# GEP-NET : rare tumour connections. Pathophysiological aspects of gastroenteropancreatic neuroendocrine tumours

Kuiper, P.

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*Pathophysiological aspects of gastroenteropancreatic neuroendocrine tumours*

Patricia Kuiper

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# GEP-NET: rare tumour connections
## *Pathophysiological aspects of gastroenteropancreatic neuroendocrine tumours*

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# Contents

# Abbreviations

| | |
|---|---|
| 5-HIAA | 5-hydroxyindoleacetic acid |
| ACTH | Adrenocorticotrophic hormone |
| APUDoma | Amine precursor uptake decarboxylase tumour |
| BB1R, BRS-1 | Bombesin-receptor type 1, Neuromedin B receptor |
| BB2R, BRS-2 | Bombesin-receptor type 2, Gastrin-releasing peptide receptor |
| BB3R, BRS-3 | Bombesin receptor subtype 3 |
| BB4R, BRS-4 | Bombesin receptor subtype 4 |
| BBS | Bombesin |
| BLP | Bombesin-like peptide |
| BSA | Bovine serum albumin |
| CBS | Central Bureau for Statistics |
| CCK | Cholecystokinin |
| CD105 | Endoglin |
| CgA | Chromogranin A |
| CNS | Central nerve system |
| CRC | Colorectal cancer |
| CT | Computed tomography |
| DAB | 3,3'-diaminobenzidine |
| DNET | Duodenal neuroendocrine tumour |
| EC | Enterochromaffin |
| ECL | Enterochromaffin-like |
| ECM | Extracellular matrix |
| ELISA | Enzyme linked immuno-sorbent assay |
| EUS | Endoscopic ultrasonography |
| Flt-1 | VEGF receptor 1 |
| F-PNET | Functioning/functional pancreatic neuroendocrine tumour |
| FSG | Fasting serum gastrin |
| GEP-NET | Gastroenteropancreatic neuroendocrine tumour |
| GI | Gastrin increase |
| GI-NET | Gastrointestinal neuroendocrine tumour |
| GIST | Gastrointestinal stromal tumour |
| GRF | Growth-hormone releasing factor |
| GRP | Gastrin releasing peptide |
| GRPR | Gastrin releasing peptide receptor |
| HRP | Horseradish peroxidase |
| IGF | Insuline-like growth factor |
| kDa | Kilo Dalton |
| KDR | VEGF receptor 2 |
| MEN-1 | Multiple endocrine neoplasia syndrome type 1 |
| MMP | Matrix metalloproteinases |
| MMP-7 | Matrilysin, matrix metalloproteinase-7 |
| MRI | Magnetic resonance imaging |
| mRNA | Messenger ribonucleic acid |
| MVD | Microvessel density |

| | |
|---|---|
| NEC | Neuroendocrine carcinoma |
| NET | Neuroendocrine tumour |
| NF-1 | Neurofibromatosis type 1 |
| NF-PNET | Non-functioning/non-functional pancreatic neuroendocrine tumour |
| NMB | Neuromedin B |
| NMBR | Neuromedin B receptor |
| NME | Necrolytic migratory erythema |
| NSE | Neuron specific enolase |
| PALGA | Nationwide network and registry for histo- and cytopathology in the Netherlands |
| PBS | Phosphate buffered saline |
| PET | Pancreatic endocrine tumour |
| PNET | Pancreatic neuroendocrine tumour |
| PP | Pancreas polypeptide |
| PPI | Proton pump inhibitor |
| ROC | Receiver operating characteristic |
| RT | Room temperature |
| s.e. | Standard error |
| SEER | Surveillance, Epidemiology and End Results database |
| SEM | Standard error of the mean |
| sEndoglin | Soluble endoglin |
| SRS | Somatostatin receptor scintigraphy |
| SSPS | Statistical Package for Social Sciences |
| St. dev. | Standard deviation |
| TBS | Tris-buffered saline |
| TGF-ß | Transforming growth factor beta |
| TNM | Tumour node metastasis |
| VEGF | Vascular endothelial growth factor |
| vHLD | Von Hippel-Lindau disease |
| VIP | Vasoactive intestinal peptide |
| WDHA | Watery diarrhea hypokalemia achlorhydria |
| WHO | World Health Organization |
| ZES | Zollinger-Ellison syndrome |

# Chapter 1

## *General Introduction*

## *and*

## *Outline of the Thesis*

**Gastroenteropancreatic neuroendocrine tumours**

Gastroenteropancreatic neuroendocrine tumours (GEP-NETs) comprise a heterogeneous group of uncommon neoplasms, including the pancreatic neuroendocrine tumours (PNETs) and gastrointestinal (GI) neuroendocrine tumours[1] (GI-NETs, Table 1).

| **Table 1. Neuroendocrine tumours** |
| --- |
| Carcinoids<br>*(gastrointestinal neuroendocrine tumours)* |
| Non-carcinoid gastroenteropancreatic tumours<br>*(pancreatic, duodenal and gastrointestinal neuroendocrine tumours)* |
| Catecholamine-secreting tumours<br>*(phaeochromocytomas, paragangliomas, ganglioneuromas, ganglioneuroblastomas, sympathoblastoma, neuroblastoma)* |
| Medullary carcinomas of the thyroid |
| Chromophobe pituitary tumours |
| Small cell lung cancer |
| Merkel cell tumours |

Table 1. All tumours which are classified and defined as 'neuroendocrine tumour'.

The total incidence is estimated at 2-5 patients per 100.000 persons per year, although recent epidemiological studies have shown that their incidence is increasing remarkably[2-5]. Nevertheless, they only comprise approximately 2% of all malignant tumours of the gastrointestinal tract.

GEP-NETs are considered to originate from the cells from the diffuse neuroendocrine system. There are at least 15 neuroendocrine cell types, scattered along the entire length of the gastroenteropancreatic tract. These cells are called neuroendocrine because their many similarities to neural cells. Not only do they have several histological similarities such as secretory granules and the expression of neuroendocrine cell markers, they also produce bioactive substances that have transmitter function. GEP-NETs are characterized by their ability to synthesize, store and secrete biogenic amines and neuropeptides. Although various neuroendocrine cell markers have been identified, the presence of chromogranin

A is nowadays widely used to identify GEP-NETs (Table 2). GEP-NETs occur mainly in the gastrointestinal tract and pancreas (2/3rd), the pulmonary system being the next most frequent location[1,6,7].

| **Table 2. Neuroendocrine cell markers** | |
|---|---|
| General markers | |
| Chromogranin A, B | |
| Pancreatic polypeptide | |
| Neuron-specific enolase | |
| Human chorionic gonadotrophin alpha/beta subunits | |
| | |
| Specific markers | |
| Insulin | (insulinoma) |
| Gastrin | (gastrinoma) |
| Glucagon | (glucagonoma) |
| Somatostatin | (somatostatinoma) |
| VIP | (VIPoma) |
| ACTH | (ACTHoma) |
| GrH | (GrHoma) |
| Serotonin | (carcinoid) |
| Calcitonin | (calcitoninoma) |

Table 2. Overview of general and specific neuroendocrine cell markers in GEP-NETs.

The clinical presentation of GEP-NETs depends on the location of the primary tumour, the presence of metastases, and the peptide(s) secreted. The diagnosis of GEP-NETs is frequently delayed, and metastases are often present when the tumour is detected. The diagnosis of GEP-NETs is based on clinical presentation, hormone assays, and pathological examination of the tumour. The detection of some biochemical markers in plasma or serum of patients with GEP-NETs raises the suspicion of a specific tumour, whereas other markers are common to several types of GEP-NETs[2] (Table 2). Commonly used imaging modalities include CT, MRI, transabdominal ultrasonography, gastrointestinal endoscopy, selective angiography, nuclear imaging such as somatostatin-receptor scintigraphy, endoscopic ultrasonography[8]. Frequently, primary tumours can not be localized, because of their small size and occult localization[2].

As GEP-NETs show a large variation in tumour behaviour and a wide spectrum of clinical manifestions, treatment of these tumours should be individualized per patient, based on the tumour type and presence of symptoms. Surgery is the treatment of choice in a large percentage of GEP-NETs, especially in patients with limited disease[2]. For patients with advanced or unresectable disease, surgery can be palliative, and even reduce morbidity and mortality. Furthermore, recent studies to medical treatment of GEP-NETs using somatostatin analogues show promising results. The prognosis of GEP-NETs varies strikingly, and is mainly dependent on the size and localization of the primary tumour, and metastatic involvement. However, GEP-NETs show less aggressive behaviour than the more common gastrointestinal carcinomas and pancreatic adenocarcinomas.

The majority of GEP-NETs are sporadic, although they can be multiple and occur as part of a hereditary syndrome, such as Multiple Endocrine Neoplasia type 1, von Hippel-Lindau disease, or neurofibromatosis type 1[9]. The model of neuroendocrine tumour development resembles that from colorectal cancer[1] (Figure 1).

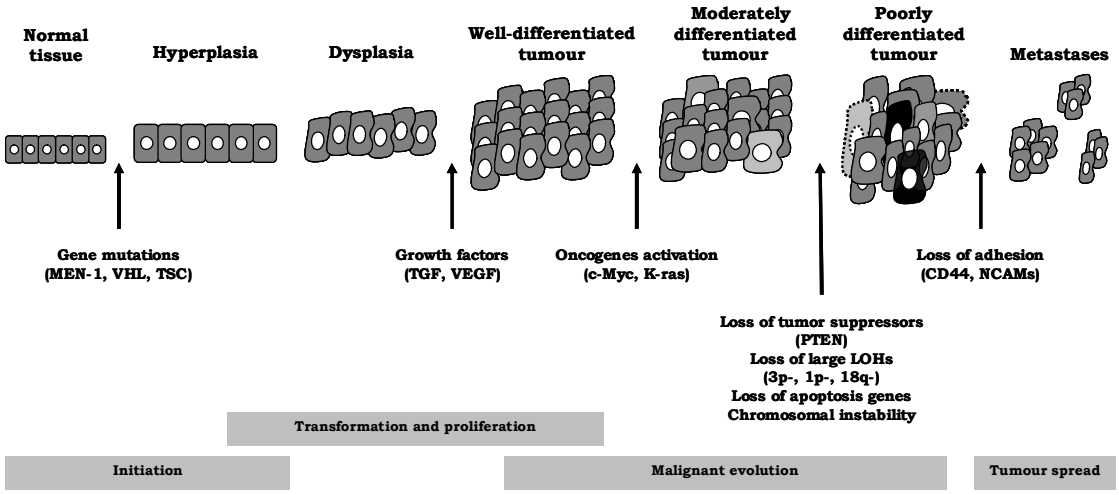


Figure 1. The neuroendocrine tumourigenesis, from normal tissue to the formation of metastases, is shown. The first step in the development of neuroendocrine tumours is the transformation of normal neuroendocrine cells into hyperplastic and/or dysplastic tissue, as a result of gene mutations. Next, the tumour differentiates into a well-, moderately or poorly differentiated tumour, in which growth factors, oncogenes and tumour suppressor genes play an important role. Eventually, tumours spread into the circulation and form metastases. Figure based on Barakat *et al*[1].

The classification of the World Health Organization (WHO) for GEP-NETs is widely used to categorize these tumours. This classification is mainly based on

histopathology and biological behaviour of tumours, divided per tumour localization, i.e., stomach, duodenum and the upper part of the jejunum, appendix, small bowel, including the second part of the jejunum, colon and rectum, and pancreas. Finally they are divided into three classifications, based on differentiation and malignant behaviour, characterized by the presence of angioinvasion and/or metastases[10] (Table 3).

| **Table 3. World Health Organization Classification for GEP-NETs** |
| --- |
| 1a. Well-differentiated neuro-endocrine tumour with benign or uncertain behaviour |
| 1b. Well-differentiated neuro-endocrine carcinoma with low-grade malignant behaviour |
| 2. Poorly differentiated neuro-endocrine carcinoma with high-grade malignant behaviour |

Table 3. Classification of the World Health Organization for GEP-NETs, introduced in 2000.

**Pancreatic neuroendocrine tumours**

Pancreatic neuroendocrine tumours (PNETs) are often referred to as pancreatic endocrine tumours (PETs), pancreatic islet cell tumours or pancreatic islet cell carcinomas. They comprise less than 2% of all pancreatic cancers, and must be distinguished from the more common pancreatic adenocarcinomas, which have a poorer prognosis[11,12]. PNETs can secrete several hormones, dependent on the cell type of origin, and are therefore divided into functional and non-functional tumours. Tumours are referred to as functional in case of the presence of a clinical syndrome resulting from hormone production, e.g., gastrin, insulin, glucagon, vasoactive intestinal peptide (VIP) or somatostatin, by the tumour. In contrast, non-functional tumours can remain clinically silent for a relatively long time and are only detected when morbidity is caused by tumour mass leading to biliary duct obstruction, bowel obstruction, and development of metastases or invasion into adjacent organs[2,12]. Although PNETs have a relatively slow growing rate, the majority of tumours are malignant. Treatment of PNETs is directed to both the tumour and the associated clinical symptoms. Medical therapies like proton pump inhibitors and somatostatin analogues can control hormonal symptoms, whereas antitumoural treatment is necessary to improve and prolong survival, and

includes chemotherapy, hepatic artery or chemo-embolisation, radioablative therapy, and surgical resection[2,13].

**Insulinomas**

Insulinomas are the most frequent occurring functional PNETs, and are primarily considered to be benign. They originate from the pancreatic beta-cells and are characterized by overproduction of the hormone insulin, leading to hypoglycemia-associated symptoms, like dizziness, lethargia and palpitations. The diagnosis of insulinoma can be established by determination of plasma insulin, proinsulin, C-peptide and glucose levels. Alternatively, a 48-72 hours fasting test can be performed to diagnose or exclude an insulin-secreting tumour[2,14,15]. About 5-10% of the insulinomas are part of the hereditary MEN-1 syndrome, while the remaining part occurs sporadically. Females seem to be slightly more affected. Most insulinomas are located in the pancreas, with an equal distribution over the pancreatic head, body and tail. The prognosis for patients with insulinomas is relatively good, showing an overall 5-year survival around 97%[16].

**Gastrinomas**

Gastrinomas are malignant gastrin-producing tumours, arising from the G-cells of the pancreas. Symptoms as dyspepsia, heart burn, diarrhea and peptic ulcers are the result of an increased gastrin production by the tumour, and are collectively named as the Zollinger-Ellison syndrome (ZES)[15]. ZES is seen more commonly in males than in females (ratio 3:2)[16]. Frequently, patients present with a long mean delay in diagnosis. With the widespread use of the proton pump inhibitors (PPIs) and other acid-suppressing medications, delays in presentation are even increasing. The diagnosis of ZES is suspected in case of increased fasting serum gastrin levels (hypergastrinemia), which have been reported to occur in 97% to 99% of the patients[17]. However, in a large percentage of patients the fasting serum gastrin levels alone are not sufficient to diagnose ZES, and therefore additional testing is needed. The secretin stimulation test is considered as the most sensitive and reliable diagnostic tool in gastrinoma patients[18].

Although the majority of gastrinomas is located in the so-called gastrinoma triangle, the anatomical area comprising the pancreatic head, superior and descending portions of the duodenum and the nearby lying lymph nodes, other primary sites of gastrinomas that have been identified are stomach, jejunum, bilitary tract, kidneys, ovaries and liver[19,20] (Figure 2). Gastrinomas occur mainly sporadic, although 30% of the tumours are part of the MEN-1 syndrome[21]. The peak incidence of gastrinomas lies between 40 and 50 years of age[17]. As gastrinomas have a relatively slow growth rate, 5- and 10-year survival rates are estimated to be 65% and 51%, respectively[16]. Even in case of metastatic disease, patients with gastrinomas have a relatively good chance of survival (5-year survival about 40% to 50%). However, patients with pancreatic gastrinomas show a worse prognosis than those with a gastrinoma located in the duodenum[22].

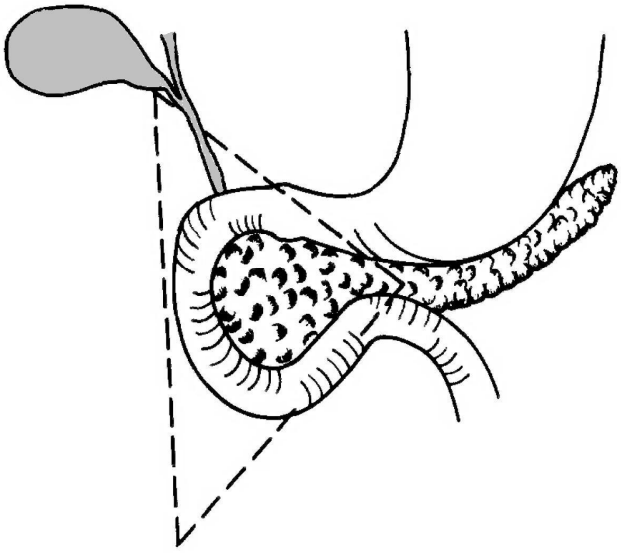

Figure 2. Gastrinoma triangle, which angles are formed by the cystic and common bile ducts, the junction of the neck and body of the pancreas, and the junction of the second and third portion of the duodenum. Figure adapted from Stabile *et al.*[19]

**Glucagonomas**

Glucagon-producing tumours, or glucagonomas, arise from the alpha-cells of the pancreas. Associated clinical symptoms are hyperglycemia, weight loss, anemia, venous thromboses and a typical skin rash called 'necrolytic migratory erythema' (NME)[15]. Glucagonomas are most frequently found in the pancreatic tail. Extrapancreatic glucagonomas are extremely rare[16]. Glucagonomas usually present with a delay in diagnosis, and are often large at first presentation (>6 cm).

At time of diagnosis, metastases are found in approximately 60% to 70% of the patients[16]. Determination of glucagon serum levels contribute to the diagnosis of a glucagonoma (>500 – 1000 pg/mL)[17].

**Somatostatinomas**

Somatostatinomas originate from the pancreatic delta-cells, and produce the hormone somatostatin. Although slow-growing, these tumours do show malignant behaviour. They occur mainly in the duodenum or pancreas, of which only tumours in the latter usually lead to a clinical syndrome[17]. Characterizing symptoms for the so-called somatostatinoma-syndrome are steatorrea, cholelithiasis, diabetes mellitus type-2 and hypochlorhydria. Somatostatinomas in the duodenum are often part of a genetic syndrome, such as the MEN-1 or neurofibromatosis (NF-1) syndrome[15]. No specific tests to establish the diagnosis of a somatostatinoma are available. Only pancreatic somatostatinomas are associated with elevated levels of somatostatin in plasma. Frequently, somatostatinomas are found by incidence, during gastrointestinal imaging studies for cholecystectomy or abdominal pain. The overall 5-years survival is about 75% or 60% in case of metastatic disease[16].

**VIPomas**

VIPomas secrete vasoactive intestinal peptide (VIP), leading to the Verner-Morrison syndrome or watery diarrhea hypokalemia achlorhydria (WDHA) syndrome. Symptoms characterized by WDHA are mainly the result of the severe secretory diarrhea, caused by the secretion of VIP, and are typically dehydration, hypokalemia and achlorhydria. Approximately 80% of VIPomas occur in the pancreas[15], in particular the pancreatic tail[47]. Females are affected more frequently than males[16]. Increased serum levels of VIP (>500 pg/mL) in combination with severe diarrhea are highly suggestive for VIPomas[17]. The 5-year survival rates for patients with VIPomas with or without metastases are estimated to be 60% and 95%, respectively[16].

**Other functional pancreatic neuroendocrine tumours**

Other functional PNETs include ACTHomas and GRFomas, which are both extremely uncommon[16]. ACTHomas secrete adrenocorticotrophic hormone (ACTH), leading to the Cushing's syndrome. GRFomas produce growth-hormone releasing factor (GRF), and are characterized by acromegaly. Furthermore, PNETs can secrete calcitonin, enteroglucagon, cholecystokinin (CKK), gastric inhibitory peptide, gastrin-releasing peptide (GRP) and ghrelin, although rare[16,17].

**Non-functional pancreatic neuroendocrine tumours**

Non-functional pancreatic neuroendocrine tumours comprise about 70% of all PNETs. These tumours are not related to any clinical syndrome caused by hormonal overproduction. However, they may show immunohistochemical positivity for hormones or neuropeptides, and frequently increased serum/plasma levels of chromogranin A or PP are found[15,23]. Whereas functional tumours cause symptoms relating to hormone production, non-functional tumours often cause tumour mass related complaints[1]. Furthermore, symptoms can be vague and aspecific, i.e., abdominal pain, anorexia, nausea and weight loss. Frequently, this leads to a delayed detection and the presence of local invasion and/or distant metastases at time of diagnosis. A small percentage of non-functional PNETs are found incidentally at surgery or autopsy[16]. The majority of non-functional PNETs can be classified as well-differentiated neuroendocrine carcinomas[23]. It is important to distinguish these tumours from the more common and aggressive pancreatic adenocarcinomas. Most non-functional PNETs are located in the head of the pancreas. Non-functional PNETs can occur as part of the MEN-1 syndrome or may be associated with Von-Hippel Lindau disease (VHL). These tumours show a more aggressive course than their functional counterparts, although 5-year survival has been reported to lie around 65%[16].

**Duodenal neuroendocrine tumours**

Duodenal NETs can generally be classified into five tumour types; gastrinomas, somatostatinomas, non-functional NETs, gangliocytic paragangliomas, and poorly

differentiated neuroendocrine carcinomas. The majority of these tumours occur in the first or second part of the duodenum. Duodenal NETs are usually small, i.e., <2cm in diameter. Although they are often limited to the (sub)mucosa, regional lymph node metastases can be found in about 40% to 60% of the patients. Liver metastases are seen less frequently (<10%). Duodenal NETs are usually single lesions. When multiple tumours are detected, the MEN-1 syndrome should be suspected. Functional syndromes are rare in these tumours, comprising mainly ZES or the carcinoid syndrome when they do occur[24,25].

**Gastrointestinal neuroendocrine tumours**

Gastrointestinal neuroendocrine tumours (GI-NETs) are heterogeneous regarding histological differentiation, hormone production and biology. Frequently, GI-NETs are referred to as carcinoids[26]. They derive from cells of the diffuse neuroendocrine system, and can be divided into serotonin-producing enterochromaffin (EC) or Kulchitsky's cells, and the gastric histamine-secreting enterochromaffin-like (ECL) cells. Carcinoids are able to produce vasoactive substances like amines (serotonin, catecholamines, and histamine) and prostaglandins[26,27]. About only 10% of the carcinoid patients actually suffer from the classical carcinoid syndrome, characterized by symptoms as flushing, hypotension, diarrhea, wheezing, and heart disease, as a consequence of the serotonin secretion. GI-NETs occur predominantly in the gastrointestinal system (70%) or pulmonary tract (25%). Other known, but rare sites of GI-NETs are the ovaries, breast, larynx, thymus and gall bladder[1]. Among the gastrointestinal system, the small intestine and appendix are most commonly affected[27-30].

Dependent on their localization, GI-NETs can remain indolent for a long time. Frequently, symptoms arise when metastases have developed[31].

Besides the determination of chromogranin A levels, 5-HIAA measurements can aid in diagnosing serotonin-producing carcinoids. Although the specificity of the 5-HIAA test is about 100%, sensitivity is only 35%. Treatment options for patients with GI-NETs include somatostatin analogues, alpha-interferon, radiation,

chemotherapy, and surgery. The decision for a medical or surgical approach is based on the location of the primary tumour, and the presence of metastases[27-29].

**Multiple endocrine neoplasia type 1 syndrome (MEN-1 syndrome)**

The multiple endocrine neoplasia type 1 syndrome (MEN-1 syndrome) is an autosomal dominant inherited disorder, caused by mutations in the MEN-1 gene, located on chromosome 11q13. This syndrome is characterized by tumours in the parathyroid, pancreas, and anterior pituitary. Familial MEN-1 is defined as one patient with MEN-1 and one first-degree relative are affected with at least one tumour in one of the three key organs[9].

In 30% to 75% of the patients with MEN-1 pancreatic tumours are seen[15]. In particular gastrinomas are associated with this hereditary syndrome (20% to 60%), followed by insulinomas (30%) and VIPomas (5%). Non-functional PNETs occur in approximately 50% of the patients with MEN-1. MEN-1 related tumours occur at a relatively earlier age, and have a better prognosis compared to sporadic tumours. They may be multiple and vary in size from small microadenomas to large tumours[23]. Other hereditary syndromes which are associated with pancreatic or gastrointestinal neuroendocrine tumours are VHL-disease and tuberous sclerosis[9].

**Neuropeptides**

GEP-NETs express a variety of peptide hormones and bioactive amines, including serotonin, chromogranin A, calcitonin, corticotrophin, neuron specific enolase, substance P, gastrin and bombesin-like peptides[28,32]. Bombesin was initially isolated from amphibian skin, and received its unusual name after the genus of the frog, i.e., Bombina bombina. Gastrin releasing peptide (GRP) and neuromedin B (NMB) are the mammalian analogs of bombesin, and belong to the family of bombesin-like peptides (BLPs)[33]. In humans, they are distributed in neural and endocrine cells, especially throughout the gastrointestinal tract. In addition to stimulating a variety of physiological responses in the human body, BLPs are involved in development and progression of several human cancers. For example, it has been shown that these peptides can stimulate the growth of lung, CNS,

breast, cervix and prostate cancer cell lines, both in vivo and in vitro[34,35]. BLPs mediate their biological actions through binding to the G-protein coupled gastrin-releasing peptide receptor (GRPR, BB2R), neuromedin B receptor (NMBR, BB1R), bombesin receptor subtype 3 (BRS3, BB3R) and bombesin receptor subtype 4 (BRS-4, BB4R). Activation of various bombesin receptor subtypes has growth effects in both normal and neoplastic tissues, and several studies have reported an upregulation of bombesin receptors in tumour samples compared to associated normal tissue[36-38].

**Angiogenesis**

Angiogenesis, the formation of new blood vessels from the existing vascular bed, is a physiological process involved in several events like wound healing and embryonic development[39,40]. Furthermore, it is a critical process for tumourigenesis, as tumours need the development of new blood vessels for their growth and further expansion[41-44]. Tumour cells stimulate mature blood vessels nearby to sprout new microvessels towards the tumour by production of angiogenic factors like transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). Obviously, angiogenesis provides the tumours with an efficient route of exit for tumour cells to leave the primary tumour, enter the blood or lymph stream and form metastases[40] (Figure 3). In various cancers, increased vascular density has been shown to be related to an increased amount of metastases and decreased survival[46].

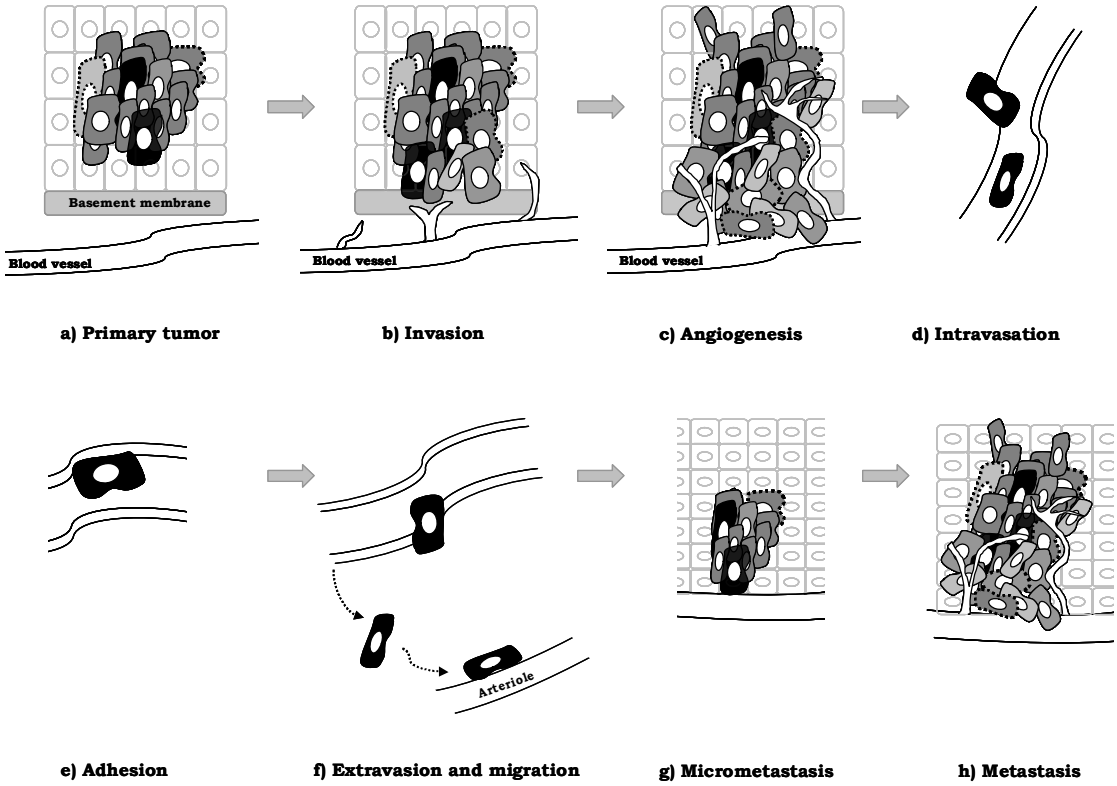


Figure 3. The process of angiogenesis in tumours step-by-step.
a) Primary tumour; b) Tumour cells induce blood vessels to form microvessels in the direction of the primary tumour; c) Angiogenesis, the formation of new blood vessels from existing ones; d) Tumour cells escape from the primary tumour, enter the circulation (intravasation), and e) adhere to other blood vessels; f) Tumour cells leave the circulation (extravasation) and migrate to other places; g+h); where they form (micro)metastases. Figure adapted from Zetter *et al.*[44]

### Vascular endothelial growth factor

One of the key factors in angiogenesis is vascular endothelial growth factor (VEGF). VEGF has numerous effects on endothelial cells, including migration and differentiation[47-49]. Its physiological effects are mediated through binding to the VEGF receptor 1 (Flt-1) and VEGF receptor 2 (KDR) [50]. Up-regulation of VEGF in tumours may result from oncogene activation, inhibition of tumour suppression factors, release of growth factors, hypoxia, or necrosis. VEGF primary acts as an endothelial cell mitogen and modulator of changes in vascular permeability, but also mediates the secretion and activation of enzymes involved in the degradation of the extracellular matrix (ECM), thereby further facilitating tumour angiogenesis[51].

