphorylates other downstream proteins, which results into the inactivation of the endogenous mTOR inhibitors, tuberous sclerosis complex 2 (TSC2) and proline-rich AKT substrate 40 (PRAS40) (79,88,89). Ultimately, this induces mTOR to modulate processes including cell metabolism, proliferation, differentiation, survival, and motility (79). mTOR exists in two forms of protein complexes: mTORC1 and mTORC2 (79,90). Activation of AKT results mainly in mTORC1 complex induction. mTORC2 complex activates AKT through phosphorylation at Ser473 (89). It is suggested that this Ser473 plays a pivotal role in the resistance to targeted therapy with selective mTORC1 inhibitors. The PI3K/AKT/mTOR pathway is closely linked and intracellular parallel located to the RAS/RAF/MEK/ERK pathway downstream of receptors (91).

RAS/RAF/MEK/ERK pathway

Growth factors can activate RTKs after receptor binding. This results in autophosphorylation. RAS-GDP is subsequently activated by adapter proteins GRB2 and SOS into RAS-GTP. Subsequently, RAS recruits RAF to the membrane (92). The proteins PKC and PMA induce activation of RAF, MEK and ERK, respectively, in the signaling pathway cascade which can lead to altered gene expression (79).

DIAGNOSTICS

After the ENETS was founded in 2004 and the North American Neuroendocrine Tumor Society (NANETS) in 2006, expertise in the field of GEP and bronchial NETs was concentrated and this has resulted into the establishment of several clinical guidelines. Patients can be scheduled for standardized diagnostic procedures and can be selected for the most optimal treatment for their GEP NETs in highly specialized centers where tumor board discussions involving specialists from a wide variety in disciplines are taking place on a regular basis (93). For making the diagnosis of a GEP NET, pathology of tumor tissue, determination of biomarkers, and radiological and nuclear imaging are pivotal (93).

Microscopic examination on a biopsy specimen

Tumor origin

First, the NE identity of the tumor has to be established, or confirmed. Generally, on a hematoxylin and eosin-stained tumor specimen, a tumor with neuroendocrine characteristics can be recognized. It is recommended that the pathology diagnosis of a NET is supported by the demonstration of positive immunohistochemical staining for the NET markers chromogranin (Cg) and synaptophysin in tumor cells (94,95).

Immunohistochemical tumor markers

1. *Chromogranin (Cg)*

Chromogranin (Cg) is a protein present in secretory granules in the cytoplasm of NE cells (93). Tumors co-secrete this protein with the amines and peptides that are present in the neurosecretory granules (96).

2. *Synaptophysin*

Synaptophysin is an integral membrane protein of small clear vesicles, which can be demonstrated in all normal and NE tumor cells (93). Immunopositivity of this immunohistochemical marker is observed in most NETs (97,98).

3. *Other immunohistochemical neuroendocrine markers*

Although there are more NET markers, application of neuron-specific enolase, CD56 and other NE markers are generally not routinely recommended because of the low specificity of the available antibodies (93).

Macroscopic examination on a resection specimen

ENETS TNM staging

Different macroscopic characteristics are used for the ENETS TNM staging (99,100). With this staging algorithm, information is collected on the primary tumor (T), the involvement of regional lymph nodes (N) and the presence, or absence of distant metastases (M). This clinical information is essential for the decision on possible treatment modalities as well as the prognosis of patients with NETs.

Tumor grading

By convention, proliferative activity and differentiation of a NET has to be determined. Distinction between a well- and poorly-differentiated GEP NETs has to be made according to the World Health Organization (WHO) GEP NET classification guidelines (12).

Proliferative activity can be assigned by counting mitoses per high-power field and/or by immunostaining for the proliferation marker Ki-67 antigen (using the MIB1 antibody), which is expressed in the nucleus (101).

Tumor grading is associated with tumor aggressiveness and is based on the Ki-67 proliferation index. The GEP NET WHO grading is subdivided into: WHO grade 1 (G1) (Ki-67 ≤2%), WHO G2 (Ki-67 3–20%) or WHO G3 (Ki-67 ≥20%) (99,100). WHO G1 and G2 GEP NETs are considered as well-differentiated NETs that display diffuse and intense expression of the NE markers chromogranin A (CgA) and synaptophysin. WHO G3 GEP NETs are grouped together with poorly differentiated neuroendocrine carcinomas (NECs). The latter usually show only positive staining for synaptophysin, but not (or only little) for CgA.

Other diagnostic options

Optional for tumoral diagnostics is the performance of immunostaining for specific hormones, and sst_{2a}. In the majority of tumor specimens of patients with G1 and G2 NETs, sst_{2a} expression is present. This also results in positive *in vivo* somatostatin receptor scintigraphy (SRS) using ^{111}In-pentetreotide, or OctreoScan® (59).

Biochemical tumor markers

Circulating tumor markers, or biochemical markers, are measurable parameters in biological sources of the human body that provide helpful information in the diagnostic work-up and therapeutic evaluation of patients with tumors.

Plasma or serum

1. *Chromogranin A (CgA)*

Seven different forms of chromogranin can be found in the circulation. CgA is the most commonly used circulating tumor marker in patients with GEP and bronchial NETs (102). This tumor marker has an age-independent reference range for both sexes (103-105). Elevated levels of CgA are demonstrated in several other non-NET-related conditions (102,106-112). Therefore, CgA has a suboptimal sensitivity of 59% and specificity of 68% (113). CgA can be secreted from both clinically functioning and non-functioning NETs, independent of their primary localization (102). CgA levels are often the highest in midgut NETs and non-functioning panNETs (102). The level of CgA correlates to tumor burden, WHO grading, and NET cell primary (102). Whereas elevated circulating levels of CgA correlate with prognosis in GEP NET patients (114,115), there is no information whether absence of CgA secretion is a favorable, or unfavorable prognostic factor in patients with stage IV GEP NETs.

2. Neuron-specific enolase (NSE)

Neuron-specific enolase (NSE) is present in the cytoplasm and secreted by NE cells (17,116-118). Plasma NSE is elevated in 30-50% of patients with GEP NETs (113,118,119). It has shown 50% correlation with tumor size (113,118). Like CgA, this tumor marker also has an age-independent reference range for both males and females and can be used as circulating biomarker for both non-functioning and functioning NETs (103-105,120,121). NSE is generally considered as a marker for dedifferentiation in NETs (113).

3. Alternative tumor markers

The gut-brain peptide ghrelin is involved in different endocrine (growth hormone secretion, insulin secretion and glucose metabolism) and non-endocrine processes (appetite, stimulation of food intake, GI motility and contribution to long term body-weight regulation) (122-128). Ghrelin can also be involved in neoplastic activities including cell proliferation, migration, invasion, inflammation, apoptosis, and angiogenesis (129-131). About 50% of circulating ghrelin is acylated (132,133). NETs express ghrelin and its growth hormone secretagogue receptor GHSR1a (126,134,135). Most NETs secrete ghrelin, but hyperghrelinemia has been reported in only 11 patients with NETs until now (136-140). The function of ghrelin in GEP NETs is not known. In general, ghrelin plays an important role in the maintenance of the body mass index, so it can be postulated that this role is similar in NET patients with elevated ghrelin levels (127,141). Total plasma ghrelin has been studied as biomarker in GEP NET patients. However, these total plasma ghrelin levels were not discriminative between patients with GEP NETs and healthy controls (136).

Studies on prognostic markers for survival and predictive markers for tumor recurrence after surgery currently focus on circulating tumor cells and tumor DNA, thymidylate synthase expression and tumor-associated macrophages (142-144). Research is ongoing

in the field to develop more specific tumor markers in which innovative techniques are used including genomic profiling, epigenetics and microRNAs (145-147).

Traditional tumor markers are tumor products that are measured according to a single-analyte approach. Lately, a new tumor test has been launched according to a new approach: the 'NETest' (148-151). In this specific blood-based multi-analyte NET gene transcript assay with algorithm analyses, the expression of 51 different genes is tested, and seems to outperform single analyte tests in the detection and follow-up of NETs (148).

Urine

1. Serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA)

Serotonin and its metabolites are often secreted by NETs. Foregut NETs can secrete 5-hydroxytryptophan (5-HTP), a precursor of serotonin. Especially midgut NETs, and to a lesser extent foregut and hindgut NETs, produce mainly 5-hydroxytryptamine (5-HT) or serotonin. Generally, hindgut NETs do not secrete serotonin, or its metabolites. Foregut, midgut and hindgut NETs and their secretion products are shown in **Figure 1** (102,152,153).

Serotonin regulates smooth muscle contraction, blood pressure and neurotransmission. It is produced and predominantly stored in NE cells of the GI tract (102). 5-hydroxyindoleacetic acid (5-HIAA) is the breakdown metabolite of serotonin and secreted in the urine. The 24-hours urinary collection of 5-HIAA is an established biomarker used in the diagnosis and follow-up of GEP and bronchial NET patients with the CS (102,154). For the CS, overall sensitivity for urinary 5-HIAA measurements is about 70% and specificity 90% (152,155). In some patients with non-metastatic NETs or CS, urinary 5-HIAA levels can also be within the reference range (156).

Imaging

Radiological imaging

The choice for a specific imaging method depends on tumor-related aspects. These include the primary tumor localization, tumor spread to local structures, relation of the tumor to adjacent organ structures, presence of regional and distant metastases and, detection of therapy effects and/or recurrent disease (13).

Computer tomography (CT), magnetic resonance imaging (MRI), ultrasound (US), and endoscopic ultrasound (EUS) are the mostly used imaging modalities (13). Detection rates of the CT and MRI scan are comparable for the visualization of panNETs, liver metastases and extrahepatic metastases. Preferentially, NET lesions in the abdomen and chest should be visualized by CT (13).

For both CT and MRI, 'Response Evaluation Criteria In Solid Tumors' (RECIST) criteria are generally used to evaluate tumors in clinical trials.

EUS is currently the imaging modality of choice for small non-metastatic panNETs.

Nuclear imaging

Somatostatin receptor scintigraphy (SRS) using OctreoScan® is a nuclear imaging modality in which mainly somatostatin receptor subtype 2 (sst_2), and to a lesser extent also sst_3 and sst_5 expression on tumor cells is visualized *in vivo* with the commercially available radiopeptide ^{111}In-pentetreotide (157). This technique was first introduced using [^{123}I-Tyr^3]octreotide (158). Using OctreoScan® and single photon emission computed tomography (SPECT), more than 75% of all GEP NETs and/or their metastases can be detected (159). However, sst expression is not entirely specific for this tumor entity and, therefore, an OctreoScan® can also be positive in other tumors or conditions (159).

The reasons to perform an OctreoScan® in patients, suspected or diagnosed with a NET, are the detection and staging of a NET and its metastases, the selection of patients who are eligible for peptide receptor radiotherapy (PPRT), to evaluate effects of PRRT, or the detection of disease recurrence.

Recently, new imaging options as positron emission tomography (PET) and PET-CT with ^{68}Ga-labeled SSAs (like ^{68}Gallium-DOTA-TOC PET/CT), but also ^{18}F-DOPA SPECT and ^{11}C-5-HTP SPECT have been introduced to further optimize the sensitivity and specificity to detect NETs (160). Future developments involve the development of somatostatin receptor antagonist-coupled isotopes and radiolabeled ligands for other receptors, like the cholecystokinin (CCK), gastrin and bombesin receptors.

TREATMENT

Tumor resection

Surgery with a complete resection of the primary NET and, if present, metastases is the only curative option for patients with a NET. Debulking surgery should be considered in patients with uncontrolled hormonal syndromes and patients with non-functioning tumors who experience symptoms which are related to tumor mass (161).

Prior to surgery, patients with the CS should be evaluated for perioperative treatments to prevent a life-threatening carcinoid crisis (45).

Interventions

Interventions such as selective (chemo)embolization, radioembolization, radiofrequency ablation, or microwave ablation of metastases can be applied at any time during the disease course in GEP NET patients with liver-dominant metastatic disease (162).

Medical therapy in advanced disease

Molecular targeted therapies

Targeted therapies are commonly used in G1-2 patients with metastasized GEP NETs in order to control tumor growth and symptoms of hormonal hypersecretion and to improve quality of live.

1. Somatostatin analogs (SSAs)

The two biologically active forms of SS, SS-14 and SS-28, cannot be used in daily clinical practice because of their short half-life in the circulation (39,163).

Octreotide was the first stable synthetic SSA that was synthesized (164). Hereafter, another SSA, lanreotide with similar affinity and activity profiles was developed (163). Octreotide and lanreotide have binding preferences to sst_2, sst_3 and sst_5 (38,39,62).

The main indications for using SSAs in NET patients are the control of hormone-related symptoms in functioning NETs and tumor growth control (38,165). SSAs can inhibit hormone release that cause the clinical syndromes and, thereby, reduce symptoms and improve quality of life in selected patients, especially those with vasoactive intestinal peptide (VIP)-secreting panNETs (VIPomas) and patients with siNETs and CS (165-168).

Octreotide LAR has been shown to significantly prolong time to tumor progression (TTP) compared with placebo in patients with (non-)functional metastatic midgut NETs (168). Treatment with lanreotide autogel in patients with metastatic G1-2 GI and pan-NETs is also associated with significantly prolonged progression-free survival (PFS) (169). Long-acting SSAs are able to induce a relief of symptoms, accompanied by a reduction in the tumor markers 5-HIAA and CgA in 30–70% of GI NETs patients. Although most patients experience a rapid relieve of symptoms, a loss of response of inhibition of hormone secretion can occur after continuous treatment of octreotide LAR and lanreotide autogel within weeks to months (20,169,170).

Patients with GEP NETs that escape from medical treatment by developing tachyphylaxis and/or resistance will eventually develop progressive disease (63,171). Tachyphylaxis is a rapidly decreasing response to a drug following continuous administration. The processes which underlie this rebound phenomenon are poorly understood. Mechanisms that are potentially involved in SSA tachyphylaxis are receptor phosphorylation, G protein uncoupling, receptor internalization, and degradation (63,171).

1a. Pasireotide

Pasireotide is a new SSA with high affinity for all sst except sst_4 (172,173). In contrast with octreotide LAR, no tachyphylaxis was observed with pasireotide LAR (173-175).

In a small phase II trial, short-acting pasireotide was effective at controlling symptoms in 27% of patients with metastatic NETs and CS inadequately controlled with octreotide LAR (176). Treatment with long-acting pasireotide LAR regretfully did no show such

an advantage. Pasireotide LAR, therefore, shows no superiority over octreotide LAR in patients with syndromic GI NETs.

1b. Telotristat etiprate

Recently, the drug telotristat etiprate has been developed. This oral serotonin synthesis inhibitor blocks tryptophan hydroxylase activity which is involved in serotonin synthesis (39). It will be positioned as supportive treatment for patients with signs and symptoms caused by hyperserotonemia like severe secretory diarrhea which is refractory to long-acting SSAs (39). In phase II studies in patients with the CS and diarrhea, this drug induced a 30% reduction in bowel movements in 28% of subjects co-treated with long-acting SSAs. This clinical response was also accompanied by a more than 50% reduction of urinary 5-HIAA excretion in 56% of the patients (177,178). Results of phase III placebo-controlled double blind trials with this drug in patients with CS and diarrhea refractory to SSAs have been reported at international meetings (39).

2. Chimeric molecules

A dopamine-somatostatin chimeric molecule, also named 'dopastatin', acts on both sst and D_2. Regretfully, with chronic administration, the dopamine-somatostatin chimeric molecule BIM-23A760 which binds preferentially to sst_2, sst_5 and D_2 was found to produce a metabolite which interfered with the activity of the parent compound (179). In the future, new dopamine-somatostatin chimeric molecules which are differently metabolized and which retain their activity might be expected.

Protein kinase inhibitors

3a. Mammalian target of rapamycin (mTOR)C1 inhibitors

Everolimus and rapamycin belong to the group of orally active mammalian target of rapamycin (mTOR) inhibitors (180). This everolimus is a tyrosine kinase inhibitor that specifically inhibits mTORC1 and modulates metabolic processes and signaling through growth factors, like IGF1, downstream of their cognate receptors (180). In panNET cell lines, mTOR inhibitors decreased cell growth (181-183). In addition, everolimus inhibits tumor growth, both *in vitro* and *in vivo* (184,185)

Everolimus is registered for the treatment of patients with progressive well-differentiated G1-2 panNETs (180,185-187) and will probably be registered for the treatment of patients with progressive well-differentiated G1-2 siNETs.

3b. Sunitinib

Sunitinib malate is a multitargeted tyrosine kinase inhibitor that blocks protein tyrosine kinases including vascular endothelial growth factor receptors 1 (VEGFR1)-3, platelet-

derived growth factor receptors alpha (PDGFRα) and beta (PDGFRβ), stem cell factor receptor (Kit), Flt-3, and colony-stimulating factor 1 receptor (CSF1R) (188). These kinases play an important role in angiogenesis (189). VEGF and their receptors are abundantly expressed in panNETs (190-194). In patients with unresectable, advanced metastatic panNETs, treatment with sunitinib prolonged PFS as compared with placebo (195).

3c. Linsitinib (OSI-906)

Recently, the dual IGF1R/IR tyrosine kinase inhibitor linsitinib (OSI-906) has been developed (196). *In vitro*, linsitinib has shown antiproliferative effects in several cell lines (196-200). Since both IGF1R and IR are important targets for therapy in NETs, dual inhibition could be a potentially novel therapy for patients with NETs. No clinical trials with OSI-906 in patients with NETs have been initiated yet.

Peptide receptor radiotherapy (PRRT)

Peptide receptor radiotherapy (PRRT) involves the use of radiolabeled SSAs in the treatment for patients with G1-2, inoperable or metastasized NETs (159). Nowadays, the most commonly used radiolabeled SSA is ^{177}Lu-DOTAtate (201).

In general, tumor response rates and PFS of PRRT with ^{177}Lu-DOTAtate are favorable as compared to alternative treatment modalities. In a study in more than 500 GEP NET patients who underwent PRRT with ^{177}Lu-DOTAtate, complete tumor response (disappearance of all target lesions) was observed in 2%, partial response (decrease in tumor size >30%) was observed in 28% and minor response (decrease in tumor size >25%–<50%) was observed in 16% of patients with metastatic inoperable GEP NETs (202). Median TTP was 40 months and median OS was 46 months (202).

Interferon (IFN)

In the past, interferon alpha (IFNα) appeared a promising treatment modality in the management of functioning midgut NETs. Interferon alpha (IFNα) binds to IFNα receptors, which are expressed on NET cells. After binding to its specific receptors, IFNα can reduce tumor cell proliferation via cell cycle blockage of the G1-S phase. This biotherapeutical also exerts immunomodulation and inhibition of angiogenesis (203). IFNα reduces hormone secretion and symptoms in about 60% of the patients with GI NETs (203,204). Bevacizumab is a monoclonal antibody that blocks angiogenesis by inhibiting vascular endothelial growth factor A (VEGF-A). In a recently published phase III trial, octreotide LAR combined with either IFNα, or bevacizumab showed comparable antitumor effects in patients with advanced small intestinal NETs (205). Two prospective randomized trials conducted in patients with stage IV GEP NETs have shown that SSAs, IFNα or their combination have comparable antiproliferative effects when used after prior disease progression (206,207). Interferon beta (IFNβ), seems more potent as

compared to IFNα in inhibiting cell proliferation of GEP NET cells *in vitro*. However, no *in vivo* data exist on the use of this drug in GEP NET patients (204,208,209).

Systemic chemotherapy

Chemotherapy is recommended in patients with poorly differentiated (G3) NETs and NECs of any site and, in progressive and/or extended panNETs (210,211).

Patients with panNETs are usually treated with combinations of streptozotocin (STZ) with 5-fluorouracil (5-FU), or doxorubicin with STZ (211). These combinations resulted in objective tumor response rates of about 40% (212,213). Temozolomide, alone or in combination with capecitabine, may be considered as an alternative therapeutic regimen (32). Patients with metastatic grade 3 GI, or bronchial NETs are generally treated with a combination of cisplatinum with etoposide (212).

AIMS OF THE THESIS

Despite the many advances in the GEP NET field reported above, there is still an unmet need for:

1) improvement of the diagnostic work-up and follow-up of GEP NET patients using sensitive and specific tumor markers and,

2) identifying new biotherapeutical options by modulating pathological pathways in panNET cells.

We have aimed to answer the following research questions in the present thesis:

Chapter 2: What is the role of serum NSE as a biomarker for tumor progression and survival in GEP NET patients?

Chapter 3: What is the clinical importance of true non-secretion of CgA as prognostic factor in patients with ENETS TNM stage IV GEP NETs?

Chapter 4: Are fasting AG and UAG potential novel biomarkers in patients with GEP NETs? Is there a relationship between fasting AG, UAG and AG/UAG ratios and biochemical and clinical parameters in GEP NET patients?

Chapter 5: Is there a relationship between serum CgA, the Ki-67 proliferation index in tumor samples and the expression of IGF-related genes in GEP NET tissues in relation to 5-year survival of GEP NET patients?

Chapter 6: What is the sst_{2a}-positivity in GEP NET samples of PRRT-treated patients? Is there a relationship between best GEP NET response to PRRT and tumoral sst_{2a} immunohistochemistry?

Chapter 7: Are panNET cell lines useful as a model for the study of the IGF pathway in GEP NETs?

Do panNET cells produce growth factors that stimulate IR-As and can SSAs and/or DAs influence the production of these growth factors?

Chapter 8: What is the effect of linsitinib alone, or in combination with the mTOR inhibitors everolimus and rapamycin, on cell migration and proliferation of panNET cells?

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2

Serum neuron-specific enolase level is an independent predictor of overall survival in patients with gastroenteropancreatic neuroendocrine tumors

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LETTER TO THE EDITOR

Serum neuron-specific enolase (NSE) is considered a tumor marker in patients with gastroenteropancreatic neuroendocrine tumors (GEP NETs) (1). It is elevated in 30-50% of GEP NET patients and correlates with tumor size (2,3). NSE has a sensitivity of 38% and specificity of 73% for GEP NET detection (2). The prognostic role of serum NSE as a biomarker for GEP NETs patients' survival is poorly studied (4).

We retrospectively studied 592 patients with sporadic (non-familial) ENETS TNM stage IV GEP NETs. Median follow-up was 58.7 months (25^{th}-75^{th} percentile: 34.02-92.98). Serum NSE was measured at first consultation, using enzyme immunoassay (NSE Cobas E602, Roche Diagnostics, Mannheim, Germany).

Cut-off values for serum NSE were: NSE ≤1× ULN (≤16.2 µg/L), NSE 1-3× ULN (16.2-48.6 µg/L) and NSE >3× ULN (48.6 µg/L).

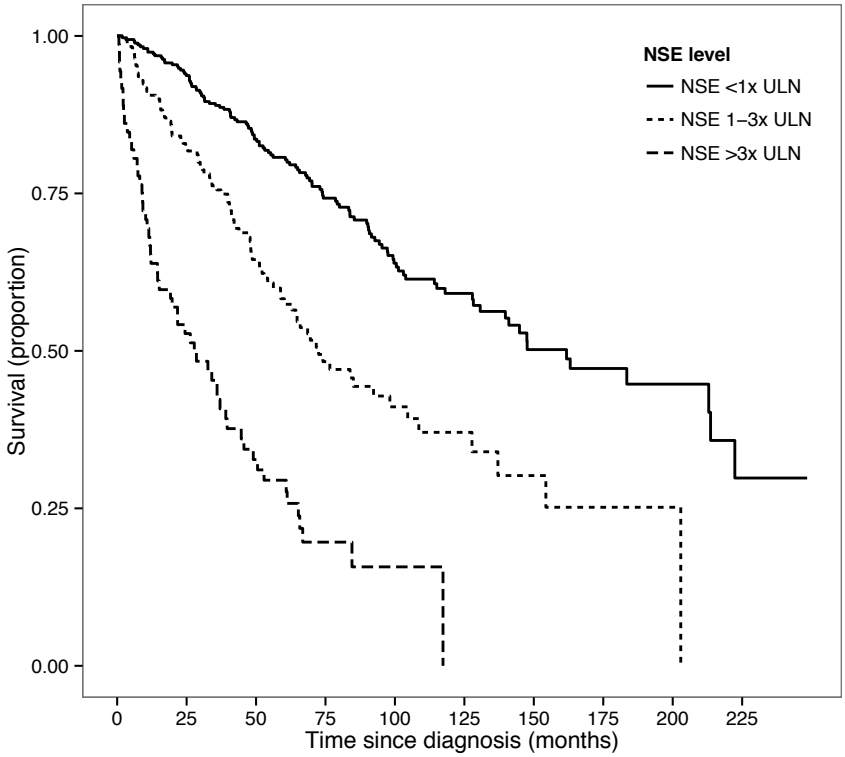
Primary outcome was overall survival, calculated from date of diagnosis to date of death by any cause, or date of last follow-up. Using statistical software R version 3.1.3 'survival' package, overall survival was estimated with the Kaplan-Meier method. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated with Cox proportional hazards models including age at diagnosis, OctreoScan® (SRS) positivity (Krenning scale ≥2 in all lesions), primary tumor site, sex and bone metastases.

Two hundred forty-two (41%) of GEP NET patients had an elevated NSE (>1× ULN). NSE >3× ULN were seen in pancreatic NETs.

Median overall survival (mOS) across all groups was 103.9 months (95% CI 92.8-137.1). mOS was 161.8 months in the NSE ≤1× ULN group (95% CI 130.7-not reached [NR]) and 72.5 months in the NSE 1-3× ULN group (95% CI 60.2-108.6; Cox proportional hazard-adjusted HR versus NSE ≤1× ULN: 1.96 [1.45-2.63], P<0.001). In the NSE >3× ULN group, mOS was 27.8 months (95% CI 15.2-44.7; HR versus NSE ≤1× ULN: 6.15 [4.36-8.69], P<0.001) (**Figure 1**). Significant contributors to our model included: age at diagnosis (HR 1.03 [1.02-1.04], P<0.001) and SRS positivity (HR 0.48 [0.28-0.83], P<0.001).

The ENETS/WHO grading system using Ki-67 staining was introduced in 2010 (5). Therefore, we used SRS positivity as a surrogate marker for ENETS/WHO tumor grading, since SRS positive GEP NETs are generally well-differentiated, ENETS/WHO grade 1-2 tumors. However, the assumption that all SRS positive patients could have ENETS/WHO grade 1-2 tumors could be considered a limitation of this study. We therefore studied the subpopulation of 367 patients with known ENETS/WHO 2010 grading (62% of all patients). In this population, the same Cox proportional hazard model with ENETS/WHO grade as an additional parameter was applied and showed that higher ENETS/WHO grade significantly contributed (P<0.001) to the model, but that NSE remained independently associated with overall survival (P<0.001). Multivariate analysis data are shown (**Supplementary Table 1**, available at Annals of Oncology online).

This study demonstrates that NSE is a biomarker for overall survival in ENETS TNM stage IV GEP NET patients. Our study cohort had a median follow-up of almost 5 years and a mOS of over 8.5 years across all groups. Elevated NSE was found in over 40% of patients, confirming published data (2,3). Elevated serum NSE indicates a more aggressive disease course and determination of NSE at first consultation could, therefore, have prognostic implications.



Number of patients at risk

NSE <1x ULN	350	325	239	159	104	64	37	21	13	4
NSE 1–3x ULN	170	138	90	41	24	13	7	2	1	0
NSE >3x ULN	72	36	20	7	1	0	0	0	0	0

Figure 1 Kaplan-Meier estimate of overall survival in NSE <1× ULN (continuous line), NSE 1–3× ULN (dotted line) and NSE >3× ULN (dashed line) level groups.

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Supplementary Table 1 Multivariate analysis model with hazard ratios (HRs) estimated through Cox proportional hazards model. P-values determined with Wald test for HR in Cox-proportional hazards regression 95% CI. Primary tumor site 'Other' includes: neuroendocrine tumors of unknown origin, stomach, duodenum and colorectal neuroendocrine tumors.

Variable	Hazard ratio	95% Confidence interval	P-value
All patients (N=592)			
NSE <1× ULN	1.00		
NSE 1-3× ULN	1.96	1.45-2.63	<0.00001
NSE >3× ULN group	6.15	4.36-8.69	<0.00001
Age at diagnosis	1.03	1.02-1.04	<0.00001
SRS positivity	0.48	0.28-0.83	0.003
Presence of bone metastasis	1.20	0.91-1.56	NS
Sex (female)	0.85	0.67-1.10	NS
Small intestinal primary	1.00		
Pancreatic primary	1.12	0.84-1.51	NS
Other primary site	1.14	0.83-1.56	NS
Patients with known WHO/ENETS grading (N=367)			
NSE <1× ULN	1.00		
NSE 1-3× ULN	2.32	1.51-3.56	0.0001
NSE >3× ULN group	10.23	6.08-17.23	<0.00001
Age at diagnosis	1.04	1.02-1.04	<0.00001
SRS positivity	1.07	0.46-2.52	NS
Presence of bone metastasis	1.21	0.82-1.79	NS
Sex (female)	0.97	0.67-1.40	NS
Small intestinal primary	1.00		
Pancreatic primary	1.01	0.65-1.58	NS
Other primary site	1.14	0.69-1.71	NS
WHO/ENETS grade 1	1.00		
WHO/ENETS grade 2	1.52	1.01-2.29	0.04
WHO/ENETS grade 3	5.42	3.07-9.57	<0.00001

3

Is true non-secretion of chromogranin A an unfavorable prognostic factor in patients with ENETS TNM stage IV gastroenteropancreatic neuroendocrine tumors?

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ABSTRACT

Introduction: Chromogranin A (CgA) is a widely used biomarker for the work-up of gastroenteropancreatic neuroendocrine tumors (GEP NETs), correlating with tumor volume and biological activity. During diagnosis and follow-up we found patients with elevated CgA levels and patients without elevated CgA levels (='true non-secretors').

Objectives: We assessed, in a tertiary referral center, whether true non-secretion of CgA is an unfavorable prognostic factor in patients with stage IV GEP NETs.

Methods: In total, 692 consecutive patients were evaluated. After exclusion of patients with concomitant proton pump inhibitors, 616 and 524 patients were included for analysis of baseline and follow-up CgA, respectively. Cut-off values for baseline and follow-up CgA groups were: normal (reference range [RR]), intermediate (≤2× upper limit of normal [ULN]), high (2-10× ULN) and very high (>10× ULN). Overall survival (OS) was estimated with Kaplan-Meier methods.

Results: Of the 692 evaluated patients, median follow-up was 61.3 months (25^{th}–75^{th} percentile: 35.7–97.5) and median OS was 104.6 months (95% CI: 94.4-136.5). OS was significantly shorter in patients with high baseline CgA (median 103.9 versus 222.4 months, $P<0.01$) and very high baseline CgA versus RR (56.2 versus 222.4 months, $P<0.0001$). For follow-up CgA, OS was only significant shorter in the very high follow-up CgA versus RR (62.9 months versus not reached). This effect remained in multivariate analysis with Cox proportional hazard models.

Conclusions: True non-secretion of CgA has shown to be a favorable biomarker for OS in patients with stage IV GEP NETs, both at first referral as well as during follow-up.

INTRODUCTION

Chromogranin A (CgA), a member of the granin family, is an acidic glycoprotein with 439 amino acids which is present in the secretory dense core granules of most neuroendocrine cells (1). Immunohistochemistry for CgA is widely used and considered to be the most valuable tissue-based biomarker in the diagnosis of neuroendocrine tumors (NETs) (2). Elevated levels of serum or plasma CgA can be found in various types of NETs, including gastrointestinal tract NETs, (non-)functioning pancreatic NETs, paragangliomas, pheochromocytomas, medullary thyroid carcinomas, pituitary and parathyroid adenomas and in some patients with small-cell lung cancer (3,4). Furthermore CgA has shown to be the best available general serum tumor marker for the work-up of gastroenteropancreatic NETs (GEP NETs) (3,5). A recent meta-analysis demonstrated that the sensitivity and specificity of elevated serum levels of CgA in the diagnosis of patients with NETs are 0.73 and 0.95 respectively (6).

The highest levels of CgA have been found in patients with metastatic small intestine NETs and non-functioning pancreatic NETs (3,7). Depending on the extent of the disease, serum CgA is elevated in >60% of patients. CgA levels may correlate with tumor volume, presence of metastases and biological activity in the tumors, but care should be taken in measuring CgA and interpreting the results. Somatostatin analogs (SSAs) are known to affect blood levels of CgA by blocking the production and release of CgA in addition to affecting tumor burden. Falsely elevated levels of CgA have also been reported in patients using proton pump inhibitors (PPIs) or histamine H_2 blockers, in patients with renal or liver failure, and in those with chronic atrophic gastritis type A or inflammatory bowel disease (7-9).

Both functionally active NETs and non-functioning NETs can co-secrete CgA with amines and peptides that are present in their neurosecretory granules (3,7). During diagnosis and follow-up we found patients with metastatic GEP NETs that secrete CgA resulting in elevated circulating CgA levels and patients with metastatic GEP NETs without elevated CgA levels. The latter we have called 'true non-secretors'. The reason why some patients with well-differentiated metastatic GEP NETs do not show elevated circulating CgA levels is not known. It is well known that poorly differentiated neuroendocrine carcinomas (NECs) lose their expression of CgA (2). On the other hand expression of CgA in non-endocrine tumors is considered a poor prognostic factor (10). In our study in neuroendocrine tumor patients, we postulated that these non-secretors would have a poorer prognosis because we considered the lack of secretion of any substance from a GEP NET to be a sign of further dedifferentiation.

Since the prognostic value of CgA in patients with metastatic NETs has not been confirmed to date (3,5), this study, conducted in a large single-center cohort, aimed to determine whether true non-secretion of CgA is an unfavorable prognostic factor in patients with metastatic, ENETS/AJCC TNM stage IV (11-13) GEP NETs. Finally, we investi-

gated whether there were any significant differences in patient and tumor characteristics between 'true non-secretors' and patients with CgA secreting GEP NETs.

PATIENTS AND METHODS

Patients

In this retrospective case study, all patients with metastatic, ENETS/AJCC TNM stage IV (11-13) GEP NETs, diagnosed between 1 January 1993 and 31 December 2012 were identified from the Erasmus MC NET database and included.

TNM stage IV indicates the presence of metastases at any distant anatomical site (including non-regional lymph nodes) (11-13). The date of diagnosis was defined as the date at which tumor tissue was collected during biopsy or surgery. Follow-up time was determined from the date of diagnosis to the date of death or the last follow-up for survivors. Patients diagnosed with the multiple endocrine neoplasia syndrome type 1 (MEN1) were excluded. In addition, to prevent influence of PPIs on CgA levels, patients with concomitant PPI use at the time of CgA measurement were excluded. Information on age, sex, location of primary tumor, OctreoScan® (SRS) positivity, presence, or absence of bone metastasis and concomitant use of PPIs was collected.

Definitions CgA groups

Patient groups were defined by first CgA level at referral or diagnosis (baseline CgA) and highest CgA level measured during follow-up (follow-up CgA). All serum CgA measurements were performed in the Erasmus MC, using an ELISA (CisBio Bioassays, Codolet, Franassay; upper limit of normal [ULN] <94 µg/L).

Four patient groups were defined by both baseline CgA and follow-up CgA levels. Cut-off values for serum CgA were: normal baseline CgA or follow-up CgA (reference range, <94 µg/L), intermediate baseline CgA or follow-up CgA (≤2× ULN, 94-188 µg/L), high baseline CgA or follow-up CgA (2-10× ULN, 188-940 µg/L) and for very high baseline or follow-up CgA (>10× ULN, >940 µg/L).

Primary outcome was overall survival, calculated from date of diagnosis to date of death by any cause, or date of last follow-up.

Statistical analysis

Continuous data were described as the mean and standard deviation (SD) and were compared by ANOVA tests. Categorical data were described as counts and percentages and were compared by χ^2 tests. Overall survival was estimated with the Kaplan-Meier method. The hazard ratios (HRs) were estimated using a Cox proportional hazards model. HRs and 95% confidence intervals (CIs) were also calculated. The proportional hazard

assumption (Schoenfeld residuals) was always satisfied. Data analysis was performed using statistical software R version 3.1.3 and is based on the survival-package. A two-sided P-value of <0.05 was considered statistically significant.

RESULTS

Patient inclusion and stratification

In total, after exclusion of 19 MEN1 patients, 692 consecutive patients were evaluated with a median follow-up of 61.3 months (25^{th}–75^{th} percentile: 35.7–97.5 months) and a median overall survival (mOS) of 104.6 months (95% CI: 94.4-136.5). After exclusion of patients with concomitant PPI use, 616 and 524 patients were included for analysis of baseline and follow-up CgA, respectively. Of these patients, 492 (79.9%) had an elevated baseline CgA level (>1× ULN) and 465 (88.7%) had an elevated follow-up CgA level (>1× ULN). Other clinical conditions which might have caused elevations in CgA were excluded.

Patient characteristics of the different groups for baseline CgA and follow-up CgA measurements can be found in **Table 1**. Highly significant differences were found for only two parameters: age at diagnosis for both baseline CgA and follow-up CgA measurement, and bone metastasis differed only significantly for follow-up CgA measurement.

Baseline CgA and survival

Median time between histological diagnosis of the GEP NET and measurement of baseline CgA was 3.2 months (25^{th}–75^{th} percentile: 0.9–17.4 months). With regard to the measurement of baseline CgA, survival analysis without concomitant PPI use (N=616) showed a mOS of 222.4 months in the normal baseline CgA group (95% CI: 141.0-not reached [NR]), and 213.0 months in the intermediate baseline CgA group (95% CI: 114.2-NR; Cox-adjusted HR versus normal CgA: 1.26 [0.79-1.99], P=0.33). Subsequently, mOS was 103.9 months in the high baseline CgA group (95% CI: 90.8-144.8; HR versus normal CgA: 1.92 [1.29-2.88], P<0.01) and 56.2 months in the very high baseline CgA group (95% CI: 49.08-65.7; HR versus normal CgA: 3.58 [2.44-5.26], P<0.0001) (**Figure 1**). Using a Cox proportional hazard model, age at diagnosis (HR 1.02 [1.01-1.03], P<0.0001), bone metastasis (HR 1.33 [1.03-1.72], P<0.05), SRS positivity (HR 0.30 [0.18-0.53], P<0.0001) and unknown/other origin of tumor (HR 1.58 [1.18-2.12], P<0.01) had a statistical significance, while sex did not contribute significantly to the model. In subanalysis, only including the 351 patients with known ENETS/WHO grading, the same Cox proportional hazard model with ENETS/WHO grade as an additional parameter was applied and showed that ENETS/WHO grade 3 significantly contributed (HR versus ENETS/WHO grade 1: 5.02 [2.92-8.65], P<0.0001) to the model. However, very high CgA remained independently associated with overall survival (HR versus normal CgA: 3.54 [2.06-6.10], P<0.0001).

Table 1 Characteristics at baseline of patient groups based upon baseline chromogranin A (CgA) measurement and follow-up CgA measurement. Differences are tested by ANOVA for age and through χ^2 for all other variables.

	Normal CgA <1× ULN (<94 µg/L)	CgA 1-2× ULN (94-188 µg/L)	CgA 2-10× ULN (188-940 µg/L)	CgA >10× ULN (>940 µg/L)	Significant difference
Baseline CgA measurement					
N	124	121	194	177	
Age at diagnosis (years±SD)	54.9±11.5	59.05±12.19	59.15±10.81	60.78±10.6	0.0001
Sex – male (%)	52.4	51.2	50.0	57.6	NS
SRS positivity (%)	87.9	93.3	96.3	92.6	NS
Bone metastasis (%)	29.0	17.35	29.38	28.81	NS
Primary tumor site					
SiNETs (%)	38.7	48.76	49.48	40.67	NS
PanNETS (%)	32.25	30.57	26.80	32.76	NS
Other (%)	29.03	20.66	23.71	26.55	NS
Follow-up CgA measurement					
N	59	86	156	223	
Age at diagnosis (years±SD)	52.97±11.15	58.12±10.97	59.21±11.89	60.66±10.5	<0.0001
Sex – male (%)	50.8	47.6	50.0	57.4	NS
SRS positivity (%)	86.4	94.1	93.6	93.7	NS
Bone metastasis (%)	33.9	15.1	28.8	30.9	0.03
Primary tumor site					
SiNETs (%)	50.8	39.53	51.28	45.73	NS
PanNETS (%)	22.03	32.55	26.92	25.56	NS
Other (%)	27.11	27.90	21.79	28.69	NS

CgA=chromogranin A, ULN=upper limit of normal, SRS=OctreoScan®
SiNETs=small intestine neuroendocrine tumors, PanNETs=pancreatic neuroendocrine tumors
Primary tumor site "Other" includes: neuroendocrine tumors of unknown origin, neuroendocrine tumors of stomach, duodenum and colorectal.

Follow-up CgA and survival

Follow-up CgA measurement during the course of the disease was used to define four groups: low, intermediate, high and very high follow-up CgA groups. Median time between histological diagnosis of the GEP NET and measurement of follow-up CgA was 18.6 months (25^{th}–75^{th} percentile: 3.9–52.1).

In the patients without concomitant PPI use (N=524), mOS was not reached in the normal follow-up CgA group, while mOS in the intermediate follow-up CgA group was 222.4 months (95% CI: 163.5-NR; HR versus normal: 1.58 [0.77-3.24], P=0.21). In the high and very high follow-up CgA group, mOS was 147.6 months (95% CI: 127.8-NR; HR versus normal: 1.55 [0.80-3.02], P=0.20) and 67.3 months (95% CI: 59.3-83.4; HR versus normal: 3.70 [1.98-6.91], P<0.001), respectively (**Figure 2**). Additional significant contributors to the used Cox proportional hazard model included: bone metastasis (HR 1.41 [1.06-1.87], P<0.05), SRS positivity (HR 0.33 [0.17-0.621], P<0.0001) and unknown/other origin of tumor (HR 1.60 [1.17-2.19], P<0.01), while sex did not contribute significantly. In sub-

analysis, only including the 302 patients with known ENETS/WHO grading, the same Cox proportional hazard model with ENETS/WHO grade as an additional parameter was applied and showed that ENETS/WHO grade 3 significantly contributed (HR versus ENETS/WHO grade 1: 4.19 [2.29-7.64], P<0.0001) to the model. In this extended model, very high CgA remained independently associated with overall survival (HR versus normal CgA: 2.99 [1.40-6.40], P<0.005).

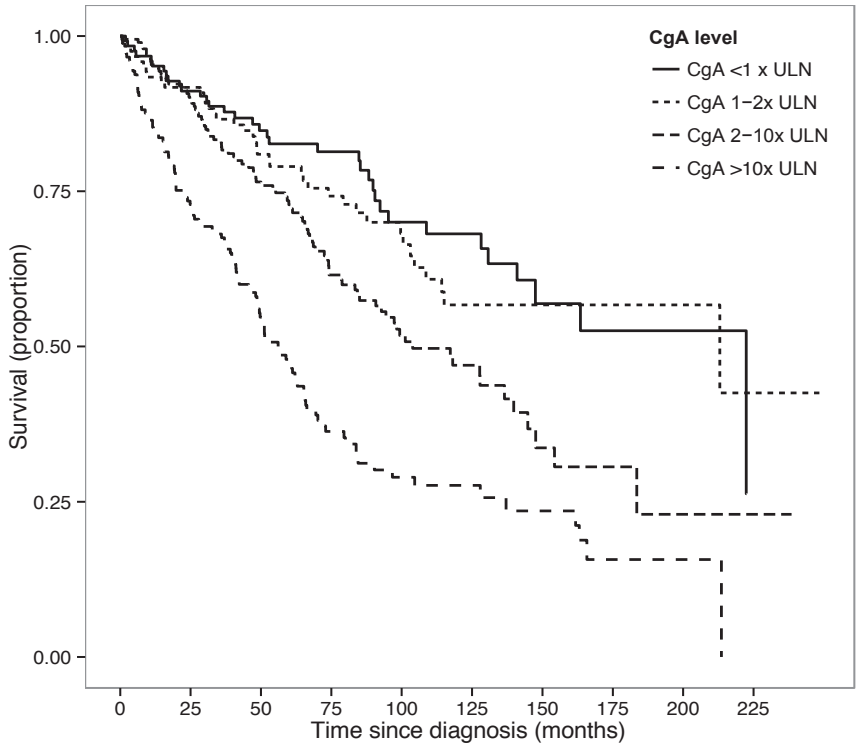
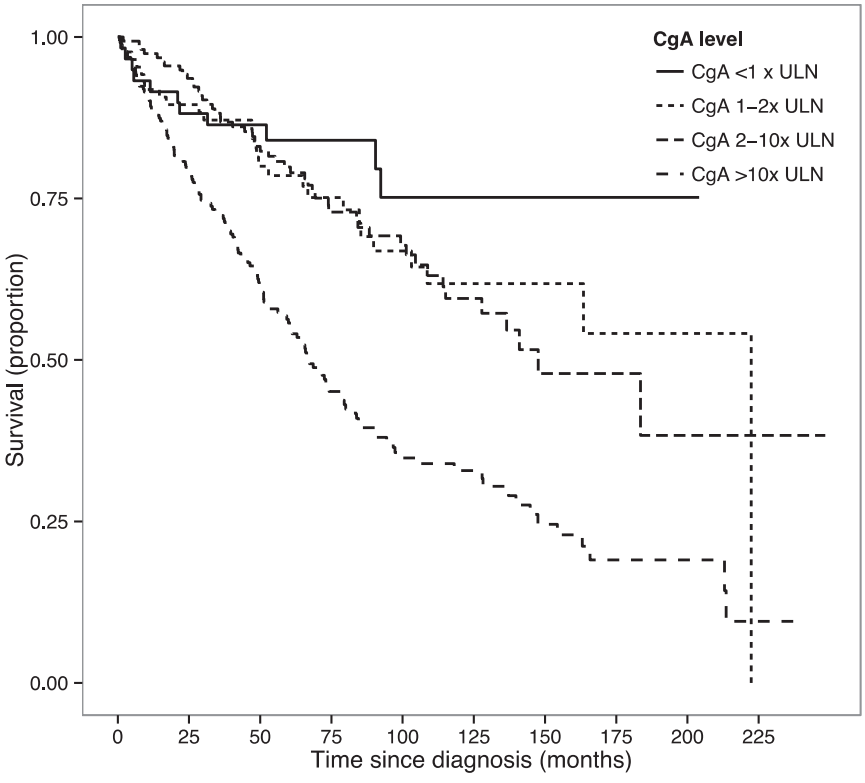


Figure 1 Kaplan-Meier estimate of overall survival in normal (<1× ULN, ---), intermediate (1-2× ULN, - - -), high (2-10× ULN, – –) and very high (>10× ULN, – ·) baseline serum chromogranin A (CgA) level groups (N=616).

DISCUSSION

This single-center retrospective study demonstrates that, contrary to our expectations, true non-secretion of CgA is not an unfavorable prognostic factor for patients with



Number of patients at risk

CgA <1 x ULN	59	52	39	27	15	11	6	5	2	0
CgA 1–2x ULN	86	76	56	40	27	17	10	5	3	0
CgA 2–10x ULN	156	144	109	67	46	28	13	5	3	2
CgA >10x ULN	223	174	120	69	42	28	16	5	4	2

Figure 2 Kaplan-Meier estimate of overall survival in normal (<1× ULN, ---), intermediate (1-2× ULN, - - -), high (2-10× ULN, - -) and very high (>10× ULN, - ·) follow-up chromogranin A (CgA) level groups (N=524).

ENETS/AJCC TNM stage IV GEP NETs, both when measured at first diagnosis as well as when measured at follow-up. Both serum baseline CgA and follow-up CgA levels show a positive correlation with overall survival.

The selected timeframe of 20 years for inclusion in this study was based upon the first availability of the most commonly used imaging techniques and treatment modalities in our institution. This included the introduction of somatostatin receptor imaging with the OctreoScan®, peptide receptor radiotherapy (PRRT) (14) and SSAs (15). Any possible bias caused by evolving imaging and treatment protocols has therefore been minimized.

Patients in our research population, referred to our hospital for PRRT, mostly have metastatic disease. For CgA measurements in patients with metastatic disease specificities of 100% have been reported (16-19).

Bone metastases in our cohort only influenced follow-up CgA levels, likely because bone metastases were not yet present at the time of diagnosis. Patients who live longer are more likely to develop bone metastases during the course of their disease. This is reflected by the relatively high frequency of bone metastases in our patients with normal CgA levels.

A recent meta-analysis demonstrated that CgA is an efficient biomarker for the diagnosis of NETs with a sensitivity of 73% and specificity of 95%, indicating that serum CgA might be helpful in the clinical management and follow-up of NETs (6). Another study by Yao and colleagues evaluated the prognostic value of CgA in patients with pancreatic NETs treated with everolimus. They confirmed the prognostic importance of baseline levels of CgA by multivariate analysis, hereby identifying CgA as an independent predictor of overall survival (9). In line with this study we confirm a significant difference in OS between true non-secretors (2) and different elevated levels of CgA secretion by not only pancreatic NETs, but also by small intestinal and other NETs and, therefore, determination of CgA at first consultation can be used for predicting prognosis in all types of GEP NETs. Up until the present study the prognostic value of CgA in patients with GEP NET had not been confirmed (3).

An elevated CgA level at baseline was found in over 80% of the patients, which is in accordance with earlier published data (3,9,20,21).

Current ENETS guidelines state that where possible, PPIs should be interrupted, leaving a clearance of at least three half-lives, prior to CgA plasma sampling (3). The potential weakness of CgA as a biomarker is that the use of PPIs can frequently cause a significant elevation in CgA levels (7,8). Since PPIs are now widely available in drugstores without a doctor's prescription, the value of future studies will likely be affected.

By selecting strict cut-off values to divide the patients in four groups, both at first measurement as highest measurement during follow-up, the impact of relatively small increases in CgA could be studied. Our study demonstrates that patients in high and very high CgA groups clearly have a worse prognosis when compared with those in the normal CgA group. Hence, an increase in CgA indicates a more aggressive disease course. The determination of CgA at first consultation can be used for predicting the prognosis. Also, CgA during the course of the disease provides additional information on tumor aggressiveness. The earlier hypothesis that GEP NETs tumors may lose CgA expression to incomplete or partial endocrine differentiation is hereby refuted.

For our data collection we did not include information on tumor grading, because Ki-67 staining on tumor samples was not routinely used for the diagnostic work-up during the entire follow-up period. After all, the ENETS/WHO grading system was introduced in 2010 and our inclusion of patients dates back to 1993 (12,13). We therefore used SRS positivity as a surrogate marker for tumor grading, since SRS-positive GEP NETs are generally well-differentiated, ENETS-WHO grade 1-2 tumors (22,23).

However, the assumption that all SRS-positive could have ENETS/WHO grade 1-2 tumors could be considered a limitation of this study. We therefore studied the sub-population of patients with known ENETS/WHO 2010 grading and demonstrated that CgA remains associated with survival.

In conclusion, true non-secretion of CgA has been proven to be an independent biomarker for overall survival in patients with stage IV well-moderately differentiated GEP NETs, both at first referral as well as perhaps more evident at follow-up.

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4

Plasma acylated and plasma unacylated ghrelin: useful new biomarkers in patients with neuroendocrine tumors?

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ABSTRACT

Introduction: To date, the value of fasting plasma acylated ghrelin (AG) and unacylated ghrelin (UAG) as potential novel biomarkers in patients with neuroendocrine tumors (NETs) is unknown.

Objectives: Of this study: are to 1) compare fasting AG and UAG levels between non-obese, non-diabetic NET patients (N=28) and age- (±3 years) and sex-matched non-obese, non-diabetic controls (N=28); and 2) study the relationship between AG, UAG, and AG/UAG ratios and biochemical (chromogranin A [CgA] and neuron-specific enolase [NSE] levels) and clinical parameters (age at diagnosis, sex, primary tumor location, carcinoid syndrome, ENETS TNM classification, Ki-67 proliferation index, grading, prior incomplete surgery) in NET patients.

Methods: Fasting venous blood samples (N=56) were collected and directly stabilized with 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride after withdrawal. Plasma AG and UAG levels were determined by ELISA. Expression of ghrelin was examined in tumor tissue by immunohistochemistry.

Results: There were no significant differences between NET patients and controls in AG (median: 62.5 pg/mL, IQR: 33.1–112.8 versus median: 57.2 pg/mL, IQR: 26.7–128.3, P=0.66) and UAG in levels (median: 76.6 pg/mL, IQR: 35.23–121.7 versus median: 64.9, IQR: 27.5–93.1, P=0.44).

Conclusions: No significant correlations were between AG, UAG, and AG/UAG ratios versus biochemical and clinical parameters in NET patients with the exception of age at diagnosis (AG: ρ=–0.47, P=0.012; AG/UAG ratio: ρ=–0.50, P=0.007) and baseline chromogranin A levels (AG/UAG ratio: ρ=–0.44, P=0.019). In our view, fasting plasma acylated and unacylated ghrelin appear to have no value as diagnostic biomarkers in the clinical follow-up of patients with NETs.

INTRODUCTION

Ghrelin is a gut-brain hormone that is produced predominantly by gastric X/A-like neuroendocrine cells (1,2). Ghrelin exists in two forms: acylated ghrelin (AG) and unacylated ghrelin (UAG). About 50% of circulating ghrelin is in the acylated form (3,4) and exerts its functions through the growth hormone secretagogue receptor type 1a (GHSR1a) (5,6). Acylation of ghrelin, which is required for its ability to activate the GHSR1a *in vivo*, is performed by the enzyme ghrelin O-acyltransferase (GOAT) (5,7,8). UAG does not activate the GHSR1a at physiological concentrations (5,9). Therefore, UAG has been considered as an inactive degradation product of ghrelin (2,10). However, as recently reported, UAG could also act as a separate hormone in several physiological and pathophysiological conditions, independent from AG (9).

AG seems to be involved in different endocrine and non-endocrine processes (2,11-16). Neoplastic effects of ghrelin have been reported as well including: cell proliferation, cell migration, cell invasion, inflammation, apoptosis, and angiogenesis (17-19).

Neuroendocrine tumors (NETs) are rare tumors characterized by the hypersecretion of several bioamines and peptides (20,21). NETs can also express ghrelin and its receptors (14,22). Although most NETs release ghrelin, hyperghrelinemia has been reported in only 11 patients with NETs (23-27).

The function of ghrelin in NETs is not well understood. It has been suggested that ghrelin could be responsible for the striking maintenance of body mass index (BMI) that can be observed in NET patients (15,28). Although total serum ghrelin levels in NET patients were reported to be positively correlated with tumor burden (29), total plasma ghrelin seems not to be a useful biomarker since total plasma ghrelin levels did not discriminate between patients with NETs and healthy controls (23). Currently, there are no data on the clinical usefulness of the two isoforms of ghrelin, AG and UAG, as potential biomarkers in patients with NETs. Examining the relationship between these two isoforms has relevance, since it is becoming clearer that levels of ghrelin acylation are regulated and not constant (4). For example, the ratio of AG/UAG has been found to be linked with metabolic status (30,31).

Therefore, the aims of this study are to 1) compare fasting AG and UAG levels between non-obese, non-diabetic NET patients (N=28) and age- (±3 years) and sex-matched non-obese, non-diabetic controls (N=28); and 2) study the relationship between AG, UAG, and AG/UAG ratios and biochemical (chromogranin A [CgA] and neuron-specific enolase [NSE] levels) and clinical parameters (age at diagnosis, sex, primary tumor location, carcinoid syndrome, ENETS TNM classification (32,33), Ki-67 proliferation index, grading, prior incomplete surgery) in NET patients.

SUBJECTS AND METHODS

NET patients

Between March 2014 and March 2015, 28 Caucasian, non-obese, non-diabetic patients who were referred with a newly diagnosed neuroendocrine tumor were recruited. The diagnosis of a NET was based on biochemical, histological and clinical parameters. The primary NETs were originating from the small intestine, pancreas, stomach, lung, or had an unknown origin.

Patients were eligible for the study if they were medical treatment naive, were not obese, and had neither metabolic syndrome nor diabetes mellitus. Prior surgery with incomplete removal of the NET was permitted for inclusion into the study.

All NET patients gave written informed consent before inclusion in this study, which was approved by the Medical Ethics Committee of the Erasmus University MC in Rotterdam.

Controls

All patients with NETs were sex- and age-matched to 28 healthy Caucasian, non-obese, non-diabetic controls (within a range of ±3 years of age). Patients were paired with age-matched controls since a study in men found an age-dependent decline in AG concentrations (34). Controls were healthy volunteers from Erasmus MC, Rotterdam. All controls were recruited prospectively between January 2015 and March 2015.

Exclusion criteria for the controls were the following: past or existent malignancies; endocrine disorders including diabetes mellitus, acromegaly, Cushing syndrome; metabolic syndrome; any active use of glucocorticoids; active inflammatory or infectious disease; past gastric surgery, kidney, or liver function abnormalities; epilepsy; and psychiatric and eating disorders.

Controls gave their written informed consent before inclusion into the study.

Materials

Vacutainers were obtained from Becton Dickinson (Breda, Netherlands; cat# 367899; 6 mL K2 EDTA). 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride (Pefabloc, SC AEBSF) was purchased from Roche Applied Science (cat# 11429876001; Almere, Netherlands). Stock solutions of AEBSF were prepared in distilled water to give a final concentration of 200 mg/mL AEBSF.

Human AG and UAG are determined by a double-antibody sandwich technique. The enzyme immunoassay (EIA) kits were obtained from Bertin Pharma (Montigny-le-Bretonneux, France; A05106 and A05119, respectively).

Blood collection, AEBSF treatment, and storage

Overnight fasting venous blood samples for the measurement of plasma AG and plasma UAG were withdrawn and collected in EDTA tubes. One 6 mL EDTA tube per patient or control was collected.

Immediately after withdrawal, AEBSF (dilution 1:100) was directly added to all blood samples to prevent des-acylation of AG (3,35). Whole blood was carefully mixed by inversion and stored on water ice (4°C) until centrifugation at 2,500 g at 4°C for 5 minutes. Plasma of these venous blood samples was then rapidly aliquoted, four 1.5 mL Eppendorf tubes with 300 mL each. All plasma samples were stored at –80°C until the assay was performed. AEBSF was stored for a maximum of 1 month after dilution.

Acylated and unacylated ghrelin EIAs

After thawning on ice, plasma samples of all NET patients (N=28) and sex- and age-matched controls (N=28) were centrifuged for 1 minute at 1,500 g, 4°C and kept on ice before transferring to the assay plates. All samples were measured in duplicate (50 µL/well) according to the manufacturer's protocol (3).