**Endoglin**

Endoglin, or CD105, is a transforming growth factor beta (TGF-β) receptor, which can bind TGF-β1 and TGF-β3 in the presence of the TGF-β receptor type II[52-54]. In the early stages of tumour formation, TGF-β inhibits the proliferation, differentiation and migration of cells, whereas endoglin counteracts these actions, thereby promoting angiogenesis[55]. Endoglin is predominantly expressed on endothelial cells of newly formed (angiogenic) blood vessels[56]. Its expression is up-regulated by hypoxia and TGF-β[57]. In several cancers, increased endoglin levels in tumours are associated with the presence of metastases and a poor survival[58-60].

**Matrilysin**

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes, involved in ECM degradation. In humans, at least 23 different MMPs are known. Based on their structure and their substrate preference, they are classified as gelatinases, collagenases, stromelysins, matrilysins, membrane-type MMPs, and others. MMPs are synthesized as pre-proenzymes. The expression of MMPs is transcriptionally controlled by inflammatory cytokines, growth factors, hormones, cell-cell interactions, and cell-matrix interactions. Next to their main function to degrade and remove ECM molecules from the tissue, MMPs are involved in pathologic processes like angiogenesis, tumour transformation and the development of metastases[61,62].

Matrilysin, or MMP-7, belongs to the subgroup of stromelysins. Matrilysin is secreted as pro-MMP-7, of which proteolytic removal of the 9 kDa prodomain from the N-terminus results in activation of the enzyme. Matrilysin is almost exclusively produced by epithelial tumour cells. Up-regulation of matrilysin in tumours is the consequence of mutations in the Wnt-signaling pathway[63]. Numerous studies have shown that matrilysin is significantly enhanced in several cancers, including breast, prostate, lung, skin, and colorectal cancer, and related to the malignant potential of the tumour[64].

**Insulin-like growth factor system**

The insulin-like growth factor (IGF) system is crucially involved in growth and development of tissues. Furthermore, by controlling cell cycle progression and preventing apoptosis, it plays an important role in tumourigenesis, tumour cell proliferation and metastatic spread[65]. The IGF-system is composed of two ligands, IGF-1 and IGF-2, three cell-surface receptors, IGF-1 receptor (IGF-1R), IGF-2 receptor (IGF-2R), and the insulin receptor (IR), and a family of six IGF binding proteins (IGFBP-1 to IGFBP-6). IGFBPs are able to regulate the bioavailabity of the IGF ligands in the circulation. IGF-1 is predominantly produced in the liver, and has numerous functions. It acts as a mitogen and an anti-apoptotic survival factor, is involved in the glucose metabolism, and promotes cell migration. The effects of IGF-1 are predominantly mediated via the type I insulin-like growth factor receptor (IGF-1R), which can also bind IGF-2. Recent studies have shown that elevation of serum IGF-1 is associated with an increased risk of tumour development. Furthermore, IGF-1R has emerged as a key regulator of mitogenesis and tumourigenicity, because of its important role in cell transformation, tumour invasion, metastasis and cell survival enhancement[65-67].

**Outline of the thesis**

Gastroenteropancreatic neuroendocrine tumours (GEP-NETs) are a group of uncommon and heterogeneous neoplasm, which show a large diversity in morphological, histocytopathological and clinical aspects. This thesis describes studies on the epidemiology, diagnosis, and pathogenesis of neuroendocrine tumours of the gastroenteropancreatic tract, in particular the pancreatic neuroendocrine tumours and the gastrointestinal carcinoids. The goal was to elucidate the mechanisms contributing to the diversity of GEP-NETs, and to investigate the role of various factors in the pathogenesis of these tumours (Figure 4).

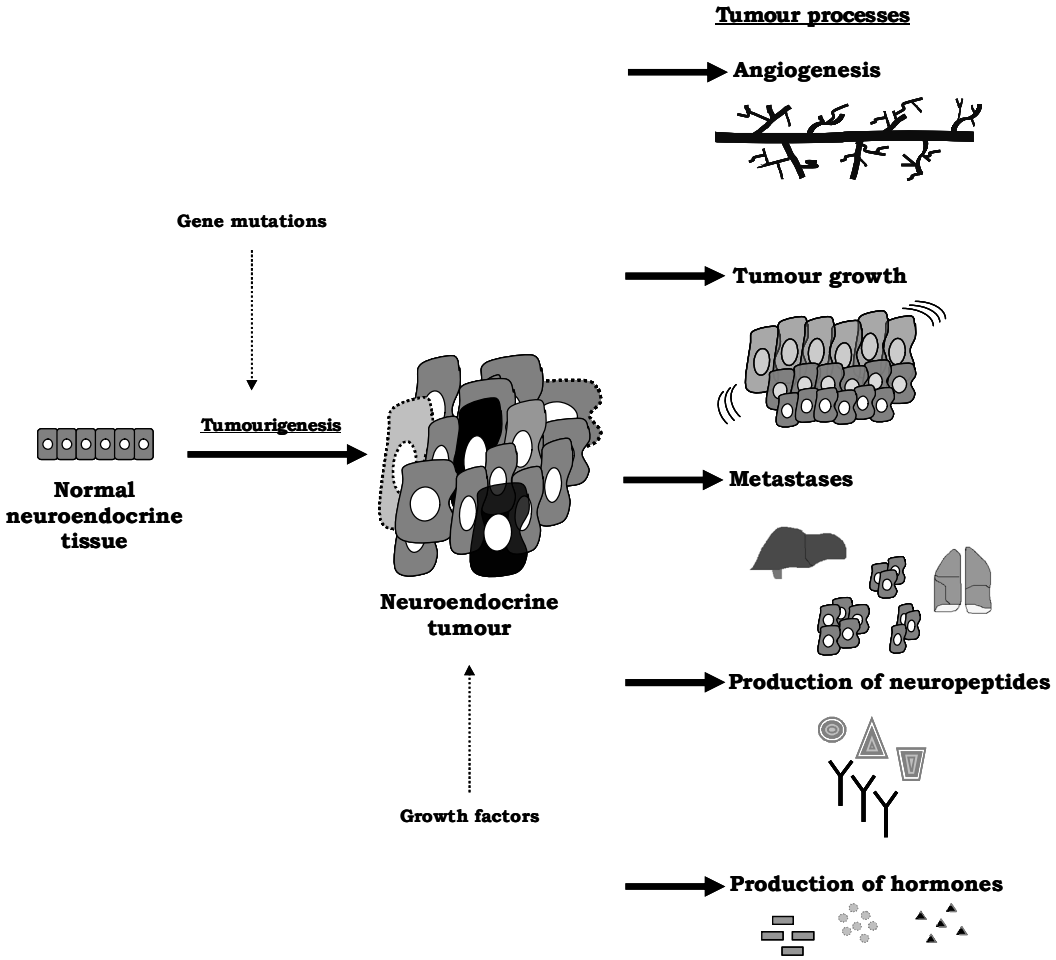


Figure 4. The processes associated with neuroendocrine tumour development, behaviour and progression, as discussed in this thesis, are depicted. As a result of gene mutations and the effects of growth factors produced by tumour cells, normal neuroendocrine tissue cells can proliferate and differentiate into a neuroendocrine tumour. Tumour processes like angiogenesis, tumour growth, metastases and the production of neuropeptides or hormones determine the clinicopathological behaviour and prognosis for the patients.

An overview of the current diagnostic approach of GEP-NETs is given in **Chapter 2**. The need for a standardized diagnostic approach of GEP-NETs is advocated by the rise in incidence of these tumours, as illustrated in **Chapter 3.** This chapter describes an epidemiological study to the incidence of duodeno-pancreatic neuroendocrine tumours from 1991 to 2009 in The Netherlands. Gastrinomas are the most frequent occurring type of malignant functional neuroendocrine tumours, usually located in the pancreatic region. However, **Chapter 4** describes a case report of a patient suffering from the Zollinger-Ellison syndrome with recurrent gastrinomas in the liver, without evidence of any tumour of another primary origin. As the existence of truly *primary* hepatic

gastrinomas is highly questionable, an overview of all liver gastrinomas defined as primary in the literature is given. The diagnosis of a gastrinoma can be established by the use of the secretin stimulation test. Although this test is currently the most used diagnostic tool for gastrinomas, several aspects of this test have been debated. **Chapter 5** describes an intra-individual comparison study using different dosages of secretin in patients and controls to investigate the most optimal criterion and secretin dosage for a positive secretin stimulation test to diagnose the Zollinger-Ellison syndrome.

GEP-NETs are characterized by their ability to secrete neuropeptides, such as gastrin releasing peptide and neuromedin B, the mammalian counterparts of bombesin. A study on the expression of these bombesin-like peptides and their receptors in carcinoids of different origin, i.e., pulmonary and intestinal origin, is reported in **Chapter 6**.

GEP-NETs are highly vascularized tumours. Angiogenesis, the formation of new blood vessels, is a crucial process in tumour development. **Chapter 7** documents an investigation on the expression and role of vascular endothelial growth factor (VEGF) and endoglin (CD105), two key players in angiogenesis, in the tumourigenesis of GEP-NETs.

In order to assess a potential growth activation process of GEP-NETs, the expression of insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 3 (IGFBP-3) and matrilysin (MMP-7) was also investigated. The role of this IGF-matrilysin network in the pathogenesis of GEP-NETs is described in **Chapter 8**.

The aim of the studies described in this thesis was to identify markers with a role in the pathogenesis of GEP-NETs, which contribute to a better understanding of the biology, histopathology and complex heterogeneity of these tumours. Ultimately, these markers might assist in improved histological grading systems and classifications, advanced diagnostics and appropriately targeted treatment for the patients, as summarized and discussed in **Chapter 9**.

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# Chapter 2

## *An Overview of the Current Diagnosis and Recent Developments in Neuroendocrine Tumours of the Gastroenteropancreatic Tract: the Diagnostic Approach*

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**Abstract**

Neuroendocrine tumours of the gastroenteropancreatic tract (GEP-NETs) comprise a group of very heterogeneous neoplasms, which are considered 'rare diseases'. Epidemiological studies on the incidence of GEP-NETs worldwide have reported a remarkable increase in the detection of these tumours.

In a recent study, based on pathology reports (PALGA) to investigate the incidence of pancreatic and duodenal neuroendocrine tumours in The Netherlands from 1991 until 2009, we also noticed a significant increase in the incidence of these tumours. In particular, the incidence of non-functioning neuroendocrine tumours had significantly increased over this period. Remarkably, a substantial discrepancy was observed between the numbers of neuroendocrine tumours diagnosed in the clinical as opposed to the pathological setting, emphasizing that these tumours provide a real diagnostic challenge. To improve the diagnosis of GEP-NETs, we advocate that these complex neoplasms should receive more specialized attention.

In this mini-review we provide an overview of the current diagnostic approach of GEP-NETs, and added the recent developments in establishing the diagnosis of these tumours, in order to increase the intelligibility and awareness of GEP-NETs among clinicians and pathologists. Early detection in order to prevent morbidity of GEP-NETs is advocated.

**Main text**

*Introduction*

Gastroenteropancreatic neuroendocrine tumours (GEP-NETs) are considered to be rare, heterogeneous and complex neoplasms[1]. They include the pancreatic (PNETs) and gastrointestinal (GI) neuroendocrine tumours (GI-NETs) or carcinoids, which share their origin of cells of the diffuse neuroendocrine system, but further show many differences regarding pathogenesis, clinical behaviour and prognostic outcome[2,3]. Characteristic for GEP-NETs is their ability to produce bioactive substances (Table 1)[4]. Based on the clinical symptoms and syndrome caused by these peptides, they can be divided into functioning (F-NETs) and non-functioning tumours (NF-NETs). Due to their heterogeneity, GEP-NETs often provide a diagnostic challenge to physicians. Although GEP-NETs are generally more indolent than carcinomas, the majority are malignant, showing aggressive tumour behaviour and presenting with metastases at diagnosis[1]. GEP-NETs can occur sporadically, or as part of a hereditary syndrome like Multiple Endocrine Neoplasia syndrome type 1 (MEN-1), von-Hippel Lindau Disease (vHLD), neurofibromatosis type 1, or tuberous sclerosis[5].

In 2007, a summit meeting on the major clinical, pathological and scientific challenges in the field of GEP-NETs was held to debate on potential solutions[6]. There was consensus between the participants that there is a worldwide substantial lack of knowledge, experience and reliable research concerning GEP-NETs. In line with these observations, we feel that also in our country, GEP-NETs indeed present a relatively unknown and underdeveloped subject with fairly limited knowledge under most physicians. However, since several epidemiological studies have shown an increase in the incidence of GEP-NETs worldwide, in combination with the fact that these tumours, when accurately managed, provide a relatively good prognosis for the patients, we feel that it can be worth to increase the awareness for and knowledge about GEP-NETs among clinicians and pathologists, in order to further increase the early detection and prevent morbidity of GEP-NETs[7-10].

In this mini-review, we describe the current diagnostic approach of GEP-NETs, in combination with several common pitfalls and some recent developments to improve the diagnosis of these tumours. In addition, we provide a diagnostic algorithm to facilitate their diagnostic approach.

*Epidemiology*

Based on pathology information from PALGA the nationwide network and registry of histo- and cytopathology in The Netherlands, we calculated incidence of GEP-NETs from 2000 till 2008 in The Netherlands[8,11]. For both pancreatico-duodenal NETs and GI-NETs a significant increase in incidence over time was noticed (Figure 1).

**Figure 1. Incidence of GEP-NETs from 2000 till 2008**

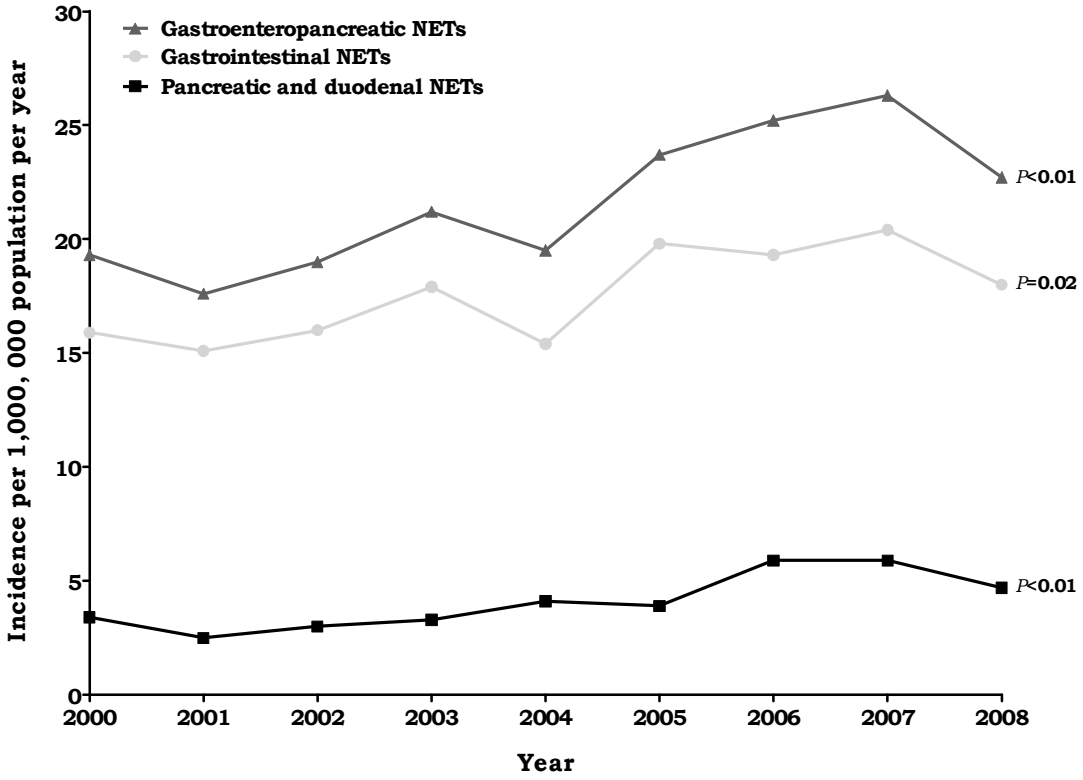


Figure 1. Current incidence of GEP-NETs in The Netherlands from 2000 till 2008. Using linear regression, trends in annual incidence rates over 2000 till 2008 were analyzed. A statistically significant increase was observed in the overall annual incidence of all GEP-NETs, and GI-NETs and pancreaticoduodenal NETs separately, over the study period.

However, these calculated incidence rates are based on pathology information only and therefore might represent an underestimation. In our study, we found that this was approximately 25%, due to the fact that some patients with clinically

diagnosed gastrinomas were not included in the PALGA database, because they had not undergone any surgery, biopsy and/or other pathological evaluation for their tumour[8]. This discrepancy between clinical and pathology incidence of GEP-NETs is an important issue concerning these tumours, which will be discussed later. Nonetheless, this pattern of increasing incidence rates indicates and confirms that GEP-NETs might not be as rare as previously thought. Whether this increase is due to improved detection methods rather than to a true rise in existence of these tumours is debatable. In that context it is important to note that we observed that 4% and 14% of the GI-NETs and pancreaticoduodenal NETs respectively, were found by incidence at autopsy, which indicates that, despite of improved detection methods, some GEP-NETs still do remain undetected.

*Current diagnostic procedure for GEP-NETs*

Symptoms of patients with GEP-NETs are in general related to the localization and hormonal production of the tumour[1]. Frequently, symptoms are vague and aspecific, although symptoms associated with a clinical syndrome may arise suspicion for a F-PNET (Table 1)[1].

Next to standard medical history and physical examination, laboratory analyses are crucial in the diagnosis[12,13]. To diagnose NETs, chromogranin A (CgA) levels can be determined in plasma/serum, or immunohistochemically[14,15]. Increased plasma/serum levels of CgA have been reported to correlate with a worse prognosis in these patients. Increased levels of 5-hydroxyindoleacetic acid (5-HIAA, the breakdown product of serotonin) can be determined in a 24-hours urine sample collection, and indicate the presence of a serotonin-producing tumour. Increased levels of hormones like insulin, indicate the presence of a hormone-secreting functioning PNET.

| **Table 1. Symptoms and syndromes associated with GEP-NETs[1-4]** | |
|---|---|
| **Gastrointestinal neuroendocrine tumours** | |
| **Functioning neuroendocrine tumours** | **Non-functioning neuroendocrine tumours** |
| **Carcinoid**<br>Flushing, diarrhoea, and wheezing | Abdominal pain, weight loss, anorexia, jaundice, nausea and vomiting, intra-abdominal haemorrhage |
| **Pancreatic neuroendocrine tumours** | |
| **Functioning neuroendocrine tumours** | |
| **Insulinoma**<br>Neuroglycopenic symptoms like headache, blurred vision, confusion, dizziness, lethargy, and amnesia. Autonomic nervous system symptoms like sweating, weakness, anxiety, tremor, palpitations, and nausea | |
| **Gastrinoma**<br>Diarrhoea, abdominal pain, heartburn, weight loss, nausea and vomiting, faecal blood loss | |
| **Glucagonoma**<br>Necrolytic Migratory Erythema, Diabetes Mellitus, cachexia | |
| **VIPoma**<br>Watery diarrhoea, hypokalemia, achlorhydria, hyperglycaemia, hypercalcemia, flushing | |
| **Somatostatinoma**<br>Diabetes Mellitus, cholelithiasis, steathorrea, anaemia, weight loss | |
| **Other (rare) pancreatic functioning neuroendocrine tumours** | |
| **ACTHoma**<br>Cushing's syndrome | |
| **GRFoma**<br>Acromegaly | |
| **PTH-RP tumour**<br>Hypercalcemia | |
| **Non-functioning neuroendocrine tumours** | |
| Abdominal pain, weight loss, anorexia, jaundice, nausea and vomiting, intra-abdominal haemorrhage | |

Table 1. Overview of all symptoms and syndromes associated with GEP-NETs.

Imaging of GEP-NETs includes endo- or gastroscopy, octreoscan, computerized tomography (CT) scan, or magnetic resonance imaging (MRI) scan[16].

Pathological examination of biopsies or surgical specimens reveal the verification of the neuroendocrine nature of the tumour by immunohistochemistry, for pan-neuroendocrine markers like keratin, CgA, neuron specific enolase (NSE), synaptophysin, grimelius, and CD56. A proliferation marker (Ki67 or MIB1) must

be used to assess the degree of differentiation and proliferation, to grade the tumours according the World Health Organization (WHO) classification[17]. Tumour characteristics like localization, size, composition, relationship to anatomic structures, resection margins, and the presence of metastases, should be assessed in order to classify the tumour along the TNM stage classification[4].

*Pitfalls in the diagnosis of GEP-NETs*

One of the major pitfalls in the nomenclature of neuroendocrine tumours is the use of the term 'carcinoid'. In 1907, Oberndorfer introduced this term for neuroendocrine tumours with a relatively 'benign' course[18]. Increasing knowledge about these tumours, however, had led to the conclusion that carcinoids also encompasses low-grade and high grade malignant tumours. Therefore, Soga *et al.* called the term 'carcinoid' a 'misnomer'[19]. In fact, this term has been used for different goals; whereas pathologists label all tumours with neuroendocrine features as a 'carcinoid', clinicians use 'carcinoid' for serotonin-producing tumours that lead to the carcinoid syndrome. Therefore, Capella *et al.* suggested replacing 'carcinoid' by 'neuroendocrine tumour' to include all tumours with neuroendocrine features, but also realized that abandoning this term completely would be too confusing, and therefore proposed to utilize it for the specification of a NET with serotonin production or producing any other substance which may lead to the carcinoid syndrome[20]. As a consensus in the use of the GEP-NETs nomenclature is highly desirable, we propose that henceforth 1) the term 'carcinoid' should be used solely in the clinical setting, and only for those tumours that lead to the carcinoid syndrome as a result of the hypersecretion of serotonin, prostaglandins, or tachykinins by the tumour, characteristic of symptoms like flushing, diarrhoea and wheezing; 2) pathologists distinguish the various types of neuroendocrine tumours; neuroendocrine tumours should be defined according to the classification of the WHO, thereby replacing 'carcinoid' by 'neuroendocrine tumour' for well-differentiated low-grade malignant carcinoids, whereas malignant carcinoids should be defined as 'neuroendocrine carcinomas'.

Another misunderstanding among pathologists and clinicians has arisen due to the lack of a standardized definition of functioning and non-functioning tumours, as pointed out by Halfdanarson *et al.* Although non-functioning tumours are characterized by the lack of a clinical syndrome, they might secrete hormonal peptides as well, but only those tumours leading to clinical symptoms are referred to as functioning[7]. For example, increased blood levels of pancreatic polypeptide or neurotensin can be found in NF-PNETs[21]. Warner *et al.* already reported that plasma hormone levels not always correlate with the presence of a clinical syndrome[22]. For example, in case of the Zollinger-Ellison syndrome, fasting serum gastrin levels may be non-diagnostic (i.e., <1000 ng/L), or symptoms might be masked by the use of proton pump inhibitors or histamin receptor antagonists, or pernicious anaemia. Furthermore, it is reported that the hormonal secretion by the tumour is not always reflected in immunohistochemical staining for this hormone at pathology[23]. For a standardized approach, we recommend that the clinical diagnosis is superior to the pathological observations concerning the designation of the tumour as 'functioning' or 'non-functioning'. In other words, in the absence of immunohistochemical positivity for a certain hormone in combination with increased serum levels of that particular hormone and/or the presence of a clinical syndrome, the tumour should be defined as 'functioning'. In the opposite situation, i.e., a positive staining at pathology, but absence of increased serum levels and/or a clinical syndrome, the clinical presentation should be decisive, and the tumour should be defined as 'non-functioning'.

Next, the existence of 'benign' GEP-NETs is disputed. Whereas the majority of GEP-NETs are considered to be malignant, insulinomas and appendiceal carcinoids are not. However, we believe that all GEP-NETs have malignant potential, and that early diagnosis of these tumours, because of the symptoms they cause, leads to the assumption that they are benign. Namely tumour size and/or invasion, and the presence of metastases, all characteristics which can be 'prevented' by early detection, makes a tumour to be referred to as malignant.[17,20] The fact that the majority of NF-NETs have a poor prognosis underlines that

absence of clinical symptoms leads to a delay in diagnosis and a consequently more progressed tumour.

Another difficulty in diagnosing GEP-NETs arises as these tumours show a relative high frequency of 'ectopic occurrence'. For example, gastrinomas, which are usually located in the pancreaticoduodenal region and lymph nodes, have been reported on ectopic locations such as ovaries, biliary tract, kidneys, stomach and liver[24]. Recently, we reported on a patient with recurrent hepatic gastrinomas, in whom no pancreatic, duodenal or other primary tumour could be detected despite of an intensive, 20-year lasting follow-up[25]. In literature, primary hepatic gastrinomas were described in about 20 patients, but real evidence for their primary origin (rather than being metastatic) was lacking. We believe that it is therefore uncertain whether these ectopic locations comprise primary gastrinomas rather than metastases of occult primaries. Furthermore, GEP-NETs have been reported on rare locations like oesophagus, gallbladder and biliary ducts, Meckel's diverticulum, ampulla of Vater, genital tract and skin[26,27]. Lack of awareness that neuroendocrine lesions can also occur on these unusual sites results in the consequence that these tumours are frequently misdiagnosed or overlooked[27]. Therefore, we recommend that when imaging is not successful to detect a neuroendocrine tumour in usual sites, an intensive search for occult tumours at ordinary sites should be started.

Additionally, it is important to realize that GEP-NETs frequently occur as or together with a second primary malignancy[28]. The presence of a simultaneous second primary or metastatic malignancy must be thoroughly examined, as several case reports describe the existence of a second tumour synchronous with a carcinoid lesion[28-32]. For example, gastrointestinal stromal tumours (GIST) are frequently seen in combination with (gastric) carcinoids[29,31]. Furthermore, patients suffering from hereditary syndromes like MEN-1, vHL-disease, neurofibromatosis type 1 or tuberous sclerosis, are at increased risk for a gastroenteropancreatic NET. Therefore, alertness for synchronous (neuroendocrine) tumours among clinicians is advocated. Furthermore, members from hereditary GEP-NET disorder families should be checked for such tumours preferably by genetic counselling, and, if

possible, DNA profile, or by measurement of markers for these or associated tumours.

*Recent developments in the diagnosis of GEP-NETs*

As CgA is produced by all types of neuroendocrine cells, it serves as a highly sensitive neuroendocrine cell marker[14,15]. In 2006, Kidd *et al.* demonstrated that also CgA mRNA and protein levels were useful in the detection of gastrointestinal carcinoids and metastases[33]. Recently, Modlin *et al.* showed that measurement of circulating mRNA of CgA (and other markers such as Tph1 and NSE) provides a promising new diagnostic method for NETs[34]. Next to CgA, several studies to other markers have been reported. In particular, investigators are interested to find markers which can discriminate between the diverse GEP-NET subtypes. Long *et al.* demonstrated that PAX8 might be a useful immunohistochemical marker in the discrimination of pancreatic and ileal NETs, as the latter lack expression of this transcription factor[35]. However, Hosoda *et al.* found that immunohistochemistry on EUS-biopsy specimens using a selected panel of markers, including CK-7, CDX-2, synaptophysin, CgA, and the KRAS mutational status, could be used to discriminate endocrine tumours from two other major types of pancreatic cancers (i.e., invasive ductal carcinoma and acinar cell carcinoma) [36]. A comparable study was performed by Burford *et al.*, who found that strong immunohistochemical expression for E-cadherin and B-catenin were characteristic for PNETs, and could be used to discriminate from solid pseudopapillary neoplasm, in which staining is absent[37]. Another selected panel, including CDX-2, NESP-55, TTF-1 and PDX-1, was described to be useful to discriminate between metastatic NETs of pancreatic, gastrointestinal and pulmonary origin, in a study of Srivastava *et al.* [38]. In contrast, Fendrich *et al.* found that PDX-1 expression was present in pancreatic but not duodenal gastrinomas, and PDX-1 expression in combination with Shh and PP expression in resected metastases might aid to locate undetected or occult primary gastrinomas[39]. However, all above mentioned studies are non-conclusive, and further research and validation studies are needed before these diagnostic tools can be used in

practice. Based on a literature review and analysis to the utility of plasma/serum CgA measurements in NETs, Modlin *et al.* concluded that CgA still serves as the most specific (86%) and sensitive (68%) biomarker in plasma/serum to diagnose NETs that is currently available[40].

The improvement of imaging techniques is one of the most probable explanations for the incidence increase of GEP-NETs. For example, in a study of Ishikawa *et al.*, endoscopic ultrasound combined with contrast enhancement showed the best results in the preoperative localization of PNETs in comparison with other imaging techniques, like CT and US[41]. Prasad *et al.* reported that occult primary NETs could be detected by PET/CT using 68Ga-DOTA-NOC receptor in 59% of patients with confirmed NETs on biopsies from metastatic lesions, which was approximately three times higher than with CT alone[42].

Also on the field of genetic and molecular pathology, research is ongoing. Previously, three detailed review articles that describe recent advances in the molecular genetics of sporadic and familial GEP-NETs, were reported[5,43,44]. Therefore, this review will not discuss this subject into detail.

*Diagnostic algorithm*

The algorithm comprises a clinical and a pathological part. Although the pathological evaluation is important in the diagnosis, the clinical presentation largely determines the definition of a NET. However, we advocate an interdisciplinary cooperation between clinicians and pathologists in the diagnostic approach of GEP-NETs.

Although research to specific biomarkers to detect GEP-NETs is ongoing, studies are still inconclusive. Therefore, we recommend CgA as a highly specific and sensitive neuroendocrine marker in the diagnosis of NETs. CgA measurement in plasma/serum, and immunostaining for this marker on biopsy or surgical specimens, should be performed routinely by clinicians and pathologists, respectively, in order to adequately diagnose (or exclude) a NET.

Imaging techniques to detect NETs are improving. The use of various imaging tools combined is advocated. In specialized centres, relatively new imaging

modalities including PET-scan can be used in the localization of a NET. Repeatedly negative imaging results in detecting a primary NET should raise the suspicion of a physician for an ectopic localized NET. Furthermore, the presence of a secondary tumour should be investigated, in particular when a hereditary syndrome is present.

For standardized documentation and in order to determine the therapeutic approach, tumours should be categorized according the WHO and TNM classification.

*Conclusion*

GEP-NETs compose a complex and heterogeneous tumour entity, which form a diagnostic challenge to physicians. In this review, we aimed to provide a clear overview of current diagnostic procedures and common pitfalls for GEP-NETs. Taking some recent diagnostic developments in account, we propose a diagnostic algorithm for GEP-NETs, to generate a more standardized diagnostic approach, facilitate the diagnosis, and eventually improve the early detection of these tumours.

Figure 2. Diagnostic algorithm for GEP-NETs. Based on the current diagnostic approach, and inclusion of several pitfalls and various recent developments in the diagnosis of GEP-NETs, we provided a diagnostic algorithm to adequately diagnose these tumours.