A cubic polynomial fitting was used to determine concentrations from the calibration curves. This resulted in r^2 values >0.99 in the majority of the assays. The intra-assay percent coefficient of variation (%CVs) for AG was 5.8 and for UAG 1.8. Interassay %CVs for AG was 14.7 and for UAG 17.3.

Immunohistochemistry

Protein expression of total ghrelin was investigated in a small intestine NET tissue sample of one patient. Normal stomach tissue was used as a positive control. Mounted sections were deparaffinized, blocked for endogenous peroxidase activity, and rinsed with water. Following antigen retrieval, sections were incubated overnight at 4°C with the antiacylated ghrelin antiserum (N-terminal antibody [#404,4-4]; dilution 1:2,000), which was kindly provided by Professor Hiroshi Hosoda. Next, sections were incubated with Brightvision poly-HRP-Anti Ms/Rb/Rt IgG kit (Immunologic, Duiven, The Netherlands), and the peroxidase activity was developed with 0.07% 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Finally, sections were counterstained with hematoxylin.

Clinical and tumor characteristics

Clinical data of NET patients, including age at diagnosis, sex, primary tumor location, carcinoid syndrome, baseline serum CgA levels, baseline serum NSE levels, ENETS TNM classification, Ki-67 proliferation index, grading, and prior incomplete surgery, were collected from patient's medical records.

Statistical analysis

Analyses were performed using SPSS software (version 21 for Windows; SPSS) and GraphPad Prism® version 6.04 (GraphPad Software). Comparisons between NET patients and controls were analyzed by Wilcoxon signed-ranks tests, Mann-Whitney U tests, and Fisher's exact tests. Correlation analyses were done using Spearman's rank correlation test. The results are expressed as median±interquartile range (IQR). P-values of <0.05 were considered to be statistically significant.

RESULTS

Characteristics of the NET patients and their tumors are summarized in **Table 1**.

Patients with NETs were matched with their controls by age and sex. Patients had a median age of 57.5 years (IQR: 50.3–66.5) and controls 58.0 years (IQR: 50.0–64.0).

Plasma AG and UAG levels in NET patients (N=28) and their sex- and age-matched controls (N=28) are shown (**Figure 1**). Levels of plasma AG (**Figure 1A**) and plasma UAG (**Figure 1B**) showed a wide range in both patients and controls. Healthy controls had 'reference levels' of AG that ranged from 10 to 273 pg/mL and plasma UAG levels of 8–331 pg/mL.

Median plasma AG levels in NET patients and controls were 62.5 (IQR: 33.1–112.8) and 57.2 (IQR: 26.7–128.3, P=0.66), respectively (**Figure 1A**). Median plasma UAG levels in NET patients and controls were 76.6 (IQR: 35.2–121.7) and 64.9 (IQR: 27.5–93.1, P=0.44), respectively (**Figure 1B**). The highest observed AG level of 973.2 pg/mL and highest UAG level of 311.2 pg/mL were observed in the same patient with a small intestine NET.

The AG/UAG ratios in NET patients and the control population are provided (**Figure 1C**). The median AG/UAG ratios in NET patients were 1.1 (IQR: 0.7–1.6), which were not significantly different from the median AG/UAG ratios in controls (1.0, IQR: 0.7–2.0, P=0.86).

Figure 2A shows the primary tumor localization in NET patients and the distribution of plasma AG. Plasma UAG levels are shown in **Figure 2B**, and AG/UAG ratio in **Figure 2C**. All data are expressed as median±interquartile range (IQR).

In **Figure 3A**, tumor grading in NET patients and the distribution of plasma AG is shown. Plasma UAG levels and tumor grading are shown in **Figure 3B**, and the AG/UAG ratio in **Figure 3C**. All data are expressed as median±interquartile range (IQR).

There were no significant differences in plasma AG levels (P=0.60), plasma UAG levels (P=0.55), and AG/UAG ratio (P=0.53) between NET patients who did not undergo surgery and those who did undergo surgery with incomplete tumor removal.

We found no statistically significant correlations in NET patients between plasma AG, UAG, and AG/UAG ratios versus biochemical and clinical parameters with the exception

Table 1 Patient and tumor characteristics (N=28). Data are expressed as median±interquartile range (IQR).

Parameters	Total
Age at diagnosis (range)	56 (35–81)
Sex – male (%)	15 (53.6)
Primary tumor location	
Small intestine	19 (67.8)
Pancreas	3 (10.7)
Stomach	1 (3.6)
Lung	4 (14.3)
Unknown	1 (3.6)
Carcinoid syndrome – yes (%)	15 (53.6)
Baseline serum CgA (µg/L) (IQR)	155.5 (56.0–493.8)
Baseline serum NSE (µg/L) (IQR)	83.0 (24.0–398.3)
Mean 24 hours urinary 5-HIAA excretion/creatinine ratio (mmol/mol)	14.9 (12.4–22.8)
Disease stage (%)	
Stage I	1 (3.6)
Stage IIA	2 (7.1)
Stage IIIB	6 (21.4)
Stage IV	19 (67.9)
ENETS TNM classification (%)	
M0	11 (39.3)
M1	17 (60.7)
Ki-67 proliferation index (IQR)	3 (2.0–10.0)
Grading (%)	
G1	9 (32.1)
G2	17 (60.7)
G3	1 (3.6)
Unknown	1 (3.6)
Prior incomplete surgical resection – yes (%)	18 (64.3)

of AG and AG/UAG ratio versus age at diagnosis (ρ=–0.47, P=0.012; P=0.46; ρ=–0.50, P=0.007) and AG/AUG/ratio versus baseline CgA levels (P=0.88, P=0.15; ρ=–0.44, P=0.019): sex (P=0.46, P=0.41, P=0.41), primary tumor location (P=0.41, P=0.38, P=0.19), carcinoid syndrome (P=0.46, P=0.41, P=0.41), baseline serum NSE levels (P=0.47, P=0.36, P=0.10), ENETS TNM classification (P=0.39, P=0.39, P=0.39), Ki-67 proliferation index

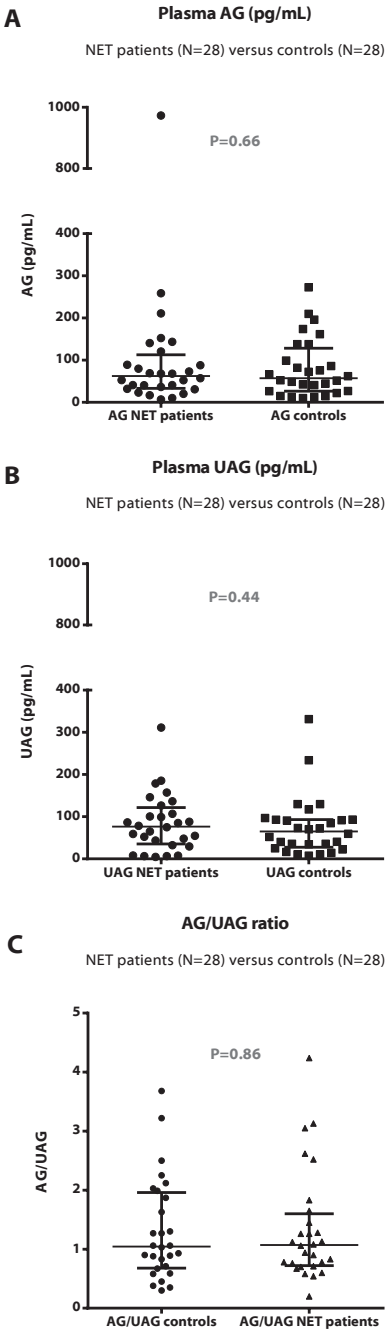

Figure 1 Plasma acylated ghrelin (AG), plasma unacylated ghrelin (UAG), and acylated ghrelin/unacylated ghrelin (AG/UAG) ratio in Caucasian, non-obese, non-diabetic NET patients (N=28) versus sex- and age-matched healthy Caucasian, non-obese, non-diabetic controls (N=28). Data are expressed as median±interquartile range (IQR).

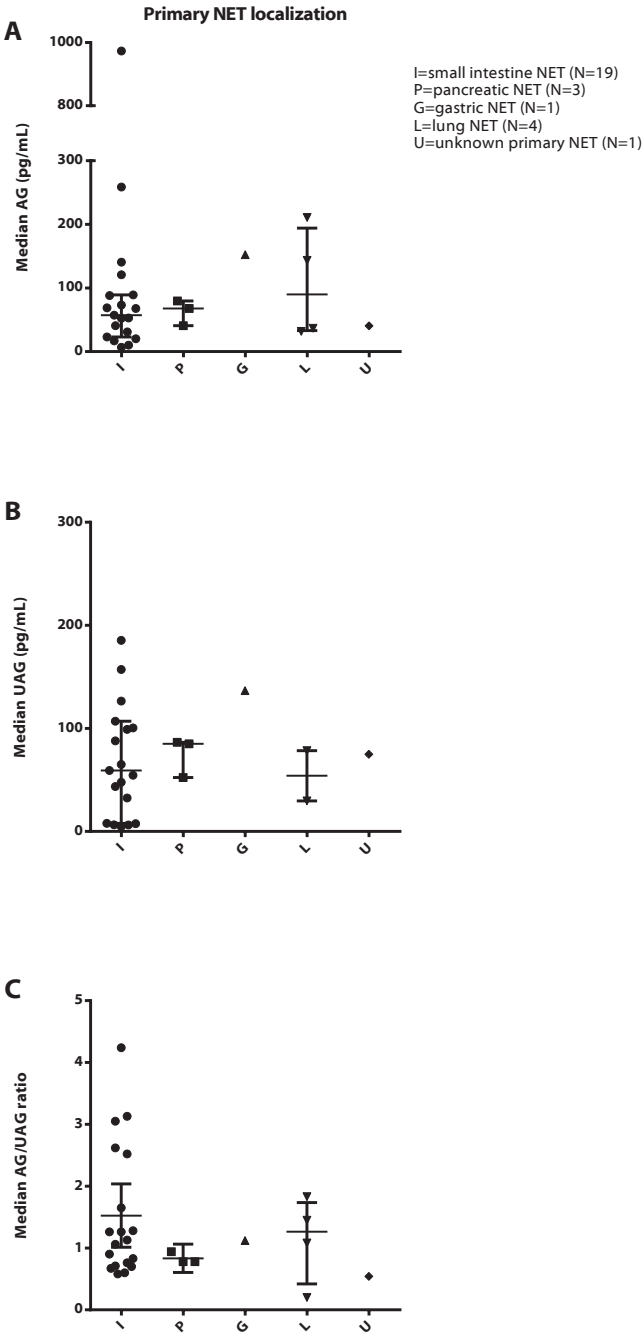

Figure 2 Primary tumor localization in patients with neuroendocrine tumors (NETs), and the distribution of plasma acylated ghrelin (AG), plasma unacylated ghrelin (UAG), and acylated ghrelin/unacylated ghrelin (AG/UAG) ratio. Data are expressed as median±interquartile range (IQR).

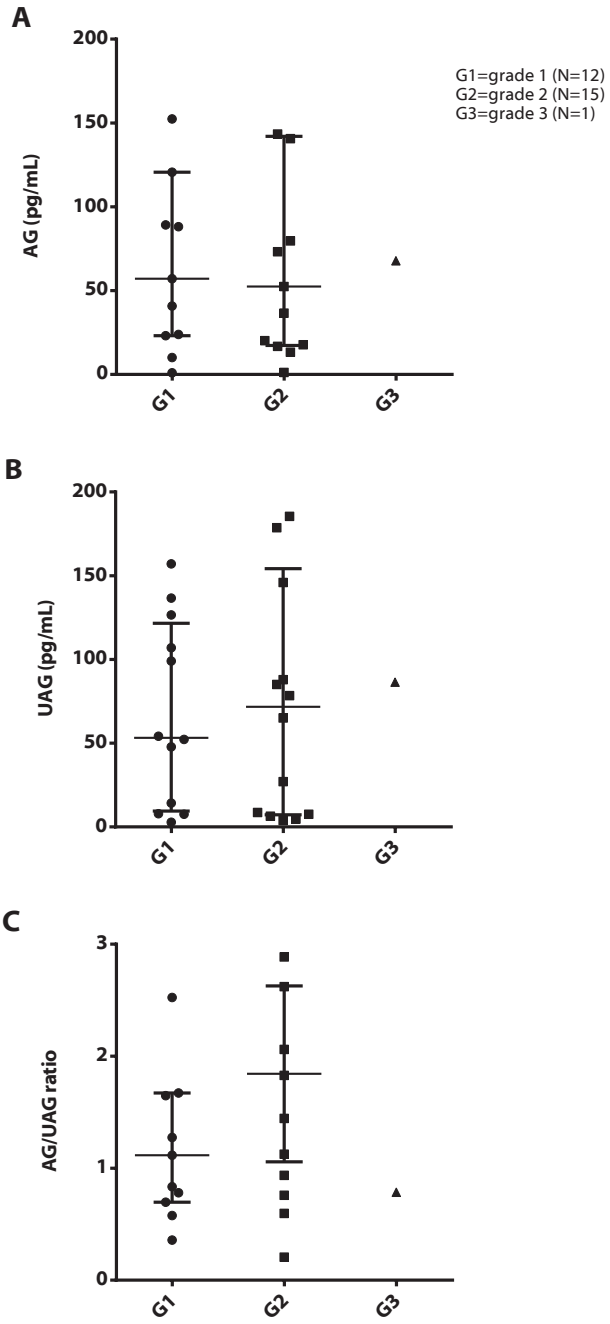

Figure 3 Plasma acylated ghrelin (AG) and plasma unacylated ghrelin (UAG) in Caucasian, non-obese, non-diabetic NET patients (N=28) distributed according to tumor grading. Data are expressed as median±interquartile range (IQR).

(P=0.92, P=0.99, P=0.65), grading (P=0.40, P=0.40, P=0.86), prior incomplete surgery (P=0.47, P=0.41, P=0.42).

Immunohistochemistry was performed on a small intestine NET tissue sample of the patient with the highest plasma AG and UAG levels. Staining showed no immunoreactive NET cells for ghrelin (**Figure 4**).

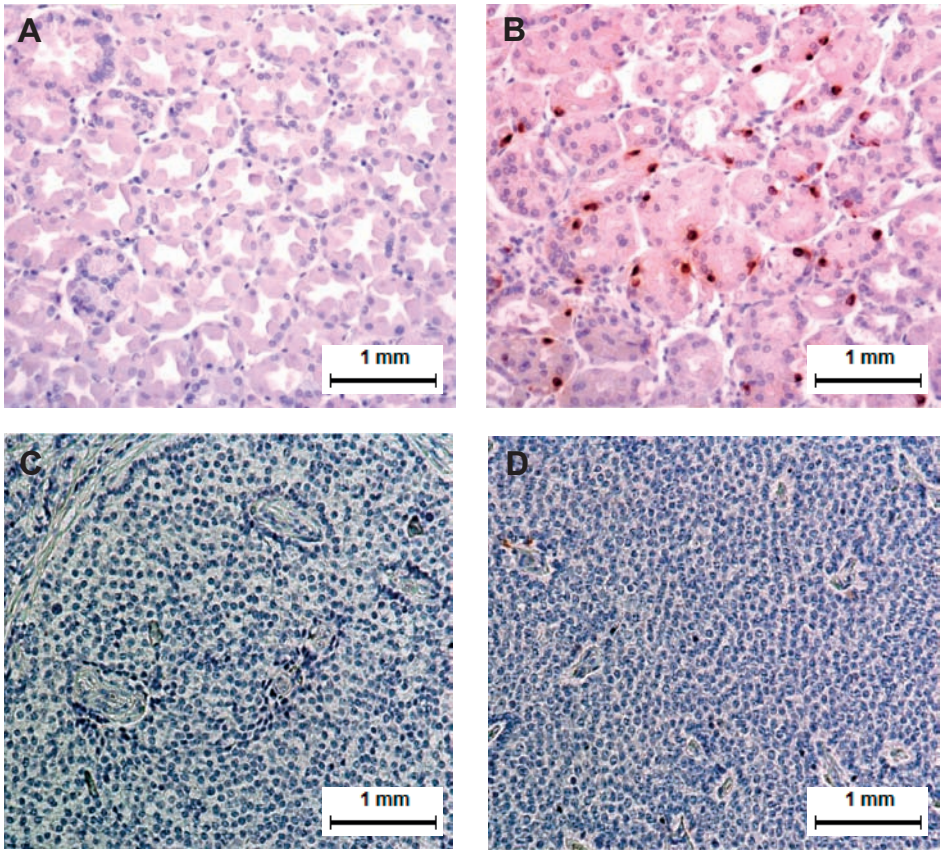

Figure 4 Immunohistochemistry: section of normal stomach tissue (panel A, magnification 10×), immunohistochemical staining of ghrelin on normal stomach tissue (panel B, magnification 10×), section of small intestine NET tissue (panel C, magnification 20×), and immunohistochemical staining of ghrelin small intestine NET tissue (panel D, magnification 20×). The scale bar is set on 1 mm.

DISCUSSION

This is the first study in which we measured fasting plasma acylated and unacylated ghrelin levels in a series of Caucasian, non-obese, non-diabetic NET patients and healthy sex- and age-matched Caucasian, non-obese, non-diabetic controls, using a sensitive assay.

Recently, we published the results of a non-interventional study in which we compared two different, commercially available, ELISA formats of AG and UAG in venous plasma stabilized or not with 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF) and stored for 0-6 months at -20°C or -80°C (3). We observed that when measured in AEBSF-stabilized plasma, the AG/UAG ratio is markedly higher than previously described and that UAG is a physiological component of the circulation. This highlights the importance of immediately stabilizing blood samples on collection for determination of both AG and UAG concentrations and provides a valuable tool for their measurement in physiological and interventional studies (3).

Trivedi and co-workers (36) showed that AEBSF may inhibit acetylcholinesterase (AChE) activity from the kits and thus may change AG levels. However, this problem of AEBSF suppressing AChE activity is circumvented using the applied processing method and Bertin Pharma EIA kits described by Delhanty and co-workers (3). Therefore, it is unlikely that because of technical issues, AG and UAG overlap and have almost the same reference range in both controls and NET groups.

Using a AEBSF-stabilized assay, our results are of interest as we found no significant differences in plasma AG levels and plasma UAG levels between NET patients and their matched controls. Therefore, we are quite certain that fasting plasma AG and UAG levels are no suitable diagnostic biomarkers in patients with NETs. Although the study was performed with a relatively small group of NET patients, we suspect that lack of power does not play a major role in our study.

We have also shown in this paper that incorrect processing of blood samples leads to raised UAG levels likely through conversion of AG to UAG. Also, we get roughly similar low levels of AG and UAG, and AG/UAG ratios to that described by Tong and co-workers (37) using a different two-site sandwich ELISA that does not use AChE as an indicator.

We observed in our series of NET patients significant negative correlations of AG and the AG/UAG ratio versus age at diagnosis. These data are in accordance with the observation of an age-dependent decline of AG concentrations found in men by Nass and co-workers. Additionally, we detected in NET patients a significant negative correlation of the AG/AUG ratio versus baseline CgA levels. However, these AG/UAG ratios were not significantly different between patients and controls. Our hypothesis is that AG/UAG ratios reflect a favorable metabolic status for NETs. AG/UAG ratio were not significantly

different between users of proton pump inhibitors (PPIs) and patients not taking PPIs (P=0.42).

Healthy controls had 'reference levels' of AG that ranged from 10 to 273 pg/mL and plasma UAG levels of 8–331 pg/mL. This is not an unusual finding in fasted subjects. For example, Liu and co-workers (4) find a range of 43-366 pg/mL for AG in four volunteers using a similar method of blood sample stabilization.

In one NET patient, the fasting plasma AG and UAG levels of 973.2 pg/mL and 311.2 pg/mL, respectively, exceeded the control 'reference values' of plasma AG and plasma UAG.

This particular 37-year-old male patient is the first case report of hyperghrelinemia associated by a small intestinal NET. He has a stage IIA, grade 2 NET that was found accidently after a short period of abdominal pain and fever. At the time of referral, he had no other clinical symptoms. Laboratory examination showed a normal baseline CgA level and normal 24-hours urinary 5-hydroxyindoleacetic acid (5-HIAA) excretion, but an elevated baseline NSE level (21.1 µg/L, maximum reference value: 16.2 µg/L). IHC on his small intestinal NET biopsy showed no immunoreactivity for ghrelin, however. This finding suggests that the NET does not seem to be directly responsible for the hyperghrelinemia. Although immunohistochemical staining on a non-representative incidentally ghrelin-negative tumor area from a tumor with ghrelin in other areas could not be excluded.

In the literature, only 11 NET patients with elevated total and/or acylated ghrelin levels have been described (23-27). Of these patients, only one patient had clinical symptoms of hypersecretion of vasoactive products by the NET. This particular patient was especially suffering from diarrhea, tiredness, and anemia, and he developed diabetes mellitus as well.

According to the authors, these symptoms were caused by the patient's ghrelinoma. However, overproduction of other more common hormones including gastrin, glucagon, vasoactive intestinal peptide, and 5-hydroxyindoleacetic acid, which could explain the signs and symptoms were not excluded. Therefore, we could not confirm that ghrelin overproduction is of clinical importance and a 'ghrelinoma syndrome' probably does not exist. This is based on our finding that plasma AG and UAG levels are not significantly different between sex- and age-matched healthy individuals and NET patients. In addition, we found no significant correlations between plasma AG and UAG levels versus biochemical and clinical parameters. We suggest that the elevated AG and UAG levels in the particular NET patient were caused by non-tumoral-related processes in which ghrelin plays a role.

In conclusion, we observed that, using a sensitive AEBSF-stabilized ghrelin assay, fasting plasma AG and UAG are useless as clinical diagnostic biomarkers in patients with a NET.

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Chromogranin A, Ki-67 index and IGF-related genes in patients with neuroendocrine tumors

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ABSTRACT

Introduction: Chromogranin A (CgA) and the Ki-67 proliferation index are considered as important biochemical and pathological markers for clinical behavior of gastroenteropancreatic neuroendocrine tumors (GEP NETs), respectively. The IGF system has been suggested as an important regulator of GEP NET proliferation and differentiation. A possible relationship between serum CgA (sCgA), Ki-67 proliferation index, and expression of IGF-related genes in patients with GEP NETs has not been demonstrated yet.

Objectives: This study investigates the relationship between sCgA, the Ki-67 proliferation index, and the expression of IGF-related genes in GEP NET tissues and their relation with 5-year survival.

Methods: Tumor and blood samples from 22 GEP NET patients were studied. Tumoral mRNA expression of IGF-related genes (IGFs: IGF1, IGF2; IGF receptors: IGF1R, IGF2R; insulin receptors: subtype A [IR-A] and B [IR-B]; IGF-binding proteins [IGFBPs]: IGFBP1, IGFBP2, IGFBP3 and IGFBP6) was measured using quantitative RT-PCR. Ki-67 proliferation index was determined using immunohistochemistry.

Results: sCgA was measured with ELISA. Five-year survival in patients with non-elevated sCgA (N=11) was 91 versus 46% in patients with elevated sCgA (N=11) (P=0.006). IR-A mRNA expression was significantly higher in tumors obtained from patients with elevated sCgA than in those from patients with non-elevated sCgA (6.42±2.08 versus 2.60±0.40; P=0.04).

Conclusions: This data suggests that sCgA correlates well with 5-year survival of GEP NET patients, and that IR-A mRNA expression correlates well with tumor mass in GEP NET patients.

INTRODUCTION

Gastroenteropancreatic neuroendocrine tumors (GEP NETs) are rare and heterogeneous tumors which may vary according to their biological, functional and clinical behavior (1). Chromogranin A (CgA) and the Ki-67 proliferation index are considered as important biochemical and pathological markers, respectively, for GEP NET clinical behavior. The insulin-like growth factor (IGF) system has been suggested as an important regulator of GEP NET proliferation and differentiation (2). Up to present, a possible relationship between serum CgA (sCgA), the cellular expression of the Ki-67 protein, and the IGF-related genes has not been studied in GEP NETs.

Deregulation of the IGF system, a complex network involved in cell growth and metabolic functions in normal tissues and tumors, plays an important role in the pathophysiology of GEP NETs (2). The IGF system consists of different IGF-related genes: two ligands (IGF1 and IGF2), two IGF receptors (IGF1R and IGF2R), two insulin receptors (IR-A and IR-B), and six IGF-binding proteins (IGFBPs). Upon binding to the IGF1R and IR-A, IGFs predominantly generate mitogenic effects. Binding to IR-B predominantly exerts metabolic effects (3,4). Almost all IGFs are bound to one of the six high-affinity IGFBPs which all differ with regard to their IGF inhibiting and potentiating actions (4-7). The functions of IGFBP1, IGFBP2, IGFBP3, and IGFBP6 have been well-characterized (6).

The Ki-67 proliferation index is generally used for grading of NETs (8-10). The ENETS/AJCC/WHO 2010 grading system consists of three categories: Grade 1 (G1)=Ki-67 proliferation index ≤2%, G2=Ki-67 proliferation index 3-20%, and G3=Ki-67 proliferation index >20% (8,9,11,12). This grading system has been shown to have relevant prognostic consequences and has been used for decision making with regard to therapeutic options in GEP NET patients (13,14).

Another important characteristic of GEP NETs is the presence of the CgA protein. CgA is co-secreted by GEP NET cells in the bloodstream with other hormones or peptides (15). CgA is the best available circulating parameter in the follow-up of tumor mass in GEP NET patients (16).

The main aim of our research was to investigate relationships between sCgA levels in GEP NET patients, cellular Ki-67 proliferation index, and the mRNA expression of IGF-related genes in their GEP NET tissues and to correlate this with their 5-year survival.

SUBJECTS AND METHODS

Patients with a GEP NET and tissue samples

A total of 22 GEP NET tissue samples from 22 non-consecutive GEP NET patients were collected before the start of any non-surgical therapy. The diagnosis of GEP NET was

based on clinical, biochemical, radiological, and histopathological characteristics. After tumor excision or biopsy, these tissue samples were immediately frozen in liquid nitrogen and stored at –80°C. The other tissues were obtained from the Erasmus MC Tissue Bank. These specimens were stored according to a standard procedure (17).

All patients gave written informed consent before inclusion in the studies, which were approved by the Medical Ethics Committee of the Erasmus MC, Rotterdam.

Biochemical parameters

Blood samples for the determination of sCgA were obtained at the time of diagnosis of the GEP NET (baseline). The sCgA levels were measured using a commercially available ELISA method (CIS Bio International, Gif-sur-Yvette cedex, France; upper limit of normal [ULN] 94 µg/L).

'Non-elevated' sCgA was defined as ≤2× the ULN (≤188 µg/L), and 'elevated' sCgA was defined as >2× ULN (>188 µg/L). These definitions were based on a previous publication and were selected to maximally exclude other confounding factors which might have caused (slight) elevations of sCgA (18).

Ki-67 immunohistochemical staining

Immunohistochemical analysis for Ki-67 was performed on 4 µm thick paraffin-embedded tissue sections according to the standardized and optimized benchmark procedure (Benchmark Ultra, Ventana, Tucson, AZ, USA). Pretreatment was performed with CC1 buffer for 64 minutes at 97°C. Primary monoclonal mouse antibodies against Ki-67 (clone MIB1, 1:200 dilution; DAKO, Glostrup, Denmark) were incubated for 32 minutes at 36°C, and were detected by a high-sensitive detection kit (UltraView Universal DAB Detection kit).

The Ki-67 proliferation index in GEP NET samples was expressed as the percentage of Ki-67 immunopositive NET cells. The counting procedure was performed by three experienced investigators according to the published guidelines (8,9,12).

In addition, all GEP NET tissue samples were classified according to the ENETS/AJCC/WHO 2010 grading system: Grade 1 (G1)=Ki-67 proliferation index ≤2%, G2=Ki-67 proliferation index 3-20%, and G3=Ki-67 proliferation index >20% (8,9).

Real-time quantitative PCR

For mRNA expression experiments, total RNA of GEP NET tissues was isolated with the ready-to-use High Pure RNA Isolation Kit (Roche Diagnostics). The cDNA synthesis and real-time quantitative PCR (RT qPCR) were performed according to previously published methods (19). Sequences and concentrations of primer-probe sets for all above-mentioned genes are listed in the **Supplementary Table 1**, see section on supplementary data given at the end of this article. The relative expression of IGF-related genes was

calculated using the comparative threshold method, $2^{-\Delta C_t}$, after efficiency correction of target and reference gene transcripts (HPRT) (20,21).

Statistical analysis

Data were analyzed using SPSS software (version 17 for Windows; SPSS, Inc.). Comparative statistical evaluations were performed by Mann-Whitney U tests. Correlation analysis was accomplished using Spearman's rank correlation tests. Survival rates were calculated using the Kaplan-Meier method, and groups were compared using the log-rank test. Kaplan-Meier curves were plotted using overall survival data. The mRNA expression data are reported as mean±SEM.

RESULTS

Patient characteristics

Patient characteristics are shown in **Table 1**. Fifty percent of the patients had non-elevated sCgA levels (N=11) and the others had elevated sCgA (N=11) with median values of 121 and 894 µg/L respectively. As compared to the elevated sCgA group, there were more female patients in the non-elevated sCgA group, these patients were younger, their primary tumor origins were less often in the small intestine and less distant metastases were found.