**I. CLINICAL DIAGNOSIS**

**1. Detailed personal history and physical examination**

See Table 1 for an overview of symptoms related to the various types of GEP-NETs.

**2. Determine localization if possible, using;**

-EUS or endoscopy in combination with CT-scan or MRI-scan

-Somatostatin Receptor Scintigraphy or Octreoscan

→ Positive imaging: Continue with 3

→ Negative imaging: Thorough search for occult tumours at unusual locations, continue with 3

**3. Measure plasma or serum CgA levels**

To verify the neuroendocrine nature of tumour

**4. Measure hormone levels in serum**

To detect possible peptide production by the tumour in order to define the tumour as 'functioning' or 'non-functioning'.

*Note:* Only define a tumour as a 'carcinoid' in case of increased serotonin serum levels and/or urinary 5-HIAA elevations, and/or the presence of the classical 'carcinoid syndrome' (Table 1).

**5. Confirm diagnosis with a specific diagnostic test**

Positive test: Diagnosis confirmed, continue with II

Negative test: consider non-functioning tumour and/or differential diagnosis, continue with II

**6. Investigate the presence of a hereditary syndrome**

-Detailed family history

-Investigation for associated tumours and/or lesions

-Gene testing

*Note:* Consider the presence of synchronous tumours in case of gastric carcinoids (GISTs) or the presence of a hereditary syndrome.

**II. PATHOLOGICAL DIAGNOSIS**

**1.Immunostaining**

-Staining for general NE markers including chromogranin A, synaptophysin, neuron specific enolase (NSE), keratin and grimelius, to determine the neuroendocrine nature of the tumour.

*Note:* For the definition of a neuroendocrine tumour, at least one of above mentioned general neuroendocrine markers should show a positive staining

-In case of a clinical (diagnosis or suspicion for) functioning tumour;
Stain for specific hormones including serotonin, gastrin, insulin, glucagon, somatostatin, and/or VIP

*Note:* Be aware that, also in case of a clinical functioning tumour, immunohistochemical staining for the particular hormone can be absent. Immunohistochemical staining should aid in determining the diagnosis, and determine the actual diagnosis.

2. **Determine WHO-classification**

-Determination of proliferation index by Ki67 or MIBG1
-Determination of mitotic count
-Investigate tumour characteristics;
*size
*histological pattern
*relation to other structures/invasion
*angioinvasion
*metastases

*Note:* Define the tumour as NET or NEC, not carcinoid. The term carcinoid should only be designed (by clinicians) to tumours with serotonin production and/or in the presence of the classical carcinoid syndrome (Table 1).

**3. Determine TNM stage**

-Determine tumour localization
-Determine tumour size
-Determine invasion of the tumour into surrounding organs/structures
-Determine the presence of lymph node metastases
-Determine the presence of distant metastases

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# Chapter 3

## *Pathological Incidence of Duodenopancreatic Neuroendocrine Tumours in the Netherlands: a Pathologisch Anatomisch Landelijk Geautomatiseerd Archief study*

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**Abstract**

Duodeno-pancreatic neuroendocrine tumours are rare, although current epidemiological studies worldwide suggest an incidence increase. We assessed the pathological incidence of duodeno-pancreatic neuroendocrine tumours over 18 years in The Netherlands.

Standardized excerpts from pathology reports of all patients diagnosed with duodeno-pancreatic neuroendocrine tumours from 1991 until 2009 were collected from PALGA and reviewed. This nationwide network and registry of histo- and cytopathology covers 100% of the pathology reports in The Netherlands.

We identified 905 patients with pancreatic (n=692) or duodenal (n=213) neuroendocrine tumours. The majority of these patients (69.4%) had a non-functional tumour. Functional tumours were diagnosed at a younger age compared to non-functional tumours (mean age ± s.d. 52.3 ± 17.7 years versus 60.0 ± 14.6 years, respectively, $P<0.01$). The average annual incidence per 1,000,000 persons over 1991 to 2009 was 2.54 for pancreatic and 0.81 for duodenal neuroendocrine tumours. The highest incidence was found in patients 65 to 79 years of age. The incidence of non-functional neuroendocrine tumours had increased significantly over two decades, $P<0.01$.

The incidence of duodeno-pancreatic non-functional neuroendocrine tumours in The Netherlands increased over 1991 to 2009. The etiology for this change includes improved diagnostic techniques and clinical awareness, as discussed.

**Introduction**

Duodeno-pancreatic neuroendocrine tumours comprise a very heterogeneous group of neoplasms, with regard to morphologic, functional and behavioral features[1]. In 2000, the World Health Organization (WHO) introduced a classification for neuroendocrine tumours (NETs) of the gastroenteropancreatic tract using histopathological characteristics and tumour behaviour to categorize these tumours per site[2]. Duodeno-pancreatic NETs are referred to as functional (or functioning) in case of the presence of a clinical syndrome resulting from ectopic hormone production, e.g., gastrin, insulin, glucagon, vasoactive intestinal peptide (VIP) or somatostatin, by the tumour, whereas non-functional NETs are not associated with a hormonal syndrome. Although these latter tumours may secrete biologic substances, like pancreatic polypeptide (PP) and chromogranin A, non-functional NETs can remain clinically silent for a relatively long time, and are only detected when morbidity is caused by tumour mass leading to biliary duct obstruction, bowel obstruction, and development of metastases or invasion into adjacent organs[3,4]. Duodeno-pancreatic NETs may be sporadic or component of the more comprehensive Multiple Endocrine Neoplasia type 1 syndrome (MEN-1), of which hyperparathyroidism and pituitary tumours are other frequent manifestations[5]. Although duodeno-pancreatic NETs have been considered as rare tumours, incidence rates have been reported to be increased substantially over the past years[6-8]. Furthermore, a high number of incidental findings of clinically silent duodeno-pancreatic NETs by autopsy studies was suggested[9,10]. Therefore, current incidence rates of duodeno-pancreatic NETs are likely to represent an underestimation. In the present study we aimed to provide insights into the epidemiology of both pancreatic and duodenal NETs in The Netherlands over a period of approximately 20 years. Therefore, we have carried out a search in the nation-wide network and registry of histo- and cytopathology in The Netherlands, abbreviated as PALGA, which is a central database for all pathology reports in our country[11].

**Materials and Methods**

*Collection of data by PALGA*

Data were collected from PALGA, the nationwide network and registry of histo- and cytopathology in The Netherlands[11]. This computerized database for pathology reports was founded in 1971, and since the participation of all pathology laboratories in 1991, national coverage was achieved. Currently, the PALGA databank contains about 50.5 million excerpts on nearly 11 million patients, with an annual addition of more than two million excerpts. A decentralized computer system collects the pathology reports from every pathology institution in The Netherlands automatically, and reports are sent to the central database on a daily basis. Reports are converted to excerpts that contain a limited number of encrypted patient data, a report identifier, (part of) the conclusions and the so called PALGA diagnosis, a coded diagnosis line based upon standard pathology terminology, containing topography (localization), morphology (nature of tissue change), etiology, function (functional abnormality), procedure and diseases. Encryption of the identifiers secures the patient's and participating laboratory's privacy.

Our search was directed to patients filed with a histological proven diagnosis of a neuroendocrine tumour in pancreas or duodenum between January 1991 and December 2008. For each excerpt, gender, date of pathology intervention, conclusion first sentences and diagnosis line were made available for retrospective analysis. Terms used for this search query were 'gastrinoma', 'insulinoma', 'glucagonoma', 'APUDoma', 'neuroendocrine tumour of digestive tract' and 'pancreas' or 'pancreatic islets' and 'duodenum' in combination with 'malignant endocrine tumour'. A query to identify patients with the MEN-1 syndrome, including hyperparathyroidism, was additionally performed under these patients.

Excerpts described several pathologic interventions, e.g., biopsies, punctures, resections autopsies or revisions of a pathologic report. Some patients had multiple excerpts included in the database, but were analyzed as one patient.

*Histological proof of tumour diagnosis*

The routine procedure for neuroendocrine tumours at pathology starts with the identification of the epithelial and neuroendocrine nature of the tumour by immunohistochemical staining, with markers like keratin, chromogranin A, grimelius, synaptophysin, etc. Based on the presence of clinical symptoms or syndrome, hormonal production by the tumour is evaluated, to exactly reveal the tumour type (i.e., gastrinoma, insulinoma, etc.). As a consequence, tumours are classified on immunopostivity for hormonal markers and clinical symptoms or syndrome as specific tumour type.

*Incidence calculations*

The incidence rates were calculated as the number of new cases per 1,000,000 persons, adjusted to general population data as obtained by the Dutch Central Bureau for Statistics (CBS)[12]. Data that were drawn from the CBS included age and sex of the total number of residents in The Netherlands per year, annual mortality rates and number of deaths caused by pancreatic malignancies. Age distribution in Table 2 was chosen referring to the distribution of the CBS, i.e., <20 years, 20-39 years, 40-64 years, 65-79 years and >80 years. The 'not reported' data refer to the use of excerpts, whereas complete pathology reports were not assessed because of privacy reasons. During the last three decades, the Leiden University Medical Centre has been the nationwide referral centre for patients with gastrinomas in The Netherlands. All patients diagnosed with or suspected of a gastrinoma, treated in our hospital, were traced and revised, to assess the extent of incidence underestimation based on pathology reports.

*Statistical analysis*

Statistical analysis was performed using Statistical Package for Social Sciences version 16 (SPSS) and GraphPad version 5. Results were reported as mean ± standard deviation (s.d.) or median, when appropriate. Using linear regression analysis, trends in annual incidence rates over the study period of 18 years were analyzed. A p-value of $<0.05$ was considered statistically significant.

## Results

*Patient characteristics*

As a result of the search query, 1529 excerpts of 1263 patients were found between 1991 and 2009. Patients with extrapancreatic or extraduodenal tumours were excluded, so that the final study cohort consisted of 692 patients with a pancreatic neuroendocrine tumour (PNET) and 213 patients with a duodenal neuroendocrine tumour (DNET) (Table 1).

**Table 1. Patient characteristics**

| | **Pancreatic NETs** | **Duodenal NETs** |
|---|---|---|
| **Age** | **Mean ± s.d. (range)** | **Mean ± s.d. (range)** |
| All tumours | 56.3 ± 16.3 (0-94) | 62.1 ± 14.1 (25-91) |
| Functional tumours | 52.59 ± 18.1 (0-98) | 51.6 ± 10.3 (38-73) |
| Non-functional tumours | 58.6 ± 14.7 (15-94) | 63.1 ± 14.1 (25-91) |
| **Sex** | **n (%)** | **n (%)** |
| Male | 322 (46.5%) | 114 (53.5%) |
| Female | 370 (53.5%) | 99 (46.5%) |
| **Tumour type** | **n (%)** | **n (%)** |
| Non-functional | 433 (62.6%) | 195 (91.5%) |
| Functional | 259 (37.4%) | 18 (8.5%) |
| Insulinoma | 202 (78.0%) | 0 |
| Gastrinoma | 21 (8.1%) | 16 (88.9%) |
| Glucagonoma | 23 (8.9%) | 0 |
| VIPoma | 6 (2.3%) | 0 |
| Somatostatinoma | 3 (1.2%) | 2 (11.1%) |
| Mixed | 4 (1.5%) | 0 |

Table 1. Characteristics of 692 patients with pancreatic neuroendocrine tumours and 213 patients with duodenal neuroendocrine tumours in the PALGA database from 1991 to 2009.

For PNETs, there was a slight female predominance, while DNETs showed a higher percentage of males. The majority of both PNETs and DNETs were non-functional tumours (Table 1). Functional PNETs comprised predominantly insulinomas (59.9% female), DNETs were mainly gastrinomas (62.5% male).

Patients with PNETs were significantly younger than patients with DNETs, $P<0.01$. This difference was largely caused by the younger age of patients with pancreatic compared to duodenal non-functional NETs, $P<0.01$. Patients with functional PNETs and DNETs were significantly younger at time of the pathologic evaluation compared to patients with non-functional PNETs and DNETs, $P<0.01$ and $P<0.01$, respectively (Table 1). Taking all PNETs and DNETs together, functional NETs were diagnosed at a younger age compared to non-functional NETs, 52.3 ± 17.7 vs 60.0 ± 14.6 years, respectively, $P<0.01$.

The MEN-1 syndrome was present in 10 patients with functional (two pancreatic glucagonomas, two insulinomas, one gastrinoma and one mixed glucagonoma/insulinoma, four duodenal gastrinomas) and 11 patients with non-functional NETs (10 pancreas, one duodenum).

*Incidence rates*

Using census statistics obtained from the Dutch Central Bureau of Statistics, the annual incidence rates per 1,000,000 population for PNETs and DNETs were calculated (Figures 1 and 2). The average annual incidence of PNETs per 1,000,000 from 1991 to 2009 was 2.54. The total incidence of PNETs increased over the years (slope 0.12 with a 95% c.i. of 0.07 to 0.18, $P<0.01$). Non-functional PNETs showed a higher incidence compared to functional tumours. The incidence increased with advancing age at time of the pathology diagnosis. The highest incidence of PNETs was found in patients from 65-79 years (Table 2). Remarkably, the incidence in patients under 40 years of age was higher for functional PNETs compared to non-functional tumours. We found a statistically significant increase in incidence of non-functional PNETs over two decades (slope 0.14 with a 95% c.i. of 0.09 to 0.19, $P<0.01$). In contrast, functional PNETs showed a slight but significant decrease in incidence over the study period (-0.01 with a 95% c.i. of -0.03 to -0.00, $P=0.05$). In the study period from 1991 to 2009, a total of 33,459 patients with malignant tumours in the pancreas were reported in the Dutch population. Crude incidences of functional and non-functional PNETs were therefore 0.008 and 0.013, respectively.

**Figure 1. Incidence rates of pancreas neuroendocrine tumours from 1991-2008 in the Netherlands**

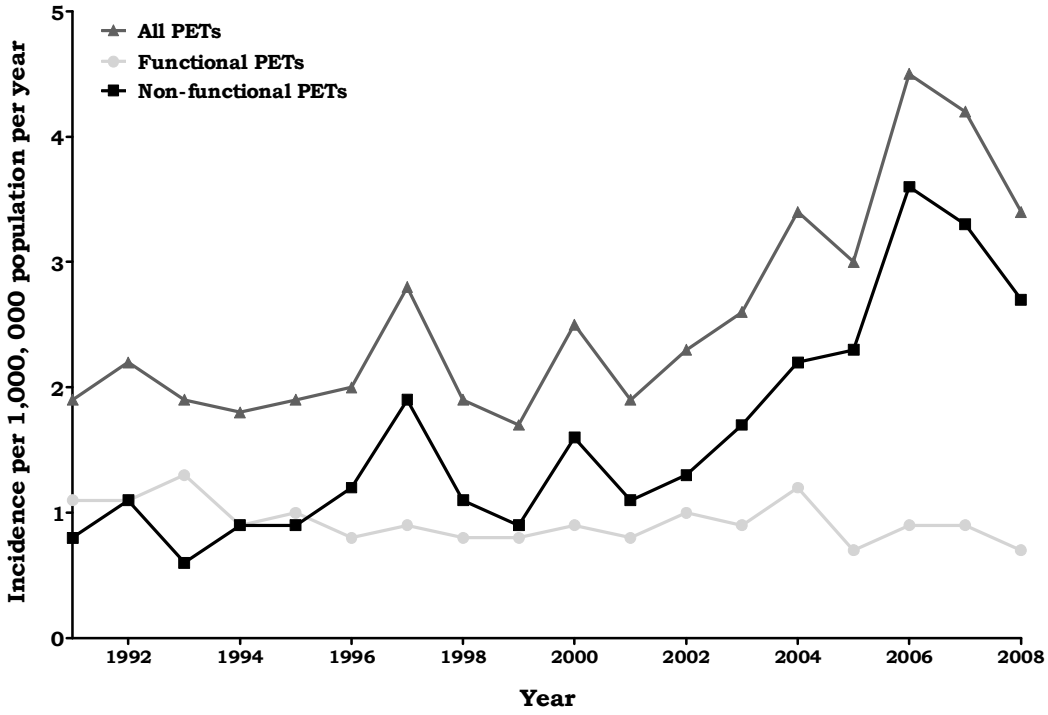


Figure 1. Annual incidence rates of pancreatic neuroendocrine tumours per 1,000,000 persons in The Netherlands from 1991 to 2009.

The average annual incidence of duodenal NETs per 1,000,000 from 1991 to 2009 was 0.81. The total incidence of these DNETs showed a similar pattern to PNETs, namely an increase over the years from 1991 to 2009 (slope 0.05 with a 95% c.i. of 0.02 to 0.07, *P*=0.003), which was mainly due to a significant increase in incidence of the non-functional duodenum NETs (slope 0.04 with a 95% c.i. of 0.02 to 0.07, *P*=0.001) while the incidence of functional tumours remained relatively stable (slope 0.00 with a 95% c.i. of -0.00 to 0.02, *P*=0.40).

**Figure 2. Incidence rates of duodenal neuroendocrine tumours from 1991 - 2008 in the Netherlands**

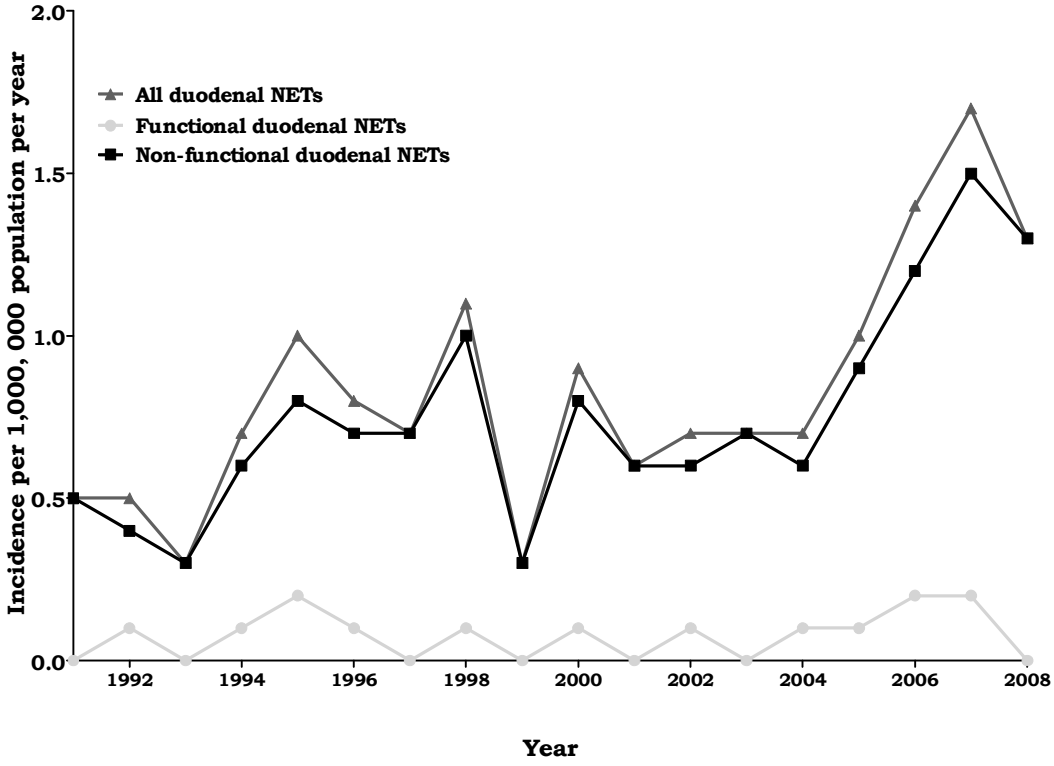


Figure 2. Annual incidence rates of duodenal neuroendocrine tumours per 1,000,000 persons in The Netherlands from 1991 to 2009.

The highest incidence of duodenal tumours was also seen in the patient group of 65-79 years of age (Table 2).

**Table 2. Incidence rates**

| Age | All tumours | | | Functional tumours | | | Non-functional tumours | | |
|---|---|---|---|---|---|---|---|---|---|
| | P | D | T | P | D | T | P | D | T |
| **<20 yrs** | 0.2 | 0.0 | 0.2 | 0.1 | 0.0 | 0.1 | 0.1 | 0.0 | 0.1 |
| **20-39 yrs** | 1.0 | 0.1 | 1.1 | 0.6 | 0.0 | 0.6 | 0.4 | 0.1 | 0.5 |
| **40-64 yrs** | 4.0 | 1.2 | 5.2 | 1.4 | 0.2 | 1.6 | 2.6 | 1.0 | 3.6 |
| **65-79 yrs** | 6.1 | 2.5 | 8.7 | 2.0 | 0.1 | 2.1 | 4.2 | 2.4 | 6.6 |
| **>80 yrs** | 4.7 | 2.4 | 7.1 | 1.5 | 0.0 | 1.5 | 3.2 | 2.4 | 5.6 |

Table 2. Incidence of pancreatic and duodenal neuroendocrine tumours per 1,000,000 persons by age at time of pathologic intervention form 1991 to 2009. P=pancreas, D=duodenum, T=total.

When pancreatic and duodenal tumours were analyzed together, a similar trend in incidence rates was seen; the incidence of non-functional tumours increased significantly (slope 0.18 with a 95% c.i. of 0.11 to 0.24, $P<0.01$) while the incidence of functional tumours slightly decreased over time (slope -0.01 with a 95% c.i. of -0.03 to -0.01, $P=0.16$).

Furthermore, 124 autopsy reports of 35 patients with functional PNETs, 75 non-functional PNETs and 14 non-functional DNETs were included. Mean age at time of death did not differ between functional PNETs (67.4 ± 14.5 years), non-functional PNETs (67.0 ± 15.0 years) and non-functional DNETs (69.4 ± 11.8 years). Patients were all younger than the mean age at death of the general population of The Netherlands (males 72.0 ± 0.8 years and females 78.2 ± 0.6 years) over the period from 1991 to 2009. When patients who where found to have a NET by incidence at autopsy were excluded from the analysis, the average annual incidence numbers were 2.17 for PNETs and 0.76 for DNETs, respectively. Furthermore, incidence numbers were still significantly increasing over the period from 1991 to 2009 (slope 0.13 with a 95% c.i. of 0.08 to 0.17, $P<0.01$ for PNETs and slope 0.04 with a 95% c.i. of 0.01 to 0.07, $P<0.01$ for DNETs).

*Tumour characteristics*

Tumour characteristics are presented in Table 3. 37.8% of PNETs and 66.7% of duodenal NETs were <2 cm in diameter. All duodenal NETs were <5 cm, but only 78.4% of the PNETs were of that size. 6.2% of the pancreatic tumours had a size of >10 cm in diameter. Tumours were <2 cm, <5 cm or >10 cm in 65.2%, 91.1% and 3.6% cases of functional PNETs and in 25.5%, 72.1% and 7.3% cases of non-functional PNETs, respectively. Tumour size of non-functional PNETs (mean 3.9 ± 3.2 cm) was significantly larger compared to tumour size of patients with functional PNETs (mean 2.3 ± 2.5 cm), $P<0.01$. Non-functional DNETs had an average size of 1.6 ± 1.2 cm, while functional DNETs were on average 0.7 ± 0.5 cm, $P=0.10$. Non-functional PNETs had a larger tumour size compared to non-functional DNETs, $P<0.01$, and functional PNETs were also significantly larger compared to functional DNETs, $P<0.01$. Mainly lymph node metastases were

present in both PNETs and DNETs. The majority of tumours were described as well-differentiated. PNETs were mainly high grade malignant, while DNETs were most often reported as low grade malignant tumours. Angioinvasion was present in the majority of tumours.

Table 3. Tumour characteristics of pancreatic and duodenal neuroendocrine tumours.

| **Table 3. Tumour characteristics** | | |
|---|---|---|
| **Tumour characteristic** | **Pancreatic NETs** | **Duodenal NETs** |
| **Tumour size** | **n (%)** | **n (%)** |
| *Reported* | *259 (37.4%)* | *39 (18.3%)* |
| <1 cm | 20 (7.7%) | 16 (41.0%) |
| 1-<2 cm | 78 (30.1%) | 10 (25.6%) |
| 2-<3 cm | 50 (19.3%) | 6 (15.4%) |
| 3-<4 cm | 36 (13.9%) | 5 (12.8%) |
| 4-<5 cm | 19 (7.3%) | 2 (5.1%) |
| 5-<10 cm | 40 (15.4%) | 0 |
| >10 cm | 16 (6.2%) | 0 |
| *Not reported* | *433 (62.5%)* | *174 (81.7%)* |
| **Metastases** | **n (%)** | **n (%)** |
| *Reported* | *239 (34.5%)* | *44 (20.7%)* |
| Lymph node | 68 (28.5%) | 24 (54.5%) |
| Liver | 46 (19.2%) | 8 (18.2%) |
| Lymph node and liver | 12 (5.0%) | 1 (2.3%) |
| Multiple or other | 28 (11.7%) | 0 |
| No metastases | 85 (35.6%) | 11 (25.0%) |
| *Not reported* | *453 (65.5%)* | *169 (79.3%)* |
| **Differentiation** | **n (%)** | **n (%)** |
| *Reported* | *103 (14.9%)* | *31 (14.6%)* |
| Well-differentiated | 83 (80.6%) | 17 (54.8%) |
| Intermediate differentiated | 17 (16.5%) | 7 (22.6%) |
| Poorly-differentiated | 3 (2.9%) | 7 (22.6%) |
| *Not reported* | *589 (85.1%)* | *182 (85.4%)* |

| **Grade** | **n (%)** | **n (%)** |
|---|---|---|
| *Reported* | *120 (17.3%)* | *23 (10.8%)* |
| Benign | 23 (19.2%) | 1 (4.3%) |
| Low grade malignant | 19 (15.8%) | 10 (43.5%) |
| High grade malignant | 64 (53.3%) | 8 (34.8%) |
| Uncertain behaviour | 14 (11.7%) | 4 (17.4%) |
| *Not reported* | *572 (82.7%)* | *190 (89.2%)* |
| **Angioinvasion** | **n (%)** | **n (%)** |
| *Reported* | *111 (16.0%)* | *10 (4.7%)* |
| Yes | 78 (70.3%) | 9 (90%) |
| No | 33 (29.7%) | 1 (10%) |
| *Not reported* | *581 (83.9%)* | *203 (95.3%)* |

The majority of PNETs was located in the pancreatic tail. Compared to non-functional PNETs, more functional PNETs were located in the pancreatic tail, but less in the pancreatic head (Table 4).

**Table 4. Detailed information on the location of the pancreatic tumour**

| **Pancreas location** | **All tumours** **n (%)** | **Functional tumours** **n (%)** | **Non-functional tumours** **n (%)** |
|---|---|---|---|
| *Reported* | *312 (45.1%)* | *112 (43.2%)* | *200 (46.2%)* |
| Caput | 105 (33.6%) | 26 (23.2%) | 79 (39.5%) |
| Corpus | 19 (6.1%) | 6 (5.4%) | 13 (6.5%) |
| Cauda | 164 (52.6%) | 70 (62.5%) | 94 (47.0%) |
| Overlapping | 24 (7.7%) | 10 (8.9%) | 14 (7.0%) |
| *Not reported* | *380 (54.9%)* | *147 (56.8%)* | *233 (53.8%)* |

Table 4. Detailed information on the location of the tumour in the pancreas

*Clinical assessment of incidence calculations*

To get an idea about the potential underestimation of the incidence calculation by this study using histocytopathological information from the PALGA database, we also assessed from our own referral centre what percentage of patients clinically suspected of or diagnosed with a gastrinoma in pancreas or duodenum, were

scored as a gastrinoma by the pathologists as well. We found that only 45.7% (16/35) of our clinical gastrinoma patients were scored accordingly by pathologists, whereas 28.6% (10/35) of the patients were scored otherwise, i.e., as undefined neuroendocrine tumour. 25.7% (9/35) of the patients had not undergone any surgery and/or other pathological evaluation for their tumour and were therefore not traceable in the PALGA database. One patient was not diagnosed in the clinical setting, but was found to have a gastrinoma by incidence at autopsy.

**Discussion**

Duodeno-pancreatic NETs are considered to be rare neoplasms with a relatively slow-growing nature[13]. Because of the common embryonic origin it is attractive to study both locations in one study. Although the majority of these tumours are malignant, they can remain indolent and undetected for a long period of time, leading to substantial delays in diagnosing. Specifically non-functional tumour patients often present with metastases and more advanced disease[4].

The present study describes the incidence rates of both pancreatic and duodenal NETs from 1991 to 2009 in The Netherlands. This study is not only the first to examine epidemiological features of NETs in The Netherlands, it is also unique in the analysis of the incidence of duodenal tumours.

In the evaluation period from 1991 until 2009, 905 patients with pancreatic and duodenal NETs were registered in PALGA. The majority was described as non-functional NETs, 69.4%. Similar to Fitzgerald *et al.* we found an increase in incidence over time for non-functional pancreatic and duodenal NETs[7]. We concur with their postulation that this increase is likely to be due to increased use and improved techniques of diagnostic modalities. Moreover, the WHO classification for neuroendocrine tumours of the gastroenteropancreatic tract, which was introduced in 2000[2], has most likely contributed as well. We assume that introduction of this classification not only resulted in more intelligibility for the nomenclature and categorization of GEP-NETs, but also raised the awareness for

the existence of these tumours. As feasible in Figures 1 and 2, incidence lines increased remarkably after 2000.

Furthermore, Fitzgerald *et al.* found that the incidence of functional PNETs over their study period of 16 years remained stable[7]. We found that the incidence of these tumours slightly decreased from 1991 to 2009. As a result of the hormonal secretion of this tumour type, functional NETs might be suspected and detected due to the clinical symptoms in these patients. The role of improved imaging techniques in the diagnosis of these tumours is only marginal, if any. In contrast, non-functional NETs are often only discovered at an advanced tumour stage, corresponding with the relatively older age of these patients at the first (pathological) diagnosis and the larger size of these tumours, compared to functional tumours, as suggested previously and confirmed in the present study[6-8]. Together, these findings imply that the increase in incidence numbers is most likely to represent an increase in detection, rather than a raise in occurrence of these tumours. The fact that in several autopsy studies neuroendocrine tumours are found by coincidence, confirms this implication[9-10]. We found that among the patients with duodeno-pancreatic NETs included in this study on autopsy reports, the majority of patients (117/124) were not included in the PALGA database for any pathologic evaluation related to a neuroendocrine disease. This suggests that in 12.9% patients (117/905) the pancreatic or duodenal neuroendocrine tumour might be an incidental finding at autopsy, not detected earlier during life. Furthermore, analysis of autopsy reports revealed that, unsurprisingly, patients with PNETs and DNETs die at a younger age, compared to the general Dutch population. However, no difference in age at time of death was found between functional and non-functional NETs.

We found that most PNETs were located in the pancreatic tail (52.6%), followed by the pancreatic head (33.6%), which is in contrast to others, who found the pancreatic head as preferred location of PNETs[14-16].

It is noteworthy to emphasize that we intentionally did not include any data on survival of the patients. Most studies which do report survival figures are based on information from the Surveillance, Epidemiology and End Results (SEER)

database, which collects cancer incidence and survival of the US population and includes data on clinical and pathology information on tumours. However, we have chosen to estimate incidence rates based on pathology data, because of several reasons. Firstly, The Netherlands Cancer Registry, which is comparable to the SEER database, does not include detailed data on (the type of) pancreatic and/or duodenal neuroendocrine tumours. Secondly, this cancer registry is partially based and dependent on information of the PALGA database. Furthermore, in the present study both benign and malignant neuroendocrine tumours were included, while in most other studies, based on information from the SEER database, only malignant tumours were covered. Therefore, we suffice with the estimation of epidemiological numbers, although a survival study might be an interesting future option.

Indeed, we are aware of the fact that the incidence rates calculated in our study might be an underestimation, as an unknown number of patients without pathology/surgical interventions were not retrievable in the PALGA database and therefore not included in our study. We assume that this mainly concerns functional NETs, as these tumours cause clinical symptoms, in contrast to non-functional tumours. From our own experience, we know that for example patients with gastrinomas can do well on medication and surgical intervention in these patients is not always necessary[17]. In the past three decades, our hospital has been the nationwide referral centre for gastrinomas in The Netherlands. Therefore, we approached the possible underestimation of incidence by exploring what percentage of patients with clinically detected gastrinomas was retrievable in the PALGA database. We found that 73.6% of the patients were present in PALGA, although only 45.7% was actually scored as a 'gastrinoma' by the pathologists. Thus the underestimation of PNETs and DNETs may be between 25% and 50%.

We further recognize that the pathological diagnosis of pancreatic or duodenal NETs is not always necessarily in agreement with the clinical symptoms of the patients. This was already noticed by Chetty[18]. As Mansour *et al.* illustrated using gastrinomas, a general pathological differentiation between different types of functional NETs is more based on the clinical background, as also

immunohistochemical staining does not often lead to conclusive evidence[19]. Therefore, we think that the combination of both clinical data and pathological findings is needed to establish the correct diagnosis in patients with NETs.

It is worth to iterate that our study is based on pathological reports, and therefore the incidence rates are most likely lower than the actual incidence when these would also be based on clinical records. However, the study period was depicted from 1991 to 2009, to warrant a 100% national coverage of all the pathologic institutions in The Netherlands by the PALGA database.

In conclusion, we explored the pathological incidence of duodeno-pancreatic NETs in The Netherlands, and found that the incidence of non-functional NETs has increased over the past two decades. However, although this effect may be due to the improvement of diagnostic tools in the clinical field, these tumours are still detected at a relatively late stage illustrated by the larger size and a diagnosis at an older age than in those patients affected by functional neuroendocrine tumours.

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# Chapter 4

## *A Case of Recurrent Gastrinoma in The Liver with a Review of 'Primary' Hepatic Gastrinomas*

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**Abstract**

In the literature only few cases of primary hepatic gastrinomas have been reported. Furthermore, most cases have a short follow-up and are limitedly documented.

We report a case of a patient suffering from the Zollinger-Ellison syndrome with recurrent hepatic gastrinomas, in whom no gastrinomas in duodenum, pancreas or other extrahepatic site could be identified, despite the use of multiple, repeatedly performed imaging techniques and explorations during the past 20 years.

A review is given on primary liver gastrinomas published since 1981. Interestingly, the present case is the only one with documented recurrent gastrinoma in the liver. None of the previously reported cases had liver gastrinomas as part of the Multiple Endocrine Neoplasia type 1 syndrome. It is further noteworthy that the risk of metastases of liver gastrinomas appears to be low. The interpretation of these hepatic gastrinomas as primary lesions can be questioned, as most cases lack an investigational and well-documented follow-up.

In this study, we report the first case of Zollinger-Ellison syndrome caused by recurrent hepatic gastrinomas in the context of what is known from the literature.

**Introduction**

The Zollinger-Ellison Syndrome (ZES) is caused by a malignant gastrin-producing neuroendocrine tumour (gastrinoma), usually located in pancreas or duodenum[1,2]. Symptoms associated with ZES are acid peptic disease, malabsorption and diarrhea[3].

Most frequently ZES occurs as a sporadic disease, while 20 to 30% of the cases is part of the Multiple Endocrine Neoplasia type 1 syndrome (MEN-1)[4,5]. This autosomal dominant disorder, caused by mutations of the MEN-1 tumour suppressor gene located on chromosome 11q13, is characterized by multiple tumours in several neuroendocrine organs and tissues. In case of endocrine symptoms in combination with a positive family history for MEN-1 and aberrant levels of calcium, prolactin, parathyroid hormone or pancreas polypeptide, MEN-1 can be suspected and confirmed by genetic analysis[6].

Gastrinomas are frequently located in pancreas (30-50%), duodenum (40-50%) or lymph nodes (19%), in the so-called gastrinoma triangle, which angle points are formed by the junction of the cystic duct and common bile duct, the junction of the second and third part of the duodenum and the junction between the neck and body of the pancreas[7]. Remaining gastrinomas (extrapancreatic, extraduodenal and extralymphatic) are called ectopic and have been reported to occur in thymus, ovaries, liver, jejunal mesenterium, stomach, heart, parathyroid glands, kidneys and common bile duct[8-10]. Although hepatic metastases of primary gastrinomas are common, primary hepatic gastrinomas are rare[11]. To date, about 16 cases of primary liver gastrinomas have been reported in the literature[9, 10, 12-25]. In a majority of these reports, the period of follow-up is short (<three years) and not well-documented. However, because primary hepatic gastrinomas are difficult to differentiate from liver metastases from an occult gastrinoma elsewhere located, an adequate and extensive follow-up is necessary.

In this case report, we describe a patient with ZES with recurrent most likely primary hepatic gastrinomas and an extended follow-up of almost 20 years after the diagnosis and more than 30 years after the first clinical presentation. Despite extensive monitoring and evaluation, including multiple physical examinations,

endoscopies and extensive imaging studies, no primary duodenal or pancreatic gastrinoma could be identified in this patient. Instead, liver tumours suspected of primary gastrinomas have been resected twice. Furthermore, we discuss the existence of primary liver gastrinomas and give an overview of all case reports of primary hepatic gastrinomas reported in literature from 1981.

**Case Report**

In 1989, a 39-year-old white male of Hispanic origin was referred to the outpatient clinic of the Gastroenterology department of the Leiden University Medical Center, for localization and treatment of a suspected gastrinoma. At that time symptoms including diarrhea, gastric complaints, pyrosis, nausea and vomiting, were present for many years. Furthermore, patient reported a remarkable weight loss of more than five kg during a period of approximately six months. Fasting serum gastrin was elevated and the secretin provocation test was positive, supporting the diagnosis of ZES. Omeprazol 80 mg/day provided relief of his symptoms. Patient was taking no other medication. About fifteen years before presentation, patient had undergone anti-reflux surgery (Nissen fundoplication). His past medical history was further unremarkable, his family history was non-contributory. Physical examination revealed no abnormalities, apart from severe scoliosis. Laboratory studies, including serum amylase, electrolytes, liver chemistry, blood cell counts and stool parameters were found normal, except for an increased fecal fat excretion (53 grams/day). Gastroduodenoscopy showed Barrett's esophagus, a small duodenal ulcer, and prominent red gastric folds and several erosions in the stomach. Further laboratory analysis revealed an increased fasting serum gastrin of 889 ng/L (424 pmol/L), an elevated basal acid output (40 mmol/hr) with a maximum acid output of 60 mmol/hr. Serum levels of calcium (2.36 mmol/L), parathyroid hormone (2.4 pmol/L), prolactin (4.9 ug/L) and pancreas polypeptide (10 pmol/L) were within normal limits, making the diagnosis of Multiple Endocrine Neoplasia type 1 (MEN-1) very unlikely.

To localize a possible gastrinoma, several imaging evaluations were performed. However, no tumour was identified at that time by conventional procedures, such

as computed tomography (CT), magnetic resonance imaging (MRI), selective arteriography and selective arterial secretin injection test. Endoscopic ultrasound did not show a tumour in gastroduodenum, pancreas or lymph nodes. Moreover, an Indium-111-somatostatin receptor scintigraphy, a technique which was at that time still in the experimental phase, was performed (University Hospital, Rotterdam). This scan revealed a possible localization of the tumour in the left liver lobe or gastric lesser curvature. To exclude a gastric localization, gastroscopy was repeated. However, no tumour was visualized. Consequently, in 1990, an explorative laparotomy was performed but again no gastrinomas were visible macroscopically. Peroperative ultrasonographic evaluation of the pancreas showed no abnormalities, while peroperative echography of the liver showed a lesion next to the inferior vena cava in the left liver lobe. Biopsies from this lesion were analyzed by immunohistochemistry, on both paraffin-embedded and frozen sections of the tumour, and were found positive for keratin, synaptophysin, gastrin and neuron specific enolase, but negative for other neuroendocrine markers. Based on these results, a liver localization of a gastrin-producing neuroendocrine tumour was suggested, and confirmed on CT. In order to localize a primary tumour, peroperative selective venous sampling for gastrin was performed[26]. No evidence of tumour localization in the duodeno-pancreatic area was found. Therefore, resection of liver segment II was performed. Postoperative histological examination confirmed that the specimen sampled from the liver contained a gastrinoma. Within five days after the partial resection, fasting serum gastrin decreased to normal (60 ng/L; 29 pmol/L) (Figure 1).

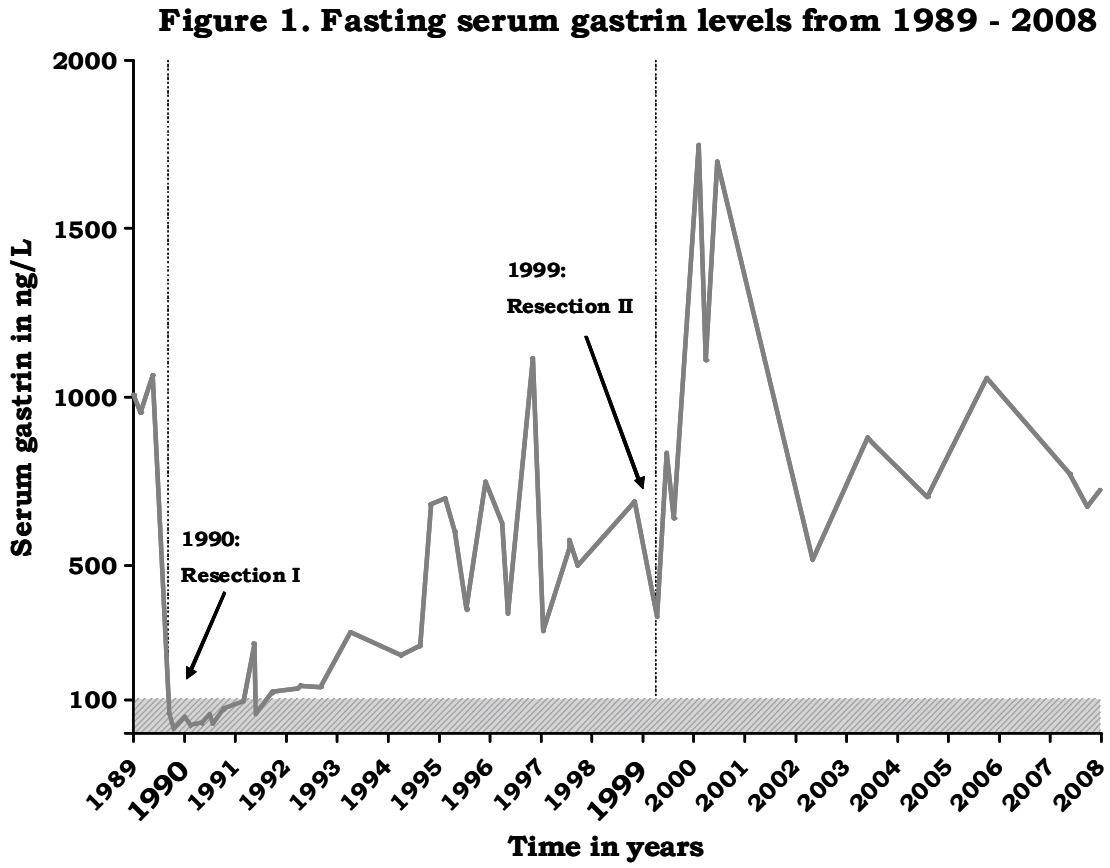


Figure 1. Fasting serum gastrin levels in ng/L, measured on multiple occasions during the evaluation from 1989 until 2008, are presented. The gray region represents the area in which serum gastrin is within normal limits (<100 ng/L). Dotted lines indicate the first and second partial liver resections in 1990 and 1999, respectively.

Approximately one and a half year after surgical excision of the liver gastrinoma, fasting serum gastrin increased to levels above the upper limit of normal (<100 ng/L). Secretin provocation test was also positive (a rise of 296 ng/L; 142 pmol/L in serum gastrin after secretin injection).

In the period from 1991 until 1999, several imaging techniques were performed without any detection of a pancreatic or duodenal gastrinoma. Multiple gastroscopies repeatedly revealed a Barrett's esophagus and edematous folds in the gastric corpus. In 1995, a selective arterial secretin injection test[27] was repeated and showed a small increased gradient of serum gastrin over the hepatic vein. In 1998, octreotide scintigraphy (SRS) revealed multiple small liver lesions, which were confirmed on CT and MRI (Figure 2).

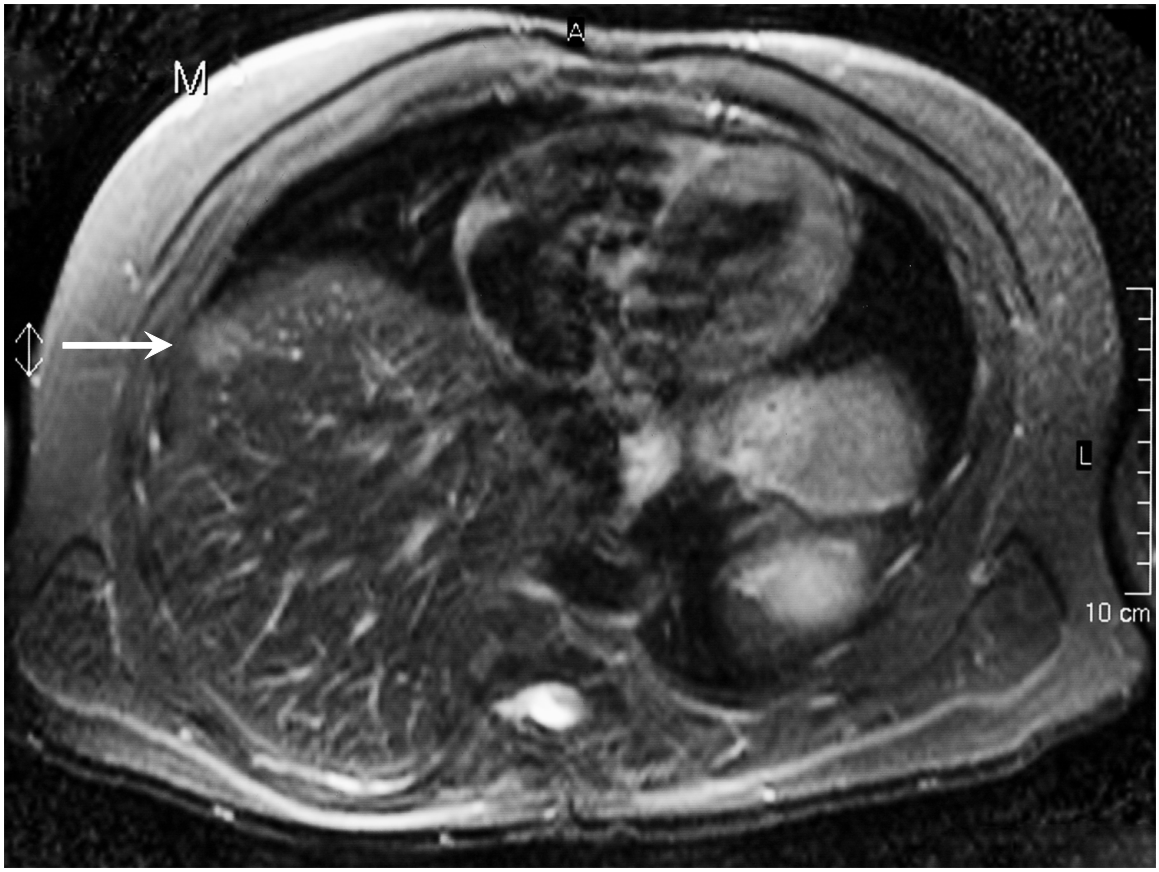


Figure 2. Magnetic resonance imaging shows a liver lesion ventrolateral in the right liver lobe (Leiden University Medical Center, Department of Radiology).

Patient had a partial resection of liver segments IVa and IVb. Postsurgically, fasting serum gastrin initially dropped from 690 (330 pmol/L) to 347 ng/L (166 pmol/L) but rapidly increased thereafter. A secretin provocation test, performed 3 months postoperatively, resulted in a postsecretin gastrin increase from 883 ng/L (422 pmol/L) to 4675 ng/L (2236 pmol/L).

The patient remained under follow-up control for the next period. Apart from surgery for a prostate adenocarcinoma, no ZES-related complaints were present. In 2003, octreotide scintigraphy (SRS) revealed a dubious accumulation of radioactivity in the ventral right liver lobe, while contrast-enhanced CT and MRI scans of the abdomen were repeatedly normal. In 2006 the liver lesion was confirmed on MRI of the abdomen. Until present date, no lesion in pancreas or duodenum has been identified. In 2008, an attempt to treat the hepatic lesion with radiofrequency ablation was performed. However, the procedure was discontinued because of failure of the needle to reach the tumour. To date, serum

gastrin levels remain increased although patient is doing well without symptoms, using 80 mg/day of Pantoprazole.

**Table 1. Review of primary hepatic gastrinomas from literature**

| Patients | | Therapy | | | Follow-up after resection | | | |
|---|---|---|---|---|---|---|---|---|
| **Ref** | **Age, sex** | **FSG pre** | **Resection** | **FSG post** | **Investigations** | **Evidence** | **m** | **FSG last** |
| 17 | 36, F | ND | No# | ND | ND | No | 24 | ND |
| 10 | 61, F | 1500 | Yes* | ND | FSG | No | 132 | 82 (1m) |
| 24 | 44, M | 2700 | Yes* | N | FSG | No | 13 | N (9m) |
| 22 | 8, M | 893 | Yes | N | FSG | No | 18 | N (18m) |
| 9 | 23, M | 670 | Yes* | ND | SPT | No | 24 | ND |
| 12 | 49, F | ND | Yes | ND | ND | No | 69 | ND |
| 19 | 13, M | 27175 | Yes | N | FSG | No | 24 | ND |
| 16 | 30, M | 572 | Yes | 64 | FSG | No | 60 | ND |
| 20 | 50, F | ND | Yes | N | CT, US, EUS, FSG, SPT | No | 24 | ND |
| 18 | 9, M | 704 | Yes | 103 | CT, FSG | No | 36 | 182 (12m) |
| 25 | 50, M | 150 | Yes | N | CT, FSG | No | 18 | N (18m) |
| 23 | 57, M | ND | Yes* | 50 | CT, SRS, FSG | No | 14 | N (14m) |
| 13 | 27, F | 1224 | Yes | N | CT, US, FSG | No | 42 | N (42m) |
| 15 | 13, M | 1141 | Yes | 30 | SRS, US, FSG | Yes | 48 | 284 (6m) |
| 14 | 29, F | 1149 | Yes | ND | CT, MRI, EUS, SRS, FSG | No | 36 | N (36m) |
| 21 | 50, M | 1500 | Yes | N | CT, SPT, FSG | No | 60 | 400 (>6m) |
| CCR | 39, M | 1065 | Yes | 60 | CT, MRI, SRS, SASI | Yes | 113 | 690 (109m) |
| +rec | 39, M | 690 | Yes | 347 | CT, MRI, SRS, EUS | Yes | 100 | 724 (105m) |

Symbols: * Plus total gastrectomy, # Streptozotocin therapy

Abbreviations: CCR + rec = Current case report + recurrence, Ref = References, m = Months, FSG pre = FSG preoperatively (ng/L), FSG post = FSG postoperatively (ng/L), FSG last = FSG last measured (months) (ng/L), FSG = Fasting serum gastrin, F = female, M = male, ND = Not done, N = in normal range (<100 ng/L), CT = Computed tomography, MRI = Magnetic resonance imaging, US = Ultrasound, EUS = Gastroduodenal endoscopic ultrasound, SRS = Somatostatin receptor scintigraphy, SASI = Selective arterial secretin injection test, SPT = secretin provocation test

Table 1. A review of primary hepatic gastrinomas from literature is presented.

## Discussion

This is an exceptional case of a ZES-patient with recurring hepatic gastrinomas, in the absence of MEN-1. As in general the majority of sporadic gastrinomas is localized in the gastrinoma triangle, an accurate investigational search to find a tumour in this area was initiated. Preoperative and postoperative techniques to

localize a gastrinoma include CT, MRI, ultrasonography, somatostatin receptor scintigraphy or octreotide scintigraphy, selective angiography, gastroduodenal endoscopic ultrasonography, and more specialized tests such as selective arterial secretin injection and selective portal venous sampling[27-33].

Although our patient was subjected to all these imaging techniques for almost 20 years, no evidence for an extrahepatic origin of a gastrinoma was found. However, we believe that it is very unlikely that any extrahepatic tumour, albeit small in size, is constantly missed. As preoperative localization techniques like MRI and CT have improved over time, it might be expected that, even if a tumour was missed repeatedly in the past, recent techniques would be able to detect gastrinomas of any size at any location. Furthermore, we believe that if any small gastrinoma would exist, in pancreas or duodenum, this tumour would grow and therefore be detected by now. However, in this patient, exclusively liver gastrinomas have been detected, resected and recurred twice. To date, a suspicious liver lesion is seen with multiple imaging modalities. Although it is not certain if this liver lesion is a new recurrence or growth of a residual tumour after the second partial liver resection, it is clear that the liver tumour has a slow growing rate. After the initial resection of the liver gastrinoma in 1990, it took about 9 years before a recurrent tumour became visible on imaging. After the resection in 1999, imaging techniques were initially negative before hepatic lesions could be visualized on MRI in 2006.

In Table 1, several cases of primary liver gastrinomas reported in the literature from 1989 until 2008 are listed. In most cases, hepatic gastrinomas were defined as primary when no extrahepatic tumour had been found pre-, intra- and postoperatively or when postsurgically serum gastrin levels decline to the normal range (<100 ng/L)[9, 12-20, 22-25]. Moreover, the suspicion of a primary liver gastrinoma could postoperatively be confirmed by immunohistochemical staining for (neuroendocrine) markers, including gastrin. In only one case report the tumour is defined as primary preoperatively, based on percutanous transhepatic venous sampling[21]. In our patient, no lesions outside the liver were found and immunohistochemical analysis of the liver lesions confirmed the diagnosis of

gastrinoma, fitting the criteria to define the liver gastrinoma as primary. In contrast, serum gastrin levels did normalize postsurgically after the first partial liver resection, but became abnormal after about one and a half year and remained increased after the second operation. Remarkably, in most reported cases the follow-up of the patient after resection of the liver gastrinoma was relatively short (<three years) [12, 19-22, 24, 25] or had a limited postoperative documentation[12, 23]. The possibility that an extrahepatic gastrinoma is present can therefore not be absolutely excluded. Our patient had undergone several imaging studies to localize a gastrinoma outside the liver, not only preoperatively, but also after the resection of the hepatic tumour and during follow-up.

Moruira *et al.* studied the cases of five primary hepatic gastrinomas from the literature and added one case, and concluded that these gastrinomas occurred in slightly younger patients when compared to patients with gastrinomas elsewhere located[20]. Furthermore, Diaz *et al.* reported that primary hepatic gastrinomas are more common in men, and are not associated with MEN-1[15]. As our patient was a relatively young male at the time of diagnosis, suffering from ZES not as part of the MEN-1 syndrome, this is in line with the interpretation of the liver lesion as a primary tumour.

In general, it is difficult to state that in patients with a supposed primary liver gastrinoma, the possibility of a pancreatic, duodenal or other localization of a primary gastrinoma is excluded. As the liver occurs to be a frequent site for metastatic gastrinomas, hepatic gastrinomas can be incorrectly interpreted as primary when no extrahepatic gastrinoma can be detected. The probability that liver gastrinomas are by mistake diagnosed as primary, is also mentioned by Tiomny *et al.*[25]. Detection of liver gastrinomas usually raises the question if this tumour is primary or metastatic. Only with long term follow-up it is possible to answer this question. However, the risk of metastases from primary hepatic gastrinomas seems to be low, as only one case reports the development of lymph node metastases after liver resection[15]. To our knowledge, recurrent tumours in the liver after surgical removal of the primary hepatic gastrinoma have not been reported before.

In some case reports listed in Table 1, patients have undergone a total gastrectomy, preventing the use of acid peptic complaints as marker for recurrence, while in other cases, the follow-up after resection relies only on the analysis of serum gastrin levels. We believe that, even in the absence of ZES-related complaints or in case of normalization of serum gastrin immediately postoperatively, recurrence may occur, although many years later. Therefore investigational imaging, such as octreotide scintigraphy or gastroduodenal endoscopic ultrasound, is required for an adequate follow-up.

In conclusion, we reviewed the literature on primary liver gastrinomas and added a patient suffering from the Zollinger-Ellison syndrome, with a liver gastrinoma without another localization of a primary gastrinoma, under evaluation for almost 20 years.

Although the absence of a primary gastrinoma outside the liver during this long follow-up is highly suggestive to define the gastrinoma in the liver as primary, the possibility of a metastasizing but not growing occult gastrinoma in the gastrinoma triangle is very unlikely, but can not excluded with absolute certainty. In general, we state that frequent measurements of serum gastrin in combination with repeated imaging investigations are indicated after resection of a liver gastrinoma. We presume that the follow-up period should last for several years, as we show in our patient that a primary hepatic tumour has a slow rate of recurrence.

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# Chapter 5

## *Diagnostic Efficacy of The Secretin Stimulation Test for The Zollinger-Ellison Syndrome: an Intra-Individual Comparison Using Different Dosages in Patients and Controls*

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**Abstract**

The secretin stimulation test is the principal diagnostic tool to identify the Zollinger-Ellison syndrome (ZES).

To investigate by intra-individual comparison which dose of secretin results in the highest diagnostic efficacy to identify the ZES.

We analyzed 57 paired secretin stimulation tests, using both 0.26 µg/kg and 0.78 µg/kg secretin, performed in 13 ZES patients and 12 controls, and confirmed the findings in a validation cohort.

In our study, a gastrin increase of >100 ng/L was found to be the most sensitive and specific criterion for a positive test, also compared to the most frequently used criteria from the literature. Using this criterion, we found that the higher gastrin increases after 0.78 µg/kg compared to 0.26 µg/kg secretin contributed to a slightly more sensitive (82.9% vs. 80.5%) but less specific (68.8% vs. 81.3%) test. Application of this criterion in a confirmative set of 98 tests, using 0.26 µg/kg secretin in 21 ZES patients and 39 controls, provided similar results. In ZES patients with normal fasting serum gastrin levels (<100 ng/L), there was no diagnostic benefit from the use of a higher secretin dose.

We conclude that the 0.26 µg/kg secretin stimulation test has the best diagnostic efficacy for the ZES.

**Introduction**

Zollinger-Ellison syndrome (ZES) is caused by a gastrin-producing neuroendocrine tumour (gastrinoma), and is characterized by symptoms of gastric acid hypersecretion, i.e., peptic ulcer disease, malabsorption and diarrhea[1]. However, symptoms can be absent for a relatively long time, for example when proton pomp inhibitors (PPI) are used[2]. ZES can be suspected when fasting serum gastrin (FSG) levels are increased, although hypergastrinemia is seen in several, more common, diseases, as well as in PPI users[3]. As a considerable number of ZES patients have FSG levels within the normal range, i.e., <100 ng/L, or FSG levels in a non-diagnostic range, i.e., 100-1,000 ng/L, determinations of FSG levels alone will not be conclusive for the diagnosis of ZES and additional diagnostic methods are needed. For this reason, several gastrin provocation tests have been developed, e.g., calcium infusion test, meal stimulation test and secretin stimulation test. The secretin stimulation test has been shown to be the diagnostic tool of choice in subjects with FSG levels < 1,000 ng/L[4,5]. In the literature, several criteria for a positive test have been reported. We first investigated which criterion for a positive secretin stimulation test results in the highest sensitivity and specificity in our study cohort and used this criterion in further analyses. Furthermore, since the introduction of the secretin stimulation test, the most optimal dose of secretin to use in this test has been disputed. While some studies have shown that a low dose of secretin is sufficient to discriminate between ZES[5,6] and other causes of hypergastrinemia, others believe that only a higher secretin dose can contribute to adequate diagnosing[7-10]. Therefore, we subjected ZES patients and non-ZES controls to sequential secretin stimulation tests with a low and high dose of secretin, and thereby obtained a per-person-comparison between different doses of secretin. To our knowledge, secretin stimulation tests have not been studied with different doses in the same patients before, except for one case report[8]. The aim of our study was to investigate whether; 1) the use of a higher dose of secretin in secretin stimulation tests leads to a higher gastrin increase, and if so, 2) does this contribute to a higher sensitivity and specificity of the secretin stimulation test for ZES, and 3) is the use of a higher secretin dose of benefit in the diagnosis of ZES in

patients with normal FSG levels (<100 ng/L). Lastly, we applied the determined criterion for a positive secretin stimulation test in a confirmative set of 98 secretin stimulation tests using the low dose of secretin in 21 ZES patients and 39 non-ZES controls to validate our initial results.