Table 1 Characteristics of 22 GEP NET patients

	Non-elevated CgA (≤2× ULN)	Elevated CgA (>2× ULN)
Number of patients	11 (50%)	11 (50%)
Sex		
Male	3 (27%)	5 (45%)
Female	8 (73%)	6 (55%)
Age at first diagnosis		
Median (years)	50	59
Range (years)	21-70	47-65
Serum CgA		
Median (µg/L)	121	894
Range (µg/L)	7-176	246-350,800
Primary tumor origin		
Small intestine	7 (64%)	9 (82%)
Pancreas	4 (36%)	2 (18%)

Tumor characteristics

In the non-elevated sCgA group, there were four G1 and six G2 patients and, there was one G3 patient. In the elevated sCgA group there were eight G1 and three G2 patients (**Table 2**).

In the non-elevated sCgA group, four patients were classified as ENETS stage IIIB and the other seven patients were classified as ENETS stage IV. In the elevated sCgA group, two patients were classified as ENETS stage IIIB and the other nine patients were classified as ENETS stage IV.

Table 2 GEP NET tissue characteristics

	Non-elevated CgA (≤2× ULN)	Elevated CgA (>2× ULN)
Number of tissues	11 (50%)	11 (50%)
GEP NET tissue		
Primary	9 (82%)	7 (64%)
Small intestine	6	7
Pancreas	3	
Metastasis	2 (18%)	4 (36%)
Lymph node	1	1
Liver	1	3
TNM classification		
$T_3N_1M_0$	4 (36%)	2 (18%)
$T_1N_0M_1$		1 (9%)
$T_3N_1M_1$	7 (64%)	8 (73%)
Grading (Ki-67 index)		
G1 (≤2%)	4 (36%)	8 (73%)
G2 (3-20%)	6 (55%)	3 (27%)
G3 (>20%)	1 (9%)	

Five-year survival of GEP NET patients

In **Figure 1**, the 5-year survival of 22 GEP NET patients categorized according to non-elevated and elevated sCgA is shown. There was a significant shorter 5-year survival in the elevated sCgA group as compared to the non-elevated sCgA group (46 versus 91%; P=0.006).

In the elevated and non-elevated sCgA groups, no statistical significant correlations could be found between the mRNA expression levels of the different IGF-related genes

and 5-year survival. Also, no statistical significant correlation could be demonstrated between the Ki-67 proliferation index and the 5-year survival in these two groups (data not shown).

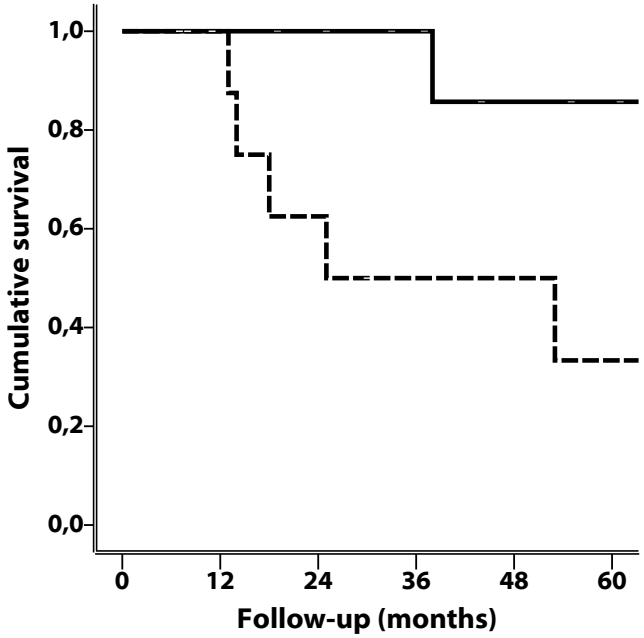

Figure 1 Five-year survival in 22 gastroenteropancreatic neuroendocrine tumor (GEP NET) patients divided according to those with non-elevated sCgA (≤2× ULN, N=11, solid line) versus elevated (>2× ULN, N=11, dashed line; P=0.006) sCgA.

Tumoral mRNA expression of IGF-related genes in GEP NET samples

In **Table 3**, the tumoral mRNA expression levels of IGF-related genes in the non-elevated and elevated sCgA groups are shown. There was a significant higher tumoral mRNA expression for IR-A in the elevated sCgA group compared with the non-elevated sCgA group (2.60±0.40 versus 6.42±2.08, P=0.04).

Correlation between IGF-related genes and Ki-67 proliferation index

No statistical significant relationship between the Ki-67 proliferation index and mRNA expression of IGF-related genes could be demonstrated (data not shown).

Table 3 Tumoral mRNA expression levels of different IGF-related genes in GEP NET tissue samples of patients with non-elevated and elevated sCgA levels

	Non-elevated sCgA	Elevated CgA	P-value
IGF1	0.89±0.32	0.41±0.13	0.30
IGF1R	0.26±0.08	0.19±0.04	0.70
IGF2	4.10±1.98	2.13±0.90	0.70
IGF2R	0.45±0.08	0.67±0.15	0.40
IR-A	2.60±0.40	6.42±2.08	0.04
IR-B	1.27±0.51	1.26±0.52	1.00
IGFBP1	1.50±1.42	1.93±1.17	0.08
IGFBP2	7.53±2.22	3.93±1.17	0.22
IGFBP3	3.15±1.07	5.40±1.94	0.61
IGFBP6	17.50±7.79	21.04±11.25	0.52

DISCUSSION

To our knowledge, this is the first study in which the relationship between sCgA levels, the tumoral Ki-67 proliferation index, and the tumoral expression of IGF-related genes has been evaluated in GEP NET patients.

Survival analysis showed a significantly shorter 5-year survival in patients with elevated sCgA levels compared with those with non-elevated sCgA levels. sCgA levels generally correlate well with tumor mass. These findings have already been confirmed by other groups (22,23).

In the elevated and non-elevated sCgA groups, no statistical significant correlations could be found between the mRNA expression levels of the different IGF-related genes and 5-year survival. Also, no statistical significant correlation could be demonstrated between the Ki-67 proliferation index and the 5-year survival in these two groups.

However, other studies have shown a significant shorter survival in grade 3 GEP NET patients (Ki-67 index >20%) (24,25). A possible explanation for our discrepant results could be the very small sample size of these heterogeneous tumor entities and the short follow-up.

Our study showed significant higher tumoral mRNA expression of the insulin receptor A (IR-A) in GEP NET patients with elevated sCgA compared with those patients with non-elevated sCgA. Increased expression of the IR-A, a mitogenic variant of the IR, is also

found in tumors arising in the colon, breast, thyroid, prostate, and fibrous tissues (26-32). Until present, these findings have not been reported for GEP NETs.

As sCgA levels correlate well with tumor bulk, our data therefore suggest that tumor mass correlates to tumoral IR-A expression in patients with GEP NETs.

No significant difference in tumoral mRNA expression levels was observed for all other IGF-related genes between patients with non-elevated versus patients with elevated sCgA. Although we have no obvious explanation for these findings, we suggest that IR-A expression has predominantly tumor-stimulating functions in more advanced tumors in contrast to other IGF-related genes, which are involved in the pathophysiology of GEP NETs regardless of the tumor stage.

In conclusion, our study could not demonstrate a relationship between IGF-related genes and the Ki-67 proliferation index in GEP NET tissues. We could confirm previous observations supporting a negative correlation between sCgA levels and 5-year survival. We could not demonstrate a relationship between the tumoral Ki-67 proliferation index and sCgA. However, our study results showed a relation between cellular IR-A mRNA expression and tumor mass.

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Supplementary Table 1 Overview with the used sequences of forward and reverse primers and, probes for IGF-related genes. Concentrations (nmol/L) used for primer-probe sets and, efficiencies are also listed.

Target gene	Sequence	Conc (nmol/L)	EFs
HPRT	Forward: 5′-CACTGGCAAAACAATGCAGACT-3′	500	1.91
	Reverse: 5′-GTCTGGCTTATATCCAACACTTCGT-3′	500	
	Probe: 5′-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3′	100	
IGF1	Forward: 5′-TTGTGATTTCTTGAAGGTGAAGATG-3′	300	1.92
	Reverse: 5′-CGTGGCAGAGCTGGTGAAG-3′	300	
	Probe: 5′-FAM-TACCTGGCGCTGTGCCTGCTCA-TAMRA-3′	200	
IGF1R	Forward: 5′-CCAAAACTGAAGCCGAGAAG-3′	300	1.85
	Reverse: 5′-GGGTCGGTGATGTTGTAGGT-3′	300	
	Probe: 5′-FAM-AAGCAGGAACACCACGGCCG-TAMRA-3′	200	
IGF2	Forward: 5′-CCAAGTCCGAGAGGGACGT-3′	300	1.98
	Reverse: 5′-TTGGAAGAACTTGCCCACG-3′	300	
	Probe: 5′-FAM-ACCGTGCTTCCGGACAACTTCCC-TAMRA-3′	200	
IGF2R	Forward: 5′-ACCGACCCCTCCACGC-3′	300	1.87
	Reverse: 5′-CCTCCAAGGCCACCTTCAG-3′	300	
	Probe: 5′-FAM-AGCAGTACGACCTCTCCAGTCTGGCAAA-TAMRA-3′	200	
IR-A	Forward: 5′-CGTTTGAGGATTACCTGCACAA-3′	300	1.86
	Reverse: 5′-GCCAAGGGACCTGCGTTT-3′	300	
	Probe: 5′-FAM- TGGTTTTCGTCCCCAGGCCATC -TAMRA-3′	100	
IR-B	Forward: 5′-CCCAGAAAAACCTCTTCAGGC-3′	300	1.92
	Reverse: 5′-GGACCTGCGTTTCCGAGA-3′	300	
	Probe: 5′-FAM-CTGGTGCCGAGGACCCTAGGCC-TAMRA-3′	200	
IGFBP1	Forward: 5′-CACAGGAGACATCAGGAGAAGAAA-3′	300	1.92
	Reverse: 5′-ACACTGTCTGCTGTGATAAAATCCA-3′	300	
	Probe: 5′-FAM-TCCAAATTTTACCTGCCAAACTGCAACAA-TAMRA-3′	200	
IGFBP2	Forward: 5′-GCCCTCTGGAGCACCTCTACT-3′	300	1.88
	Reverse: 5′-TCTTGCACTGTTTGAGGTTGTACA-3′	300	
	Probe: 5′-FAM-ACATCCCCAACTGTGACAAGCATGGC-TAMRA-3′	200	
IGFBP3	Forward: 5′-AGTCCAAGCGGGAGACAGAAT-3′	300	1.89
	Reverse: 5′-CACATTGAGGAACTTCAGGTGATT-3′	300	
	Probe: 5′-FAM-TGGTCCCTGCCGTAGAGAAATGGAAGA-TAMRA-3′	200	
IGFBP6	Forward: 5′-GGCCCATGCCGTAGACATC-3′	300	1.87
	Reverse: 5′-CTACCGGAAGCGGCAGTGCCG-3′	300	
	Probe: 5′-FAM-CTACGTGCCCAATTGTGACCATCGAG-TAMRA-3′	200	

Abbreviations: Conc=used concentration (nmol/L), EFs=efficiency factors

6

Is there an additional value of somatostatin receptor subtype 2a immunohistochemistry over somatostatin receptor scintigraphy uptake in predicting gastroenteropancreatic neuroendocrine tumor response?

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ABSTRACT

Introduction: It is unknown whether tumoral somatostatin receptor subtype 2a (sst_{2a}) immunohistochemistry (IHC) has additional value compared to somatostatin receptor scintigraphy (SRS) uptake using OctreoScan® in predicting response to peptide receptor radiotherapy using ^{177}Lu-octreotate (PRRT) in patients with gastroenteropancreatic neuroendocrine tumors (GEP NETs).

Objectives: Of this study were 1) to establish the percentage of sst_{2a} immunopositivity in GEP NET samples of PRRT-treated patients, 2) to determine the relationship between best GEP NET response using RECIST 1.0 criteria 1 year after PRRT and tumoral sst_{2a} IHC, and 3) to compare characteristics of patients with sst_{2a} IHC-negative and -positive tumors.

Methods: All 73 consecutive patients were selected for PRRT based on a positive SRS. Radiological response was scored according to RECIST 1.0 criteria. Sst_{2a} status was detected on tumor samples by IHC.

Results: In total, 93% of GEP NET samples showed sst_{2a} IHC-positivity. No statistically significant relationship was observed between *in vitro* sst_{2a} expression and *in vivo* best GEP NET response 1 year after PRRT (P=0.47). Sex, primary tumor site, disease stage, ENETS TNM classification, Ki-67 index, highest serum chromogranin A level, and highest neuron-specific enolase level were not significantly different between patients with negative and positive sst_{2a} tumoral IHC with the exception of age at diagnosis (P=0.007).

Conclusions: Sst_{2a} IHC on tumor samples has no additional value compared to SRS uptake using OctreoScan® in predicting tumor response after PRRT.

INTRODUCTION

The majority of gastroenteropancreatic neuroendocrine tumors (GEP NETs) express somatostatin (sst) receptor subtypes. The somatostatin receptor subtype 2a (sst_{2a}) is the most common receptor subtype in GEP NETs and is expressed in about 90% of these tumors (1-4). Sst_{2a} can be used both as a diagnostic and as a therapeutic target in patients with GEP NETs. Although sst_{2a} can be visualized *in vivo* by sst receptor scintigraphy (SRS) using ^{111}In-pentetreotide scintigraphy (OctreoScan®) and *in vitro* on tumor samples using immunohistochemistry (IHC), there is no consensus on how best to identify sst_{2a} expression on GEP NETs (5).

Peptide receptor radiotherapy with ^{177}Lu-octreotate (PRRT) is increasingly used for the palliative treatment of inoperable or metastasized GEP NETs expressing sst_{2a} (6-8). Prior to PRRT, uptake on the OctreoScan®, equal or higher than the liver uptake as judged from planar images (defined as Krenning scale 2-4), is generally required (9). Of all GEP NET patients who receive PRRT, about 80% achieved a partial response or stable disease (9). The rest of these patients developed progressive disease following PRRT (9). Therefore, it would be helpful if it was possible to determine which patients with GEP NETs will have a beneficial response prior to PRRT.

A strong and positive association between SRS uptake and *in vitro* sst receptor IHC in tumor samples was demonstrated in several studies (10-12). However, the potential superiority of IHC in demonstrating tumoral sst_{2a} expression compared to SRS uptake in predicting GEP NET response to PRRT has not been studied. Since IHC studies have shown that sst_{2a} expression could be demonstrated in approximately 50% of GEP NETs which were not visualized by SRS, sst_{2a} IHC could potentially have an advantage (5).

Therefore, we 1) established the percentage of sst_{2a} immunopositivity in GEP NET samples of patients who had been treated to PRRT, 2) studied the relationship between the best GEP NET response at 1 year after PRRT and tumoral sst_{2a} expression, and 3) compared 8 characteristics of GEP NET patients with negative and positive sst_{2a} IHC in tumors: sex, age at diagnosis, primary tumor site, disease stage, ENETS TNM classification (13,14), Ki-67 index, highest serum chromogranin A level, and highest neuron-specific enolase level.

The purpose of this study was to investigate the additional value of tumoral sst_{2a} expression using IHC in tumor samples compared to SRS uptake in predicting best GEP NET response to PRRT after 1 year.

MATERIALS AND METHODS

Patients with GEP NETs

We retrospectively recruited 73 GEP NET cases from our Erasmus MC NET database. All these patients were selected for PRRT based on the fact that they all had a positive SRS and Krenning scale 2-4 uptake. Patients were eligible if they had undergone a baseline CT scan and at least 2 follow-up CT scans at 6 six weeks and 3 or 12 months after 4 completed treatment cycles with PRRT. In addition, their tumoral sst_{2a} status was determined on GEP NET tissue samples, which were obtained prior to PRRT.

All GEP NET patients treated at the Erasmus MC, Rotterdam, gave written informed consent before inclusion in the PRRT study, which was approved by the Medical Ethics Committee of the Erasmus MC, Rotterdam.

Tumoral sst_{2a} expression

Formalin-fixed paraffin-embedded sections of 4 μm were cut and immunostained with primary rabbit monoclonal antibody directed against sst_{2a} according to the protocol provided by the manufacturer (Bio-Trend, clone SS-8000-RM, dilution 1:25, CC1 buffer, BenchMark Ultra strainers; Ventana Medical Systems, Tucson, Ariz., USA) for 64 minutes at 97°C. IHC was uniformly performed on all GEP NET samples obtained in the Erasmus MC, Rotterdam, between March 2000 and July 2013.

GEP NET tissue samples were obtained by surgical resection (N=36) or tumor biopsy (N=37). Normal human pancreatic tissues served as positive controls. Two investigators independently determined the immunoreactivity score (IRS) of the IHC stainings and were blinded to the tumor response results. The tumor samples were scored according to an intensity score (+1=weak staining; +2=intermediate staining; +3=strong staining) and proportion score (0=no positivity; +1=less than 1/3 tumor cell positivity; +2=1/3 to 2/3 tumor cell positivity; and +3=more than 2/3 tumor cell positivity). The sum of both scores was between 0 and 6 (15).

Tumor response

'Best GEP NET response at 1 year' was defined as the best tumor response that was confirmed at a second follow-up CT scan. This best GEP NET response was determined on 2 and, if available, on 3 CT scans that were evaluated at 6 weeks and 3 and/or 12 months after the fourth completed cycle of PRRT.

Target lesions were measured according to RECIST version 1.0 (16). Two trained investigators independently measured tumor response and scored all GEP NET responses; they were blinded to the IHC results. Discrepancies were resolved by a consensus review with a third expert.

Definition

SRS using Octreoscan® is a nuclear imaging method in which tumoral sst_{2a} expression is visualized using ^{111}In-pentetreotide scintigraphy.

Statistical analysis

Analyses were done with non-parametric tests using SPSS software (version 21 for Windows; SPSS Inc., Chicago, Ill., USA) and GraphPad Prism® version 6.04 (GraphPad Software, San Diego, Calif., USA). Comparative statistical evaluations between groups were done by one-way ANOVA, Fisher's exact tests, and independent-samples t tests. Overall survival was calculated using the Kaplan-Meier method, and groups were compared with the log-rank test. Univariate analysis was performed to study the relationship between the different patient characteristics and progressive disease. We judged values as significant at a P-value <0.05.

RESULTS

Sixty-eight patients (93%) with GEP NETs had positive sst_{2a} IHC in their tumor samples. In the tumor samples from the remaining 5 patients (7%), sst_{2a} IHC was negative.

The best GEP NET responses at 1 year after the last PRRT cycle are shown in **Table 1** for patients with positive and negative sst_{2a} IHC in their tumor samples. No significant relationship was observed between the *in vitro* sst_{2a} expression and the *in vivo* best GEP NET response 1 year after PRRT (P=0.47). For all GEP NETs and tumor samples, the best tumor response 1 year after PRRT and the corresponding IRS of tumoral sst_{2a} expression were determined (**Table 2**).

Table 1 Best GEP NET response 1 year after PRRT of patients with positive and negative sst_{2a} immunohistochemical tumor expression

Best GEP NET response at one year (%)	sst_{2a} IHC-positive tumors (N=68, 93%)	sst_{2a} IHC-negative tumors (N=5, 7%)
Partial response (PR)	8	0
Stable disease (SD)	55	4
Progressive disease (PD)	5	1

Table 2 Best GEP NET response 1 year after PRRT and corresponding IRS' of sst_{2a} IHC on tumor samples (N = 73)

	IRS							Total (%)
Best GEP NET response at one year (%)	0	1	2	3	4	5	6	
Partial response (PR)	0	0	1	0	0	4	3	8 (11)
Stable disease (SD)	4	0	1	13	19	17	5	59 (81)
Progressive disease (PD)	1	0	0	2	1	0	2	6 (8)
Total	5	0	2	15	20	21	10	73 (100)

In **Figure 1**, IRS' with the corresponding best GEP NET responses are illustrated. There was no significant difference between IRS and best GEP NET response 1 year after PRRT (P=0.14). The mean IRS of tumoral sst_{2a} expression for patients with partial response, stable disease, and progressive disease was 5±0.5, 3.9±0.2 and 3.7±0.9, respectively.

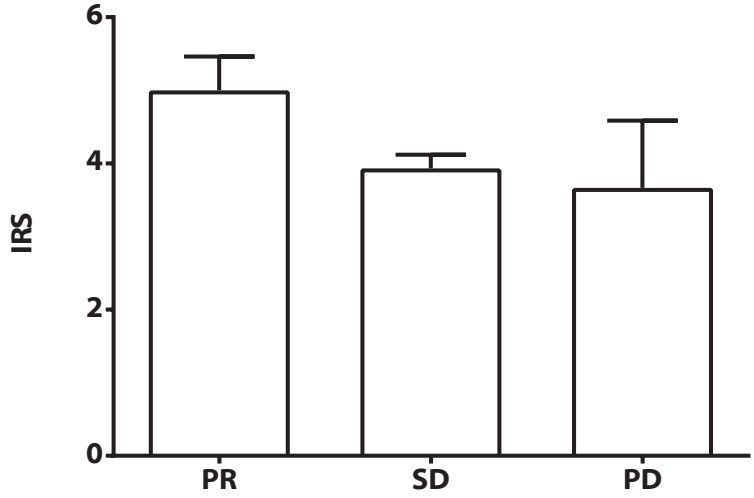

Figure 1 IRS' with corresponding best GEP NET response. Data are expressed as means±SEM. PR=partial response; SD=stable disease; PD=progressive disease.

There was no statistically significant difference in overall survival between patients with positive and negative sst_{2a} IHC in their tumor samples (P=0.91; **Figure 2**). Patient and tumor characteristics including sex, age at diagnosis, primary tumor site, disease stage, ENETS TNM classification, Ki-67 index, highest serum CgA level, and highest NSE level were compared between patients with sst_{2a} IHC-positive and -negative tumors. Patients with negative sst_{2a} IHC in their tumor samples had a significantly lower age at diagnosis (P=0.007) as compared to patients with positive sst_{2a} IHC. However, there were

no statistically significant differences for the other characteristics between patients with positive and negative sst_{2a} IHC. These results are shown in **Table 3**.

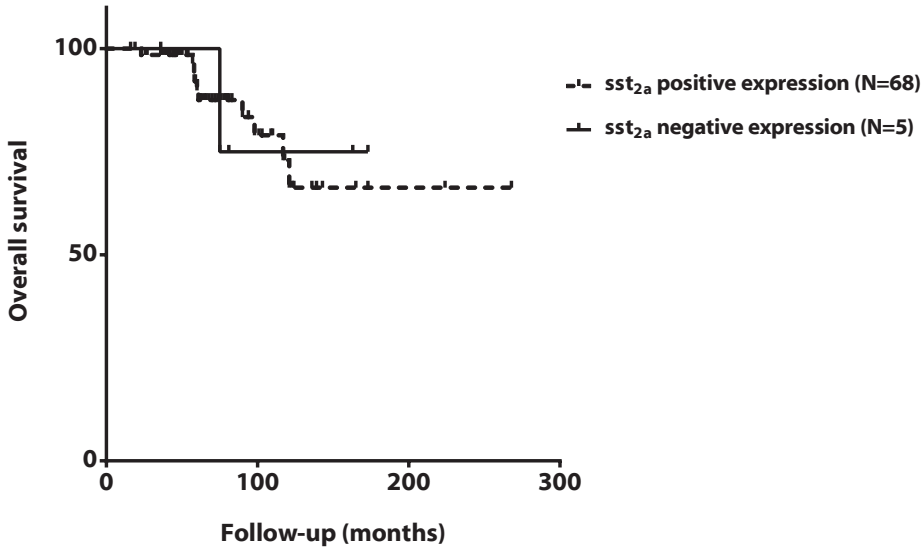

Figure 2 Overall survival of 73 patients with GEP NETs.

Five of 68 patients (7%) with positive sst_{2a} IHC in their tumor samples and 1 of 5 patients (20%) with negative sst_{2a} IHC in their tumor samples developed progressive disease. These percentages were not significantly different (P=0.27). The only characteristic which significantly predicted progressive disease after PRRT in our study group was an advanced disease stage (P=0.0001).

Table 3 Characteristics of GEP NET patients (N=73)

Parameters	Positive sst_{2a} IHC (N=68, 93%)	Negative sst_{2a} IHC (N=5, 7%)	P-value
Sex – male (%)	62	80	0.42
Age at diagnoses (range)	60.1±1.2 (30.7-77.9)	47.3±6.4 (32.2-64.2)	0.007
Primary tumor (%)			0.76
Intestine	31 (45.69)	1 (20.0)	
Pancreas	23 (33.8)	4 (80.0)	
Other	14 (20.6)	0 (0)	
Disease stage (%)			0.65
Stage IIIB	4 (5.95)	0 (0)	
Stage IIIB-IV	2 (2.9)	0 (0)	
Stage IV	62 (91.2)	5 (100)	
ENETS TNM classification (%)			0.58
M0	(5.9)	0 (0)	
M1	64 (94.1)	5 (100)	
Ki-67 index (%)	5.0±0.7	12.5±5.9	0.29
Grading (%)			0.56
G1	9 (13.2)	1 (20.0)	
G2	556 (80.9)	2 (40.0)	
G3	1 (1.5)	1 (20.0)	
Unknown	3 (4.4)	1 (20.0)	
Cumulative doses of PRRT (GBq)	29.8±0.4	30.0±0.7	0.18
Krenning Scale	3.2±0.1	3.2±0.2	0.91
Highest serum CgA level (µg/L)	3,509.2±1,289.5	2,195±1,148.0	0.79
Highest serum NSE level (µg/L)	69.0±18.6	175.6±105.4	0.37

DISCUSSION

We investigated whether there is an additional value of tumoral sst_{2a} IHC in tumor samples compared to SRS uptake in predicting GEP NET response to PRRT. Sst_{2a} immunopositivity was demonstrated in 93% of the tumor samples from GEP NET patients treated with PRRT. These data are in accordance with earlier published data (5). It was previously shown that 93% of the GEP NET patients with positive SRS had sst_{2a} or sst_5 expression in their tumors. However, 7% of the tumors which could be visualized using

SRS had no sst_{2a} expression using IHC (5). No statistically significant relationship was observed between tumoral sst_{2a} expression using IHC and best GEP NET response to PRRT at 1 year. In addition, there was no significant association between sst_{2a} expression using IHC and overall survival in our patient group. However, in other studies, positive sst_2 expression using IHC was associated with improved overall survival in patients with GEP NETs (17,18). Apparently, tumoral sst_{2a} expression is an independent predictor for survival but has no greater value than SRS in predicting GEP NET response to PRRT.

In our study, 5 patients (7%) received PRRT based on sufficient uptake on SRS but had negative sst_{2a} IHC in their tumor samples. Other research groups have also found that both methods have a high concordance rate in demonstrating sst_{2a} expression (5,19-21). Therefore, in countries with no accessibility to an OctreoScan®, IHC could be a useful method for the demonstration of tumoral sst_{2a} expression prior to PRRT.

Our group of 5 patients (7%) with sst_{2a}-negative IHC in their tumor samples all had in common that the tumor samples were tissue biopsies. In about half (N=36) of the patients with positive sst_{2a} IHC, these studies were performed on tumor biopsies. However, there could be a sampling error using biopsies from sst_{2a}-negative lesions in a patient presenting with both sst_{2a}-positive and -negative lesions. Alternatively, there could be a sampling problem using biopsies from non-representative incidentally sst_{2a}-negative tumor areas from tumors with abundant sst_{2a} expression in other areas.

Possible pitfalls in assessing sst_{2a} IHC *in vitro* could be the reliability of the sst_{2a} antibody, a suboptimal IHC procedure, and cross-activity with other antigens. In addition, tumoral sst_{2a} receptor levels in the whole tumor were based on IHC performed in only a slice of tumor tissue.

In a study in medullary thyroid carcinoma patients, no correlation could be demonstrated between tumoral sst expression on IHC and patients' age. In this series, about a half of the medullary thyroid carcinoma samples displayed sst_2-positive IHC (22). Another study showed no significant difference in median age between GEP NET patients with low versus high tumor uptake on SRS (23). Although we found a significantly lower age at diagnosis in patients with a negative tumoral sst_{2a} expression, this might be explained by the low number of patients in this group. Therefore, we believe that this observation was a chance finding and has no clinical significance.

In only one publication, GEP NET patients with sst_2-positive tumors on SRS who were treated with PRRT were subdivided into two subgroups: those with progressive disease versus those with no progressive disease (24). In this study, the only parameter which was significantly different between these two patient groups was baseline tumor progression. Unfortunately, this parameter was not investigated in our study.

In three different studies, the percentages of NETs that were sst_{2a} negative on SRS and sst_{2a} positive on IHC on tumor samples (sst_{2a}: IHC+/SRS–) varied between 12 and 33%. The percentages NETs that were sst_{2a} positive on SRS but sst_{2a} negative on IHC on tumor

samples (sst_{2a}: IHC–/SRS+) varied between 0 and 15% (10,25,26). Other sst receptor subtypes than sst_2 showed no significant relationship between IHC on tumor samples and *in vivo* SRS uptake on Octreoscan® (10,25,26). Since these receptor subtypes are less frequently expressed on GEP NETs as compared to sst_{2a}, we focused only on sst_{2a} IHC in our study (1-4).