**Materials and Methods**

*Patients*

Sequential 0.26 μg/kg and 0.78 μg/kg secretin stimulation tests in 25 subjects, suspected of ZES based on increased FSG levels (hypergastrinemia) or because of clinical suspicions, were performed in our Gastroenterology Department of the Leiden University Medical Centre. In total, thirteen patients suffering from ZES, of whom four as part of the MEN-1 syndrome, and twelve non-ZES controls suffering from MEN-1 but not ZES (n=3), atrophic gastritis (n=2) or a non-ZES-related (mainly other gastroenteropancreatic) disease (n=7), were included. In the majority of patients (12/13), the diagnosis of ZES was confirmed by identification of a tumour on imaging or at surgery. Thirteen patients were female and twelve patients were male. In a subset of patients, the secretin stimulation test was performed multiple times for follow-up. Therefore, the total number of tests exceeds 25. For a validation study, an additional group of 60 patients, suspected of ZES, was included. In total, 98 secretin stimulation tests with 0.26 μg/kg of 21 ZES patients (20/21 confirmed with imaging or at surgery) and 39 controls were analyzed, using the criterion for gastrin increase of >100 ng/L for a positive test. Seven patients suffered from ZES as part of MEN-1, while ten controls had the MEN-1 syndrome without ZES. It must be noted that in this validation group, fourteen patients (nine ZES patients and five non-ZES controls) were included who also had been tested in the study group, although at different time points.

This study was performed according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Centre in compliance with the Helsinki Declaration.

*Secretin stimulation tests*

Secretin stimulation tests were done in patients after an overnight fast and acid-suppressing medications were continued, except for the day before and on testing, when possible. The secretin stimulation test was performed by the procedure as described previously[11]. Before, during and after intravenous injection of 0.26 μg secretin (Secretin, ClinAlfa, 255 ng is estimated to be 1 clinical unit) per kg of body weight during 30 seconds, blood samples were collected at -5, 0, +1, +5, +10, +15 and +30 minutes. Serum gastrin levels were measured by a radioimmunoassay, using an antibody raised in rabbits against synthetic human gastrin I (unsulfated gastrin-17) covalently coupled to bovine serum albumin. Labeled gastrin $^{125}$I-Tyr$^{12}$-gastrin-I (human) was purchased by PerkinElmer, USA. The antibody binds to all known circulating gastrin fragments. The upper limit of the normal range for fasting state was taken as 100 ng/L, samples were diluted with repeated measurements as necessary to generate gastrin levels in a measurable range. After a minimum delay of at least 60 minutes, the test was repeated using 0.78 μg of secretin per kg body weight. The basal fasting serum gastrin is calculated as the average of two fasting blood samples before secretin injection. The increase in gastrin levels in ng/L after stimulation was calculated by:

*[maximal value after secretin injection] – [basal fasting value prior to secretin stimulation].*

In daily practice, according to our hospital protocol, a gastrin increase of more than 50% of basal value with a minimum rise of 100 ng/L was defined as a positive test.

*Statistical analysis*

Statistical analysis was performed using Statistical Package for Social Sciences version 16 (SPSS). The Wilcoxon signed ranks test was performed for comparison of differences between serum gastrin levels before administration of distinct secretin doses. Linear regression analysis was used to evaluate the linear relationship between the different doses of secretin. In particular, an orthogonal regression was used, to minimize the orthogonal or perpendicular distances from the data points to the fitted regression line. A receiver operating characteristic

(ROC) curve was used to determine the discrimination threshold of gastrin increase for a positive secretin stimulation test. A p-value of <0.05 was considered to be statistically significant.

## Results

*Patient characteristics*

An overview of included patients in the initial study group (n=25) and confirmation group (n=60) is presented in Table 1.

| **Table 1. Patient characteristics** | | | | |
|---|---|---|---|---|
| | **Initial study group** | | **Confirmation group** | |
| | **25 patients** | **57 tests** | **60 patients** | **98 tests** |
| Sex | n | n | n | n |
| Male | 12 | 25 | 31 | 46 |
| Female | 13 | 32 | 29 | 52 |
| Age at test | yrs | yrs | yrs | yrs |
| Mean | 51.9 | - | 46.4 | - |
| St. dev. | 11.7 | - | 13.7 | - |
| Diagnosis | n | n | n | n |
| ZES | 13 | 41 | 21 | 56 |
| Preoperatively | 7 | 10 | 13 | 18 |
| Postoperatively | 10 | 31 | 12 | 38 |
| Non-ZES controls | 12 | 16 | 39 | 42 |
| MEN-1 present | n | n | n | n |
| ZES patients | 3 | 4 | 7 | 19 |
| Non-ZES controls | 3 | 4 | 10 | 13 |

Table 1. Patient characteristics of study group and validation group.

*Determination of the optimal criterion for a positive secretin stimulation test*

In our hospital daily practice, a secretin stimulation test is defined as positive in case of a gastrin increase of more than 50% of basal value with a minimum rise of 100 ng/L. In the literature, several criteria for a positive secretin test have been described. Therefore, the most sensitive and specific secretin stimulation test was

assessed in our study population (Table 2). To determine the most optimal criterion for differentiation between Zollinger-Ellison syndrome and non-Zollinger-Ellison disease within our study group, a ROC curve analysis was performed. The optimal cut-off point for absolute gastrin increase with 0.26 µg/kg secretin was found to be 100 ng/L, with a sensitivity and specificity of 80.5% and 81.3% respectively. For 0.78 µg/kg secretin the cut-off point was found to be 95 ng/L, with a sensitivity and specificity of 82.9% and 68.8% respectively, but for the cut-off point of 100 ng/L identical results were found. Therefore, in this study, an absolute gastrin increase >100 ng/L as the uniform criterion with the highest sensitivity and specificity was chosen and used in our further analysis. In combination, we found that the criterion of a gastrin increase >100 ng/L is optimal for the diagnostic effectiveness for ZES, as this criterion led to equal or higher sensitivity and specificity compared to other criteria. Only when 0.78 µg/kg of secretin is used in the secretin stimulation test, the criterion of a gastrin increase of >100 ng/L + >50% leads to a slightly higher sensitivity and specificity.

*Fasting serum gastrin analysis*

We were also interested whether a higher dose of secretin contributes to a more diagnostic efficiency of the secretin stimulation test, and therefore 57 secretin stimulation tests were sequentially performed with two doses of secretin. For optimal comparison of gastrin increases after stimulation with 0.26 µg/kg or 0.78 µg/kg secretin, it is favorable that FSG levels before administration of secretin are comparable. We found that the FSG concentrations (mean 339 ng/L, range 7.5-43200 ng/L and 289 ng/L, range 5-47850 ng/L for 0.26 µg/kg and 0.78 µg/kg, respectively) did not significantly differ in the paired analysis and that there was a significant correlation (Spearmans rho = 0.9854 with $P<0.01$) between FSG levels before the use of 0.26 µg/kg and 0.78 µg/kg of secretin.

| Table 2. Determination of optimal criterion | | | |
|---|---|---|---|
| **Gastrin increase** | | **Sensitivity** | **Specificity** |
| **0.26 µg/kg secretin** | >100 ng/L[#] | 80.5% | 81.3% |
| | >110 ng/L[$] | 80.5% | 81.3% |
| | >120 ng/L[&] | 78.0% | 81.3% |
| | >200 ng/L* | 58.5% | 81.3% |
| | >50%[+] | 80.5% | 75.0% |
| | >100%[¶] | 65.9% | 81.3% |
| | >100 ng/L+>50%[§] | 68.3% | 87.5% |
| **0.78 µg/kg secretin** | >100 ng/L | 82.9% | 68.8% |
| | >110 ng/L | 82.9% | 68.8% |
| | >120 ng/L | 80.5% | 68.8% |
| | >200 ng/L | 73.2% | 68.8% |
| | >50% | 95.1% | 68.8% |
| | >100% | 78.0% | 81.3% |
| | >100 ng/L+>50% | 80.5% | 87.5% |
| Criteria of Lamers *et al.* (#,§)[11], Deveney *et al.* ($)[12], Berna *et al.* (&)[5], McGuigan and Wolfe (*)[10], Lamers and van Tongeren (+)[13], and Modlin *et al.* (¶)[14]. | | | |

Table 2. Specificity and sensitivity, using different criteria as reported in the literature, calculated for secretin tests using 1 and 3 clinical units per kg of secretin for diagnosing ZES. Remarkably, sensitivity was higher for 0.78 µg/kg compared to 0.26 µg/kg of secretin for each criterion, while specificity showed an opposite pattern, resulting in higher specificity when the secretin stimulation test is performed using 0.26 µg/kg of secretin.

*Gastrin increase analysis*

In Figure 1, gastrin increase levels after the use of 0.26 µg/kg of secretin are plotted on a logarithmic scale against gastrin increase levels after the use of 0.78 µg/kg of secretin. To determine if the use of 0.78 µg/kg of secretin leads to a higher gastrin increase, an orthogonal regression analysis was performed. For the total group of 57 tests, this resulted in a slope of 1.400 ± 0.0770 with a 95% confidence interval between 1.245 and 1.554, indicating that the use of 0.78 µg/kg leads to a higher gastrin increase compared to the use of 0.26 µg/kg of secretin. This effect was also found when ZES patients were analyzed separately; 1.403 ±

0.0929 with a 95% confidence interval between 1.215 and 1.591. Furthermore, in these ZES patients a previous low dose secretin provocation did not affect the response to the high secretin dose as illustrated by the significantly higher maximum gastrin level (mean 10,920 ng/L, range 29-110,000 ng/L versus 13,740 ng/L, range 38-188,000 ng/L, respectively; $P<0.03$). The resulting mean maximum gastrin level ratio of 1.17 was similar to that observed in two patients where the two secretin stimulation tests were performed with an approximately two-week interval having a ratio of 1.16.

In contrast, orthogonal regression analysis of non-ZES controls (n=16) revealed a slope of 0.6743 ± 0.0616, with a 95% confidence interval between 0.5421 and 0.8064, lower than 1. No points in Figure 1 are located in the right lower quadrant representing a gastrin increase with 0.26 μg/kg >100 ng/L and with 0.78 μg/kg <100 ng/L, which means that in none of the tests in this quadrant the use of 0.78 μg/kg of secretin was superior to the use of 0.26 μg/kg. In contrast, there are three points (two controls and one ZES patient) located in the left upper quadrant representing a gastrin increase with 0.26 μg/kg <100 ng/L and with 0.78 μg/kg >100 ng/L, indicating that in one ZES patient the 0.78 μg/kg secretin stimulation resulted in a positive test, while the 0.26 μg/kg secretin stimulation test was falsely negative, but this was also the case in two non-ZES patients indicating false-positive results with 0.78 μg/kg in these patients.

To asses whether this increase was clinically relevant, sensitivity and specificity were compared between 0.26 μg/kg and 0.78 μg/kg secretin stimulation tests. Hereby the secretin stimulation test was defined as positive when gastrin increase was >100 ng/L. This led to a higher number of truly positives for 0.78 μg/kg secretin stimulation tests, but to a higher number of false positives as well. Therefore, sensitivity was slightly higher in tests using 0.78 μg/kg compared to 0.26 μg/kg secretin stimulation tests (82.9% vs. 80.5%), but specificity was lower (68.8% vs. 81.3%).

**Figure 1.**

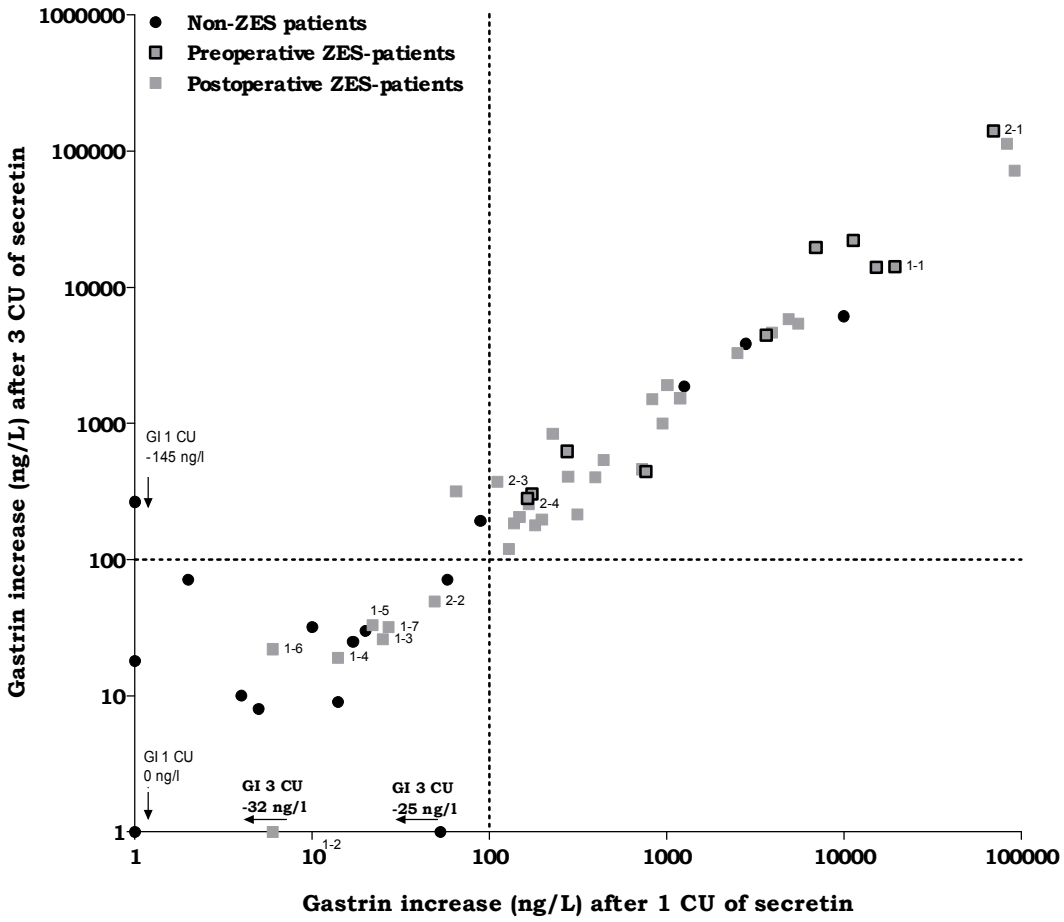


Figure 1. Gastrin increase levels after the use of 0.26 µg/kg of secretin are plotted against gastrin increase levels after the use of 0.78 µg/kg of secretin. A logarithmic scale is used. Arrows indicate gastrin increase levels of values which would originally fall outside the graph, therefore exact values are mentioned above the arrows. Numbers next to individual points represent 2 patients with an exceptional disease course; 1-1 till 1-7 represent a patient with normal postoperative serum gastrin levels without any symptoms or signs of recurrence; 2-1 till 2-5 represent a patient with initially normal but thereafter increased postoperative serum gastrin levels, while tumour recurrence could not be confirmed on imaging studies.

*Effect of the use of 0.78 µg/kg on ZES patients with normal fasting serum gastrin levels*

To assess whether the use of 0.78 µg/kg of secretin is more contributory to diagnose ZES in patients with normal FSG concentrations (<100 ng/L), this group of ZES patients was analyzed separately (Figure 2a and b). In total, 12 tests of four patients after resection were examined. A Chi-square analysis revealed no significant difference between groups, as the use of 0.78 µg/kg led to an almost equal number of true positive tests (6/12 vs. 5/11, Figures 2b and 2a, respectively) as the use of 0.26 µg/kg in the secretin stimulation test when a cut-off for gastrin increase of 100 ng/L was used. Thus, when gastrin increase are <100 ng/L or

when FSG levels are >100 ng/L, the diagnosis remains uncertain but in the case of normal FSG levels <100 ng/L) in combination with a gastrin increase >100 ng/L, the diagnosis of ZES is highly likely.

**Figure 2a.**

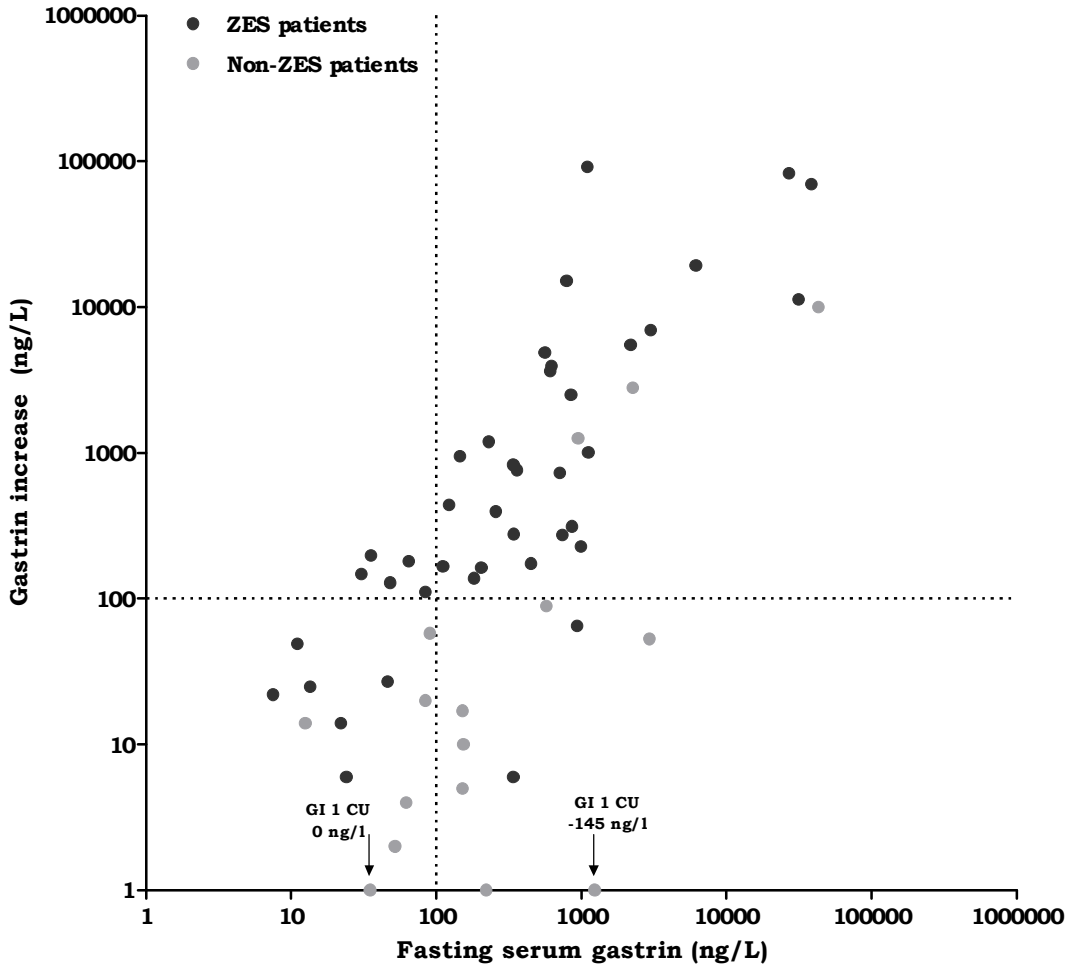

**Figure 2b.**

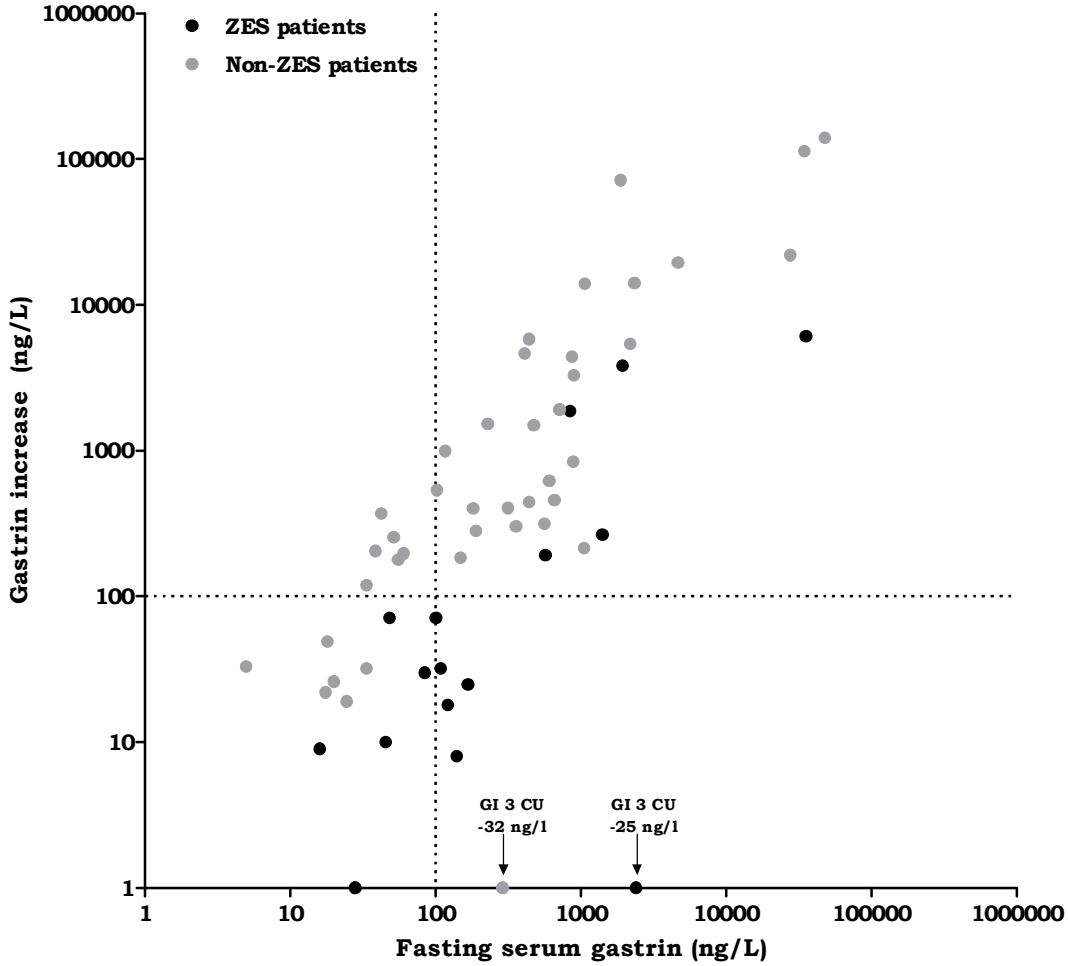


Figure 2a,b. Fasting serum gastrin levels are plotted against gastrin increase after stimulation with a low (0.26 µg/kg, 2a.) or high (0.78 µg/kg, 2b.) secretin dose are presented. A logarithmic scale is used. Arrows indicate gastrin increase levels of values which will originally fall outside the graph, therefore exact values are mentioned above the arrows.

*Validation study*

Based on the results described above, we concluded that an absolute gastrin increase >100 ng/L leads to the highest sensitivity and specificity in the study group. Therefore, this criterion was validated in 60 patients suspected of ZES, by performing 98 secretin stimulation tests using 0.26 µg/kg of secretin. Using the criterion of a gastrin increase of >100 ng/L for a positive test, 35/42 tests of 39 non-ZES controls were indeed negative, while 45/56 tests of 21 ZES patients were truly positive. This led to a sensitivity of 80.3% and specificity of 83.3%, comparable to the sensitivity and specificity of the initial study cohort (80.5% and 81.3%, respectively).

**Discussion**

Hypergastrinemia is a common characteristic of the Zollinger-Ellison syndrome, although the extent of hypergastrinemia can differ considerably between patients. Furthermore, the use of acid suppressing medications may delay the diagnosis of ZES, by masking the symptoms in ZES patients or mimic ZES by causing hypergastrinemia in patients without ZES. Therefore, FSG levels alone are not conclusive in a considerable number of ZES patients. Particularly, in case of mild to moderate hypergastrinemia (100 – 1,000 ng/L), additional diagnostics are required to confirm or exclude the diagnosis of ZES[3]. The secretin stimulation test is preferred above the calcium or meal stimulation tests, as the secretin stimulation test is more sensitive, easy to perform and less inconvenient for patients[14]. However, several aspects of the secretin stimulation test have been disputed, e.g., the criterion for a positive test and the dose of secretin to be used. In most previous publications, serum gastrin responses to secretin of > 200 ng/L, introduced by McGuigan and Wolfe, are used as the criterion for a positive secretin stimulation test[10]. Recently, Berna *et al.* studied the secretin stimulation test in 293 patients and 537 patients from the literature, and recommended to use a gastrin increase of 120 ng/L[4,5]. We investigated the most optimal criterion (sensitivity) for a positive test to diagnose ZES in our study group and not the best criterion to exclude the disease in other patients and controls (specificity). By both sensitivity/specificity determinations and ROC analysis we found a post-secretin gastrin elevation of >100 ng/L to be the most optimal discriminating value between ZES and non-ZES patients. Applying this criterion in a validation cohort of 21 ZES patients and 39 non-ZES controls led to a similar sensitivity and specificity for this criterion and confirmed the initial findings.

As also the most optimal dose of secretin has been disputed, we investigated whether a higher dose of secretin would lead to more sensitive and specific tests, by subjecting ZES patients and non-ZES controls to sequential secretin stimulation tests using both a low and a high dose of secretin. Comparison-studies for secretin doses have been reported before, but, except for a case report in which two doses of secretin were compared in one patient, these have all been based on the

comparison with patients from literature and were not performed in the same subjects[8]. Although the number of included patients in this study is relatively low, this is the first study in which Zollinger-Ellison patients and non-ZES patients are subjected to multiple secretin stimulation tests with different doses, making this an intra-individual comparison. We found that the use of 0.78 µg/kg of secretin provokes a higher post-secretin serum gastrin increase, resulting in a higher number of true-positive ZES patients but also in a higher number of false-positives, leading to a higher sensitivity but a decrease in specificity, compared to the use of 0.26 µg/kg of secretin. Therefore, we concluded that a higher dose of secretin did not contribute to a better discrimination between ZES patients and non-ZES controls in secretin stimulation tests. In general, a relatively small group of Zollinger-Ellison patients have FSG levels in the normal range (<100 ng/L), often after gastrinoma excision, and are therefore hardly recognized as (recurrent) ZES. In the present study, patients suffering from ZES having normal FSG levels (<100 ng/L), had no diagnostic benefit from the use of a higher secretin dose in the secretin stimulation test. Hence, the use of 0.26 µg/kg of secretin seems to be appropriate. From a financial point of view, the use of a low dose of secretin is preferential, as a three times higher dose leads to higher costs, but does not contribute to a more valuable secretin stimulation test.

It is generally known that the use of PPIs or other acid suppressing medications can lead to elevated fasting serum gastrin levels, and therefore might falsely suggest ZES. Indeed, Hirschowitz *et al.* have shown that longterm use of PPIs in non-ZES-patients increases FSG-levels but does not lead to a further gastrin increase in the ZES patients[15]. In addition, a recent case report by Goldman *et al.*, suggests that the use of a PPI can also lead to a false positive secretin stimulation test resulting in diagnosing ZES in non-ZES controls[16]. In our study, however, 39% (7/18) of the PPI-using non-ZES controls, with a FSG level of >100 ng/L, had a false positive secretin stimulation test, as opposed to 30% (3/10) of those free of acid-suppressing medication. These findings illustrate that there is no direct relation between PPI use and a false positive secretin stimulation test. Therefore,

we believe that it is not necessary to discontinue acid-reducing medications for the secretin stimulation test, also to reduce the risk of developing ulcer complications.

In conclusion, we found that a gastrin increase after stimulation with secretin of >100 ng/L leads to the highest sensitivity and specificity to diagnose ZES. Applying this criterion in our study revealed that the use of a higher dose of secretin did not contribute to a more valuable secretin stimulation test in diagnosing ZES. Therefore, we recommend the use of 0.26 µg/kg of secretin in secretin stimulation tests to diagnose or exclude ZES, with a gastrin increase >100 ng/L as the optimal criterion for a positive secretin stimulation test.

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# Chapter 6

## *Expression and Ligand Binding of Bombesin Receptors in Pulmonary and Intestinal Carcinoids: The role of bombesin in carcinoids*

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**Abstract**

Carcinoids are mainly found in the gastrointestinal (65%) and bronchopulmonary tract (25%). These neuroendocrine tumours secrete a wide range of bioactive peptides, including gastrin releasing peptide and neuromedin B, the mammalian analogs of bombesin. The purpose of this study was to investigate the quantity and localization of bombesin receptors in gastrointestinal and pulmonary carcinoids, and to reveal whether bombesin-like peptides and their receptors are of any value in distinguishing pulmonary carcinoids from carcinoids of intestinal origin.

Carcinoid tumours with pulmonary (n=9) and intestinal (n=15) localizations were analyzed by immunohistochemistry, autoradiography and radioimmunoassay, to examine the presence of bombesin receptor subtypes and determine bombesin-like peptide levels in these tumours.

All three bombesin receptor subtypes (GRPR, NMBR and BRS-3) were present on pulmonary and intestinal carcinoids by immunohistochemistry. In pulmonary carcinoids, low receptor ligand binding densities together with high and low bombesin-like peptide levels were found. Intestinal carcinoids showed predominantly high receptor ligand binding densities in combination with low bombesin-like peptide levels.

The expression of bombesin receptor subtypes is independent from the carcinoid tumour origin, and is therefore not recommended as a distinction marker, although carcinoids of pulmonary and intestinal origin possess different receptor binding affinities for bombesin and dissimilar bombesin-like peptide levels. The combined presence of bombesin and its receptors might suggest the presence of a paracrine or autocrine growth loop in carcinoids.

**Introduction**

Carcinoids are tumours from the diffuse neuroendocrine system, which derive predominantly from serotonin-producing enterochromaffin (Kulchitsky's) cells or gastric histamin-secreting enterochromaffin-like cells and comprise more than 50% of the well-differentiated neuroendocrine tumours (NETs). The majority of carcinoids are located in the gastrointestinal tract (65%) or bronchopulmonary system (25%). Their clinical course and prognosis is mainly dependent on the site of the primary tumour[1]. Initially, carcinoids were classified according to their embryonic origin, into foregut (originating from esophagus to pancreas, lungs included), midgut (originating from third part of the duodenum to ascending colon) and hindgut tumours (originating from transverse colon to rectum)[2]. Currently, based on histological classifications of the World Health Organization (WHO), neuroendocrine tumours are arranged according to tumour localization and differentiation[3].