About 10% of all SRS' for the localization and staging of GEP NETs can show false-positive uptake, mostly in non-tumor-related areas (27). Potential sources for this false-positive uptake on SRS are: thyroid disease, breast disease, granulomatous lung disease, inflammatory diseases like respiratory infections, recent operation sites, lymphomas, meningiomas, paragangliomas, and accessory spleens (28-32). None of these causes could explain SRS positivity in our 5 patients with negative tumoral sst_{2a} IHC.

The radioligands pentetreotide and octreotate, which are used for the Octreoscan® and PRRT, respectively, have the highest binding affinity for sst_{2a} but also bind with lower affinity to sst_3 and sst_5 (33). Theoretically, it is possible that SRS visualized sst_3 and/or sst_5 in the patients with negative tumoral sst_{2a} IHC.

In conclusion, we have shown that the assessment of tumoral sst_{2a} expression using IHC has no additional value compared to SRS using OctreoScan® in predicting the *in vivo* GEP NET response to PRRT. Ninety-three percent of our GEP NET patients with a positive SRS and Krenning scale 2-4 uptake prior to PRRT had a positive sst_{2a} tumor sample staining on IHC. We, therefore, suggest that if there is no accessibility to the OctreoScan®, sst_{2a} IHC in a tumor sample is also a suitable investigational tool for the selection of suitable patients for PRRT.

Further research is needed to investigate other potential markers that can predict best GEP NET response to PRRT.

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Effects of somatostatin analogs and dopamine agonists on insulin-like growth factor 2-induced insulin receptor isoform-A activation by gastroenteropancreatic neuroendocrine tumor cells

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ABSTRACT

Introduction: Gastroenteropancreatic neuroendocrine tumors (GEP NETs) express insulin-like growth factor (IGF)-related factors (IGF1, IGF2; insulin receptor [IR]-A, IR-B; IGF-binding protein [IGFBP]1–3) as well as somatostatin (sst) and dopamine receptor type 2 (D_2).

Objectives: To 1) compare mRNA expression of IGF-related factors in human pancreatic NET (panNET) cell lines with that in human GEP NETs to evaluate the usefulness of these cells as a model for studying the IGF system in GEP NETs, 2) determine whether panNET cells produce growth factors that activate IR-A, and 3) investigate whether somatostatin analogs (SSAs) and/or dopamine agonists (DAs) influence the production of these growth factors.

Methods: In panNET cells (BON1 and QGP1) and GEP NETs, mRNA expression of IGF-related factors was measured by quantitative real-time PCR. Effects of the SSAs octreotide and pasireotide (PAS), the DA cabergoline (CAB), and the dopastatin BIM-23A760 (all 100 nM) were evaluated at the IGF2 mRNA and protein level (by ELISA) and regarding IR-A bioactivity (by kinase receptor activation assay) in panNET cells.

Results: PanNET cells and GEP NETs had comparable expression profiles of IGF-related factors. Especially in BON1 cells, IGF2 and IR-A were most highly expressed. PAS+CAB inhibited IGF2 (–29.5±4.9%, $P<0.01$) and IGFBP3 (–20.0±4.0%, $P<0.01$) mRNA expression in BON1 cells. In BON1 cells, IGF2 protein secretion was significantly inhibited with BIM-23A760 (–23.7±3.8%). BON1- but not QGP1-conditioned medium stimulated IR-A bioactivity. In BON1 cells, IR-A bioactivity was inhibited by BIM-23A760 and PAS+CAB (–37.8±2.1% and –30.9±4.1%, respectively, $P<0.0001$).

Conclusions: 1) The BON1 cell line is a representative model for studying the IGF system in GEP NETs, 2) BON1 cells produce growth factors (IGF2) activating IR-A, and 3) combined sst and D_2 targeting with PAS+CAB and BIM-23A760 suppresses IGF2-induced IR-A activation.

INTRODUCTION

The insulin-like growth factor (IGF) system is considered to play an important role in gastroenteropancreatic neuroendocrine tumors (GEP NETs) (1-3). The IGF system is involved in cell metabolism, growth, differentiation and survival (4-6). Known proteins that are part of this IGF system include IGF1 and IGF2, IGF receptor 1 (IGF1R), IGF2R, insulin receptors (IR) isoform A (IR-A) and B (IR-B), and IGF-binding proteins 1, 2 and 3 (IGFBP1-3).

The tumor promoting role of IGF1, IGF2, and the IGF1R in cancer has previously been explored (7-9). IGFs can also exert their effects after binding to IR-A and IR-B. IR-A has mainly mitogenic effects and IR-B is involved in metabolic activities (7,9). We have recently shown that, compared to IGF1R and IR-B, IR-A was the most predominantly expressed receptor in GEP NETs (10). In addition, we have shown that BON1 pancreatic NET (panNET) cells produce growth factors (IGF2) that stimulate the IGF1R in an autocrine/paracrine manner (11). To the best of our knowledge, the functional role of IR-A has not been studied in this respect.

GEP NET cells also express somatostatin receptors (sst) and dopamine type 2 receptors (D_2), which are known to inhibit the secretion of many factors/hormones (12-14). Sst and D_2 are highly, but variably, expressed in most GEP NETs, and their expression may depend on the stage of tumor dedifferentiation (12-14). Of the sst, sst_{2a} is the most abundantly expressed subtype. Somatostatin analogs (SSA) such as octreotide (OCT) and lanreotide, which act primarily via sst_2, are used in the treatment of GEP NETs and were previously shown to control symptoms related to the overproduction of hormones and bioactive substances, and more recently to control tumor progression as well (15,16).

In theory, targeting sst and/or D_2 could result in lowering of the production of factors that interact with IR-A. Heterodimerization of sst and D_2 can result in receptors with an enhanced functional activity (17,18). As such, the combination of single-receptor ligands as dopamine agonists (DAs) and SSAs, and also somatostatin-dopamine (SS-DA) chimeric compounds, could have synergistic effects by targeting these co-expressed receptors in GEP NETs. Beneficial effects of chimeric compounds and multiligand SSAs were already shown in a subgroup of patients with NETs and growth hormone/prolactin-secreting pituitary adenomas (19-22). In one study, antiproliferative effects were observed in the small intestine NET (siNET) cell line KRJ1 after incubation with multiligand SSAs but not with SS-DA, because KRJ1 cells lack D_2 expression (23).

To the best of our knowledge, there are no studies in GEP NET cells in which the effect of targeting sst and D_2 on the production of IGF-related factors has been evaluated. The main aims of our study were: 1) to compare the expression of the IGF system in human panNET cells (BON1 and QGP1) and a series of GEP NET tissues, and to investigate in which aspect the human panNET cell models reflect the human IGF system in GEP NETs;

2) to evaluate whether panNET cells produce growth factors that are able to activate IR-A, and 3) to investigate whether SSAs and/or DAs can influence the production of these growth factors.

MATERIALS AND METHODS

Cell lines and culture conditions

For functional experiments, we used the human panNET cell lines BON1 and QGP1. The BON1 cell line that was established from a lymph node metastasis of a human functional panNET (24) and was a kind gift of Dr. C.M. Townsend (The University of Texas Medical Branch, Galveston, Tex., USA). The QGP1 cell line, which was derived from a pancreatic islet cell carcinoma, was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (25).

In kinase receptor activity (KIRA) bioassays, we utilized the human embryonic kidney (HEK) cell-line Flip-in™-293 from Invitrogen (Breda, The Netherlands), which was stably transfected with plasmids (pNTK2) containing a cDNA insert of the human IR-A gene, using Fugene® transfection reagens according to manufacturer's protocol (26). The IR-A plasmid was kindly provided by Axel Ullrich (Martinsried, Germany).

Cell lines were routinely cultured in 75 cm^2 cell culture flasks from Corning (Amsterdam, The Netherlands). BON1 cells were cultured in culture medium consisting of a 1:1 mixture of DMEM and F-12K medium, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.5 mg/L fungizone, and 2 mM L-glutamin. QGP1 cells were cultured in RPMI 1640 culture medium enriched with 10% FBS and 100 U/mL penicillin. HEK IR-A cells were grown in DMEM containing 10% FBS, 100 U/mL penicillin, 100 µg/L streptomycin, and 500 µg/mL geneticin from Invitrogen.

The cell lines were passaged weekly by trypsinization with trypsin/EDTA (0.05%/0.53 mM) and resuspended in medium. Trypan blue staining was used to assess cell viability, which always exceeded 95%. Before plating, cells were counted microscopically in a standard hemocytometer. Periodically, cells were confirmed as free of *Mycoplasma*. The cell culture conditions in the incubator were kept at a humidified atmosphere of 5% CO_2 at 37°C.

Cell experiments for mRNA expression and IR-A bioactivity (KIRA assay)

For all mRNA expression and IR-A bioactivity experiments, both panNET cell lines were seeded at a density of 100,000 cells/well in 12-multiwell culture plates (Corning). After 48 and 72 hours, the media were refreshed with serum-free medium.

In order to test whether growth factors produced by panNET cells could influence tyrosine kinase activity of IR-A, 72-hours conditioned medium of BON1 cells and QGP1

cells was collected. Since QGP1-conditioned medium showed no tyrosine kinase IR-A bioactivity, we did not further evaluate the effects of SSA/DA on this cell line. Therefore, all further experiments were performed with BON1 cells only.

BON1 cells were incubated for 72 hours without or with SSAs and/or DAs at a concentration of 100 nM. After 72 hours of incubation, supernatant of the cells was collected, stored at -20°C, and later used for IR-A bioassays. The same control and treated BON1 cells were used for total RNA isolation. The samples were stored at –20°C until analysis. The ability of BON1-secreted factors to stimulate IR-A phosphorylation was measured using an in-house IR-A KIRA assay according to a previously published method (27). Bioactivity was expressed relative to a standard curve of insulin. The treatment groups were tested in quadruplicate.

Test substances

Regarding SSAs, we tested OCT (Novartis Pharma AG, Basel, Switzerland) and the multi-receptor-binding SSA pasireotide (PAS), also known as SOM230 (28). PAS was a gift from Novartis. The DA used was cabergoline (CAB; Pharmacia-Pfizer, New York, N.Y., USA). The SS-DA chimeric compound BIM-23A760 was provided by Biomeasure Inc./IPSEN (Milford, Mass., USA). Cells were treated with either single drugs or with drug combinations, namely OCT+CAB and PAS+CAB. The sst and D_2 binding affinities of all compounds are listed in online **Supplementary Table 1** (for all online supplementary material, see www.karger.com/doi/10.1159/000444280) (22,28-33). Stock solutions of SSAs were prepared in 0.01 M acetic acid and 0.1% bovine serum albumin. CAB was dissolved in 70% ethanol. All stock solutions were aliquoted at concentrations of 10^{-4} M and stored at -20°C. For each experiment, fresh working solutions were diluted in serum-free medium.

GEP NET tissues

The diagnosis of a GEP NET was based on both clinical parameters and histology. Samples of GEP NETs were immediately frozen after surgery in liquid nitrogen and stored at –80°C until further analysis. Tissues obtained from the Erasmus MC (MC) Tissue Bank were stored according to a standardized protocol (34). Approval from the Medical Ethical Committee of the Erasmus MC, as well as informed consent to use the tumor tissues for research purposes, was obtained.

Quantitative real-time PCR

Total RNA of panNET cells and GEP NET samples was isolated to determine mRNA expression of the IGF-related factors according to the manufacturer's protocol with a High Pure Isolation Kit (Roche Diagnostics, The Netherlands).

Poly A^+ mRNA isolation for detection of sst and D_2 mRNA in panNET cells was performed according to a previously used method (35). Sequences and concentrations of

the primes and probes that were used have been described previously (10). The synthesis of cDNA and quantitative real-time PCR was conducted as previously described (36).

The used primer probe sets of all IGF-related factors, including their sequences and concentrations, have been previously published (10). Relative mRNA expression of IGF-related factors was calculated using the comparative threshold method after efficiency correction of target and reference gene (HPRT) transcripts (37,38). The tested compounds did not significantly change expression of HPRT after 72 hours of incubation (data not shown).

IGF2 protein assay

To test whether inhibition of IR-A activation could be clarified by modulation of IGF2 secretion, we used a 'two-step' sandwich-type immunoassay with a Non-Extraction IGF-II Enzyme-Linked Immunosorbent Assay Kit (DSL Germany GMBH-Benelux, Assendelft, The Netherlands). The assay was performed according to the protocol supplied by the manufacturer. Intra- and interassay coefficients of variability were 5.2 and 6.9%, respectively.

IGF2 immunohistochemistry

Expression of the IGF2 protein in GEP NET tissues was measured by immunohistochemistry (IHC) using a polyclonal goat antibody (1:500; Santa Cruz Biotechnology, Dallas, Tex., USA) as described previously (36). The immunoreactivity of IGF2-stained GEP NET tissues was interpreted in a semiquantitative manner and expressed as an immunoreactivity score (IRS) between 0 and 6 (39). The IGF2 staining and IRS counting procedure were done by 2 independent researchers, and any discrepancy was resolved by a consensus review.

Statistical analysis

For statistical analysis, GraphPad Prism® version 6.04 (GraphPad Software, San Diego, Calif., USA) was used. Comparative statistical evaluations between groups were accomplished with unpaired t tests and one-way ANOVA followed by Tukey's tests for multiple post hoc comparisons. Correlation analysis was performed using Spearman's rank correlation tests. Each drug condition of an experiment was tested in quadruplicate, with the exception of the IGF2 ELISA, which was done in triplicate. All experiments were carried out at least 2 times and gave comparable results. Outliers were excluded by Grubbs' test with the GraphPad QuickCalcs outlier calculator. Data are reported as means±SEM. In all analyses, a two-sided P-value of $P<0.05$ ($^{*}P<0.05$, $^{**}P<0.01$) was considered statistically significant.

RESULTS

Levels of mRNA expression of sst and D_2 in panNET cells

Figure 1A shows mRNA levels of sst and D_2 in BON1 cells. The sst subtypes were expressed in the following order: sst_5>sst_1>sst_2>sst_3 (0.57±0.093, 0.47±0.058, 0.081±0.011, and 0.036±0.0065). D_2 mRNA expression levels were 0.27±0.011. Of all receptors, sst_5 was expressed most highly.

In QGP1 cells, the order of expression was the same as in the BON1 cells, but the expression of sst_3 was not detectable (sst_5: 0.05±0.02; sst_1: 0.038±0.022; sst_2: 0.005±0; sst_3: not detectable). D_2 in the most highly expressed receptor in QGP1 cells, and is expressed at the same level as in BON1 cells (0.16±0.08) (**Figure 1B**).

mRNA expression of IGF-related factors in panNET cells

mRNA expression levels of the IGF-related factors were measured in BON1 (**Figure 1C**) and QGP1 cells (**Figure 1D**). The results are expressed as relative expression (normalized to HPRT). In BON1 cells, IGF2 was expressed at the highest level (292.8±34.60). BON1 cells expressed statistically significant higher mRNA levels of IGF2 than of IGF1 ($P<0.01$). Of the IGF-related receptors, IR-A had the highest mRNA expression level (0.27±0.016). IR-A was significantly more highly expressed (14.3-fold) than IR-B (0.27±0.016 versus 0.019±0.0016, $P<0.01$). In addition, IR-A was expressed at a higher level (1.2-fold) than IGF1R (0.27±0.016 versus 0.22±0.0093, $P<0.05$). No statistically significant difference of mRNA expression levels was observed between the IR-A and IGF2R (0.27±0.016 versus 0.24±0.019, $P>0.05$) or between IGF1R and IGF2R (0.22±0.0093 versus 0.24±0.019, $P>0.05$). With respect to IGFBPs, IGFBP2 (0.76±0.034) was expressed at the highest level, followed by IGFBP3 (0.35±0.023) and IGFBP1 (0.023±0.0019).

IGF-related factors were expressed in a relatively comparable pattern in QGP1 cells, but their expression levels were considerably lower than in BON1 cells. PanNET IGF2 (292.8±34.60 versus 0.26±0.15, $P=0.01$) and IR-A (0.27±0.016 versus 0.064±0.005, $P=0.0003$) were significantly more highly expressed in BON1 cells than in QGP1 cells. In addition, in QGP1 cells there was no detectable expression of IGFBP1 and IGFBP3.

mRNA expression of IGF-related factors in GEP NET tissues

The mRNA expression levels of IGF-related factors were investigated in primary human GEP NETs originating from the small intestine (N=18; **Figure 2A**: IGF-related receptors, **Figure 2C**: IGF-related proteins) and pancreas (N=7; **Figure 2B**: IGF-related receptors, **Figure 2D**: IGF-related proteins).

Expression data on a subset of these GEP NETs were previously reported (10). All genes were expressed in highly variable amounts. Of the IGFs, IGF2 was most highly expressed (siNET: 3.60±1.31; panNET: 1.05±0.56). IGF2 was expressed at a higher level than IGF1

(siNET: 3.60±1.31 versus 0.71±0.20; panNET: 1.05±0.56 versus 0.35±0.25). IR-A was the most prominently expressed IGF-related receptor in this series of GEP NET tissues; it was expressed at a higher level than IR-B (siNET: 4.34±0.69 versus 1.50±0.42; panNET: 1.87±0.41 versus 0.47±0.18) and IGF1R as well (siNET: 0.34±0.11; panNET: 0.12±0.06) (**Figure 2A, B**). Although the expression levels varied, overall siNETs and panNETs showed mRNA expression patterns of IGF-related factors comparable to those of the panNET cell lines. Results of logarithmic gene expression levels are shown.

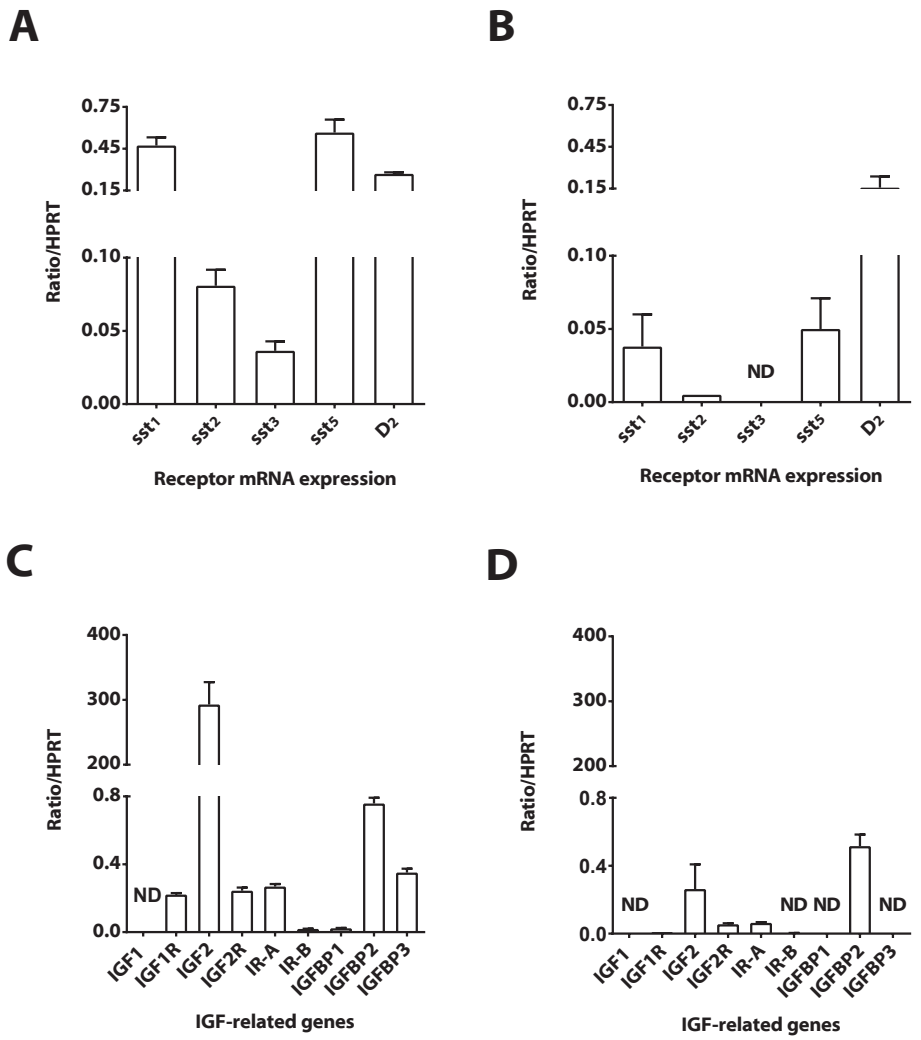


Figure 1 mRNA expression profiles of sst and D_2 (A, B) and IGF-related factors (C, D) in BON1 (A, C) and QGP1 cells (B, D), expressed as relative expression normalized to HPRT. For all samples: N=2. ND=Not detectable.

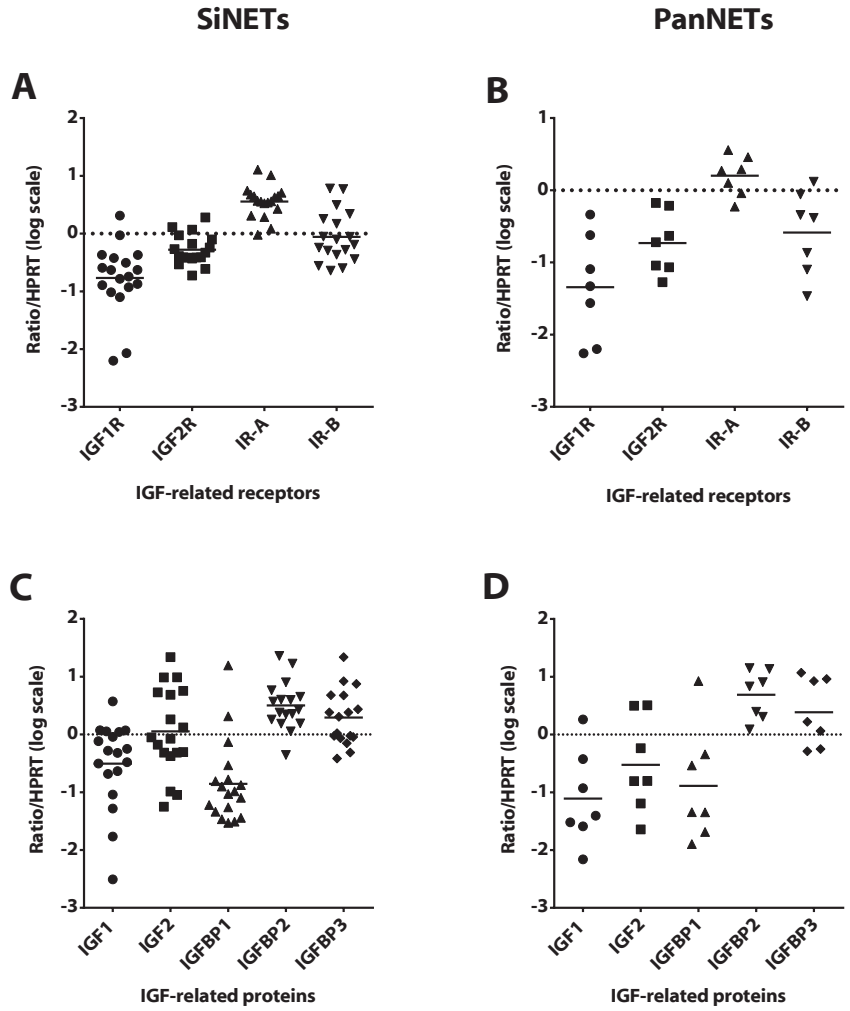


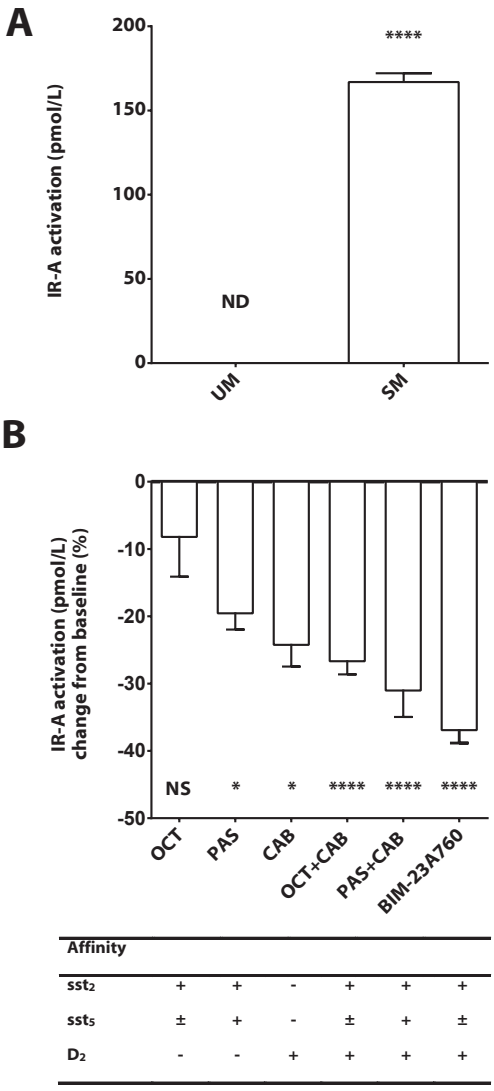
Figure 2 mRNA expression patterns of IGF-related receptors (A, B) and proteins (C, D) in siNETs (A, C; N=18) and panNETs (B, D; N=7), expressed as log relative expression normalized to HPRT. Horizontal bars represent means.

Receptor bioactivity after stimulation with serum-free conditioned BON1 cell medium

Conditioned medium of BON1 cells stimulated IR-A bioactivity, whereas control, unconditioned medium did not (**Figure 3A**). Conditioned medium of QGP1 cells did not show any detectable bioactivity of IR-A (data not shown).

In the IR-A KIRA assay, the strongest inhibition of BON1 conditioned medium-induced IR-A bioactivity was observed after treatment for 72 hours with the SS-DA chimera BIM-23A760 (-37.8±2.1%, P<0.0001). Other compounds or combinations of compounds

(all tested at 100 nM) that induced a statistically significant decrease in conditioned medium-induced IR-A bioactivity were: PAS+CAB (-30.9±4.1%, P<0.0001), OCT+CAB (-26.5±2.1%, P<0.01), CAB (-24.1±3.4%, P<0.05), and PAS (-19.4±2.6%, P<0.05). No statistically significant differences in IR-A bioactivity were observed after treatment with OCT (-8.0±6.1%). Data, expressed as percentage change from baseline of IR-A activation, are



Affinity						
sst_2	+	+	-	+	+	+
sst_5	±	+	-	±	+	±
D_2	-	-	+	+	+	+

Figure 3 A IR-A bioactivity of unconditioned BON1 cell medium (UM) and supernatant of conditioned BON1 cell medium (CM). ND=Not detectable. B IR-A bioactivity after treatment with single or combinations of compounds (all at concentrations of 100 nM), expressed as percentage change from baseline. *P<0.05, **** P<0.0001 versus control.

shown in **Figure 3B**. None of the compounds had a direct effect on IR-A phosphorylation (data not shown), indicating that the effects involved inhibition of the secretion of growth factors produced by BON1 cells. Under the conditions that we used, concentrations of IGF2 between 0.25 and 0.5 nM induced an IR-A activation comparable to that of BON1 conditioned medium (data not shown).

mRNA levels of IGF-related factors after treatment with SSAs and DAs

First, mRNA levels of IGF-related factors were measured after 6, 24, and 72 hours of treatment. After 72 hours, the mRNA data showed the most significant changes. Therefore, all experiments were accomplished at that time point. A significant decrease in IGF2 mRNA was observed after treatment with PAS+CAB (-29.5±4.9%, P<0.01). The results are shown in **Figure 4**. IGFBP3 expression as well was significantly decreased after treatment with PAS+CAB (-20.0±4.0%, P<0.01; data not shown). No other statistically significant effects on mRNA were found for the remaining IGF-related factors. None of the other drugs or combinations were able to modulate mRNA expression levels.

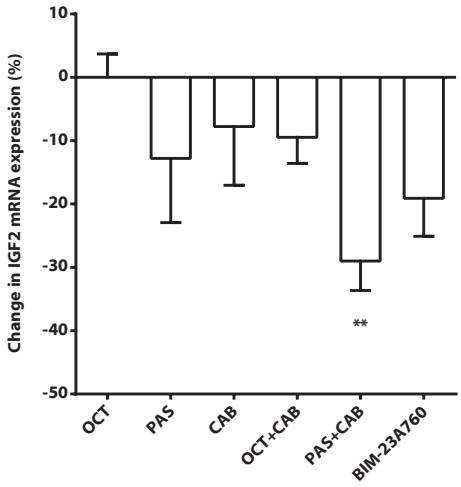


Figure 4 Change in mRNA expression of IGF2 after 72 hours of treatment with single or combinations of compounds in BON1 cells. **P<0.01 versus control.

IGF2 ELISA

Total IGF2 protein levels were quantitatively measured in conditioned medium of BON1 cells. A significant decrease in IGF2 protein levels was observed after 72 hours of incubation with BIM-23A760 (-23.7±3.8%). Inhibitory but statistically non-significant effects were observed with the other compounds (OCT: -12.5±5.3%; CAB: -12.1±4.8%; PAS+CAB: -9.8±8.2%; PAS: -12.0±3.3%; OCT+CAB: -5.7±12.4%). The results are shown in **Figure 5**.