Carcinoids are able to produce and secrete a variety of biogenic amines and peptides, including serotonin, chromogranin A, neuron specific enolase, substance P, gastrin and bombesin[4]. Bombesin and its mammalian analogs gastrin releasing peptide (GRP) and neuromedin B (NMB) belong to the family of bombesin-like peptides (BLPs), which were initially isolated from amphibian skin[5]. In humans, they are distributed in neural and endocrine cells, especially throughout the gastrointestinal tract. In addition to stimulating a variety of physiological responses in the human body, BLPs are involved in development and progression of several human cancers. For example, it has been shown that these peptides can stimulate the growth of lung, CNS, breast, cervix and prostate cancer cell lines, both in vivo and in vitro[6,7]. BLPs mediate their biological actions through binding to the G-protein coupled gastrin-releasing peptide receptor (GRPR, BB2R), neuromedin B receptor (NMBR, BB1R), bombesin receptor subtype 3 (BRS3, BB3R) and bombesin receptor subtype 4 (BRS-4, BB4R). Activation of various bombesin receptor subtypes has growth effects in both normal and neoplastic tissues, and several studies have reported an upregulation of bombesin receptors in tumour samples compared to associated normal tissue. Gonzalez *et al.* speculated that

'Bombesin receptors are one of the most frequent receptor classes that are overexpressed or ectopically expressed by human cancers'[8]. By binding to their membrane-bound receptors on tumour cells, BLPs are able to activate autocrine loops, leading to growth of the tumour. In pulmonary neuroendocrine tumours (small cell and non-small cell lung cancer), an autocrine loop involving BLPs has been suggested[9,10]. Also in colon cancer, BLPs have been reported to act both as morphogens and mitogens[11]. Aims of this study were 1) to investigate the quantity and localization of bombesin receptors in combination with bombesin-like peptide level expression on pulmonary and gastrointestinal carcinoids, and 2) whether carcinoids of different origin, i.e., from the pulmonary or gastrointestinal system, can be distinguished based on bombesin (receptor) expression. We used three different techniques, i.e., storage phosphor autoradiography, radioimmunoassay and immunohistochemistry, to determine presence of three different bombesin receptor subtypes and bombesin peptide levels in carcinoids of pulmonary and intestinal origin.

**Material and Methods**

*Patients and tissues*

After surgical removal, tumour tissues were collected at the department of Gastroenterology, Leiden University Medical Center, Leiden, (n=4), the department of Pathology, Antoni van Leeuwenhoek Hospital, Amsterdam, (n=11) and the department of Pathology, The Netherlands Cancer Institute - Academic Medical Center, Amsterdam, (n=9), frozen at -80 ◦C for ligand binding studies or embedded in paraffin for immunohistochemical staining. In total, 24 tissues from patients with carcinoids were studied. Carcinoids included primary typical (n=8) and atypical (n=1) lung carcinoids, primary small bowel well-differentiated neuroendocrine carcinomas (n=9), primary duodenum well-differentiated neuroendocrine carcinoma (n=1), primary rectum neuroendocrine carcinoma (n=1), liver metastases from a primary small bowel well-differentiated neuroendocrine carcinoma (n=1), and lymph node metastases from primary small bowel well-differentiated neuroendocrine carcinomas (n=3). Neuroendocrine

tumours were classified according to the World Health Organization Classification for Neuroendocrine Tumours[3], and in this study further referred to as 'carcinoids'.

*Immunohistochemical analysis*

The primary antibody for BRS-3 was a polyclonal rabbit antibody raised against a synthetic peptide, corresponding to the carboxy terminal tail of the BRS-3. This antibody was provided by Schulz *et al.*[6]. The primary antibody against the NMBR was a polyclonal rabbit antibody, raised against the carboxy-terminus of the human NMBR (Biotrend Chemikalien GmbH, Cologne, Germany). The primary antibody for GRPR was a polyclonal rabbit antibody, raised against a synthetic peptide conjugated to KLH, corresponding to the 2nd extracellular loop (Abcam Inc., Cambridge, UK). Immunohistochemistry was performed as follows. Briefly, tissues were fixed in formalin, embedded in paraffin and cut into seven µm sections. After deparaffinization and rehydration, endogenous peroxidases were blocked in methanol containing 0.3% $H_20_2$ (Merck). Antigen retrieval was performed by boiling in citrate buffer pH 6.0 for 12-17 minutes. Primary antibodies were diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA, for NMBR and GRPR antibodies) or Tris-HCl-PBS pH 6.4 with 0.5% Thimerosal (Sigma-Aldrich, for BRS-3 antibody) and incubated overnight at room temperature (RT). Incubation with biotinylated goat anti-rabbit immunoglobulins for 30 minutes at RT was followed by incubation with horseradish peroxidase (HRP)-streptavidin complex (both Dako, Glostrup, Denmark) for 30 minutes at RT. Staining was visualized using 0.05% diaminobenzidine (DAB, Sigma-Aldrich) containing 0.0038% H202 in 0.05 M Tris-HCl buffer pH 7.6. For NMBR staining, the visualization step was intensified by addition of 0.15% Imadizole.

Cervix carcinomas were used as positive controls[12], whereas for negative controls primary antibodies were omitted. Representative photomicrographs were taken with a Olympus BX-51TF microscope with a DP23-3-5 camera.

Specificity of the primary antibodies against NMBR and BRS-3 was confirmed by preincubation with a 10-fold surplus of the immunizing peptide for four hours at RT before incubation of the slides. For BRS-3, addition of 1% goat serum was

necessary to avoid aspecific binding of the formed antibody-blocking peptide complex. Further protocol was performed as described above. No immunizing peptide for our GRPR antibody was available but incubations without primary antibodies were negative, and Western blot analysis performed previously[13] confirmed the specificity for this antibody.

*Immunohistochemistry scoring*

Evaluation of immunohistochemical staining for the different antibodies was performed by two independent observers. The following characteristics were investigated; presence/absence of specific staining on tumour cells of carcinoids, staining intensity and comparison between carcinoid groups. Intensity of staining (0=negative, 1=slightly positive, 2=moderately positive, 3=positive, 4=strongly positive, 5=exceptionally positive) and the number of cells showing immunopositivity (0=no positive cells, 1=0-25% positive cells, 2=25-75% positive cells, 3=75-100% positive cells) were included in the final score for each antibody. Maximum achievable score was therefore eight.

*Storage Phosphor Autoradiography*

Before use, tissues were cut into 14 µm sections at -20 °C using a cryostat microtome and mounted on gelatin coated glass slides. Slides were dried overnight at -80 °C to stimulate adhesion. Storage phosphor autoradiography was performed as described previously[14,15]. Briefly, slides were pre-incubated with 50 nM Tris-HCl containing 0.5% Bovine Serum Albumin (BSA, Sigma-Aldrich, Steinheim, Germany) at RT at pH 7.0 for 20 min. Then slides were incubated with pre-incubation buffer and Bacitracine 0.25 mg/ml, Leupeptine 4 µg/ml, Chymostatin 2 µg/ml, NaCl 130 mM, KCl 7.7 mM, $MgCl_2$ 5 mM and EGTA 1mM, together with 75 pM [$^{125}$I]Tyr$^4$-bombesin (2200 Ci/mmol: PerkinElmer, Inc. Boston MA, USA) for 3 hr. For each slide, an alternating slide was incubated with the previous in combination with the addition of 1 µM non-radioactive bombesin, to determine aspecific binding. Slides were washed in the pre-incubation buffer three

times for 5 minutes at 4 ◦C, rinsed in distilled water and dried at RT under a cold stream of air.

To determine receptor density, dried tissue sections were placed in a storage phosphor cassette for 48 h at RT and subsequently scanned by Phosphorlmager. Images were processed with ImageQuant software (Amersham Biotech - Molecular Dynamics, Inc. Sunnyvale, CA, USA). Slides with two drops of 10 ul 100 pM labeled bombesin were included for standardization. Human intestinal smooth muscle cells of non-carcinoid patients were used as positive controls. Receptor binding was calculated as fmol of radioactive peptides bound per mg protein (with an estimation of 10 mg protein per 100 mg wet tissue). Tumour localization was confirmed on consecutive Hematoxylin-Eosin stained slides.

For a subset of samples, radiolabeled ligands for NMB (1132.3 g/mol, Bachem, Inc. USA) and GRP (2859.4 g/mol, Bachem) were used for cold saturation inhibition curves to identify the bombesin receptor subtypes present in the samples. The procedure was performed as described above.

*Radioimmuno assay*

After frozen tumour sections were trimmed, tumour tissues were homogenized in 0.1M TrisHCl 0.1% Tween80 buffer pH 7.5 on ice. Levels of BLP were measured in the supernatant. The radioimmunoassay was performed in a two-step incubation; samples, or bombesin as standard, and diluted anti-bombesin antibody were first incubated for 48 hours at 4 ◦C. This antiserum K162 was generated by immunization of rabbits with synthetic bombesin. Final dilution of the bombesin antibody used in the assay was 1:40.000. After 48 hours, labeled bombesin ($^{125}$I[D-Tyr$^{4}$] bombesin, 200Ci/mmol: PerkinElmer, Inc. Boston MA, USA) was added and further incubated for 24 hours. Bound and free ligands were separated by precipitation with sheep anti-rabbit antibody coupled to microsepharose beads (Pharmacia decanting suspension 3) and counted in a γ-counter. Concentrations were calculated from the bombesin standard curve.

*Statistical analysis*

Statistical analysis was performed using Statistical Package for Social Sciences version 16 and GraphPad Prism version 5. Data were summarized as median. Mann-Whitney U-test was used to compare mean receptor densities, bombesin-like peptide levels and immunohistochemical scores for bombesin receptor subtypes between pulmonary and intestinal carcinoids. Fisher Exact test was used to examine the significance of association of receptor densities and bombesin-like peptide levels between pulmonary and intestinal carcinoids. Spearman correlations were used to investigate significant correlations between various parameters. A p-value of $<0.05$ was considered statistically significant.

**Results**

*Immunohistochemistry*

Staining of BRS-3, GRPR and NMBR on carcinoids was found to be predominantly cytoplasmic, with some incidental membrane-bound staining (Figure 1), as in the positive controls, i.e., cervix carcinomas. For BRS-3 also nuclear immunopositivity was seen but considered aspecific, as the addition of a blocking peptide to the primary antibody did not lead to clearance of this staining. In total, 20 of 24 carcinoids expressed BRS-3, whereas 21 of 24 carcinoid tumours expressed NMBR. All 24 carcinoids were positive for GRPR. Immunostaining for all three bombesin receptor subtypes simultaneously was seen in 20/24 carcinoids.

**Figure 1. Immunostaining on carcinoids**

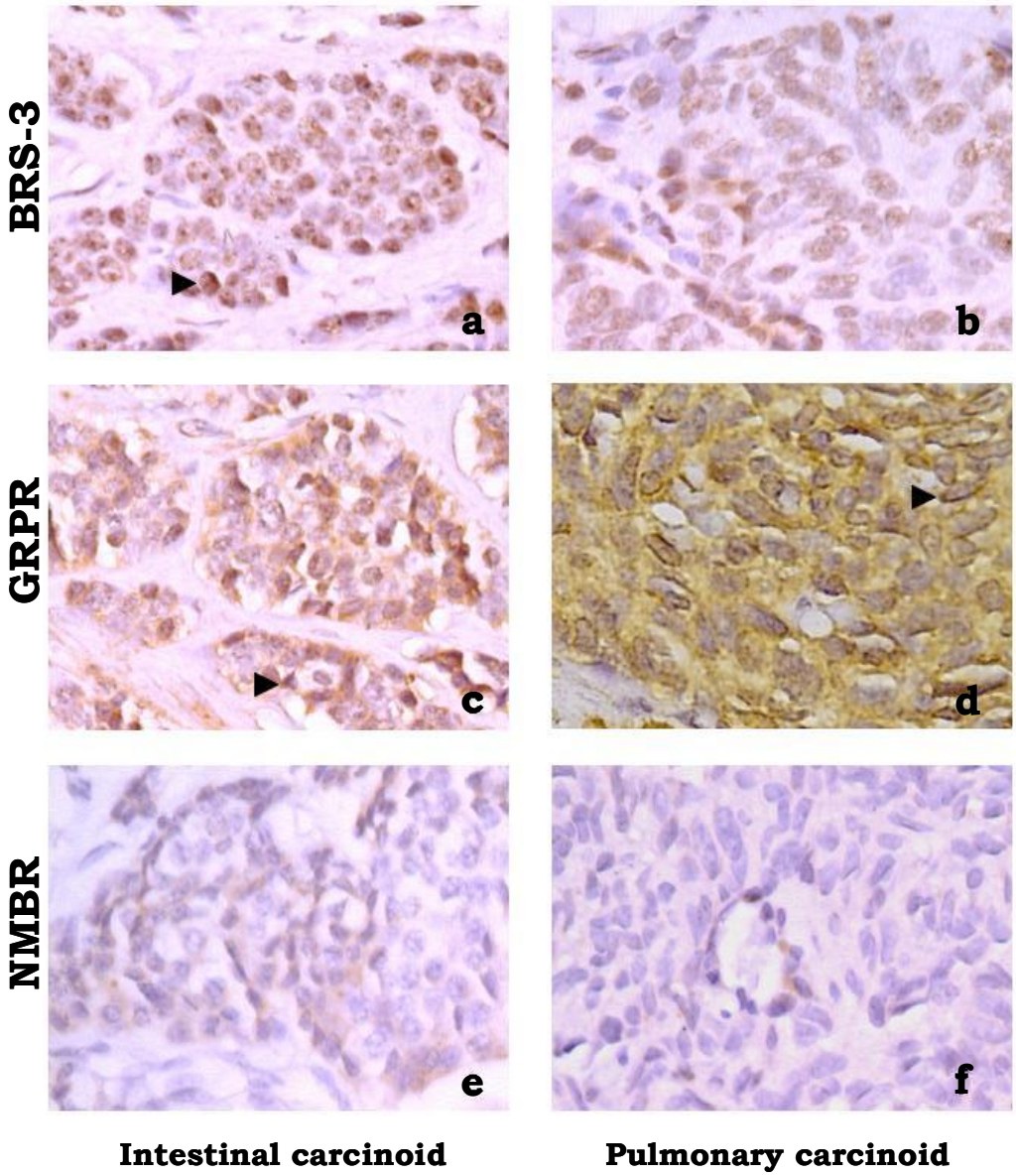


Figure 1. Immunostaining for the three bombesin receptors on an intestinal and a pulmonary carcinoids Magnification x 400. The carcinoids are stained with antibodies for BRS-3 (a,b), GRPR (c,d) and NMBR (e,f). Incidental membrane-bound staining is indicated with ►. Immunohistochemical scores were 3 for BRS-3 (a), 5 for GRPR (c) and 4 for NMBR (e) in the intestinal carcinoid, and 4 for BRS-3 (b), 8 for GRPR (d) and 3 for NMBR (f) in the pulmonary carcinoid.

Comparison of immunohistochemical scores between groups revealed that in pulmonary carcinoids (n=9), GRPR staining was significantly higher compared to intestinal carcinoids (n=15, median scores 6 and 5 respectively, *P*=0.02, Figure 2). This was mainly due to a higher GRPR staining intensity (median scores 3 and 2, *P*=0.02). Median total scores for BRS-3 were equal in pulmonary and intestinal carcinoids, also for BRS-3 staining intensity and number separately. Although

NMBR total score, staining intensity and number were higher in pulmonary carcinoids compared to intestinal carcinoids, this did not reach statistical significance. Furthermore, inter-antibody comparison of the immunohistochemical scores for the different bombesin receptor subtypes was not applicable because of the intrinsic differences between the antibodies.

**Figure 2. Immunohistochemical staining scores**

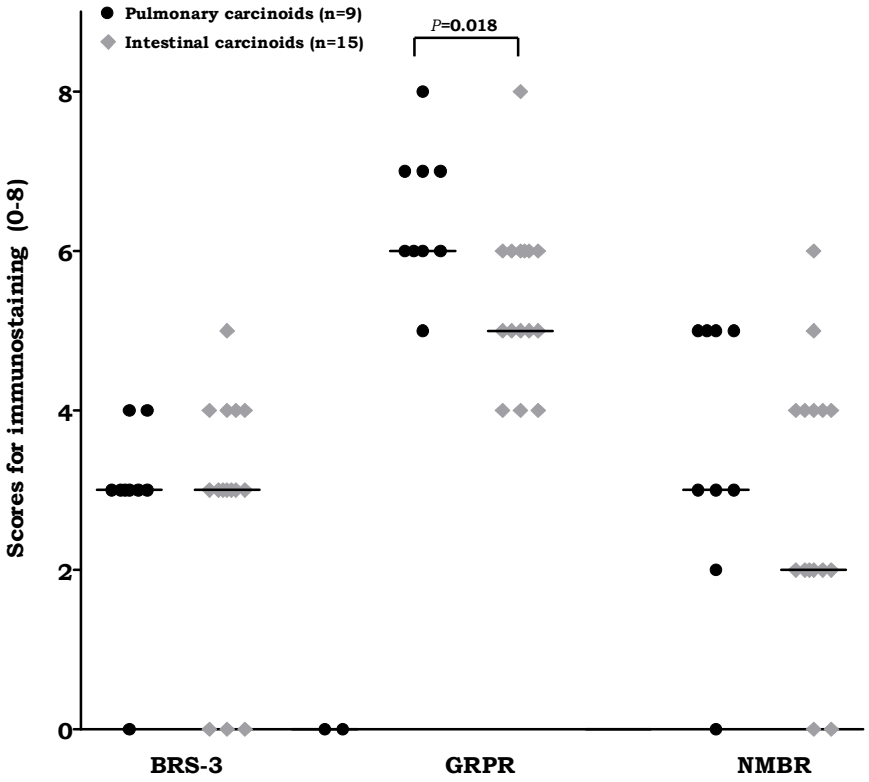


Figure 2. Graphic presentation of immunohistochemical scores for BRS-3, GRPR and NMBR staining in pulmonary (n=9) and intestinal (n=15) carcinoids. Median scores are indicated by bars. Scores are composed of staining intensity (0-5) and number of cells with immunopositivity (0-3), total scores may therefore range from 0 (no staining) to 8 (very positive).
Of note; GRPR scoring is remarkably higher compared to scoring for BRS-3 and NMBR immunostaining, which is mainly due to antibody characteristics, it is of no value to compare immunoexpression between the different bombesin receptor subtypes.

*Quantification of binding sites for bombesin on carcinoids*

Storage Phosphor Autoradiography was used to identify the presence of bombesin receptors on 24 carcinoids. Autoradiographic ligand binding on these tumour tissues was found to be diffuse. Receptor densities were found in a range of 0 to 87 pmol/g tissue. Receptors were present, i.e., detectable, in 13/15 and 7/9 intestinal and pulmonary carcinoids, respectively. In pulmonary carcinoids, receptor

densities were high (≥10 pmol/g tissue) in 1/9 samples, while in intestinal carcinoids 7/15 samples showed high receptor densities. Median receptor binding was almost significantly higher in intestinal carcinoids (n=15) compared to pulmonary carcinoids (n=9), *P*=0.07 (Table 1).

**Table 1. Receptor densities (fmol/mg tissue)**

| | Minimum | Maximum | Mean ± SEM | Median |
|---|---|---|---|---|
| Pulmonary carcinoids (n=9) | 0 | 32 | 5.11 ± 3.401 | 2 |
| Intestinal carcinoids (n=15) | 0 | 86 | 15.93 ± 6.076 | 6 |

SEM = Standard error of the mean

Table 1. Overview of receptor densities and bombesin-like peptide levels in pulmonary and intestinal carcinoids.

Pictures of autoradiographic ligand binding are shown in Figure 3.

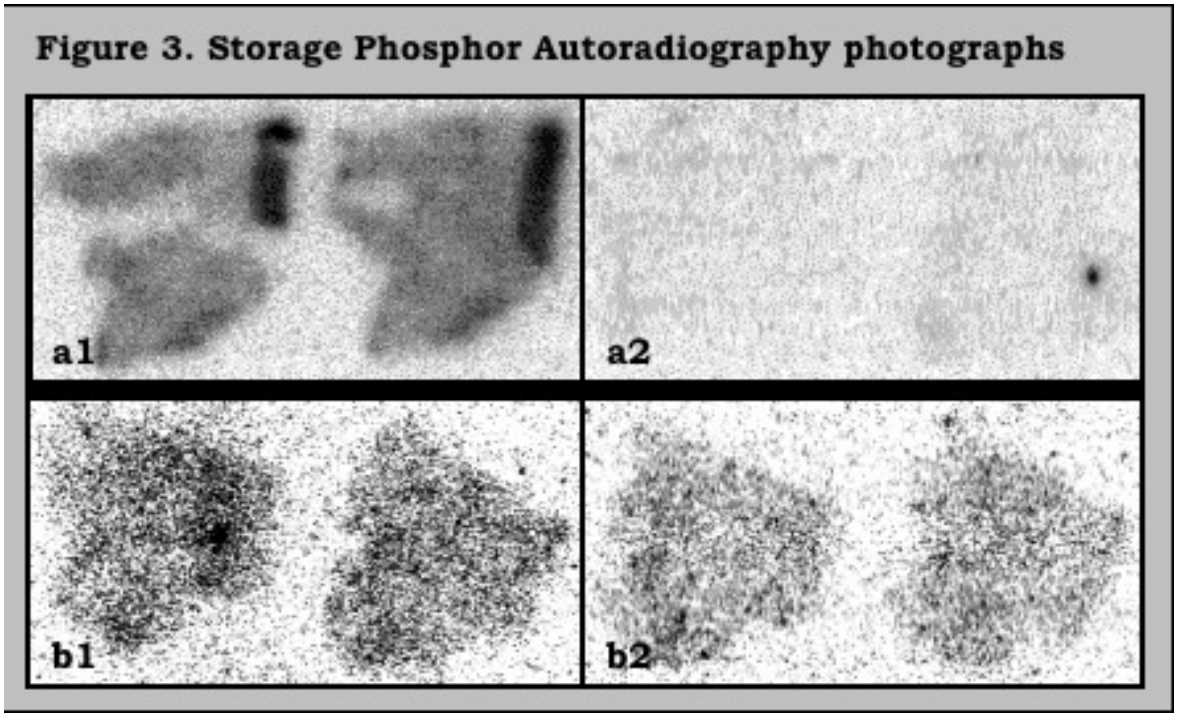


Figure 3. Autoradiographs of ligand binding of I-[D-Tyr]-bombesin in two carcinoid tissues. a1, a2) Carcinoid in abdominal lymph node (metastasis). b1, b2) Carcinoid in distal ileum. Binding detected with storage phosphor autoradiography was quantified using ImageQuant software. Total binding represented by a1 and b1. Aspecific binding represented by a2 and b2. To calculate specific binding, aspecific binding was subtracted of total binding.

*Quantification of bombesin-like peptide levels in carcinoids*

BLP levels in 24 carcinoid tissue homogenates were determined using a radioimmunoassay. BLP levels were detectable in all carcinoids, range 1-148 ng/g

tissue. Low (<10) levels were detected in 14/15 intestinal and 5/9 pulmonary carcinoids, respectively. Median BLP levels were significantly higher in pulmonary carcinoids (n=9) compared to intestinal carcinoids (n=15), *P*=0.02 (Table 1).

*Receptor densities and bombesin-like peptide levels in carcinoids*

In 4/9 pulmonary carcinoids, low (<10 pmol/g tissue) receptor densities in combination with high (≥10 ng/g) BLP levels were found, 4/9 pulmonary carcinoids had both low receptor densities and BLP levels. Only 1/9 pulmonary carcinoids had a high receptor density and low BLP levels. The majority of intestinal carcinoids (14/15) had low BLP levels, eight of these in combination with low receptor densities and six with high receptor densities. One intestinal carcinoid, originally located in rectum, had both high receptor densities and high BLP levels. A significant negative correlation, Spearman rho=-0.47, was found between receptor densities and BLP levels for all carcinoids, *P*=0.02, with a similar trend when pulmonary and intestinal carcinoids were separately analyzed (rho -0.32 and -0.39, respectively). Overall Chi Square analysis revealed a significant difference in receptor status in combination with BLP levels between pulmonary (n=9) and intestinal (n=15) carcinoids (*P*=0.03) (Figure 4).

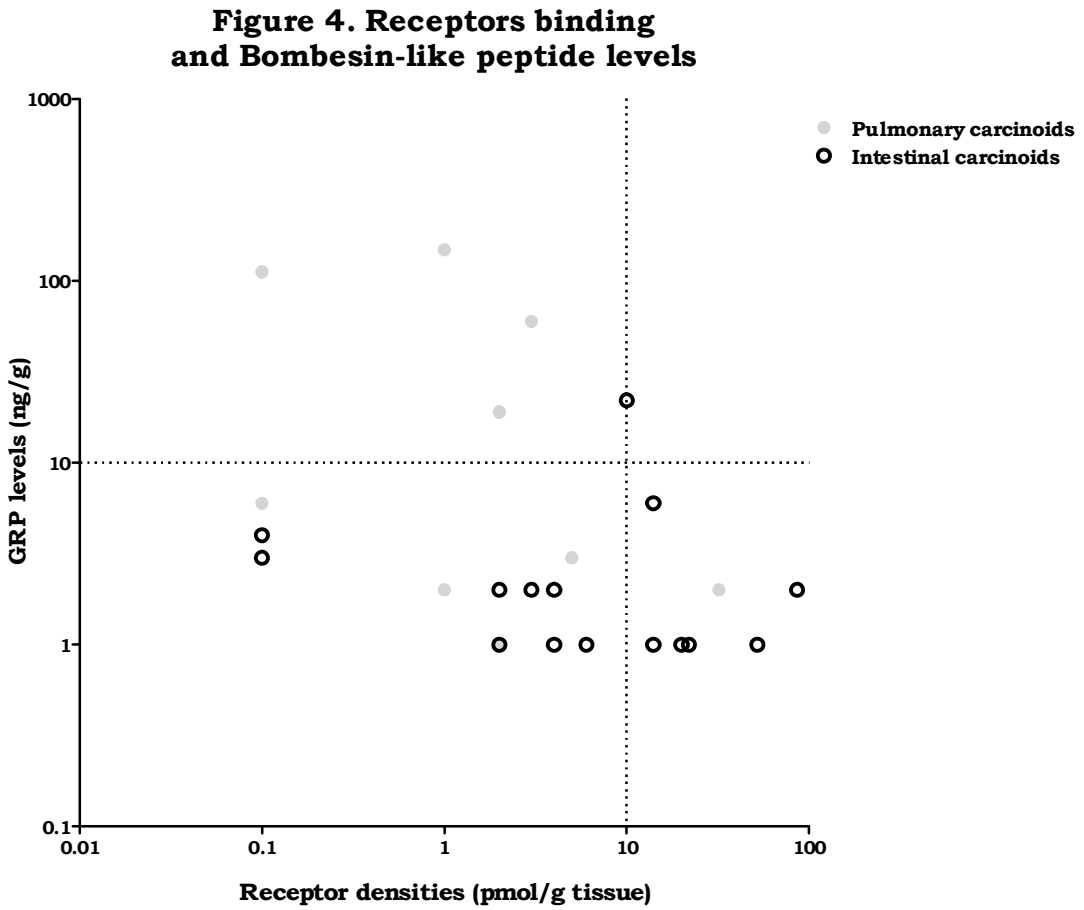


Figure 4. Receptor densities and bombesin-like peptide levels in pulmonary and intestinal carcinoids. A logarithmic scale is used.

## Discussion

Carcinoids can arise in many places of the human body, but are mainly situated in the pulmonary or gastrointestinal tract. Not only localization, but also hormonal production, histological differentiation and clinical behaviour of the tumour contributes to the heterogeneity of these neuroendocrine tumours[3]. We evaluated the quantity and localization of the different bombesin receptor subtypes GRPR, NMBR and BRS-3 in carcinoids of lung and intestine, to reveal whether carcinoids of different locations, i.e., from pulmonary or gastrointestinal system, can be distinguished by bombesin peptide and receptor characteristics. We found that in the majority of carcinoids (83.3%) all three receptor subtypes were present immunohistochemically. Therefore, overall bombesin receptor expression seems not to be a very useful marker to distinguish carcinoids based on tumour origin. In the remaining carcinoids, staining for at least one of the receptors was found. Both

GRPR and NMBR immunostaining scores were higher in pulmonary carcinoids compared to intestinal carcinoids, only the first being statistically significant ($P$=0.02). Immunopositivity for GRPR, BRS-3 and NMBR was diffuse and predominantly cytoplasmic, with some membrane-bound staining, identical to the staining of GRPR on cervix carcinomas as described by Cornelia *et al.*[12], on gastrointestinal carcinoids by Scott *et al.*[16] and on pancreatic and prostate carcinomas by Schulz *et al.*[6], although the latter also showed strong membrane-bound staining in breast cancer. Whereas immunohistochemistry gives information about the presence or absence of bombesin receptors on carcinoid tissues, no clues about the binding affinity of these receptors for bombesin can be obtained from this expression.

Therefore, we subjected carcinoid tumours to autoradiographic ligand binding. Previous study on ileal and colonic tissue showed no binding sites with high affinity for GRP, suggesting that GRPR, but not NMBR and BRS-3, was present in human ileum and colon[15]. We found an almost significantly higher median receptor binding density in intestinal carcinoids compared to pulmonary carcinoids ($P$=0.07), suggesting diversity in the receptors present on the two types of carcinoids and suggesting that, although many receptors were demonstrated immunohistochemically in pulmonary carcinoids, not all these receptors have a high binding affinity for the bombesin ligand. The expression of bombesin receptors for GRP and NMB have been studied on carcinoids before by Reubi *et al.*[17], who studied 51 bronchial and intestinal carcinoids by autoradiography, and found a preferential expression of NMBR in intestinal carcinoids while BRS-3 expression was highest in bronchial carcinoids. Because in the current study, a universal ligand for bombesin receptors was used to study ligand binding by autoradiography, it is not possible to determine which bombesin receptor subtype is present, but similar to Reubi *et al.*[17] the carcinoids were found to have diverse BLP binding capacities. However, no endogenous BLP levels were determined in their study.

To investigate whether the binding affinity of bombesin receptors correlated with peptide levels of BLP, we also performed a radioimmunoassay for the latter. A

significant negative correlation between receptor ligand binding and BLP levels was found for all carcinoids with a similar trend when pulmonary and intestinal carcinoids were analyzed separately. BLP levels were significantly higher ($P$=0.02) in pulmonary carcinoids compared to intestinal carcinoids. The higher ligand binding in combination with lower BLP levels on gastrointestinal carcinoids compared to pulmonary carcinoids, might be due to occupancy and/or inactivation of the receptor on the latter group of carcinoids, leading to impairment of the ligand binding.

From several studies, it is known that in lung carcinomas, including SCLC and NSCLC, and colon cancer, autocrine growth loops for GRP and NMB exist[9-11]. By these loops, tumour cells are able to stimulate their own growth and proliferation. Our observation that both bombesin receptors and peptide are present in the same tissues, provides circumstantial evidence that a paracrine or autocrine growth loop for BLPs exists in carcinoid tumours, although more studies are needed to further explore this hypothesis.

The aim of this study was to investigate whether carcinoids of pulmonary and intestinal origin could be distinguished by bombesin (receptor) characteristics. Other studies have also tried to find differences in bombesin expression patterns to distinguish carcinoids of different origin; whereas Reubi *et al.* concluded that (by autoradiography) BRS-3 is preferentially but not exclusively found in lung carcinoids as opposed to NMB-receptors in gastrointestinal carcinoids[17], Granberg *et al.* suggested that, when GRP immunoreactivity is found, the primary tumour is most probable of pulmonary origin[18]. Based on our results, with a relatively low number of patients, which can mainly be ascribed to the rarity of neuroendocrine tumours in general, we conclude that in both pulmonary and intestinal carcinoids, all three bombesin receptors are present, although the quantity and ligand binding affinities are diverse on carcinoids of different origin; apparently on pulmonary carcinoids, bombesin receptors have a low binding affinity for bombesin, while intestinal carcinoids possess predominantly receptors with a high ligand binding affinity.

**Acknowledgements**

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# Chapter 7

## *Angiogenic markers Endoglin and Vascular Endothelial Growth Factor in Gastroenteropancreatic Neuroendocrine Tumours*

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**Abstract**

Gastroenteropancreatic neuroendocrine tumours (GEP-NETs) are uncommon, heterogeneous neoplastic lesions. Angiogenesis, the process of new blood vessel formation, is required for tumour growth, progression and the development of metastases. This process is induced by several growth factors, including vascular endothelial growth factor (VEGF), and transforming growth factor beta 1 (TGF-β1). Endoglin is a co-receptor for TGF-β1 and a marker for angiogenic endothelial cells. The aim of the present study was to evaluate the expression and potential prognostic role of VEGF and endoglin in GEP-NETs.

Microvessel density (MVD) in GEP-NETs was evaluated using endoglin and CD31 immunohistochemistry. In addition, tissue levels of endoglin and VEGF were determined in homogenates by ELISA.

Endoglin was highly expressed on tumour endothelial cells. CD31 microvessel density in GEP-NETs was significantly higher compared to endoglin MVD. Two to four-fold higher tissue levels of endoglin and VEGF were seen in tumours compared to associated normal tissue. This increased endoglin tissue expression in tumours was significantly related to tumour size, presence of metastases and a more advanced tumour stage, whereas expression of VEGF was not.

Based on these findings, we suggest endoglin to be a potential marker to detect present and to predict future metastases. Assessment of endoglin tumour levels provides information on tumour aggressiveness which might be useful in the post-resection therapeutic approach of patients with GEP-NETs.

**Introduction**

Gastroenteropancreatic neuroendocrine tumours (GEP-NETs), including gastrointestinal carcinoids and pancreatic neuroendocrine tumours (PNETs), comprise a very heterogeneous group of neoplasia, with respect to tumour biology, histocytopathology and prognosis[1]. Despite a slow-growing nature, they are primarily malignant[2]. Angiogenesis, the formation of new blood vessels from the existing vascular bed, is a crucial process in tumour progression. When tumours reach a size of approximately 1 or 2 mm, they become dependent on neovascularisation, not only to provide them with nutrients and oxygen, but also as an exit route for metabolic waste products, further growth of the primary tumour, and eventually, metastatic spread[3]. One of the key factors in angiogenesis is vascular endothelial growth factor (VEGF) which has numerous effects on endothelial cells (ECs), including induction of migration and differentiation[4]. Several studies have addressed the prognostic implications of VEGF in patients with GEP-NETs, and trials investigating the action of the anti-VEGF antibody bevacizumab in patients with GEP-NETs are ongoing[5,6].

Another important growth factor, with a pivotal role in angiogenesis is transforming growth factor beta 1 (TGF-β1), a multifunctional cytokine that is involved in numerous physiological and pathological processes[7]. Endoglin (CD105) is a co-receptor for TGF-β1. As a result of its principal expression on ECs of newly formed blood vessels, several studies have suggested that endoglin is a specific marker of neovascularisation in various cancer types[8-10]. In pancreatic carcinomas, high endoglin microvessel density (MVD) has been found to be related to shorter survival and therefore, is suggested to be a prognostic marker[11]. In colorectal cancer, the vessel count by positive endoglin staining is able to identify patients at high risk of metastases[12].

In the present study, we assessed the tissue expression and levels of two key players in the process of angiogenesis, namely endoglin and VEGF, to assess their potential clinical implications in patients with GEP-NETs.

**Materials and Methods**

***Patients***

After surgical removal, tumour tissues were collected at the Department of Gastroenterology, Leiden University Medical Centre (LUMC), Leiden, and either frozen at -80◦C and/or embedded in paraffin for immunohistochemical staining. Sixty-eight homogenates (27 tumour samples and 41 normal samples) of 27 patients were available for the determination of tissue levels of endoglin. For the measurement of VEGF levels, one tumour sample was exhausted; therefore, the total number of tumour samples comprises 26. For CD31 and endoglin immunostaining, 50 and 49 samples, respectively, of 39 patients, were available. For most patients, but not all, both homogenates and paraffin slides were available. In total, 41 patients with GEP-NETs were included. GEP-NETs comprised pancreatic neuroendocrine tumours (PNETs) and gastrointestinal neuroendocrine tumours, which were also referred to as 'carcinoids'.

Clinicopathological information was obtained by evaluation of patients' medical files and pathology reports, when available. According to the classification of the World Health Organization for GEP-NETs, tumours were categorized into well-differentiated neuroendocrine tumour (NET), well-differentiated neuroendocrine carcinoma (NEC), or poorly differentiated NEC[13]. From some patients, the WHO classification was not assessable due to the lack of specified classification. This study was performed according to the guidelines of the Medical Ethics Committee of the LUMC in compliance with the Helsinki Declaration.

***Immunohistochemistry***

Immunohistochemistry was performed as follows. Tissues were fixed in formalin, embedded in paraffin and cut into 5 µm sections. After deparaffinisation and rehydration, endogenous peroxidases were blocked in methanol containing 0.3% $H_20_2$ (Merck, Darmstadt, Germany). Antigen retrieval was performed by boiling in 0.01M citrate buffer pH 6.0 for 10 minutes. Slides were incubated overnight at room temperature (RT) with primary antibodies: biotinylated goat anti-human endoglin (1:200, R&D Systems Europe, Abingdon, UK), or mouse monoclonal anti-

CD31 (1:400, Dako, Glostrup, Denmark) diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA), as described previously[14]. Immunodetection was performed with a biotinylated goat anti-mouse antibody (for CD31) and horseradish peroxidase (HRP)-streptavidin complex (both Dako) for 30 minutes at RT. Staining was visualized using 0.05% 3,3'-diaminobenzidine (DAB, Sigma, Darmstadt, Germany) containing 0.0038% $H_20_2$. Colon carcinomas were used as positive controls. Negative controls were included by omitting the primary antibodies. Representative photomicrographs were taken with an Olympus BX-51TF microscope equipped with a DP23-3-5 camera.

The endoglin and CD31 MVD in the tumour-bearing area were quantified by computerized analysis. Four representative tumour areas for either endoglin or CD31 were selected and photographed at a 100x magnification. Images were binarized and the extent of staining was quantified using ImageJ 1.43u (National Institutes of Health, Bethesda, MD, U.S.A.). Finally, the average MVD out of four photographs was taken. The microvessel quantification was performed blinded, that is, without knowledge of patients or tumour characteristics, and expressed as the number of pixels per field x 1,000.

***Quantitative human endoglin and VEGF determinations in tissue samples***

Tissues were homogenized and protein concentrations were determined according to Lowry *et al.*[14,15]. Endoglin levels were determined in tissue homogenates, using a commercially available quantitative immunoassay (ELISA) for human endoglin, performed according to the manufacturer's instructions (R&D Systems), as described before[14]. VEGF tissue levels were determined using a commercially available duoset (R&D Systems) as described before[16]. Endoglin and VEGF levels were expressed as ng/mg and pg/mg protein, respectively.

***Statistical analysis***

Statistical analysis was performed using Statistical Package for Social Sciences version 16 (SPSS) and GraphPad Prism version 5. Unpaired *t* test and one-way ANOVA were used to compare mean levels of endoglin and VEGF between

various data sets. Orthogonal regression analysis and Pearson's correlation (*r*) were used to explore the relationship between two variables. Survival curves were plotted using the method of Kaplan and Meier. Results are reported as mean ± S.E. A p-value of <0.05 was considered statistically significant.

**Results**

Overall, 41 patients with NETs were included (Table 1) of which the majority were female. Most patients (28/41) had a solitary primary tumour, while 13/41 patient had multiple primaries. Primary tumours of 23/41 patients were localized in the pancreas, 5/41 in the duodenum, 10/41 in the small bowel , 1/41 in the appendix, 1/41 in the sigmoid, and in one patient, the exact primary tumour location was unknown. Functional tumours were mainly insulinomas (42.1%) and gastrinomas (52.6%). Tumour size was significantly different between the groups, *P*=0.01, with a smaller tumour size for functional PNETs. Metastases were seen in the majority of patients, with an almost equal distribution of lymph node or liver location. Angioinvasion was present in only 18.3% of the tumours.

Endoglin and VEGF tissue levels were measured in 27 tumour samples from 18 patients with GEP-NETs. Endoglin and VEGF levels were significantly increased in tumours compared to (associated) normal tissues (Table 2). However, among the various types of GEP-NETs, both endoglin and VEGF levels were comparable. Interestingly, metastatic tumours showed significantly higher endoglin levels compared to those in primary lesions. VEGF levels were also increased in metastases, although not significantly. Furthermore, well-differentiated NECs showed significantly higher endoglin levels compared to well-differentiated NETs. Again, this difference in VEGF levels was not statistically significant, although levels in well-differentiated NECs were also increased. Of particular interest, we observed that primary tumour tissues of patients who had developed (lymph node or liver) metastases displayed significantly higher endoglin levels than from those without metastases. Neither endoglin nor VEGF levels were (significantly) related to other clinicopathological parameters including patients' age, sex, the

hormonal status (i.e., functional or non-functional) of the PNETs, or the presence of angioinvasion.

| **Table 1. Patient and tumour characteristics** | | | |
|---|---|---|---|
| **Patients (n=41)** | | **Tumours (n=60)** | |
| **Age** | **Years** | **Primary or metastatic** | **n (%)** |
| Mean ± s.d. | 47 ± 14 | Primary | 45 (75.0%) |
| Range | 20 - 77 | Metastasis | 15 (25.0%) |
| **Sex** | **n (%)** | **Angioinvasion** | **n (%)** |
| Male | 17 (41.5%) | Present | 11 (18.3%) |
| Female | 24 (58.5%) | Absent | 49 (81.7%) |
| **Tumour type** | **n (%)** | **Tumour size** | **Mean ± s.d. (cm)** |
| Carcinoid | 12 (29.3%) | Carcinoids | 3.4 ± 2.7 |
| Functional PNET | 19 (46.3%) | Functional PNETs | 1.9 ± 1.7 |
| Non-functional PNET | 10 (24.4%) | Non-functional PNETs | 3.6 ± 2.4 |
| **Tumour grade** | **n (%)** | | |
| Well-differentiated NET | 13 (31.7%) | | |
| Well-differentiated NEC | 26 (63.4%) | | |
| Poorly differentiated NEC | 1 (2.4%) | | |
| Unknown | 1 (2.4%) | | |
| **Metastases** | **n (%)** | | |
| Present | 26 (63.4%) | | |
| Lymph node only | 9 (34.6%) | | |
| Liver only | 7 (26.9%) | | |
| Both | 10 (38.5%) | | |
| Absent | 15 (36.6%) | | |

Table 1. Patient and tumour characteristics.

Endoglin tissue levels, but not tissue levels of VEGF, were found to increase with tumour size (Figure 1). Finally, endoglin tumour levels showed no significant correlation with VEGF tumour levels ($r$=0.11 with $P$=0.59).

**Figure 1. Orthogonal linear regression analysis of tumour size and endoglin levels**

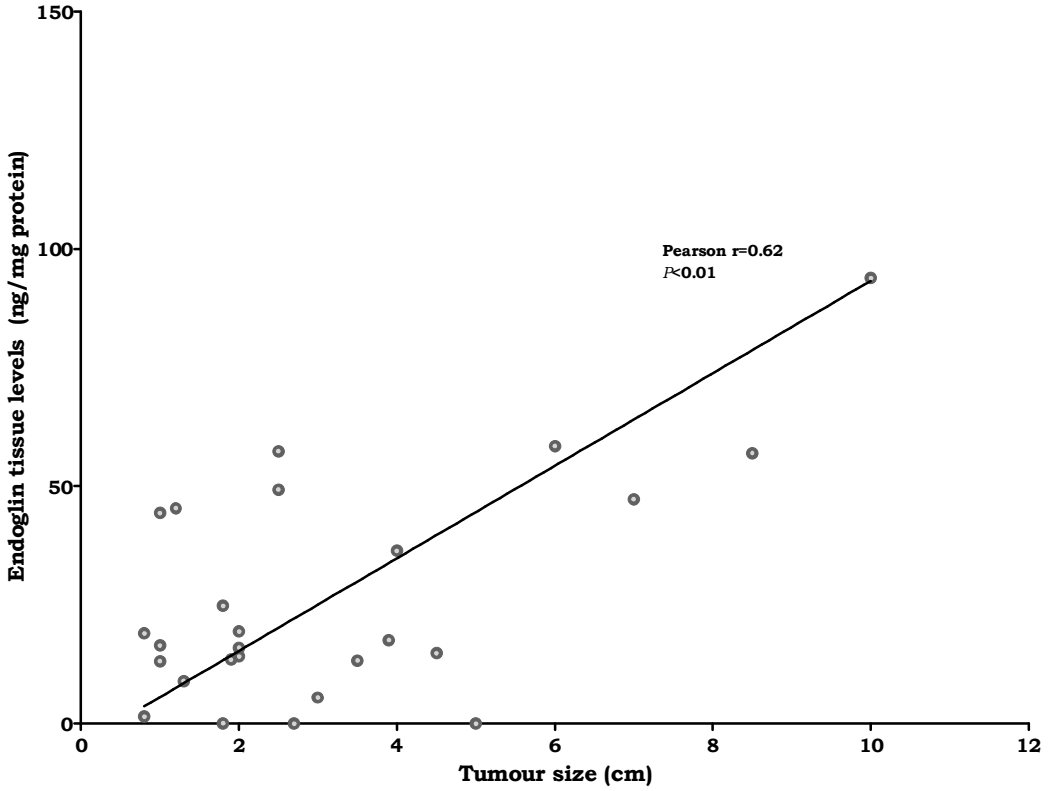


Figure 1. Orthogonal regression analysis of endoglin tissue levels and tumour size (n=26). Increasing endoglin levels in tumours are significantly correlated with a greater tumour size, r=0.62 with p<0.01.

The immunohistochemical expression of endoglin and CD31 was analyzed in 39 patients with GEP-NETs. All tumours showed expression for CD31 and endoglin on intratumour vascular ECs. Endoglin expression was mainly observed on ECs of small tumour-associated blood vessels, while its expression in normal, non-tumourous tissue was weak or negative, in contrast to CD31 staining (Figure 2).

**Figure 2. Immunostaining of gastroenteropancreatic neuroendocrine tumours**

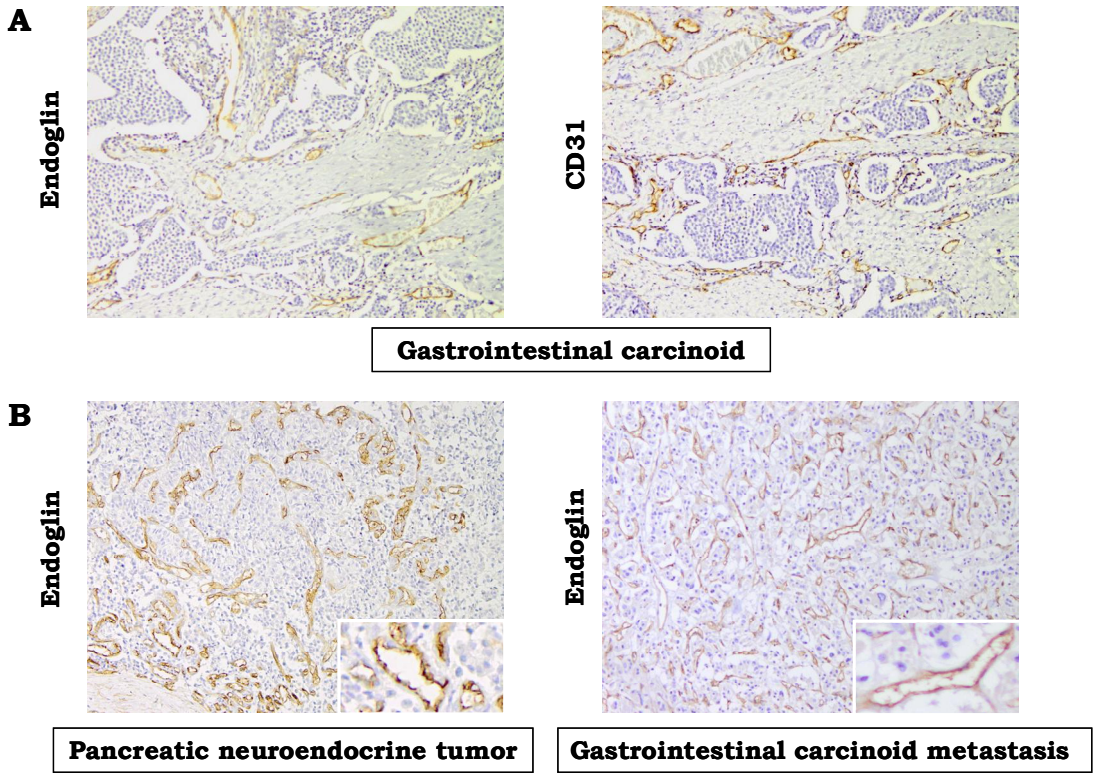


Figure 2. Immunostaining of endoglin and CD31 on peritumoural and intratumoural vessels in GEP-NETs. A) Endoglin staining is limited to angiogenic vessels, whereas CD31 stains both old and new blood vessels in tumour tissue. Magnification 100x. B) Representative endoglin staining in a pancreatic neuroendocrine tumour and a gastrointestinal carcinoid metastasis (mesenterium of small bowel). Magnification 100x. Inserts show a higher magnification at 200x.

The CD31 MVD was found to be significantly higher than the endoglin MVD in 73% of the tumour samples, $P<0.01$. No significant differences in endoglin and CD31 MVD were observed between carcinoids and PNETs (Table 3). Furthermore, both endoglin and CD31 MVD were not significantly related to clinicopathological parameters such as patients' age, sex, tumour size, functionality, and angioinvasion.

**Table 2. Mean endoglin and VEGF levels in GEP-NETs in relation to clinicopathological parameters**

| | Endoglin (ng/mg) | | | | VEGF (pg/mg) | | | |
|---|---|---|---|---|---|---|---|---|
| | *n* | Mean | S.E. | *P* | *n* | Mean | S.E. | *P* |
| **Tissues** | | | | | | | | |
| Normals | 38 | 12.1 | 2.0 | **<0.01** | 38 | 75.0 | 9.5 | **<0.01** |
| Tumours | 27 | 26.8 | 4.5 | | 26 | 316.8 | 46.0 | |
| **Tumour type - tumours** | | | | | | | | |
| Carcinoid | 8 | 35.3 | 11.4 | 0.37 | 8 | 354.9 | 72.0 | 0.67 |
| Functional PNET | 14 | 25.4 | 4.7 | | 13 | 274.4 | 46.7 | |
| Non-functional PNET | 5 | 16.8 | 8.7 | | 5 | 366.2 | 186.8 | |
| **Origin** | | | | | | | | |
| Primary tumours | 19 | 18.8 | 3.9 | **<0.01** | 18 | 293.2 | 52.0 | 0.45 |
| Metastastic tumours | 8 | 45.7 | 9.0 | | 8 | 369.9 | 95.8 | |
| **WHO classification** | | | | | | | | |
| Well-differentiated NETs | 6 | 7.6 | 5.2 | **0.02*** | 6 | 200.2 | 52.8 | 0.21* |
| Well-differentiated NECs | 20 | 32.9 | 4.0 | | 19 | 328.5 | 60.2 | |
| Poorly-differentiated NECs | 1 | 19.0 | ND | | 1 | 795.0 | ND | |
| **Primary tumours: Metastases** | | | | | | | | |
| Present | 12 | 24.8 | 5.2 | **0.04** | 11 | 339.5 | 76.4 | 0.28 |
| Absent | 7 | 8.5 | 3.5 | | 7 | 220.6 | 54.8 | |

Table 2. Mean values of endoglin and VEGF levels in GEP-NETs in relation to major clinicopathological parameters. Bold p-values are considered statistically significant.
*Result of unpaired t-test to compare well-differentiated NETs with well-differentiated NECs.

| **Table 3. MVD scores in GEP-NETs in relation to clinicopathological parameters** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **MVD-endoglin** | | | | **MVD-CD31** | | | |
| | ***n*** | **Mean*** | **S.E.*** | ***P*** | ***n*** | **Mean*** | **S.E.*** | ***P*** |
| **Tumour type - tumours** | | | | | | | | |
| Carcinoid | 11 | 55 | 107 | 0.30 | 13 | 123 | 23 | 0.75 |
| Functional PNET | 24 | 65 | 8 | | 23 | 106 | 18 | |
| Non-functional PNET | 14 | 85 | 18 | | 14 | 100 | 17 | |
| **Origin** | | | | | | | | |
| Primary tumours | 36 | 66 | 8 | 0.58 | 37 | 111 | 13 | 0.69 |
| Metastatic tumours | 13 | 75 | 15 | | 13 | 101 | 24 | |
| **WHO classification** | | | | | | | | |
| Well-differentiated NETs | 13 | 69 | 18 | *0.93*** | 13 | 76 | 12 | *0.08*** |
| Well-differentiated NECs | 33 | 67 | 7 | | 34 | 121 | 15 | |
| Poorly-differentiated NECs | 1 | 212 | x | | 1 | 82 | x | |
| **Primary tumours: Metastases** | | | | | | | | |
| Present | 19 | 66 | 9 | 0.96 | 20 | 138 | 18 | **0.05** |
| Absent | 17 | 67 | 14 | | 17 | 88 | 15 | |

Table 3. MVD determined by endoglin and CD31 in GEP-NETs in relation to clinicopathological parameters. Bold p-values are considered statistically significant. *Values x 1,000 pixels per area. ** Result of unpaired *t* test to compare well-differentiated NETs with well-differentiated NECs.

Endoglin and CD31 MVD were significantly correlated with endoglin tumour levels; $r$=0.64 with $P$<0.01 (Figure 3) and $r$=0.58 with $P$<0.01, respectively. VEGF tumour levels were not correlated with endoglin MVD ($r$=0.28 with $P$=0.25), but were borderline significantly correlated with CD31 MVD, $r$=0.43 with $P$=0.07.

**Figure 3. Correlation between MVD and tissue levels of endoglin in tumours**

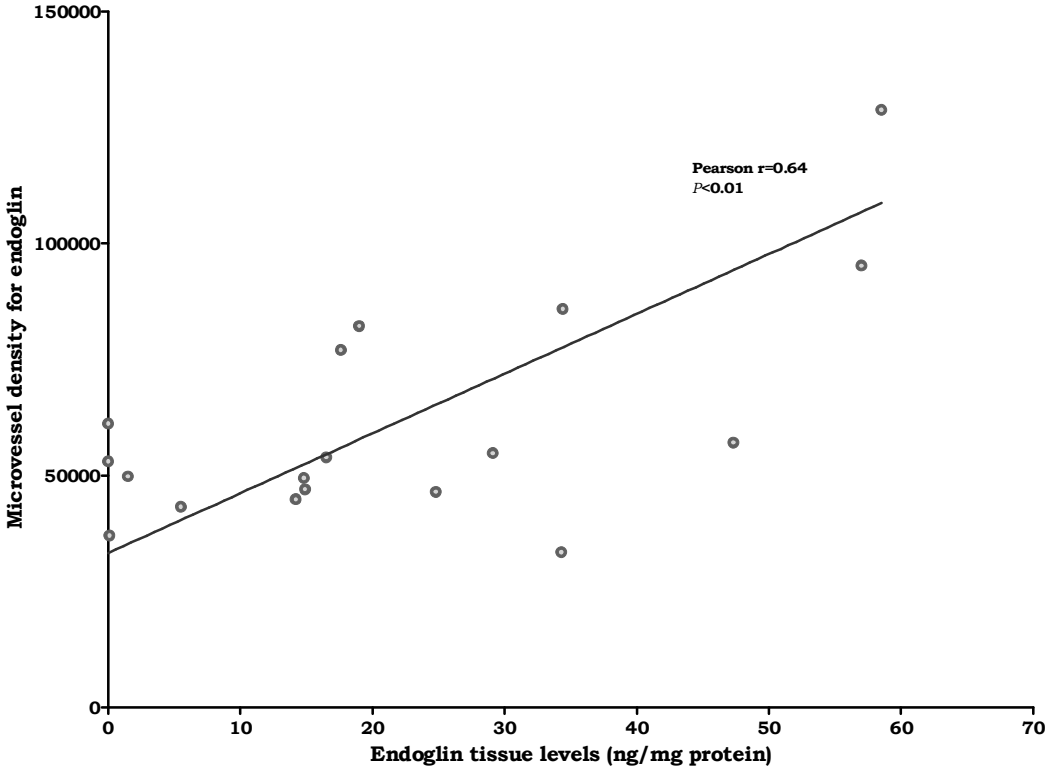


Figure 3. Correlation analysis of the endoglin MVD and endoglin tissue levels in tumours (n=17). For one patient in whom endoglin tissue levels were assessed, no paraffin slides for MVD determination was available. Endoglin MVD is significantly correlated with tumour levels of endoglin, r=0.64 with p<0.01.

To evaluate the prognostic potential of endoglin and VEGF tissue levels, Kaplan Meier survival analysis was performed (Figure 4) by dividing the patients into two groups (i.e. low versus high) using the mean value of endoglin and VEGF tumour levels (Table 2). Both endoglin and VEGF tissue levels were not significantly related to patient survival. Furthermore, patients were divided into two groups based on the MVD of endoglin and CD31. Both parameters were not significantly correlated with overall survival of these patients.

**Figure 4a. Survival analysis on tissue levels**

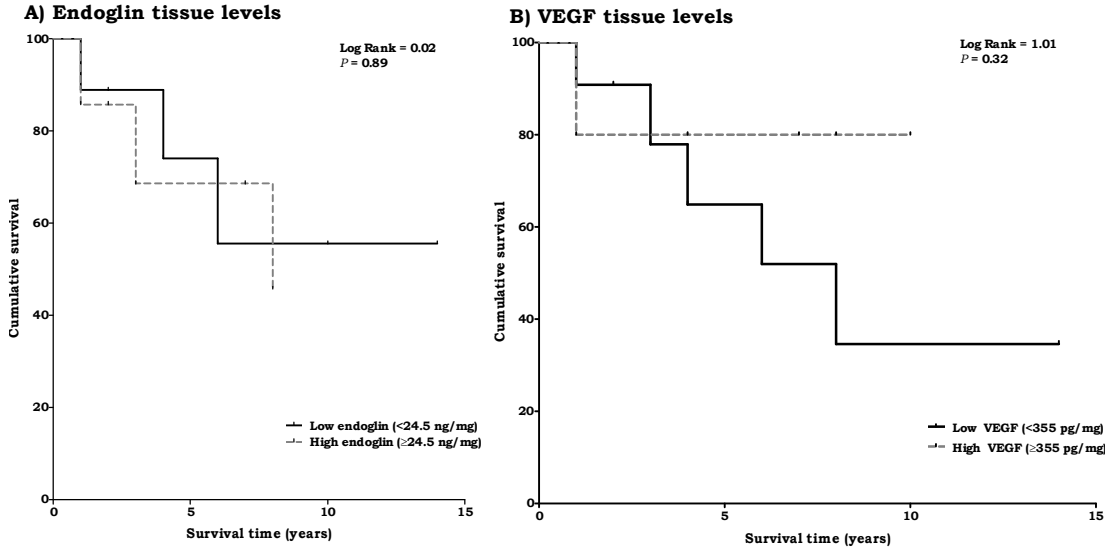


**Figure 4b. Survival analysis on MVD**

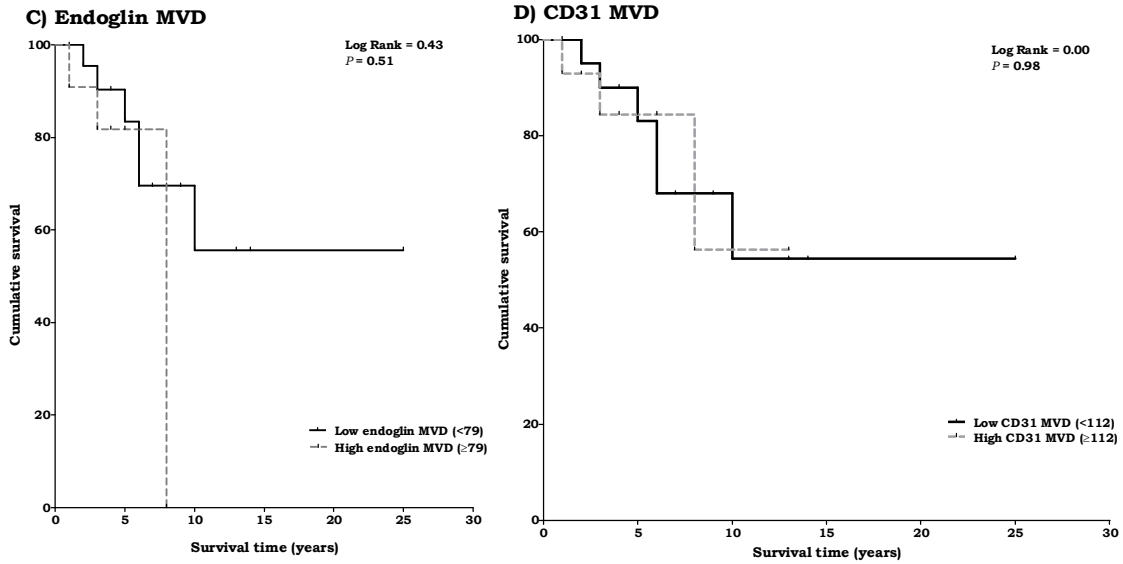


Figure 4. Kaplan Meier survival analysis for endoglin tumour levels (a), VEGF tumour levels (b), endoglin MVD (c) and CD31 MVD (d). Patients were divided into two groups based on mean tumour levels (a,b) or mean MVD-scores (c,d). None of the parameters showed a significant relation with survival of the patients.

## Discussion

In this study, we observed that the expression of the angiogenic cell marker endoglin was related to tumour size, aggressiveness and metastatic potential in patients with GEP-NETs, whereas expression of another key player in angiogenesis, namely VEGF, was not.