IGF2 immunohistochemistry

IGF2 IHC was determined in 25 tissue samples of siNETs (N=18) and panNETs (N=7) in order to examine IGF2 protein expression. Most of the tumors expressed a significant amount of IGF2 protein, although its expression was variable. The intensity and proportion of the IGF2 IHC staining were heterogeneous in most of the GEP NET tissues. In online **Supplementary Table 2**, the IGF2 mRNA and protein expression levels of both siNETs and panNETs are listed. No significant correlation was observed between IGF2 mRNA and IGF2 protein in siNETs (ρ=0.17, P=0.49) and panNETs (ρ=0.44, P=0.33), expressed as IGF2 IRS. **Figure 6** shows exemplary photomicrographs of staining of IGF2 in GEP NET samples with a IRS of 2, 4 and 6, respectively. There was no statistically significant association between the proliferation marker Ki-67 and IGF2 IRS in siNETs (ρ=–0.09, P=0.79) and panNETs (ρ=0.89, P=0.11).

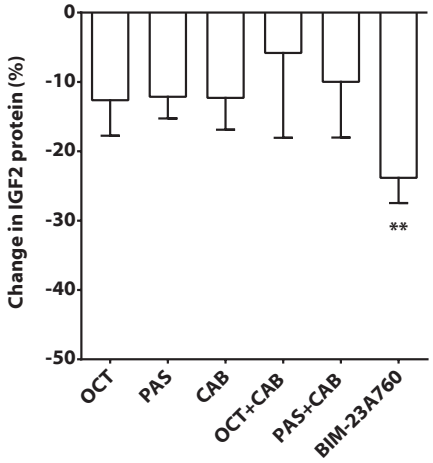


Figure 5 Change in IGF2 protein secretion in BON1 cells after 72 hours of treatment with single or combinations of compounds. **P<0.01 versus control.

DISCUSSION

To the best of our knowledge, this is the first study having gathered evidence that the human BON1 cell line is a model that reflects in many respects the typical characteristics of the IGF system in human GEP NETs. We showed that especially IGF2 and IR-A are expressed at high levels in our series of GEP NETs as well as in the BON1 cell model. In addition, we demonstrated that SSAs and DAs modulate the secretion of growth factors (e.g. IGF2) produced by BON1 cells that are capable of activating IR-A.

We measured mRNA levels of IGF-related factors (IGF1, IGF2, IGF1R, IGF2R, IR-A, IR-B, and IGFBP1-3) in both panNET cell lines and GEP NETs. In earlier publications, the expres-

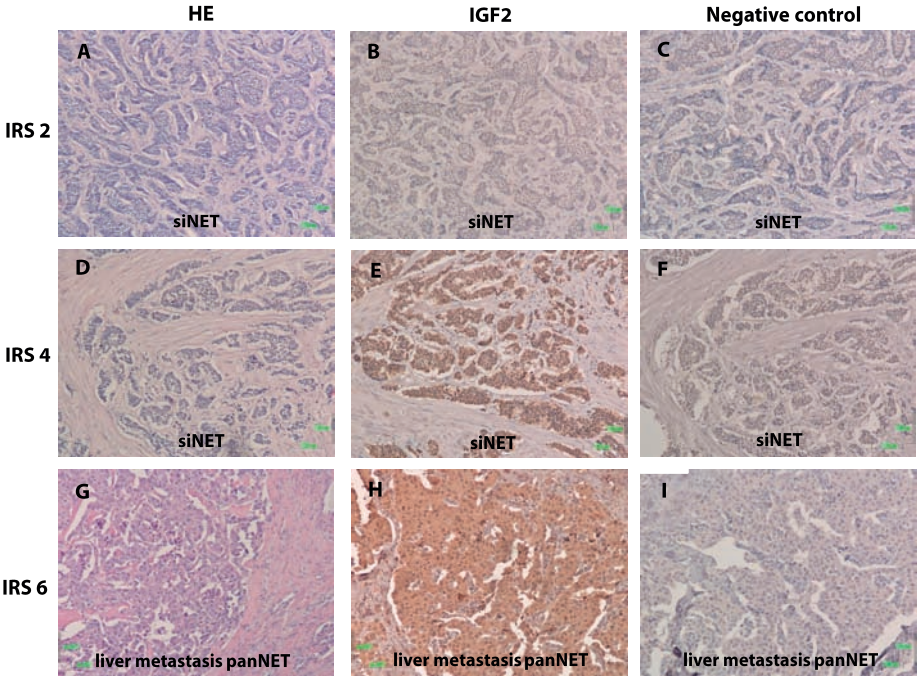


Figure 6 Exemplary cases of IGF2 IRS of GEP NET tissues. A–F siNETs (N=18). G–I panNETs (N=7). A, D, G HE. B, E, H IGF2. C, F, I Negative controls.

sion of these genes has been studied in human NETs (2,10). However, the quantitative expression of factors of the IGF system, and modulation of the expression of these growth factors (both at the mRNA and the protein level, during treatment with SSAs, DAs, or their combinations), have not been studied so far.

Overall, BON1 and QGP1 cells displayed an expression pattern of IGF-related factors which was relatively comparable to that of GEP NETs, both siNETs and panNETs. However, BON1 cells did not express detectable levels of IGF1, and QGP1 cells did not express detectable levels of IGF1, IGFBP1, and IGFBP3. In BON1 cells, IGF2 mRNA levels were expressed 1,000-fold more highly, and IR-A levels 5-fold more highly, than in QGP1 cells. These relatively low mRNA expression levels of IGF2 (and IR-A) in QGP1 cells may explain the absence of effects on conditioned medium of QGP1 on IR-A bioactivity. Therefore, the QGP1 cell line appears not a suitable model for investigating whether SSAs and/or DAs can modulate the production of these growth factors. On the other hand, both cell lines are panNET cell lines, and the difference between the cell lines might reflect the heterogeneity is this tumor group.

In both BON1 and QGP1 cells, we observed higher mRNA expression levels for sst_1 than for sst_2. The siNET cell line KRJ1 demonstrated equal mRNA expression levels for

sst_1 and sst_2 (3). In most studies where the quantitative mRNA expression levels of sst were studied, sst_2 was more highly expressed than sst_1 (40-44). In general, there is a predominant expression of sst_1 and sst_2 mRNA in NETs, with a highly variable mRNA expression levels (40,45). We suggest that these differential findings again represent the heterogeneity of these tumors. No sst/DA mRNA expression levels were determined in our series of GEP NETs, since expression profiles of these receptors have already been extensively investigated (40,42).

For all experiments, we used the concentration of 100 nM of OCT, PAS, CAB, and/or BIM-23A760. With this supraphysiological concentration, we expected to observe effects that could answer our primary research question, i.e. to investigate whether the different SSAs and/or DAs used were able to activate sst subtypes and D_2 resulting in a maximal biological response. At such a concentration, it is not fully possible to make statements about the specific involvement of individual sst subtypes in this context.

In a previous study, modulation of the IGF2/IGF1R autocrine loop was demonstrated in BON1 cells using neutralizing IGF2 antibodies (27). To assess IGF2-mediated activity of the IR-A, we used an IR-A KIRA bioassay developed in-house. In the current study, we focused on IR-A bioactivity, as stimulation of IR-A by IGF2 may play a role in signal transduction in tumorgenesis (7,9). With the IR-A bioassay, we found that stimulation of phosphorylation of tyrosine residues of the IR-A by conditioned medium of BON1 cells was as potent as a stimulation of 167 pM recombinant human insulin. As indicated above, a significant IR-A bioactivity of conditioned medium of QGP1 cells was not observed. This may be explained by the reduced IGF2 mRNA expression in QGP1 cells, which was approximately 1,000-fold lower than in BON1 cells.

Since BON1 cells only produce IGF2 but not IGF1, the most likely explanation for the observed IR-A activation in BON1 cells is the production of IGF2. After 72 hours of incubation, BON1 cells treated with PAS+CAB showed a significant decrease in IGF2 mRNA, while no effects were observed on mRNA expression after incubation with any of the other components. PAS and CAB monotherapy had less effect on mRNA expression. There was only a borderline reduction in IGF2 mRNA after treatment with BIM-23A760. Although sst_1 was the most highly expressed sst_1 subtype in our BON1 cell line, a 72-hours incubation of BON1 cells with BIM-23926 (sst_1 analog) did not result in a statistically significant change in IGF2 mRNA expression compared to untreated BON1 cells (+113.80±19.31%, P=0.52) (unpublished data). Apparently, targeting sst_1 is not effective in modulating IGF2 mRNA levels. Overall, these results suggest that the sst subtypes 2 and 5 and D_2 may play a role in modulating IGF2 mRNA levels.

BIM-23A760 treatment resulted in a significant decrease in secreted IGF2, while no effect was seen after any of the other treatments. Treatment with PAS+CAB or BIM-23A760 induced a significant decrease in IR-A bioactivity. In the IR-A KIRA bioassay, all compounds or combinations, except OCT, were able to suppress the activation of IR-A.

This result indicates that BON1 conditioned medium-induced IR-A activation can be modified by the (combined) activation of D_2 and sst subtypes 2 and 5. The absence of an effect of OCT may be explained by the very low sst_2 expression in BON1 cells.

While IGF2 expression has previously been demonstrated in GEP NET tissue at the mRNA level, there are no large studies that have evaluated IGF2 protein expression. In order to study whether IGF2 is also highly expressed in GEP NET tissues, we performed IHC. The GEP NET tumors also expressed IGF2 protein at a significant but variable level. Protein expression of sst has already previously been examined in GEP NET cells (46). No significant correlations were observed by IHC between IGF2 mRNA expression and IGF2 protein positivity in GEP NET tissues. Nonetheless, our study suggests that IGF2 is expressed at significant levels in almost all GEP NETs. No significant association between IGF2 IRS and Ki-67 index was found as well, which may be explained by the small sample size of our GEP NET series.

Although the BON1 and QGP1 cell lines are both originating from panNETs, discrepancies in results of experiments between the panNET cell lines indicate that these cell lines represent two different tumor subtypes, namely tumors with a low IGF2 production and panNETs with high levels of IGF2 secretion.

In conclusion, the human BON1 panNET cell line, and to a lesser extent the QGP1 cell line, appears to be a suitable model for studying the role of the IGF system in human panNETs. Of all the IGF-related factors, IGF2 and IR-A seem the most important players in human BON1 panNET cells and human GEP NETs. We found that most GEP NET tissues express IGF2 protein as well. In our hands, therapies with the combination of PAS+CAB or with the SS-DA chimeric compound BIM-23A760, which act through D_2 and sst subtypes 2 and 5, showed especially inhibitory effects on autocrine/paracrine (IGF2)-induced IR-A activation. Our study suggests that combinations of SSAs and DAs and/or chimeric SS-DA ligands are treatment options showing promise for the treatment of GEP NETs, and they should be in the focus of future research.

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Supplementary Table 1 Human somatostatin receptor (sst) and dopamine receptor subtype 2 (D_2) binding affinities of octreotide, pasireotide, cabergoline and BIM-23A760 (nM).

Compound	sst_1	sst_2	sst_3	sst_4	sst_5	D_2
Octreotide	280-1,140	0.38-0.6	7.1-34.5	>1,000	6.3-7	ND
Pasireotide	9.3	1.0	1.5	>100	0.16	ND
Cabergoline	ND	ND	ND	ND	ND	1.27
BIM-23A760	142-853	0.03-0.2	40-160	471->1,000	3.1-42	5-15

ND=not determined

References: octreotide (31, 47), pasireotide (28, 47), cabergoline (48), BIM-23A760 (49)

Supplementary Table 2 Insulin-like growth factor 2 (IGF2) mRNA levels (relative expression, normalized to HPRT) and corresponding IGF2 immunoreactivity score (IRS) in gastroenteropancreatic neuroendocrine tumors (GEP NETs, N=25) of which are small intestine (siNET, N=18) and pancreatic NETs (panNETs, N=7). ND=Not detectable.

Primary GEP NET	IGF2 mRNA	IGF2 IRS
SiNET (N=18)	0.06	2
	0.09	4
	0.42	4
	0.48	1
	0.48	6
	0.50	4
	0.67	4
	0.84	3
	0.89	4
	1.32	4
	1.37	3
	1.83	2
	4.86	5
	5.36	5
	5.69	5
	9.70	2
	9.74	5
	21.82	3
PanNET (N=7)	0.16	5
	0.16	2
	0.28	3
	0.31	4
	0.58	2
	3.15	4
	3.21	6

8

Differential effects of linsitinib and mTOR inhibitors on cell migration and proliferation of pancreatic neuroendocrine tumor cells

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ABSTRACT

Introduction: Effects of dual insulin-like growth factor 1 receptor (IGF1R)/insulin receptor (IR) kinase inhibitor linsitinib (LIN) and mammalian target of rapamycin (mTOR) inhibitors on cell proliferation and migration in pancreatic neuroendocrine tumors (panNETs) are unknown.

Objectives: 1) To study whether LIN (100 nM) and/or mTOR inhibitors everolimus (EVE 10 nM) and sirolimus (SIR 10 nM) can modulate panNET cell (BON1, QGP1) proliferation and migration and, 2) to investigate the underlying mechanism to exert panNET cell migration and proliferation via selective blockade of the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways with AKT1/2/3 inhibitor AZD5363 (AZ) and MEK1/2 inhibitor PD0325901 (PD).

Methods: Scratch assays were used to measure cell migration. DNA measurements were used as a measure of cell proliferation. Statistical analyses were performed by one-way ANOVA and Tukey's multiple comparison tests.

Results: Significant inhibition of panNET cell migration was observed after an 8-hours incubation with LIN ($P<0.0001$), not with mTOR inhibitors ($P>0.05$) in both panNET cell lines. Significant additive effects on panNET proliferation were observed after a 7-days incubation with LIN or EVE versus LIN+EVE and LIN+SIR, in QGP1 only ($P<0.0001$). AZ (100 nM) and PD (10 nM) significantly inhibited, also additive, migration ($P<0.0001$) but not proliferation in both cell lines.

Conclusions: 1) Linsitinib plus mTOR inhibition differentially affects cell migration and proliferation in panNETs cells. 2) Cross-talk between the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways is essential to accomplish panNET cell migration and proliferation.

INTRODUCTION

Pancreatic neuroendocrine tumors (panNETs) are historically considered to be relatively rare neoplasms. However, their incidence and prevalence is increasing (1). About half of the patients with panNETs present with liver metastases at initial examination or during the disease course (1,2). The development of metastases in panNETs is a complex process in which different steps are involved including cell proliferation and migration (3). Activation of growth factors such as the insulin-like growth factor 1 receptor (IGF1R) and/or insulin receptor (IR) may play a key role in proliferative and migratory processes. This activation results into downstream induction of the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway and the RAS/RAF/Mitogen-activated protein kinase/ERK kinase/extracellular-signal-regulated kinase (RAS/RAF/MEK/ERK) pathway (4-8). However, the exact contribution of these signaling pathways to the development of metastases remains unclear.

Nowadays, a commonly used first-line therapy for patients with progressive or symptomatic well- and moderately differentiated unresectable, locally advanced and metastasized panNETs is the mammalian target of rapamycin (mTOR) inhibitor everolimus (9,10). However, panNETs frequently escape everolimus therapy and patients will consequently develop progressive disease (11-13).

Recently, a potential role for the dual IGF1R/IR tyrosine kinase inhibitor linsitinib or 'OSI-906' has been established (14). Linsitinib has shown antiproliferative effects *in vitro* in different cell lines (14-18). In clinical trials, linsitinib has been used for several types of cancer (19-21). Currently, only one phase III trial on adrenocortical carcinomas has been completed but showed no significant increase in overall survival (19). However, preliminary data showed promising results with regard to tumor response and tolerability in advanced solid tumors and linsitinib might be a potential new drug for treatment of panNETs (14,20,21). To the best of our knowledge, effects of linsitinib, alone or combined with mTOR inhibitors, on panNET cell proliferation and migration have not been studied so far.

Co-targeting the PI3K/AKT/mTOR pathway and RAS/RAF/MEK/ERK pathway has already shown antiproliferative effects in castration-resistant prostate cancer and, head and neck squamous cell carcinomas tumors (22,23). Combined everolimus with a tyrosine kinase inhibitor (TKI) has demonstrated antitumor effects in neuroendocrine tumors (NETs) as well (24). Since mTOR inhibitors have already proven their antiproliferative effects on panNET, we conducted a study to examine whether the dual IGF1R/IR kinase inhibitor linisitinib has additive or synergistic effects when combined with the mTOR inhibitors everolimus and sirolimus in panNET cells.

Further aims of our study were: 1) to assess whether the dual IGF1R/IR kinase inhibitor linsitinib and/or mTOR inhibitors everolimus and rapamycin can modulate panNET cell

proliferation and cell migration in the human panNET cell lines BON1 and QGP1 and 2) to investigate the underlying mechanism of the effects of linsitinib and mTOR inhibitors on proliferation and panNET cell migration by selective blocking of the PI3K/AKT/mTOR pathway and RAS/RAF/MEK/ERK pathway, in both panNET cell lines, with the AKT1/2/3 inhibitor AZD5363 and MEK1/2 inhibitor PD0325901.

MATERIALS AND METHODS

Cell lines and culture conditions

We used two human panNET cell lines for our experiments: BON1 and QGP1. The BON1 cell line was a kind gift of Dr. C.M. Townsend (The University of Texas Medical Branch, Galveston, USA). QGP1 cells, originating from a pancreatic islet cell carcinoma, were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. Identity of both cell lines was confirmed using short tandem repeat profiling (25). PanNET cell lines were routinely cultured in 75 cm^2 cell culture flasks from Corning (Amsterdam, The Netherlands). BON1 cells were cultivated in culture medium consisting of a 1:1 mixture of DMEM and F-12K medium, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.5 mg/L fungizone, and 2 mmol/L L-glutamin. QGP1 cells were grown in RPMI 1640 culture medium containing 10% FBS and 100 U/mL penicillin from Invitrogen (Breda, The Netherlands).

Both cell lines were harvested weekly by trypsinization with trypsin (0.05%)-EDTA (0.53 mM) and resuspended in culture medium. Trypan blue staining was used to measure cell viability and always exceeded 95%. Cells were counted microscopically in a standard hemocytometer. Cell culture conditions in the incubator were kept at a humidified atmosphere of 5% CO_2 at 37°C.

In all experiments, seeded cells were plated in 10% FBS-enriched culture medium. After three days, this culture medium was replaced by FBS-free culture medium with 0.1% bovine serum albumin (BSA). Subsequently, all experiments were performed under these serum-free culture conditions.

Drugs and reagents

To test modulation of cell migration and cell proliferation in panNET cell lines, we used two groups of compounds including the IGF1R/IR tyrosine kinase inhibitor (TKI) linsitinib (LIN), also known as 'OSI-906', and the mTOR inhibitors everolimus (EVE) and sirolimus (SIR) (all from LC Laboratories Inc., Woburn, MA, USA). Both panNET cell lines were also treated with the drug combinations linisitinib+everolimus (LIN+EVE) and linisitinib+sirolimus (LIN+SIR) to investigate additive drug effects.

Stock solutions of the IGF1R/IR TKI and mTOR inhibitors were prepared in 40% dimethylsulfoxide (DMSO), aliquoted at concentrations of 1 mM and stored at -20°C. For each experiment, fresh working solutions were diluted in 40% of DMSO to (supra) pharmacological concentrations.

Tested concentrations were: LIN 1 nM and 100 nM, EVE 10 nM and SIR 10 nM.

Both panNET cell lines were also incubated with the phase II AKT1/2/3 inhibitor AZD5363 (AZ; Selleckchem, Huissen, The Netherlands) and phase II MEK 1/2 inhibitor PD0325901 (PD; Pharmacia Pfizer, New York, USA). In combination experiments IC_{50} concentrations of AZ (100 nM) and PD (10 nM) were used according to manufacturer's data (26,27).

Scratch assays

Scratch assays or 'wound healing' assays were used as model system in order to study panNET cell migration under conditions that more accurately mimic steps of the metastatic pathway. The *in vitro* cell migration was measured by a previously described scratch assay method with some modifications (28).

PanNET cells were seeded in 12-multiwell plates coated with poly-L-lysin (final concentration 10 µg/mL), respectively 700,000 cells per well and 1,300.000 cells per well for BON1 and QGP1 cells in 10% FBS-enriched culture medium. This culture medium was refreshed after 2 days. After 3 days, cells formed a uniform monolayer throughout the whole well. A sterile plastic 0-200 µL pipet tip was used to generate a homogeneous scratch in the shape of a cross in the cell monolayer. Peeled off cells were removed with three washes in FBS-free medium with 0.1% BSA. Subsequently, panNET cells were incubated for 8 hours with the test compounds in FBS-free culture medium with 0.1% BSA.

Cells that migrated into these scratched areas were evaluated on pictures made by camera (Canon PowerShot A640, zoom operation ×1.0) under light microscopy (Carl Zeiss 42616, magnification 5×). The scratch widths were measured with ImageJ Software (version 1.46j, National Institute of Health, USA). The ability of cells to migrate was measured after 8 hours. Scratch widths (mean at t=8 hours – mean t=0 hours) were compared to corresponding areas in control cells incubated with vehicle DMSO (final concentration 0.4%).

Cell proliferation assay

Effects of drugs were tested following 7 days of incubation. PanNET cells were plated in 24-multiwell culture plates in FBS-containing medium at 6,000 (BON1) cells and 20,000 (QGP1) cells per well, respectively. After 3 days, medium was refreshed and cells were incubated with the compounds in medium with 0.1% BSA for 3 days. After 3 days, medium and compounds were refreshed and cells were incubated for 4 additional days. Controls received vehicle only (0.4% DMSO). After 7 days, cells were collected for DNA measure-

ment. Measurements of total DNA content were determined with the bisbenzimide fluorescent dye (Hoechst 33258; Sigma-Aldrich, Zwijndrecht, The Netherlands) as previously described (29).

Statistical analysis

Data were analysed using SPSS software (version 17 for Windows; SPSS Inc., Chicago, Illinois) and GraphPad Prism Software (version 7.01; San Diego, California, USA). Comparative statistical evaluations between groups were done by one-way ANOVA, two-way ANOVA and Tukey's multiple comparison tests. Result were considered significant when $P<0.05^{*}$, $P<0.01^{**}$, $P<0.001^{***}$ and $P<0.0001^{****}$. Migration assays were performed three times and each assay was done in triplicate. Proliferation tests were done three times and each experiment executed in quadruplicate.

RESULTS

PanNET cell migration: dose-dependent effects of LIN, EVE and SIR

All compounds were tested in the concentrations: 1 nM, 10 nM, 100 nM and 1,000 nM. LIN showed dose-dependent effects in both panNET cell lines. Statistically significant inhibition of BON1 and QGP1 cell migration was already observed with LIN 1 nM and LIN 10 nM, respectively (**Figure 1A** and **1B**). Both mTOR inhibitors had no statistically significant effect on panNET cell migration.

LIN 1 nM and 100 nM are the concentrations to obtain 50% (IC_{50}) and maximal reduction in QGP1 cell migration respectively with LIN. In BON1 cells we tested with the same concentrations for comparability.

Dose-dependent effects of LIN, EVE and SIR on panNET cell proliferation

In both cell lines, cell proliferation was strongly and significantly inhibited after treatment with both mTOR inhibitors (**Figure 1C** and **1D**). These inhibitory effects on the panNET cell lines were dose-dependent and, stronger in higher pharmacological concentrations. However, only in higher pharmacological concentrations, LIN inhibited cell proliferation in BON1 cells as well, but not in QGP1 cells.

Additive effects on panNET cell proliferation and migration

Effects of LIN (100 nM) and EVE/SIR monotherapy (10 nM), as well as additive effects of LIN+EVE and LIN+SIR were compared on panNET cell proliferation and migration. Results are shown in **Figure 2A-D**. No significant additive effects on panNET cell migration were observed with LIN versus LIN+EVE and LIN+SIR in BON1 cells (**Figure 2A**) and QGP1

cells (**Figure 2B**). An 8 hours-incubation of LIN 1 nM versus LIN 1 nM+EVE 10 nM and LIN 1 nM+SIR 10 nM resulted in comparable result (data not shown).

For BON1 cells, no additive antiproliferative effects of combination therapy were observed compared to LIN or mTOR monotherapy (**Figure 2C**). Compared to LIN monotherapy (100 nM) and EVE (10 nM) alone, the combination of LIN 100 nM+EVE 10 nM and LIN 100 nM+SIR 10 nM resulted in a statistically significant additive reduction in proliferation in QGP1 cells (**Figure 2D**).

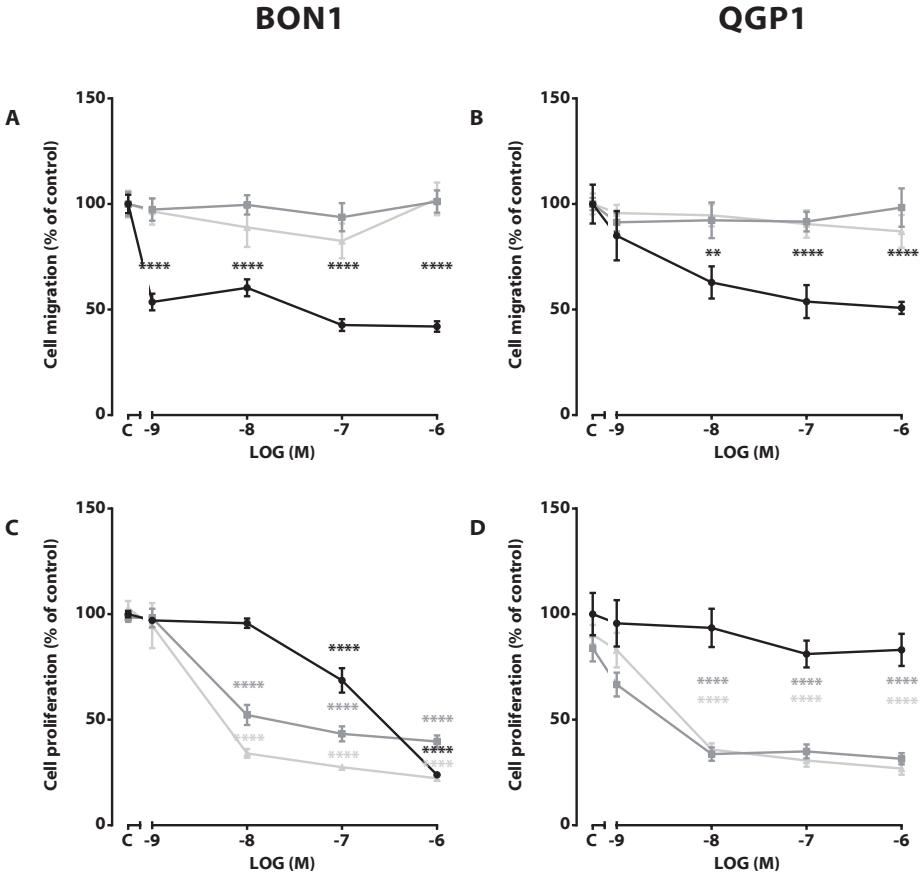

Figure 1 Dose-dependent effects for linsitinib (LIN), everolimus (EVE) and sirolimus (SIR) on pancreatic neuroendocrine tumor (panNET) cell migration (8-hours incubation) and proliferation (7 days of incubation): A BON1 cell migration, B QGP1 cell migration, C BON1 cell proliferation, D QGP1 cell proliferation. Results are significant when: $P<0.01^{**}$, and $P<0.0001^{****}$.

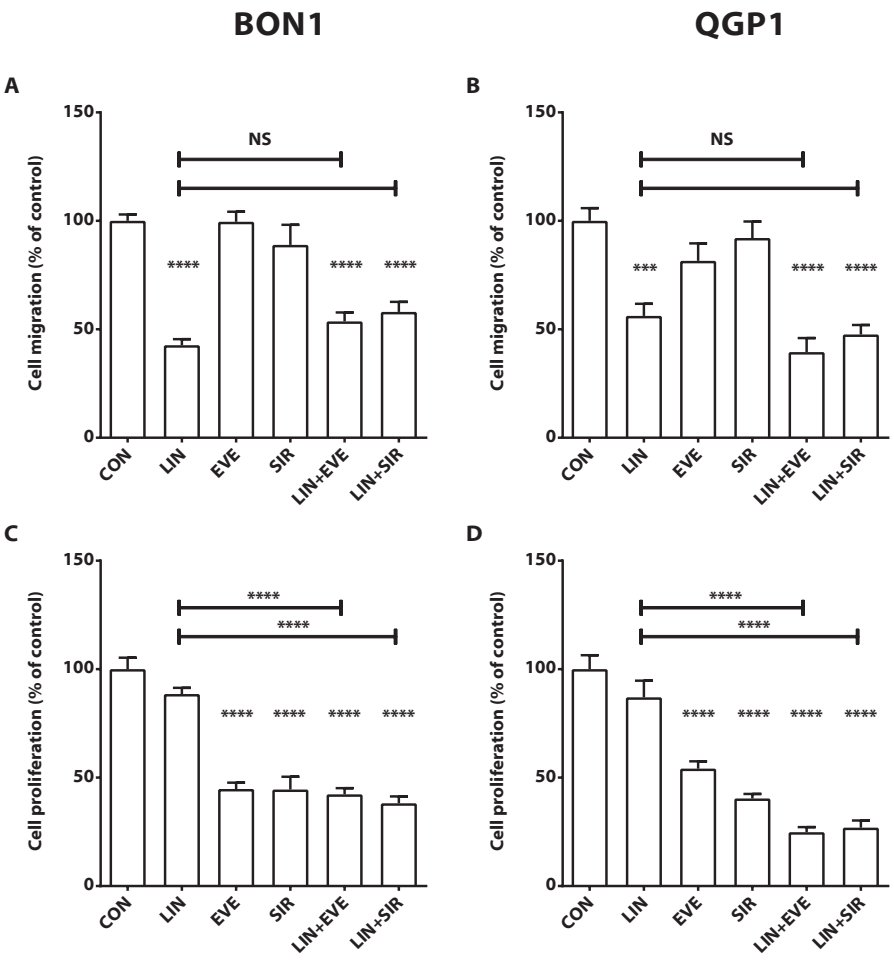

Figure 2 Partial additive effects of linsitinib (LIN, 100 nM) plus mammalian target of rapamycin (mTOR) inhibitors everolimus (EVE, 10 nM) and sirolimus (SIR, 10 nM) in panNET cells: A BON1 cell migration, B QGP1 cell migration, C BON1 cell proliferation and, D QGP1 cell proliferation. Significant results: $P<0.001^{***}$, and $P<0.0001^{****}$.

Effects of AZD5363 on panNET cell proliferation and migration

In order to understand the underlying mechanism of LIN and the mTOR inhibitors EVE and SIR to exert their differential effects on panNET cell migration and proliferation, we treated BON1 and QGP1 cells with AKT1/2/3 inhibitor AZD5363 (AZ) and MEK1/2 inhibitor PD0325901 (PD). Results are displayed in **Figure 3**.

AZ treatment resulted in a dose-dependent, statistically significant, inhibition of panNET cell migration. The strongest significant effects were observed with the highest tested pharmacological concentration (**Figure 3A**: BON1 cells, **Figure 3B**: QGP1 cells).

No significant inhibition on BON1 cell migration was observed with AZ 1 nM, but this concentration stills inhibits QGP1 cell migration.

No statistically significant inhibition on proliferation was observed after incubation with AZ on BON1 (**Figure 3C**) and QGP1 cells (**Figure 3D**). AZ, however, showed a growth-stimulating trend.

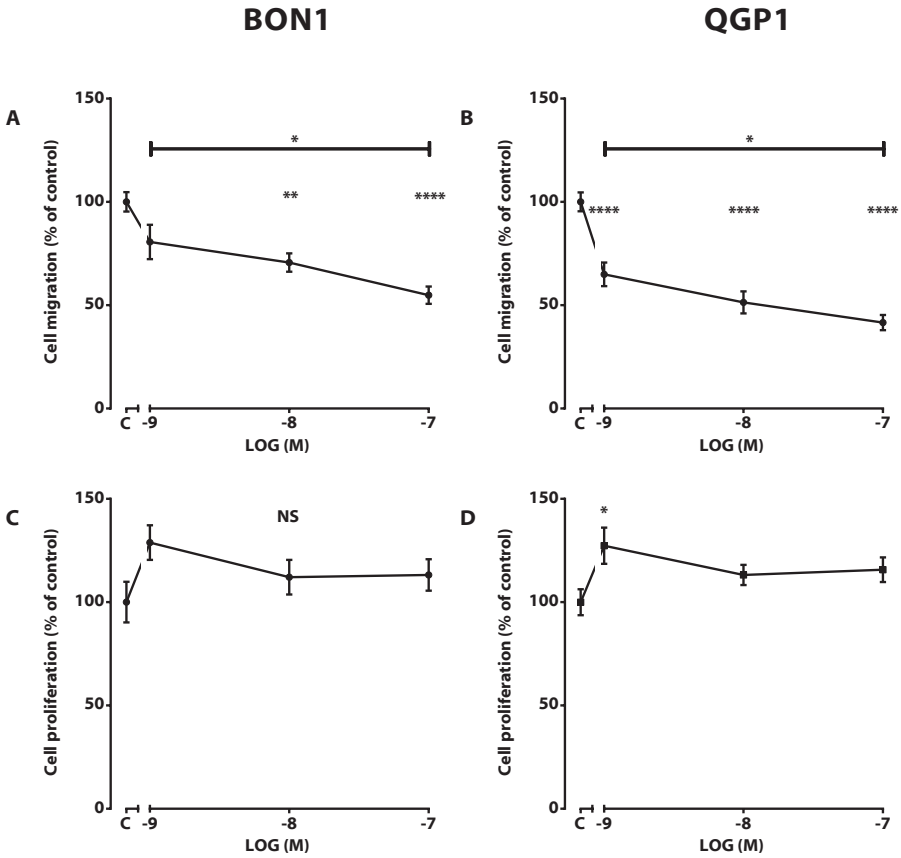

Figure 3 Effect of the AKT1/2/3 inhibitor AZ5363 (AZ) on panNET cells: A BON1 cell migration, B QGP1 cell migration, C BON1 cell proliferation and D QGP1 cell proliferation. Significant are P-values: $P<0.05^{*}$, $P<0.01^{**}$, and $P<0.0001^{****}$.

Effects of PD0325901 on panNET cell proliferation and migration

In both cell lines, there was a significant dose-dependent inhibition of cell migration. (**Figure 4A**: BON1, **Figure 4B**: QGP1). In the QGP1 cell line, but not in the BON1 cells, migration was significantly inhibited already at 0.1 nM.

A strong and statistically significant dose-dependent inhibition of cell proliferation was also observed after treatment with PD in both cell lines (**Figure 4C**: BON1, **Figure 4D**: QGP1).

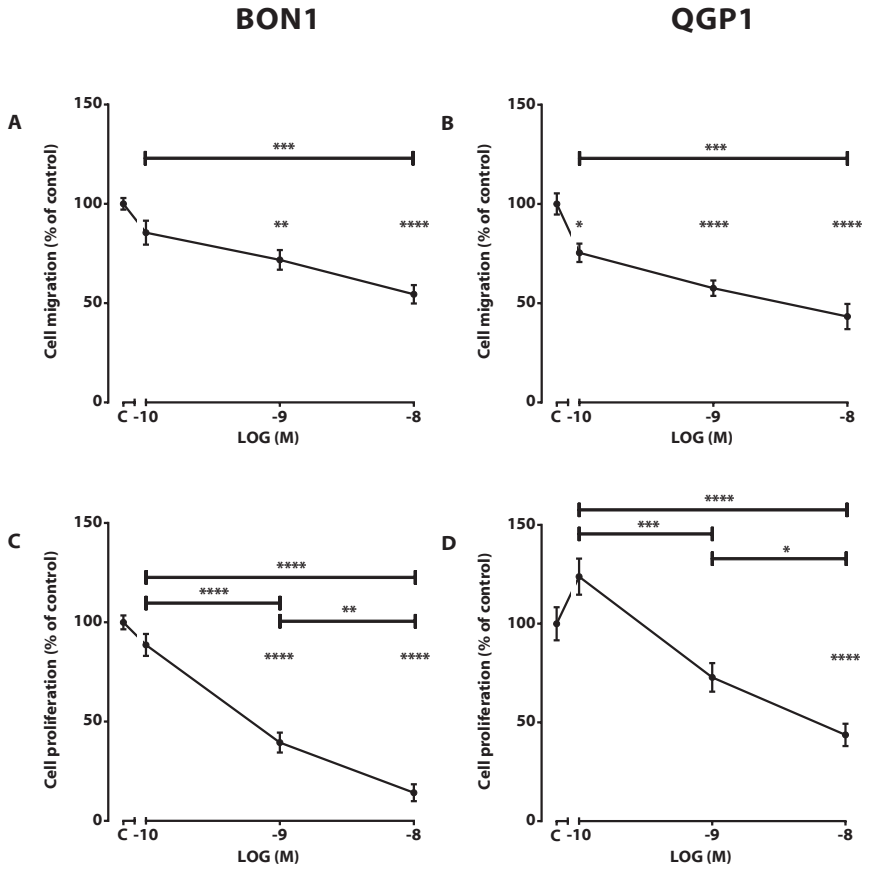

Figure 4 Effects of the MEK1/2 inhibitor PD0325901 (PD) on panNET cells: A BON1 cell migration, B QGP1 cell migration, C BON1 cell proliferation and D QGP1 cell proliferation. Result were considered significant when $P<0.05^{*}$, $P<0.01^{**}$, $P<0.001^{***}$ and $P<0.0001^{****}$.

Additive effects of AZ and PD on panNET cell migration and proliferation

In both panNET cell lines, incubation with AZ (100 nM)+PD (10 nM) resulted in a statistically significant and, additive inhibition of migration compared to AZ or PD monotherapy (BON1: **Figure 5A** and QGP1: **Figure 5B**).

Effects of AZ (100 nM)+PD (10 nM) are shown on BON1 cell (**Figure 5C**) and QGP1 cell proliferation in **Figure 5D**. AZ monotherapy (100 nM) has no significant effect on panNET cell proliferation. Addition of AZ (100 nM) to PD (10 nM) has no additional effect to the already strong inhibition of PD on panNET cell proliferation in BON1 and QGP1 cells.

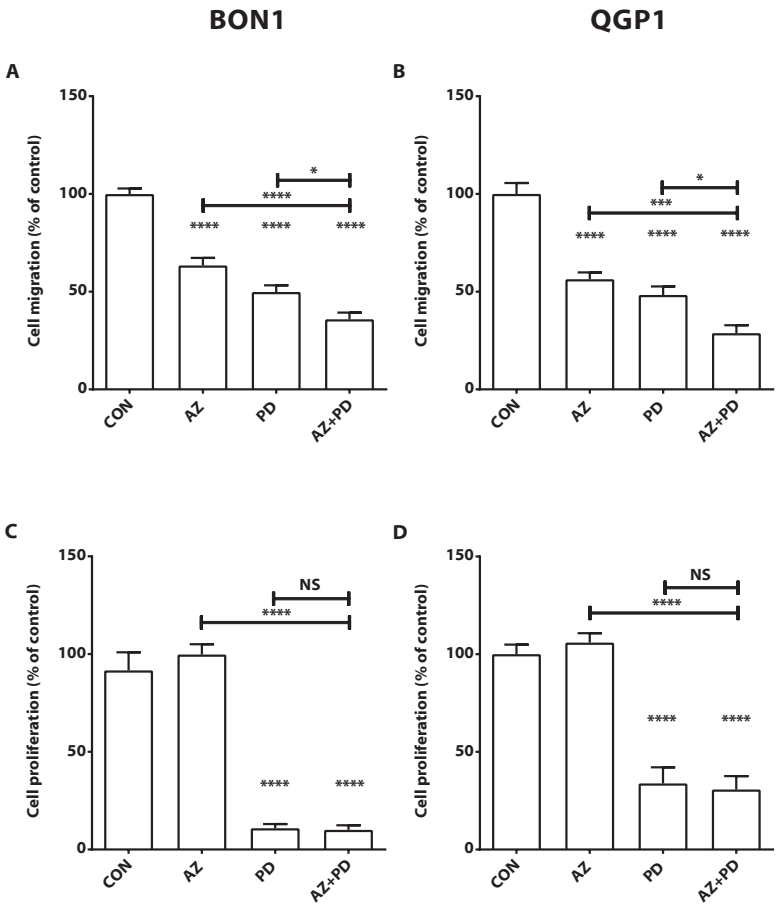

Figure 5 Partial additive effects of AKT1/2/3 inhibitor AZ5363 (AZ, 100 nM) and MEK1/2 inhibitor PD0325901 (PD, 10 nM) on panNET cells: A BON1 cell migration, B QGP1 cell migration, C BON1 cell proliferation and D QGP1 cell proliferation. Significant are P-values: $P<0.05^{*}$, $P<0.001^{***}$ and $P<0.0001^{****}$.

DISCUSSION

Cell proliferation and cell migration are important processes involved in tumor progression and development of metastases (3). The mTOR inhibitor EVE, which is used in the treatment of patients with progressive and/or symptomatic well- and moderately differentiated unresectable and metastasized panNETs, has shown significant antiproliferative effects on panNET cell lines and NET tissues (4,30,31). Additionally, in the RADIANT-3 trial tumor shrinkage was observed in 64% of the patients treated with EVE as compared to 21% of patients in the placebo group (32). Modulation of cell migration with EVE had been investigated in some studies, however, both inhibitory and absence of inhibitory

effects were observed in cell lines (33-36). One study, performed in bronchial NET cells, showed a decrease of cell migration after three days of treatment with EVE (24). In our opinion, after such a timeframe, effects of cell proliferation could be involved as well. Therefore, in our experiments, migration effects were studied during an 8-hours incubation period. To date, the other tested mTOR inhibitor SIR has not been studied in clinical trials for the treatment of panNET patients.

Cell proliferation and migration can also be modulated via upstream inhibition of mTOR by IGF1R/IR TKIs like LIN. One study investigated effects of an IGF1R/IR TKI on cell proliferation, but not cell migration, in a bronchial NET cell line (37). Therefore, this is the first study in which we examined the ability of the dual IGF1R/IR TKI LIN and the mTOR inhibitors EVE and SIR to modulate both panNET cell proliferation and migration as processes of tumor progression. Migration was studied using *in vitro* scratch assays. In these scratch assays, or 'wound healing assays', a scratch was created in panNET cell monolayers in order to measure cell migration. Since cell migration is one step in the complex processes of developing metastases, this model system could provide important information with regard to tumor progression.

We tested mTOR inhibitors using a concentration of 10 nM that can be reached in the circulation and which is well-tolerated in patients (38,39). In our study, we observed a potent inhibition of panNET cell proliferation after treatment with mTOR inhibitors in this concentration. However, mTOR inhibitors had, even in suprapharmacological concentrations no effect on cell migration. LIN showed clear differential effects, with inhibition of panNET cell migration already after low-dose treatment and, in relatively higher doses inhibition of cell proliferation in both panNET cell lines. Combination therapy of LIN and an mTOR inhibitor might therefore modulate panNET progression with dual inhibition of cell migration and proliferation. This drug combination that differentially modulates different signalling pathways, might be a potential new therapeutic option for patients with well- to moderately differentiated metastasized panNET.

In vitro studies showed that LIN completely blocks the phosphorylation and activation of the IGF1R at IC_{50} 0.024 μM and the IR at IC_{50} 0.039 μM. Antitumor activity was seen with EC_{50} ranging from 0.02-0.81 μM (14). In a phase I trial, the oral administration of 150 mg LIN twice daily resulted into antitumor activity in patients with advanced solid tumors, which correlates with maximal plasma concentrations ranging between 1.71-3.11 μM. These concentrations exceed the minimum predicted concentrations for antitumor activity of 0.021 μM (21). The above-mentioned concentrations for antitumor activity correspond well with results in our study of LIN 10-100 nM. With concentrations of LIN 100 nM, statistically significant inhibitory effects were observed on both panNET cell migration and proliferation. In addition, in our experiments these relatively low concentrations of LIN already induced migration inhibitory effects. Therefore, lower

dosages of LIN may be equally effective *in vivo* with potentially less side effects. This needs, however, further investigation (19).

In our experiments, we demonstrated in BON1 cells that LIN in lower pharmacological concentrations inhibited panNET cell migration and in higher concentrations also cell proliferation. These effects on proliferation were not observed in QGP1 cells. On the other hand, in QGP1 cells, combined treatment of LIN with an mTOR inhibitor resulted in significant additive inhibitory effects on proliferation. This was not observed in BON1 cells. These different effects of LIN combined with mTOR inhibitors on both cell lines might reflect the heterogeneous behavior of these panNET cells.

The PI3K/AKT/mTOR pathway plays an important role in the pathophysiology of panNET cell proliferation and migration (4,5,32,40-42). mTOR inhibitors target the mTOR complex 1 (mTORC1), not mTORC2, which results in a negative feedback loop to maintain PI3K activity and upregulate AKT (8,13). The mTORC2 is involved in cell migration (8,13,40). The influence of RAS/RAF/MEK/ERK pathway has not been studied for panNET cell migration, only for cell proliferation (4,43). In a single study, the RAS/RAF/MEK/ERK pathway was shown to be involved in melanoma cell migration (44). Therefore, our hypothesis was that the PI3K/AKT/mTOR pathway is preferentially involved in cell proliferation and that the RAS/RAF/MEK/ERK pathway is predominantly associated with cell migration. In order to test this pathway selectivity, we conducted experiments with an AKT1/2/3 inhibitor (AZ) and MEK1/2 inhibitor (PD).

PD seems to modulate both cell migration and cell proliferation, and we observed that the migration system is much more sensitive to AZ, as compared to the proliferation system, which is almost not affected. Although PD was used to investigate pathway selectivity, MEK 1/2 is a potentially interesting target that could modulate both panNET cell migration and proliferation.

Upstream inhibition could be compensated via intracellular downstream activation of the PI3K/AKT/mTOR pathway and thereby escape from cell proliferation. Probably cross-talk between signalling pathways plays a key role in this process. In our experiments, we demonstrated no effect on panNET cell proliferation with AZ, but cell proliferation might be modulated via interaction of AKT with other pathways than the RAS/RAF/MEK/ERK pathway.

In conclusion, we demonstrate that mTOR inhibitor monotherapy inhibits at low pharmacological concentrations panNET cell proliferation, but not migration. In contrast, LIN modulates in low pharmacological concentrations both panNET cell migration and in higher pharmacological concentrations cell proliferation. Therefore, we suggest that combination therapy of LIN plus mTOR inhibitors EVE or SIR, both in a low dose, could effectively influence panNET cell migration and proliferation as important processes of tumor progression.

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9

General discussion

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GENERAL DISCUSSION

Gastroenteropancreatic neuroendocrine tumors (GEP NETs)

Since its foundation, the European Neuroendocrine Tumor Society (ENETS) and the North American Neuroendocrine Tumor Society (NANETS) have put effort to standardize diagnostic work-up and treatments in patients with gastroenteropancreatic neuroendocrine tumors (GEP NETs) and these will undergo continuous renewal on the basis of evolving insights (1).

The main aims of this thesis were to: 1) improve the diagnostic work-up and follow-up of GEP NET patients using sensitive and specific tumor markers and, 2) to identify new biotherapeutical options by modulating pathological pathways in pancreatic NET (panNET) cells.

The importance of the findings in the studies that are described in this thesis and their implications for the current and future diagnostic work-up and treatment of patients with GEP NETs are discussed below.

Biomarkers

Currently used circulating biomarkers like serum chromogranin A (CgA) and neuron-specific enolase (NSE) have major limitations because of a relative lack of sensitivity, specificity and predictive capacity in the diagnosis of GEP NET patients (2-4). Immunohistochemical markers in tumor cell specimens have these restrictions to a certain extent as well. New biomarkers for these patients are needed to provide better diagnostic and prognostic information. The prognostic value of the two most frequently used single-analyte serological biomarkers NSE and CgA was further investigated.

First, we have studied serum NSE as an independent predictor for overall survival (OS) in patients with GEP NETs (**Chapter 2**). We have demonstrated that NSE is a predictive biomarker for OS independent of the primary GEP NET. Although less than half of the GEP NET patients have elevated NSE levels, in those patients with elevated NSE levels, any increase of NSE above reference levels is indicative of a poor prognosis. Thereby, only a single NSE measurement can already provide prognostic information.

Second, in **Chapter 3** we have investigated the prognostic role of serum CgA levels in patients with stage IV GEP NETs. In this study, we have clearly demonstrated that patients in the high and very high CgA groups have a worse prognosis than patients in the normal CgA group. Therefore, as with NSE, strongly elevated CgA levels in GEP NET patients are also indicative for a poor prognosis. It is know that some GEP NET tumor cells do not express Cg as a manifestation of tumor dedifferentiation. In contrast we have demonstrated that patients with stage IV GEP NETs with circulating CgA levels within the reference range did have a better prognosis as compared to those patients with elevated CgA levels. It would be of interest to further investigate the clinical implications

of this finding, e.g. whether and how patients with a CgA-secreting GEP NET should be treated differently as compared to patients having a non-CgA secreting NET. It has also been shown that serum CgA and NSE can produce additional prognostic information when both are considered in combination. GEP NET patients with combined elevated serum CgA and NSE had a shorter median progression-free survival (PFS) than those with combined normal levels (5). Future research is needed to investigate whether the addition of more single-analyte circulating tumor markers to these two biomarkers can lead to the development of a diagnostic multi-analyte test with superior metrics. Recently, the specific *in vitro* multi-analyte NETest® with algorithm analyses has been launched. This test is further discussed in 'Future perspectives in diagnostics'.

In this thesis we have also aimed at identifying potential new single-analyte biomarkers. In **Chapter 4**, we have studied whether fasting plasma acylated (AG) and plasma unacylated ghrelin (UAG) could be new useful biomarkers in GEP NET patients. However, we could not find significant differences in fasting plasma AG and UAG levels between GEP NET patients and their sex- and age-matched controls. Therefore, fasting plasma AG and UAG do not seem suitable diagnostic biomarkers in patients with GEP NETs.

In order to test whether combined use of single-analyte serological and immunohistochemical markers could offer additional prognostic information, we have studied in **Chapter 5** the relationship between serum CgA, the Ki-67 proliferation index and the expression of insulin-like growth factor (IGF)-related genes in GEP NET tissues and their relation with patient survival. We found significant higher tumoral mRNA expression of insulin receptor isoform A (IR-A) in GEP NET patients with elevated serum CgA as compared to those with non-elevated serum CgA levels. As serum CgA levels correlate well with tumor mass, our data suggest that tumor mass correlates with tumoral IR-A expression in patients with GEP NETs. Whether tumoral IR-A could be used as a prognostic tumor marker for patients with GEP NETs has not been investigated yet and would be an interesting future research focus. *In vivo* research might provide information on whether the dual IGF1 receptor (IGF1R)/IR tyrosine kinase inhibitor (TKI) linsitinib, or a more specific IR-A TKI, are potential therapeutic tools to modulate tumoral IR-A and clinical symptoms in those patients with CgA-producing GEP NETs expressing high IR-A levels.

The somatostatin receptor subtype 2a (sst_{2a}) is a commonly expressed receptor in GEP NETs. As shown in **Chapter 6**, we found that tumoral sst_{2a} expression using immunohistochemistry on tumor samples had no significant additional value compared to somatostatin receptor scintigraphy (SRS) using ^{111}In-pentetreotide (OctreoScan®) in predicting the *in vivo* GEP NET response to peptide receptor therapy (PRRT) using ^{177}Lu-DOTAtate. Although there was no additional value of immunohistochemical sst_{2a} expression in GEP NET tissues in this selected study population, tumoral sst_{2a} and its

clinical relevance as an independent prognostic predictor were recently demonstrated by another research group (6).

The studies in this thesis on currently available serological and immunohistochemical single-analyte biomarkers and/or their combinations have revealed new prognostic insights with clinically relevant information.

Future perspectives in diagnostics

NETest®

The recently commercially available multi-analyte NETest® is significantly more sensitive and specific (>93%) than single-analyte assays which are used for the diagnostic work-up and follow-up of patients with GEP NETs (7). This test affords information on tumor behavior, treatment and their effectiveness (7-10). For this non-invasive blood test, expression data of 51 genes are combined with clinical parameters of patients from whom blood samples are analyzed. Gene expression of genomic clusters (sst-ome, proliferome, growth factor signalome, metabolome, secretome, epigenome, plurome, apoptome) is captured in a NET score. This NET score is calculated from four different prediction algorithms based on PCR data sets of individual studies that are mathematically scaled to different scales of disease activity (9,11-13). Activity levels correlate with clinical status, e.g. stable or progressive disease. At present, this test is not routinely available and is only performed in a single laboratory. Until now, this test has only been studied in retrospective settings (14-16). Therefore, a prospective clinical controlled study is needed to further validate this test.

Circulating Tumor Cells (CTCs)

Circulating Tumor Cells (CTCs) are potential biomarkers in different solid tumors including NETs. CTCs are cells that are grown into the vasculature or lymphatic system of a primary tumor and can be measured in the systemic circulation (17,18). Clinically validated are the United States Food and Drug Administration (FDA) approved CellSearch® CTC Test and CE-approved CellCollector® (19). The usefulness of CTCs as biomarker is currently under investigation in an ongoing phase IV CALM-NET study in patients with midgut NETs (NCT02075606). A potential problem for CTC tests in G1-2 GEP NETs is the relatively low proliferation rate of these tumors and only very low numbers of CTCs could be detected until now in blood samples of GEP NET patients.

Genomic and microRNA (miRNA) profiling

Genomic and microRNA (miRNA) profiling are other promising tumor markers for GEP NETs. The miRNAs are small non-coding RNAs that play a central role in diverse pathological processes. Large differences in miRNA expression profiles have been

demonstrated between different types of NETs (20-22). At the moment, the feasibility of genomic and miRNA profiling has to be demonstrated in clinical studies with GEP NET patients. Currently, a clinical study is ongoing to investigate molecular profiling in NETs (NCT02586844).

Epigenetic modifications

Epigenetic modifications have been demonstrated in GEP NETs, but it is still unclear whether NETs are driven by epigenetic changes (23-25).

Biotherapy

Biotherapy plays a significant role in the treatment of patients with GEP NETs when surgery or local therapies are no suitable options. Most patients with metastasized GEP NETs benefit from treatment with non-radiolabeled 'cold' somatostatin analogs (SSAs). These drugs can inhibit tumor growth and symptoms caused by hormonal secretion and, eventually improve quality of life (26-29). Radiolabeled 'hot' SSAs applied as PRRT for patients with inoperable or metastasized NETs has also shown significant tumor responses (30). Results of the randomized controlled phase III NETTER-trial may lead to formal worldwide registration of PRRT using ^{177}Lu-DOTAtate in patients with metastatic midgut NETs (NCT02705313). The mammalian target of rapamycin (mTOR) inhibitor everolimus and multikinase inhibitor sunitinib have proven efficacy in patients with advanced progressive panNETs as well (31-34).

Although clear progress in the treatment of patients with well- and moderately differentiated metastatic GEP NETs has been made over the last 30 years, the therapeutic options are still limited. GEP NETs may escape from medical treatment by tachyphylaxis and/or the development of resistance and patients will eventually develop progressive disease (35,36). We have, therefore, investigated the use of biotherapeutical agents in panNET cells.

Combination biotherapy

D_2 expression has been demonstrated *in vitro* in GEP NET cell lines and tissues, however, this receptor is currently not used as therapeutical target (37-39). Dopamine-somatostatin chimeric molecules or 'dopastatins' have shown strong inhibitory effects on hormonal hypersecretion in growth hormone secreting adenomas, and antiproliferative effects in other tumor cell types as compared to octreotide and cabergoline, and both sst_2 and sst_5 monospecific analogs (40-42).

We have shown favorable effects of the combination of SSAs and dopamine agonists (DAs), either or not as chimeric compound, on mainly IGF2-induced IR-A activation in panNET cells (**Chapter 7**). Our data suggested a role of targeting $sst_{2/5}$ and D_2 in modulating IGF2 production in GEP NETs. Our data support the necessity of more *in*

vivo research with dopastatins. Unfortunately, in a previous initial clinical trial, chronic administration of dopastatin BIM-23A760 produced a metabolite with dopaminergic activity that gradually accumulates and interferes with the activity of the parent compound (43).

In another study described in **Chapter 8**, we have demonstrated that combination therapy of linsitinib plus low doses of the mTOR inhibitors everolimus or sirolimus could effectively influence both panNET cell migration and proliferation, which are important indicators of tumor progression. Modulation of cell migration as measure for disease progression was investigated in panNET cells and may be a new concept for the treatment of GEP NET patients. Our data indicate that more *in vivo* research is needed to investigate whether combined therapy of linsitinib plus low doses of an mTOR inhibitor would influence disease progression in GEP NET patients. In addition, we have shown significant inhibition on panNET cell proliferation and migration with the MEK1/2 inhibitor PD0325901 (PD). Therefore, this agent should be considered as a potential new target for TKI inhibition in GEP NETs as well.

Future perspectives of biotherapy

Clinical trials are ongoing in the field of GEP NETs and will be expected in the future to result into new biotherapeutical agents. Some of these agents are already approved for other types of cancer and could therefore be of interest for GEP NETs as well.

Dual mTOR inhibitors

The mTORC1 inhibitor everolimus has shown limited efficacy in patients with panNETs (34). Dual inhibition of both mTORC1 and mTORC2 might have stronger anti-proliferative properties. In an ongoing multicenter, open-label phase I-II study, safety and tolerability are assessed of the oral mTOR inhibitor CC-223, which is a competitive inhibitor of the mTOR kinase that targets mTORC1 and mTORC2 in patients with advanced non-pancreatic NETs (NCT01177397) (44).

Immunotherapy

New immunological agents, immune-checkpoint inhibitors, have shown to prolong survival in patients with inoperable or metastasized types of cancer (45-47). These FDA proven monoclonal antibodies target the cytotoxic T-lymphocyte-associated protein 4 receptor (CTLA4, ipilimumab) and the programmed cell death protein 1 receptor (PD1, nivolumab) (48,49), which are both proteins that down regulate the immune system. After binding to the respective receptors, these monoclonal antibodies activate the immune system and result into antitumor responses leading to tumor cell apoptosis (48,49). Clinical trials with these drugs in gastroenteropancreatic neuroendocrine car-

cinoma (GEP NEC) patients are in preparation (www.netrf.org/net-research-foundation-launches-major-immunotherapy-initiative).

Multikinase inhibitors

Besides sunitinib, registered multikinase inhibitors for other types of cancer and multikinase inhibitors in development are potential new therapies for patients with GEP NETs. Sorafenib tosylate, an inhibitor of vascular endothelial growth factor receptors (VEGFRs)1-3 and platelet-derived growth factor receptors (PDGFRs), and lenvatinib which targets mainly VEGFRs, PDGFRs and fibroblast growth factor receptors (FGFRs), have shown to inhibit tumor growth and progression and, increase PFS in other cancers (50-55). Results are expected of the phase II trial in which sorafenib tosylate was investigated in the treatment of patients with progressive metastatic NETs (NCT00131911). A prospective, multicenter phase II trial is ongoing in order to test lenvatinib efficacy in metastatic NETs (TALENT) (NCT02678780).

Combination therapy

An increasing number of studies is focusing on combined treatments of SSAs, PRRT, everolimus, multikinase inhibitors and, chemotherapy in the field of NETs.

With regard to studies on combined therapy with SSAs, there is a randomized double-blinded phase II SUNLAND trial, in which patients are recruited with progressive advanced midgut NETs to study lanreotide acetate with sunitinib malate versus lanreotide acetate with placebo regarding PFS (NCT01731925). In addition, results of an ongoing randomized double-blind phase II trial are expected on octreotide LAR plus VEGFR1-3 multikinase inhibitor axitinib versus placebo in patients with progressive advanced well-differentiated non-pancreatic NECs (NCT01744249).

A number of trials is ongoing on combination therapy with PRRT. Recently, results of the phase I NETTLE trial have been published in which acceptable safety profiles were investigated of PRRT and everolimus in GEP NET patients (56). These results should be further studied in a phase II-III trial.

Moreover, studies are ongoing on the combined effects with everolimus as well. In a phase I trial with everolimus plus sorafenib, beneficial effects were shown in patients with advanced NETs. These data need to be further studied in a phase II-III trial. In an ongoing phase II study, the safety and efficacy of everolimus plus erlotinib, an epidermal growth factor receptor TKI, is under investigation in patients with G1-2 NETs (NCT00843531). The large ENETS randomized, open label phase II SEQTOR trial has been initiated in which the efficacy and safety are compared of everolimus followed by streptozotocin and 5-fluorouracil (STZ-5FU), or the reverse sequence, upon progression in patients with advanced panNETs (NCT02246127). This trial is an important step forward in the search for the recommended sequence of treatments for GEP NETs with different

disease stages, since at present decisions with regards to therapy are mainly based on personal or expert opinions (57).

Concluding remarks

In the reported studies, we have first investigated the use of sensitive and specific tumor markers in order to improve the diagnostic work-up and follow-up for patients with GEP NETs. We investigated existing serological and immunohistochemical markers (CgA, NSE; sst_{2a}, Ki-67 proliferation index) and found new applications for clinical follow-up. Potential new markers (fasting plasma AG and UAG; IGF-related genes) appeared not to be of added diagnostic value for GEP NET patients. Primarily, our focus was on single or combined analytes. Since GEP NETs can behave very heterogeneously and secrete a variety of biologically active products, future diagnostic approaches might take into account more than one parameter to improve both the sensitivity and specificity of assays. Very recently, the multiple-analyte assay NETest®, in which more than 50 genes are investigated, has been introduced. Preliminary results show promising test metrics (7,8,15,16). The concept of multiple-analyte analyses might be a step forward to the further improvement of the diagnostic work-up and follow-up in GEP NET patients.

Secondly, we examined new biotherapeutical options by influencing pathological pathways in panNET cells. In the last decades, treatment for GEP NET patients has evolved into targeted therapies which modulate receptors and deregulated pathways, in order to improve clinical symptoms, control tumor size and, improve quality of life and PFS. In our view, future research on the treatment of this patient group with metastasized disease will focus more on multiple receptor targeting in order to tackle the multiple pathways that are involved in tumor-promoting processes and to overcome tachyphylaxis and/or resistance to treatment. Therefore, linsitinib (combined targeting of the IGF1R and IR), and combined SSAs and DAs (acting on both sst and D_2), preferentially using chimeric compounds, are potential novel therapeutical options for patients with GEP NETs (58). The importance of targeting other expressed receptor types, either or not in combination with IGF1R/IR receptors, could be of interest as well. Tumor heterogeneity characterizes, but also impedes the clinical success of novel therapies. Therefore, it will be a future challenge to develop new drugs with both antiproliferative and antisecretory effects with clinical feasibility for the whole GEP NET patient population. Finally, future studies correlating drug responses to circulating tumor markers or molecular markers expressed in tumors might help to better select the appropriate drug to treat individual patients (e.g. tailored drug treatment).

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Summary/Samenvatting

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SUMMARY

Introduction

Neuroendocrine tumors (NETs) arise from the diffuse neuroendocrine (NE) cell system. These NETs are considered rare tumors and they generally show a very heterogeneous manifestation. The majority of NETs are gastroenteropancreatic NETs (GEP NETs) and lung NETs. NETs originating from the gastrointestinal tract and pancreas are considered as separate tumor entities. GEP NETs are historically subdivided according to their embryologic origin into foregut, midgut or hindgut NETs.

GEP NETs are characterized by tumoral production of metabolically active substances causing distinct clinical syndromes. Among these clinical syndromes, the carcinoid syndrome (CS) is the most well known. Besides presenting with a functional syndrome, these tumors, can also present as non-syndromic or non-functioning NETs.

NETs express somatostatin (sst) and dopamine (DA) receptors. Co-expression of these receptors may generate so-called chimeric receptors or hybrid receptors with altered functional properties. Sst receptors are used in the clinic as important therapeutic targets for the inhibition of hormonal secretion and cell proliferation in GEP NETs using somatostatin analogs (SSAs).

Several pathways including the insulin-like growth factor (IGF) pathway, the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway and the RAS/RAF/mitogen-activated protein kinase kinase/extracellular-signal-regulated kinase (RAS/RAF/MEK/ERK) pathways are known to be involved in the pathophysiology of NETs. Under normal, non-pathological conditions, these signaling pathways are crucial for cell metabolism, proliferation, migration, differentiation and survival. How these different dysregulated pathways exactly are involved in the formation of NETs is presently unknown.

Diagnosis

The diagnosis of a GEP NET is based on: pathology of tumor tissue, radiological and nuclear imaging and, circulating biomarkers. Pathological evaluation of tumor tissue is essential to establish or confirm the NE entity of the tumor, for ENETS TNM staging and, WHO grading, which are important for treatment decisions and prognosis.

Besides general imaging techniques including computer tomography (CT), magnetic resonance imaging (MRI), ultrasound (US), and endoscopic ultrasound (EUS), somatostatin receptor scintigraphy (SRS) using OctreoScan®, or ^{68}Ga-DOTATATE-PET are used for staging in GEP NET patients.

The most relevant immunohistochemical biomarkers are chromogranin (Cg) and synaptophysin. The most widely used serological biomarkers are chromogranin A (CgA) and neuron-specific enolase (NSE). The 24-hours urinary collection of the serotonin

breakdown metabolite, 5-hydroxyindoleacetic acid (5-HIAA), is another established biomarker used in the diagnosis and follow-up of GEP and bronchial NET patients with the CS. However, these available single-analyte immunohistochemical and serological biomarkers have major limitations.

In this thesis, the first main aim was to improve the diagnostic work-up and follow-up of GEP NET patients using sensitive and specific tumor markers. We have re-investigated the prognostic test properties of the two most frequently used single-analyte serological biomarkers NSE and CgA. *New insights on existing single-analyte tumor markers were presented*. We have shown that the biomarker NSE is a predictive biomarker for overall survival independent of the primary tumor in GEP NET patients. Our data suggest that patients with non-CgA-secreting metastatic stage IV GEP NETs have a favorable prognosis as compared to those with CgA-secreting stage IV metastatic GEP NETs.

No new sensitive and specific suitable single-analyte biomarkers were identified. Fasting plasma acylated and plasma unacylated ghrelin appeared not to be the expected suitable biomarkers. No significant association was found between the combined serological and immunohistochemical single-analyte biomarkers serum CgA, the Ki-67 proliferation index in tumor specimens and, the expression of IGF-related genes in GEP NET tissues on one hand and patient survival on the other hand. The clinical relevance of the observed significant higher tumoral mRNA expression of insulin isoform-A (IR-A) in GEP NET patients with elevated serum CgA, as compared to those patients with non-elevated serum CgA levels, might become an interesting topic for future clinical research.

Finally, the expression of the somatostatin receptor sst_{2a} on tumor samples had no significant additional value as compared to SRS using OctreoScan® in predicting the *in vivo* GEP NET response to peptide receptor radiotherapy (PRRT) with ^{177}Lu-DOTAtate. However, this relatively cheap diagnostic tool might become helpful in those geographic areas of the world where there is no or very limited availability of SRS to select patients for PRRT with radiolabeled somatostatin analogs (SSAs).

Since GEP NETs can behave very heterogeneously and secrete a variety of biologically active products, future diagnostic approaches might take into account the concept of evaluating more than one parameter with multiple-analyte analyses to improve both the sensitivity and specificity of assays for the diagnostic work-up and follow-up in GEP NET patients.

Treatment

Surgery with complete resection of the primary NET and if present its metastases, is the only curative option for patients with a NET.

Medical therapy in advanced well- to moderately differentiated grade (G)1-2 NETs might include: 1) molecular targeted therapies with the synthetic SSAs, octreotide and

lanreotide, the new SSA pasireotide, and the serotonin synthesis inhibitor telotristat etiprate. 2) Protein kinase inhibitors including the mTOR inhibitor everolimus, the multikinase inhibitor sunitinib and potentially the dual insulin-like growth factor 1 receptor (IGF1R)/insulin receptor (IR) tyrosine kinase inhibitor linsitinib. 3) PRRT with ^{177}Lu-DOTATATE and 4) interferon. Dopamine-somatostatin chimeric molecules, or 'dopastatins', acting on both sst and D_2 are not yet available for clinical use. Systemic chemotherapy is recommended in patients with poorly differentiated G3 NETs and neuroendocrine carcinomas of any site and, in progressive and/or extended panNETs.

Progress had been made the last 30 years in the treatment of patients with advanced NETs. However, therapeutic options are still limited since NETs escape from medical treatment with SSAs by tachyphylaxis, which is the loss of response to chronic SSA administration, and/or develop resistance to the different drugs used, and patients will eventually develop progressive disease. We have, therefore, investigated the use of new potential biotherapeutical agents for the treatment of panNET cells.

In this thesis, the second aim was to identify new biotherapeutical options by modulating pathological pathways in panNET cells. *We showed that especially combination therapy of SSAs and dopamine agonists (DAs), either or not as chimeric compound, can have favorable effects on panNET cell function*. We identified favorable effects on the combination of SSAs and DAs on IGF2-induced IR-A activation in panNET cells. In another study, we demonstrated that combination therapy of linsitinib plus mTOR inhibitors everolimus or sirolimus both in a low dose could effectively influence panNET cell migration and proliferation as important parameters of tumor progression. In addition, significant inhibition of both panNET cell proliferation and migration were observed with the MEK1/2 inhibitor PD0325901. These agents should therefore be considered as potential new therapies in patients with GEP NETs.

We expect that future treatment for patients with GEP NETs will consist of combined therapies of SSAs, DAs, immunotherapy, protein and/or multikinase receptor inhibitors, either or not as a chimeric compound. These therapeutical approaches will focus on simultaneous multiple receptor targeting in order to target multiple pathways that are involved in tumor-promoting processes. A next step would be to find effective therapies that can overcome tachyphylaxis and/or drug resistance as well. Finally, future studies correlating drug responses to circulating tumor markers or molecular markers expressed in tumors might help to better select the appropriate drug to treat individual patients (e.g. tailored drug treatment).

SAMENVATTING

Introductie

Neuroendocriene tumoren (NETs) zijn zeldzame tumoren die jaarlijks bij 5 op de 100.000 personen worden vastgesteld. NETs ontstaan uit het diffuse neuroendocriene celsysteem. Ongeveer tweederde van de NETs bevindt zich in de maag, darmen en de alvleesklier (Gastro-Entero-Pancreas=GEP) en éénderde in de long. Darm en alvleesklier NETs maken deel uit van de groep GEP NETs, maar kunnen zich over het algemeen heel verschillend gedragen. Als vanouds wordt er een onderverdeling aangehouden, afhankelijk van hun embryologische oorsprong: voordarm, middendarm of achterdarm GEP NETs.

GEP NETs worden gekenmerkt door productie van hormoonachtige stoffen welke verschillende klachtenpatronen (syndromen) kunnen veroorzaken. Van deze syndromen is het carcinoïd syndroom het meest bekend. Bij dit syndroom produceert de NET grote hoeveelheden van het hormoon serotonine. Patiënten kunnen hierdoor last krijgen van opvliegers, kortademigheid, buikpijn en diarree. Wanneer door tumorproductie van deze hormoonachtige stoffen klachten worden veroorzaakt, spreekt men van een zogenaamde 'functionele' NET. Een NET die geen hormonaal syndroom veroorzaakt, kan in een later stadium door ingroei van omliggende structuren pas (pijn)klachten veroorzaken en dan spreken we van 'niet-functionele' NETs.

Receptoren zijn eiwitten die het slot vormen waarop passende sleuteleiwitten kunnen binden. NETs hebben somatostatine (sst) en dopamine (DA) receptoren op hun celoppervlak. Deze, maar ook andere receptoren op het celoppervlak van NETs, kunnen door verbinding met elkaar 'chimeer' of 'hybride' receptoren vormen waardoor ze ook andere eigenschappen krijgen.

Somatostatine receptoren worden als therapeutisch "doelwit" gebruikt. 'Somatostatine analoga' (SSAs) binden aan deze somatostatine receptoren. Dit zijn medicijnen die de celgroei remmen van GEP NETs en ook de hormoonproductie. Uiteindelijk wordt dan verlichting van klachten bereikt.

Van verschillende signaalpaden zoals het insuline-achtige groei factor (IGF) signaalpad, het PI3K/AKT/mTOR signaalpad en het RAS/RAF/MEK/ERK signaalpad, is bekend dat deze betrokken zijn bij de ontregelde groei van tumoren bij patiënten met GEP NETs. Onder normale, niet-zieke omstandigheden, spelen deze signaalpaden een onmisbare rol voor de cel en met name bij de groei en uitrijping ervan, maar ook bij de stofwisseling, celmigratie en celoverleving. Het is tot nu toe nog onbekend hoe de verschillende verstoorde signaalpaden precies betrokken zijn bij het ontstaan van NETs.

Diagnose

De diagnose van een GEP NET is gebaseerd op: onderzoek van tumorweefsel, radiologische en nucleaire beeldvorming en circulerende biomerkers (tumorstoffen welke gemeten kunnen worden in het bloed van de patiënt). De beoordeling van tumorweefsel door een ervaren patholoog is essentieel om vast te stellen of te bevestigen dat het een NET betreft en geen andere soort tumor. Ook is tumorweefselonderzoek van belang om de tumorgroeisnelheid (Ki-67 proliferatie index) in kaart te brengen volgens de zogenaamde WHO gradering. Beeldvorming is nodig om de tumoruitbreiding volgens de ENETS TNM stadiëring in beeld te brengen. Met deze gegevens kan een keuze gemaakt worden voor een bepaalde therapie. Ook worden deze gegevens gebruikt om een inschatting te kunnen maken van de prognose.

Naast algemene beeldvormende technieken zoals computer tomografie (CT), beeldvorming met magnetische resonantie (MRI), echo en endoscopische echografie is somatostatine receptor scintigrafie (SRS) met OctreoScan® of een ^{68}Ga-DOTATATE-PET scan belangrijk voor het in beeld brengen van de tumoruitbreiding bij GEP NET patiënten.

De meest relevante biomerkers op tumorweefsel zijn chromogranine (Cg) en synaptofysine. De meest gebruikte biomerkers gemeten in bloed zijn het chromogranine A (CgA) en het neuron-specifiek enolase (NSE). De 24-uurs urine verzameling van het serotonine afbraakproduct 5-hydroxyindolazijnzuur is een andere biomerker welke wordt gebruikt bij het stellen van de diagnose en voor het vervolgen van het succes van de therapie bij GEP en long NET patiënten met het carcinoïd syndroom. Momenteel hebben de meest gebruikte biomerkers die worden bepaald in bloed, maar ook in tumorweefsel, hun beperkingen. Zo kunnen de biomerkers ook verhoogd zijn bij andere omstandigheden en in andere gevallen kan de biomerker niet worden aangetoond terwijl een patient wel een NET heeft.

In dit proefschrift was het eerste onderzoeksdoel om op een betere manier de diagnose te kunnen stellen en het succes van de therapie te vervolgen bij GEP NET patiënten gebruikmakend van bestaande biomerkers. We hebben de prognostische waarde van de twee meest gebruikte biomerkers in bloed, namelijk CgA en NSE, opnieuw bekeken. *Er werden nieuwe inzichten gevonden bij bestaande tumormerkers.* We hebben laten zien dat NSE een voorspellende biomerker is voor de overleving voor GEP NET patiënten en dit is onafhankelijk van de tumorlocatie in het lichaam. Onze onderzoeksresultaten tonen ook aan dat patiënten met uitgezaaide GEP NETs die geen CgA produceren een gunstigere prognose hebben vergeleken met patiënten met uitgezaaide GEP NETs die wel CgA produceren. *Ons onderzoek leverde geen geschikte nieuwe biomerkers op.* Het nuchter gemeten stofwisselingshormoon ghreline bleek in twee verschillende verschijningsvormen niet de geschikte biomerker te zijn welke we dachten te vinden. Er werd geen belangrijk verband gevonden tussen de combinatie van CgA bepaald in bloed, de Ki-67 proliferatie index onderzocht op tumorweefsel, en de aanwezigheid van

verschillende insuline-achtige genen in GEP NET weefsels. Wel werd een duidelijk verhoogde hoeveelheid van de insuline receptor A (IR-A) aangetoond in tumoren van GEP NET patiënten met een verhoogd CgA gehalte in het bloed in vergelijking met tumoren van patiënten met niet-verhoogd CgA in het bloed. Deze bevinding is een interessant onderwerp voor toekomstige onderzoek.

Tenslotte had het vaststellen van de aanwezigheid van de somatostatine receptor sst_{2a} op tumorweefsel geen belangrijk toegevoegde waarde, vergeleken met het aantonen van deze receptor met somatotatine receptor scintigrafie gebruik makend van de OctreoScan®, om het resultaat van peptide receptor radiotherapie met ^{177}Lu-DOTAtaat (PRRT) te kunnen voorspellen. PRRT is behandeling met een SSA welke gekoppeld is, of 'gelabeld' is, aan een radioactieve stof. Het aantonen van de somatostatine receptor sst_{2a} op tumorweefsel is wel een relatief goedkopere methode die toepasbaar zou kunnen zijn in die gebieden waar geen of nauwelijks beschikbaarheid is van SRS om patiënten te selecteren voor PRRT met radioactief gelabelde SSAs.

Zoals genoemd kunnen GEP NETs zich heel divers gedragen en veel verschillende soorten hormonen uitscheiden. Bij de ontwikkeling van toekomstige biomerkers waarmee we de diagnose kunnen stellen en behandelingseffecten kunnen vervolgen bij GEP NET patiënten, moeten we daarom rekening houden met de bepaling van niet één, maar meerdere geschikte biomerkers.

Therapie

De enige behandeloptie voor GEP NETs, met als doel genezing, is een operatie waarbij de tumor en ook alle eventueel aanwezige uitzaaiingen volledig worden verwijderd.

NETs worden naar aanleiding van hun tumorgroeisnelheid (Ki-67 proliferatie index op tumorweefsel) in drie groepen ingedeeld: goed (=graad 1), matig (=graad 2) en slecht gedifferentieerde (=graad 3) NETs. Deze graad 3 tumoren worden tegenwoordig onderverdeeld in NETs en neuroendocriene carcinomen (NECs).

Medicamenteuze therapie voor patiënten met graad 1-2 NETs kan bestaan uit: 1) gerichte therapieën met de SSAs octreotide en lanreotide, de nieuwe SSA pasireotide, en de serotonine aanmaakremmer telotristat etiprate. 2) Proteïne kinase remmers: de mTOR remmer everolimus, de multikinaseremmer sunitinib en mogelijk de insuline-achtige groeifactor 1 receptor (IGF1R)/insuline receptor (IR) tyrosine kinase remmer linsitinib. 3) PRRT en 4) interferon alfa. Dopamine-somatostatine chimere moleculen of 'dopastatines' binden aan zowel de sst als de D_2 en worden momenteel nog niet gebruikt in de praktijk. Chemotherapie wordt aanbevolen bij patiënten met NECs.

De laatste 30 jaar is er vooruitgang geboekt bij de behandeling van patiënten met uitgezaaide NETs. Hoewel er meerdere behandelopties zijn, zijn deze tumoren de behandeling soms te slim af waardoor deze therapieën niet langer meer effectief blijken. Eén van de processen waarmee tumoren dat voor elkaar krijgen heet 'tachyfylaxie'.

Tachyfylaxie is het verschijnsel dat er bij langdurige SSA toediening geen remming meer van tumorgroei of hormoonproductie is. Ook treedt er onder sommige omstandigheden resistentie voor een bepaalde therapie op. Resistentie is het proces dat de tumor ongevoelig wordt voor het medicijn. Als het medicijn geen effect meer heeft, zal de tumor ondanks behandeling verder groeien en zal er uitbreiding van ziekte ontstaan. Om deze reden hebben we de bruikbaarheid van nieuwe middelen onderzocht in pancreas NET cellen.

In dit proefschrift was het tweede onderzoeksdoel om nieuwe middelen te onderzoeken die de verstoorde signaalpaden in pancreas NET cellen kunnen beïnvloeden. *We hebben aangetoond dat in het bijzonder combinatietherapie van SSAs en dopamine agonisten (DAs), al dan niet als chimeer (twee medicijnen in een toedieningsvorm), gunstige effecten kunnen hebben op de pancreas NET celfunctie.* Met de combinatie van SSAs en DAs hebben we een duidelijke afname gezien van de door de pancreas NET cellen geproduceerde, tumorgroei stimulerende insuline-achtige stof IGF2. De effecten van IGF2 kunnen deels worden uitgevoerd via de insuline receptor IR-A die aanwezig is op de pancreas NET cellen. In een andere studie hebben we aangetoond dat de combinatie van een lage dosis insuline receptor en IGF1 receptor remmer linsitinib en een lage dosis van de mTOR remmers everolimus of sirolimus een duidelijke remming geeft van de pancreas NET celgroei en –migratie. Deze twee eigenschappen vormen een belangrijke maat voor tumoruitbreiding. Bovendien werd er in deze studie een duidelijke remming gezien van zowel pancreas NET celgroei als -migratie met de MEK1/2 remmer (specifieke remmer van het RAS/RAF/MEK/ERK signaalpad) PD0325901. Bovengenoemde middelen kunnen daarom mogelijk in de toekomst als nieuwe therapieën worden ingezet voor patiënten met GEP NETs.

In de toekomst verwachten we dat patiënten met GEP NETs behandeld zullen worden met combinatietherapieën met SSAs, DAs, immunotherapie, en eiwit en/of multi-eiwit receptor remmers. Deze medicamenten zullen dan, al dan niet als chimeer eiwit, worden toegepast. Nader onderzoek zal zich focussen op gelijktijdige activatie van meerdere receptoren met als doel meerdere signaalpaden, welke betrokken zijn bij tumor stimulerende processen, aan te pakken. De volgende stap zal als doel hebben om effectieve therapieën te vinden die ook tachyfylaxie en/of resistentie kunnen overwinnen. Toekomstig onderzoek waarbij de effectiviteit van geneesmiddelen bij patiënten met GEP NETs wordt gekoppeld aan circulerende tumor merkers of aan merkers die in tumorweefsel aanwezig zijn, kan leiden tot het selecteren van het juiste geneesmiddel voor de juiste patiënt.

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List of publications

PhD portfolio

About the author

Dankwoord

LIST OF PUBLICATIONS

1. Chromogranin A, Ki-67 index and IGF-related genes in patients with neuroendocrine tumors.
   van Adrichem RC, Hofland LJ, Feelders RA, De Martino MC, van Koetsveld PM, van Eijck CH, de Krijger RR, Sprij-Mooij DM, Janssen JA, de Herder WW.
   Endocrine Connections 2013 2 172-177

2. Parathyroid hormone-related peptide (PTHrP) secretion by gastroenteropancreatic neuroendocrine tumors (GEP NETs): clinical features, diagnosis, management, and follow-up.
   Kamp K, Feelders RA, van Adrichem RC, de Rijke YB, van Nederveen FH, Kwekkeboom DJ, de Herder WW.
   The Journal of Clinical Endocrinology and Metabolism 2014 99 3060-3069

3. Is there an additional value of using somatostatin receptor subtype 2a immunohistochemistry compared to somatostatin receptor scintigraphy uptake in predicting gastroenteropancreatic neuroendocrine tumor response?
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4. Serum neuron-specific enolase level is an independent predictor of overall survival in patients with gastroenteropancreatic neuroendocrine tumors.
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5. Effects of somatostatin analogs and dopamine agonists on insulin-like growth factor 2-induced insulin receptor isoform-A activation by gastroenteropancreatic neuroendocrine tumor cells.
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   Neuroendocrinology 2016 103 1-11

6. Plasma acylated and plasma unacylated ghrelin: useful new biomarkers in patients with neuroendocrine tumors?
   van Adrichem RC, van der Lely AJ, Huisman M, Kramer P, Feelders RA, Delhanty PJ, de Herder WW.
   Endocrine Connections 2016 5 143-151

7. Limited value for urinary 5-HIAA excretion as prognostic marker in gastrointestinal neuroendocrine tumors.
   Zandee W, Kamp K, van Adrichem RC, Feelders RA, de Herder WW.
   European Journal of Endocrinology 2016 175 361-366

8. Is true non-secretion of chromogranin A an unfavorable prognostic factor in patients with ENETS TNM stage IV gastroenteropancreatic neuroendocrine tumors?
   Kamp K[*], van Adrichem RC[*], Vandamme T, de Rijke YB, Peeters M, Feelders RA, de Herder WW.
   Manuscript submitted

9. Differential effects of linsitinib and mTOR inhibitors on cell migration and proliferation of pancreatic neuroendocrine tumor cells.
   van Adrichem RC, van Koetsveld PM, Feelders RA, Vandamme T, de Herder WW, Hofland LJ.
   Manuscript in preparation

[*] joint first authors

PhD PORTFOLIO

Courses	Year	ECTS
38th Erasmus Endocrinology Course	2012	0.7
Introductory course on statistics & survival analysis	2012	0.5
Biomedical English Writing and Communication	2014	3.0
Integrity in science	2014	0.3
Knowledge Network programme	2014	1.0
Basis course for clinical investigators (BROK®)	2015	0.9
Oral presentations		
Dutch Endocrine Meeting, Noordwijkerhout	2013	0.8
NETWORK Europe, Munich	2013	1.0
Dutch Endocrine Meeting, Noordwijkerhout	2014	0.8
EYES meeting, Belgrade	2014	1.0
Dutch Endocrine Meeting, Noordwijkerhout	2015	0.8
Poster presentations		
Science Days Internal Medicine, Antwerp	2013	0.8
ENETS, Barcelona	2013	1.0
Science Days Internal Medicine, Antwerp	2014	0.8
ENETS, Barcelona	2014	1.0
Science Days Internal Medicine, Antwerp	2015	0.8
ENETS, Barcelona	2015	1.0
Clinical activities		
Grand rounds	2012-2015	9.8
Tumor board meeting	2012-2013	4.6
Co-investigator COOPERATE-2 phase II study	2011-2014	3.0
Co-investigator CBEZ235F2201 phase II study	2013-2014	1.2
NET zorgpad	2012-2015	0.4
Teaching activities		
Attending seminars of the Department of Internal Medicine/Erasmus MC lectures	2012-2015	4.0
Supervising bachelor course hypercortisolism	2013-2014	0.4
Supervising bachelor course thyroid gland	2013-2014	0.4

ABOUT THE AUTHOR

Roxanne Caresse Samantha van Adrichem was born on March 20th 1986, in Moordrecht. In 2004, she completed secondary school at the Goudse Scholengemeenschap Leo Vroman in Gouda, after which she started her medical studies at the Erasmus University Medical School, Rotterdam. As a part of the Master of Science programme Clinical Research, she attended in 2008 a summer programme at the Johns Hopkins Bloomberg School of Public Health, at the Johns Hopkins University in Baltimore, United States of America. She finished her graduation project of the Master of Science programme Clinical Research entitled 'The role of somatostatin analogs and dopamine agonists on the IGF system in neuroendocrine tumors' in 2009 under supervision of Prof.dr. L.J. Hofland. After obtaining her medical degree cum laude in 2011, she worked at the Department for Internal Medicine at the IJsselland Hospital in Capelle a/d IJssel while awaiting for her PhD position. In this PhD project she investigated the preclinical and clinical aspects of gastroenteropancreatic neuroendocrine tumors under the supervision of Prof.dr. W.W. de Herder and Prof.dr. L.J. Hofland. During the PhD project, she was co-investigator in two clinical trials: COOPERATE-2 and CBEZ235F2201. In September 2015 she started her training residencies in Internal Medicine at the IJsselland Hospital in Capelle a/d IJssel under the supervision of Dr. H.E. van der Wiel, which will be continued in the future at the Erasmus Medical Center, Rotterdam under supervision of Dr. S.C.E. Klein Nagelvoort-Schuit.

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