In general, GEP-NETs are highly vascularised. In recent years it has become clear that angiogenesis has important effects on tumour progression in several cancers,

and the therapeutic role of angiogenesis inhibitors in the treatment of cancers is increasing[17,18]. In this study, we investigated whether endoglin and VEGF were related to any clinicopathological characteristics of GEP-NETs and evaluated their potential prognostic implications.

By immunohistochemistry, we observed high endoglin expression on vascular ECs in tumour tissues of GEP-NETs. In contrast to CD31, immunopositivity of endoglin was mainly observed on newly formed blood vessels, which indicates that endoglin is more representative of tumour neovascularisation than the pan-endothelial marker CD31.

Furthermore, we found that endoglin tissue levels were significantly higher in tumours compared to normal tissues. Interestingly, we observed that an increased endoglin expression was indicative of metastatic disease. Endoglin levels were higher in metastases compared to primary tumours, and primary tumours with metastases showed higher endoglin levels compared to tumours without metastases. Additionally, endoglin levels were increased in well-differentiated NECs compared to well-differentiated NETs, and higher endoglin levels were related to larger tumour size in patients with GEP-NETs. In several cancers, the extent of tumour angiogenesis was shown to be reflective of their potency to become invasive and form metastases[19,20]. Our data indicate that tissue endoglin may serve as a potential assessment marker for the tumour aggressiveness (i.e., NEC versus NET) and the presence of metastases following tumour resection. In the context of anti-cancer therapy, anti-endoglin treatment might provide a new effective anti-angiogenic strategy for GEP-NETs, but more research is needed. However, several promising *in vivo* and *in vitro* studies using anti-endoglin antibodies for anti-cancer treatment have recently been published[21].

In the present study, we did not evaluate the immunohistochemical expression of VEGF. High immunoexpression of VEGF on GEP-NETs has already been shown by others, but opposing results regarding the prognostic role of VEGF in these tumours have been reported; Takahashi *et al.* found no correlation of VEGF-A immunoexpression with growth of blood vessels, haematogenous spread or tumour growth in pancreatic endocrine tumours. In contrast, Zhang *et al.* have

revealed that strong expression of VEGF was associated with increased angiogenesis and poor prognosis in patients with GEP-NETs [22,23]. However, we determined tissue VEGF expression in GEP-NETs and found that VEGF tissue levels showed a similar pattern to endoglin, but were not significantly related to any clinicopathological parameter. Therefore, we assume that, although VEGF is most likely to be involved in the process of neoplastic blood vessel formation in GEP-NETs, this key mediator of angiogenesis is not the appropriate prognostic marker in these tumours. In contrast, our data suggest that endoglin can function as a predictive marker for the development of metastases in GEP-NETs. Endoglin is a co-receptor for TGF-β1. Among the various members of the TGF-β family, TGF-β1 is mostly involved in cancer, and has been shown to stimulate angiogenesis[24]. Endoglin is an important modulator of the TGF-β response, particularly in tumour pathogenesis[25]. In another study by our group, strongly increased tissue levels of endoglin were observed in colorectal cancers, whereas premalignant lesions displayed endoglin levels comparable to those in normal tissues, which supports the pivotal role of endoglin in tumour progression[14].

The fact that neither endoglin nor VEGF levels were associated with patient survival might be due to the relatively good prognosis of the patients. Gastrointestinal carcinoids show a 5-year survival rate of about 70%, whereas PNETs have a reported 5-year survival rate ranging from 25 to 100%, even in the case of (unresectable) liver metastases[26,27]. In our study cohort, 10/18 patients in whom endoglin or VEGF levels were determined were still alive at the end of the study (median survival 8 years), which makes it unlikely to use one of these parameters as a predictor of outcome or survival marker. However, our data support a role for endoglin in identifying patients with GEP-NETs at risk for metastasis.

It is worth reiterating that the current study involved a relatively small number of patients. Nevertheless, GEP-NETs are a rare disease with a low incidence, which leads to general scarcity of patients and samples. However, we believe that the significant differences observed here are representative and illustrate the differential expression pattern of endoglin and VEGF among GEP-NETs.

In conclusion, we suggest that endoglin is a potential marker to predict present and future metastases, which might help to optimize the therapeutic approach in patients with GEP-NETs.

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# Chapter 8

## *The IGF-matrilysin network in gastroenteropancreatic neuroendocrine tumours*

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**Abstract**

The Insulin-like Growth Factor (IGF) system plays an important role in the tumour development, growth, and spread of several cancers.

Matrilysin (MMP-7) has been implicated in tumour processes like invasion and metastasis. Recently, matrilysin was found to be able to cleave IGF binding proteins (IGFBPs), thereby increasing the bioavailability of IGFs.

The aim of the present study was to investigate the expression of IGF-1, IGFBP-3 and matrilysin in gastroenteropancreatic neuroendocrine tumours (GEP-NETs), and their relation to the pathogenetic factors of the tumours.

Tissue expression and levels of IGF-1, IGFBP-3, and matrilysin were analyzed by immunohistochemistry and ELISA, respectively.

IGF-1 and IGFBP-3 tissue levels were increased in tumours compared to associated normal tissue. This increased IGFBP-3 expression in tumours was related to a larger tumour size and the presence of metastases, whereas IGF-1 was not related to any clinicopathological parameter. Matrilysin expression was found to be down-regulated in tumours, and negatively correlated to the expression of IGFBP-3.

These findings suggest that IGFBP-3 plays a role in the pathogenesis of GEP-NETs, whereas matrilysin might indirectly be involved via regulation of this IGFBP-3 expression. Further studies are indicated to assess the contribution of this IGF-matrilysin network in the etiopathogenesis of GEP-NETs.

**Introduction**

Neuroendocrine tumours comprise a heterogeneous group of neoplasms, arising from enterochromaffin cells widespread distributed throughout the gastrointestinal and bronchopulmonary system[1,2]. In this study, we focus on gastroenteropancreatic neuroendocrine tumours (GEP-NETs), including the pancreatic neuroendocrine tumours (PNETs) and the gastrointestinal carcinoids (GI-NETs). Although slowly-growing, the majority of GEP-NETs are malignant and characterized by angioinvasion and the presence of metastases[3].

The insulin-like growth factor (IGF) system, composed of two IGF ligands (IGF-1 and IGF-2), three receptors and six binding proteins (IGFBPs), plays an important role in growth and development[4]. Furthermore, this system is involved in tumour cell processes like proliferation, survival and growth[5]. Increased levels of insulin-like growth factor-1 (IGF-1) have been reported to be related to the development of cancer of the breast, lung, colon and prostate[6-9]. In NETs, mRNA levels of several components of the IGF-system were found to be variable in different types of NETs[10]. Furthermore, increased expression of IGF-1 and its receptor IGF-1R in gastrinomas were found to be associated with higher tumour aggressiveness[11].

MMPs, or matrix metalloproteinases, constitute a family of more than 20 proteolytic enzymes, with similar protein sequences and domain structures, but diverse substrate specificities, which are involved in remodeling of the extracellular matrix under both physiological and pathological conditions[12]. Matrilysin, or MMP-7, belongs to the subgroup of stromelysins. Like other MMPs, matrilysin is secreted as a proenzyme, of which proteolytic removal of the 9 kDa prodomain from the N-terminus leads to activation of the enzyme. Uniquely, matrilysin is produced by epithelial rather than stromal cells[13]. Various studies have shown that matrilysin is significantly enhanced in cancer of the breast, prostate, lung, skin, and colorectum[14-19]. Furthermore, matrilysin expression has been related to the presence of lymph node metastases in gastric cancer patients[20].

In addition, several studies have shown that matrix metalloproteinases indirectly participate in controlling the levels of IGFs, through proteolytic cleavage of the IGFBPs which form complexes with the IGFs in the circulation[21-23]. For example,

Miyamoto *et al.* have recently shown that proteolysis of the IGFBP-3 by matrilysin increases the bioavailability of IGF-1, leading to enhanced cell survival[24]. In the present study, we aimed to evaluate the expression of matrilysin, IGF-1 and IGFBP-3 on GEP-NETs, to assess whether such a growth-activation cascade also exists in these tumours.

## Material and methods

*Patients*

After surgical removal, tumour tissues were collected at the Department of Gastroenterology, Leiden University Medical Centre (LUMC), Leiden, and either frozen at -80 ◦C and/or embedded in paraffin for immunohistochemical staining.

Fifty-one homogenates (23 tumour samples and 28 normal samples) of 25 patients were available for the determination of tissue levels of matrilysin, IGF-1 and IGFBP-3. For immunostaining of IGF-1, IGFBP-3 and matrilysin, 44, 44 and 36 samples respectively, of 35 patients, were available.

GEP-NETs comprised pancreatic neuroendocrine tumours (PNETs) and gastrointestinal neuroendocrine tumours (GI-NETs), which were also referred to as 'carcinoids'. Clinicopathological information was obtained by evaluation of patients' medical files and pathology reports, when available. According to the classification of the World Health Organization for GEP-NETs, tumours were categorized into well-differentiated neuroendocrine tumour (NET), well-differentiated neuroendocrine carcinoma (NEC), or poorly differentiated NEC[25]. This study was performed according to the guidelines of the Medical Ethics Committee of the LUMC in compliance with the Helsinki Declaration.

*Quantitative determination of matrilysin, IGF-1 and IGFBP-3 in tissue samples*

Tissues were homogenized and protein concentrations were determined according to Lowry *et al.*[26]. Matrilysin, IGF-1 and IGFBP-3 levels were determined in tissue homogenates, using commercially available quantitative immunoassays (ELISA) for human matrilysin, IGF-1 and IGFBP-3, respectively, performed according to

the manufacturer's instructions (R&D Systems). Matrilysin, IGF-1 and IGFBP-3 levels were expressed per mg protein.

*Immunohistochemistry*

Immunohistochemistry was performed as follows. Tissues were fixed in formalin, embedded in paraffin and cut into 5 µm sections. After deparaffinisation and rehydration, endogenous peroxidases were blocked in methanol containing 0.3% $H_2O_2$ (Merck, Darmstadt, Germany). Antigen retrieval was performed by boiling in 0.01M citrate buffer pH 6.0 for 10 minutes. Slides were incubated overnight at room temperature (RT) with primary antibodies: monoclonal mouse anti-human MMP-7 (1.25 µg/mL), polyclonal goat anti-human IGF-1 (10 µg/mL), and polyclonal goat anti-human IGFBP-3 (5 ug/mL, all R&D Systems Europe, Abingdon, UK), diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). Incubation with goat-anti-mouse (for MMP-7) and rabbit-anti-goat (for all IGF-system components) immunoglobulins for 30 minutes at RT was followed by incubation with horseradish peroxidase (HRP)-streptavidin complex (all Dako, Glostrup, Denmark) for 30 minutes at RT. Cervix carcinomas were used as positive controls. Negative controls were included by omitting the primary antibodies. Representative photomicrographs were taken with an Olympus BX-51TF microscope equipped with a DP23-3-5 camera.

*Immunohistochemical evaluation*

Staining for matrilysin, IGF-1, and IGFBP-3 in tumour cells was scored semiquantitatively, according to a system proposed by Ruiter *et al.*[27]. As final score, the mean result of 2 independent individuals (P.K. and E.J.M.) was used. The percentage of tumour cells that stained positive were scored as follows: **0**, absent; **1**, 1–5% sporadic; **2**, 6–25% local; **3**, 26–50% occasional; **4**, 51–75% majority and **5**, 76–100% large majority. The intensity of tumour cell staining was scored as: **0**, no; **1**; weak; **2**, moderate and **3**, intense staining. A total score was calculated by adding the scores for percentage and intensity, resulting in values from 0 to 8.

*Statistical analysis*

Statistical analysis was performed using Statistical Package for Social Sciences version 16 (SPSS) and GraphPad Prism version 5. Unpaired *t* test and one-way ANOVA were used to compare mean levels between various data sets. Pearson's correlation (*r*) was used to explore the relationship between two variables. Results are reported as mean ± standard error (S.E.). A p-value of <0.05 was considered statistically significant.

**Results**

*Patient and tumour characteristics*

The majority of the patients in this study were female (59%). Primary tumours of nine patients were localized in the pancreas, one in the duodenum, and six in the small bowel. Functional tumours were four insulinomas, two gastrinomas and one glucagonoma. The majority of tumours (95.7%) were well-differentiated. Tumours were classified according to the WHO classification into five well-differentiated NETs, seventeen well-differentiated NECs, and one poorly differentiated NEC. Carcinoid tumours were larger in size compared to pancreatic neuroendocrine tumours, mean size 4.9±1.2 cm vs. 2.7±0.6 cm, *P*=0.08. Metastases were seen in the majority of patients (76.5%); one patient with liver metastases, four patients with lymph node metastases, and eight patients with both liver and lymph node metastases. Angioinvasion was present in only 21.7% of the tumours.

*Tissue levels of matrilysin, IGF-1 and IGFBP-3*

IGF-1, IGFBP-3 and matrilysin tissue levels were measured from 25 patients with GEP-NETs (Table 1). Both IGF-1 and IGFBP-3 levels were found to be increased in tumours compared to (associated) normal tissues, only the latter being statistically significant, *P*<0.01. In contrast, tumour levels of matrilysin were somewhat lower compared to matrilysin levels in normal samples. Among the various types of GEP-NETs, i.e., carcinoids versus functional PNETs and non-functional PNETs, levels of IGF-1 and IGFBP-3 were not significantly different. Matrilysin levels, however, were significantly higher in non-functional PNETs compared to

functional PNETs and carcinoids, *P*=0.03. IGFBP-3 levels were somewhat higher in metastatic tumours compared to primary tumours, *P*=0.06. IGFBP-3 levels in primary tumours with and without metastases were not significantly different. Both IGF-1 and matrilysin levels were higher in primary tumours compared to metastases, and lower in primary tumour tissues of patients who had developed metastases than those without metastases, although not significant. IGF-1, IGFBP-3 and matrilysin levels were not related to other tumour parameters like tumour size or the presence of angioinvasion.

**Table 1. Tissue levels of IGF-1, IGFBP-3 and matrilysin in GEP-NETs**

| | **IGF-1 (pg/mg)** | | **IGFBP-3 (ng/mg)** | | **Matrilysin (pg/mg)** | |
|---|---|---|---|---|---|---|
| | **Mean±S.E.** | **P-value** | **Mean±S.E.** | **P-value** | **Mean±S.E.** | **P-value** |
| *Tissues* | | | | | | |
| Normals (n=28) | 52.1±10.1 | | 0.6±0.2 | | 206.6±56.7 | |
| Tumours (n=23) | 85.4±19.3 | 0.12 | 6.9±1.6 | **<0.01** | 163.0±48.1 | 0.57 |
| *Tumour types* | | | | | | |
| Carcinoid (n=7) | 51.4±12.7 | | 3.5±2.1 | | 46.7±14.9 | |
| Functional PNET (n=12) | 115.3±33.7 | | 8.0±2.1 | | 146.3±52.2 | |
| Non-functional PNET (n=4) | 54.9±27.3 | 0.28 | 9.5±5.5 | 0.35 | 416.8±192.7 | **0.03** |
| *Origin* | | | | | | |
| Primary (n=16) | 96.6±26.7 | | 5.0±1.7 | | 196.5±67.2 | |
| Metastasis (n=7) | 59.6±16.3 | 0.39 | 11.2±3.0 | 0.06 | 86.4±26.3 | 0.30 |
| *Metastases* | | | | | | |
| Present (n=11) | 79.1±29.3 | | 4.7±2.1 | | 120.6±57.6 | |
| Absent (n=5) | 135.2±57.2 | 0.35 | 5.65±2.8 | 0.81 | 363.4±160.4 | 0.09 |

Table 1. Mean values of IGF-1, IGFBP-3 and matrilysin tissue levels in relation to clinicopathological parameters. Significant p-values are in bold.

*Immunohistochemical expression of matrilysin, IGF-1 and IGFBP-3*

The results of the immunohistochemical evaluation is shown in Table 2 and Figure 1. For IGF-1, IGFBP-3 and matrilysin the cytoplasmic staining of tumour cells was generally strong. Remarkably, staining of matrilysin was stronger in tumour-associated normal tissue compared to tumour tissue in 61.5% of the samples, in particular pancreatic and hepatic NETs. IGF-1 and IGFBP-3 staining were

generally absent in normal tissue, and when present, the staining was less strong than in tumour tissue. Nuclear staining of tumour cells by IGF-1 was seen in 75% of the tumours. For IGFBP-3, membrane staining was found in more than 50% of the tumours.

| **Table 2. Immunohistochemical evaluation** | | | |
|---|---|---|---|
| | ***IGF-1*** | ***IGFBP-3*** | ***Matrilysin*** |
| **GEP-NETs** | ***n=44*** | ***n=44*** | ***n=36*** |
| Mean total score | *6* | *6* | *7* |
| (range) | *(0-8)* | *(2-8)* | *(6-8)* |
| **Staining present** | ***%*** | ***%*** | ***%*** |
| Cytoplasm | *100* | *97.7* | *97.2* |
| Nucleus | *75.0* | *2.3* | *0* |
| Membrane | *0* | *52.3* | *0* |
| **Normal tissue** | ***n=34*** | ***n=34*** | ***n=26*** |
| Staining present | *38.2%* | *14.7%* | *84.6%* |
| N>T | *32.3%* | *8.8%* | *61.5%* |

Table 2. Immunohistochemical staining results for IGF-1, IGF-2, IGF-1R, IGFBP-3 and matrilysin on tumour cells in 36<n<44 gastroenteropancreatic neuroendocrine tumours.

Staining scores for matrilysin did not differ between carcinoids and the two types of PNETs (all 7). For IGF-1 and IGFBP-3, staining scores in carcinoids and F-PNETs were both 6, whereas for NF-PNETs these were both 7.

Total staining scores were mutually correlated (Table 3). IGF-1 expression was significantly related to the expression of IGFBP-3. Interestingly, matrilysin expression was negatively correlated to the expression of IGF-1 and IGFBP-3, although only the latter being significant, $P$=0.02. Furthermore, staining scores were evaluated in relation to clinicopathological parameters, such as tumour size, angioinvasion, WHO classification, and the presence of metastases. Interestingly, a larger tumour was correlated to more IGFBP-3 staining in tumour cells (r=0.45, $P$=0.002). No other significant correlations were found. Finally, tumour levels of IGF-1, IGFPBP-3 and matrilysin were not significantly correlated with the immunohistochemical staining scores for these proteins (-0.143<r<0.413).

| **Table 3. Mutual correlations of immunostaining scores** | | | |
|---|---|---|---|
| | ***IGF-1*** | ***IGFBP-3*** | ***Matrilysin*** |
| ***IGF-1*** | | *r=0.44*<br>***P<0.01*** | *r=-0.23*<br>*P=0.15* |
| ***IGFBP-3*** | *r=0.44*<br>***P<0.01*** | | *r=-0.37*<br>***P=0.02*** |
| ***Matrilysin*** | *r=-0.23*<br>*P=0.15* | *r=-0.37*<br>***P=0.02*** | |

Table 3. Mutual correlations between immunohistochemical staining results for IGF-1, IGFBP-3 and matrilysin on tumour cells of gastroenteropancreatic neuroendocrine tumours. Bold p-values indicate a significant correlation.

**Figure 1. Immunostaining of IGF-1, IGFBP-3 and matrilysin on GEP-NETs**

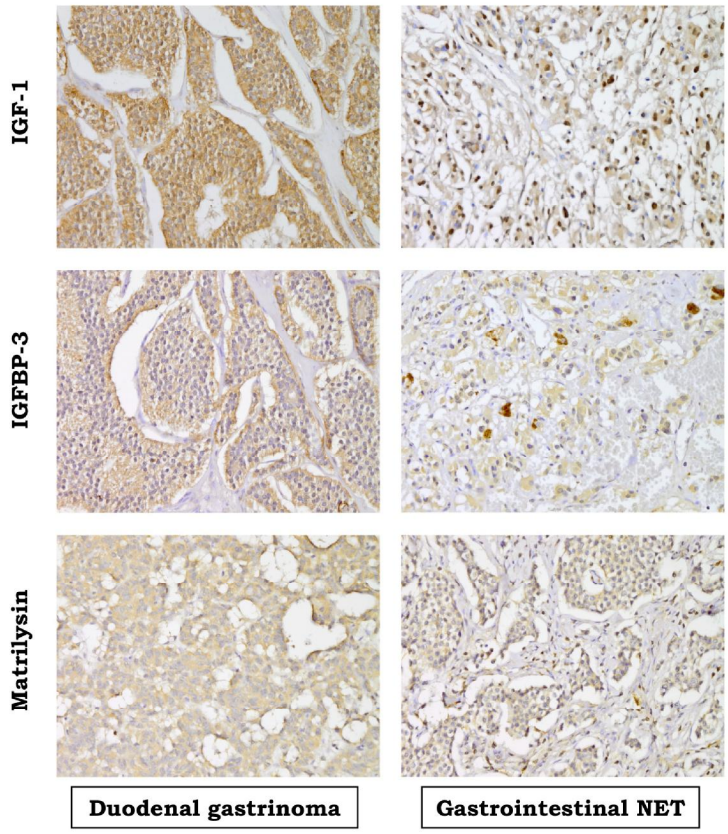


Figure 1. Immunohistochemical staining for a, b) IGF-1, c,d) IGFBP-3 and e,f) matrilysin on tumour cells of a duodenopancreatic gastrinoma (a, c, d) and gastrointestinal NET (carcinoid) (b, c, e). Magnification x100. In the insert of d) membrane staining of IGFBP-3 is shown at a higher magnification (x200).

**Discussion**

In this study, we examined the expression of IGF-1, IGFBP-3, and matrilysin in GEP-NETs, and find indications of an interrelated role for IGFBP-3 and matrilysin in the pathogenesis of these tumours.

The IGF system is of particular interest in cancer, as it is involved in many processes related to tumour growth[4]. IGF-1 has been described to have important functions in tumour development, such as the inhibition of apoptosis, the promotion of tumour growth, the inducement of transformation, and the promotion of metastasis in several cancers[28]. In gastrinomas, IGF-1 mRNA levels were found to be increased, and related to tumour growth, aggressiveness and curability[11]. IGF-1 is mainly present in the circulation, where it is bound to IGFBPs that act to protect IGF-1 from degradation by proteases[28,29]. IGFBP-3 is the most abundant IGFBP in the circulation[24]. The IGFBPs have both stimulating and inhibiting effects on IGFs. Gigek *et al.* described that when IGFBP-3 binds IGF-1, it inhibits its binding to one of the IGF receptors, thereby counteracting the actions of IGF-1[30]. In a study of Miyamoto *et al.*, a correlation between high levels of IGF-1 and low levels of IGFBP-3 was found[24]. Matrilysin has been shown to be involved in tumour cell invasion and the development of metastases[20]. In several cancers of the digestive tract, including gastric, oesophageal, pancreatic and colorectal cancer, matrilysin tissue levels were upregulated and related to malignant behaviour and a poor prognosis of the patients[20,31-34]. From previous studies it is known that matrix metalloproteinases are able to serve as proteinases for the various IGFBPs[21-23,35]. McGaig *et al.*, for example, have shown that *Helicobactor pylori*-associated epithelial-derived matrilysin cleaves IGFBP-5, thereby liberating IGF-2, which in turns stimulates epithelial cell proliferation, suggested to contribute to the progression to gastric cancer[36]. In addition, matrilysin was shown to be able to cleave IGFBP-3, thereby increasing the bioavailability of IGF-1 to cancer cells[24].

We evaluated IGF-1, IGFBP-3, and matrilysin in GEP-NETs to assess whether they are part of a similar growth activation process in these tumours.

Using ELISA, we measured tissue levels of matrilysin, IGF-1, and IGFBP-3 in the various neuroendocrine tumours of the gastroenteropancreatic tract. Both IGF-1 and IGFBP-3 tumour levels were increased compared to levels in normal tissue, although only the latter was significant. In addition, we found that IGFBP-3 levels were up-regulated in metastatic tumours samples compared to primary tumours. Furthermore, a higher IGFBP-3 staining was be indicative of a larger tumour size. Together, these findings suggest that IGFBP-3 might play a role in the tumourigenesis of GEP-NETs, independent of IGF-1. Although the expression of IGF-1 and IGFBP-3 were significantly correlated, IGF-1 expression in the tumour alone showed no association with any clinicopathological parameter. Similarly, Wulbrand *et al.* found no relation between IGF-1 expression and the presence of metastases in GEP-NETs, whereas Furukawa *et al.* previously showed that enhanced levels of IGF-1 in gastrinomas were related to tumour growth, aggressiveness and extent[10,11].

Remarkably, matrilysin levels were lower in tumours compared to associated normal tissues of patients with GEP-NETs. By immunohistochemical staining of matrilysin, we observed a similar pattern. Furthermore, tissue levels of matrilysin were lower in metastatic tumours and in metastases compared to primary tumours. Although a high matrilysin expression in tumours has been related to a more malignant phenotype and a poor prognosis in several cancer types, the results of our study suggest that matrilysin is not directly involved in the pathogenesis of GEP-NETs[14-20]. However, matrilysin might indirectly be related to malignant tumour behaviour, as a negative correlation between matrilysin and IGFBP-3 expression on tumour cells by immunohistochemistry was observed. So when the expression of matrilysin on tumour cells was high, a low expression of IGFBP-3 was found, and vice versa. Possibly matrilysin regulates the expression of IGFBP-3, thereby indirectly effecting the tumour's extent of malignancy. One explanation could be that matrilysin acts as a protease that cleaves IGFBP-3 present on the tumour cells. The observation that IGFBP-3 staining is also present on the membrane of tumour tissue supports this assumption. Further *in vitro* studies are required to determine IGFBP-3 levels in the medium of

gastroenteropancreatic neuroendocrine tumour cells, to reveal whether these cells secrete IGFBP-3, and if so, whether this process is mediated by matrilysin. In combination with serological analyses of GEP-NET patients these studies will elucidate whether matrilysin regulates secretion, complex formation and breakdown of IGFBP-3 in these tumours.

In summary, we found that the levels of IGF-1 and IGFBP-3 were increased in GEP-NETs, whereas matrilysin was decreased. Higher IGFBP-3 expression was related to the presence of metastases and a larger tumour size, which might indicative of a more malignant tumour. For matrilysin, an opposite trend was observed. Together, these findings suggest that IGFBP-3 plays a direct role in the pathogenesis of GEP-NETs, whereas matrilysin might indirectly be involved via regulation of this IGFBP-3 expression. Further studies are required to investigate this potential growth mechanism in more detail.

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# Chapter 9

## *Summarizing Discussion*

Summary main observations

Epidemiology of GEP-NETs in The Netherlands

Gastrinomas

Heterogeneity and tumour markers in GEP-NETs

Concluding remarks

References

**Summary main observations**

Clinical behaviour of gastroenteropancreatic neuroendocrine tumours (GEP-NETs) varies strikingly, both in terms of symptoms and outcome[1-3]. An understanding of the basic biology unique to GEP-NETs is necessary for optimal management of patients with these complex tumours. Although markers for GEP-NETs exist, sensitive and specific markers that predict tumour growth and behaviour are lacking[4]. The general purpose of the studies described in this thesis was to investigate the epidemiology, diagnosis and pathogenesis of GEP-NETs in The Netherlands, to reveal insights in the underlying mechanisms contributing to the development and progression of these tumours. The major findings reported in this thesis are highlighted in Figure 1.

**Epidemiology of GEP-NETs in The Netherlands**

Although GEP-NETs were considered as rare tumours, incidence rates have been reported to increase substantially in recent years[5-7]. Furthermore, a relatively high number of incidental findings of clinically silent NETs by autopsy studies was suggested in literature[8,9]. We calculated the current incidence of gastrointestinal carcinoids and duodeno-pancreatic NETs in The Netherlands, by the use of the PALGA database (**Chapter 2** and **Chapter 3**)[10]. Interestingly, the incidence of non-functional pancreatic and duodenal NETs showed a significant increase from 1991 till 2009, whereas the incidence of gastrointestinal carcinoids increased significantly over 2000 to 2009 as well. Although this increase in incidence of GEP-NETs is likely to be the result of improved diagnostics rather than an actual increase in occurrence of these tumours, non-functional tumours are still detected at a relatively late stage illustrated by the larger size and a diagnosis at an older age than in those patients affected by a functional neuroendocrine tumour. In **Chapter 2**, we provided an overview on recent developments in the diagnosis of GEP-NETs, to increase the intelligibility and awareness of these tumours among clinicians and pathologists, in order to facilitate earlier detection and to prevent morbidity of GEP-NETs.

**Gastrinomas**

Gastrinomas, after insulinomas, are the most common type of functional neuroendocrine tumours. They are frequently located in the pancreas and duodenum[11]. However, gastrinomas can also occur at ectopic sites[12-14]. In **Chapter 4** we described a unique case of recurring hepatic gastrinomas in a patient suffering from the Zollinger-Ellison syndrome (ZES), in whom no other (primary) tumour was detected, even though with a follow-up of almost 20 years. In this context, we reviewed the literature on primary liver gastrinomas, and found 16 studies in which gastrinomas in the liver were defined as primary. However, we believe that the interpretation of hepatic gastrinomas as primary lesions can still be questioned. Nonetheless, our study showed that a gastrin-producing tumour in the liver can recur. As most cases lack an investigational and well-documented follow-up, we recommend a long-lasting follow-up including frequent serum gastrin measurements and repeated imaging investigations in case of a suspected hepatic gastrinoma.

Gastrinomas produce and secrete gastrin, a hormone normally produced by G-cells in the stomach to stimulate the acid secretion. Patients with gastrinomas therefore suffer from symptoms related to hyperacidity, such as acid reflux, abdominal pain, recurrent ulcers, and diarrhoea. Together these symptoms are called the Zollinger-Ellison syndrome (ZES)[15]. Usually, ZES is suspected in case of increased fasting serum gastrin levels and/or the presence of symptoms. However, the increased use of proton pump inhibitors or other acid reducing medications often masks symptoms, contributing to a delay in diagnosis in these patients[16]. Furthermore, serum gastrin levels can be non-conclusive. The secretin stimulation test has widely been used to diagnose ZES[17]. In the literature, however, the dosage of secretin and the criteria for a positive test have been disputed[18-22]. We discussed the diagnostic efficacy of the secretin stimulation test in patients with ZES by comparison of two different doses of secretin and selecting the most optimal criteria for a positive secretin test (**Chapter 5**). We found a gastrin increase of >100 ng/L to be the most sensitive and specific criterion for a positive secretin stimulation test. When this criterion was applied to both our

study and confirmation group, we found that a higher dose of secretin (0.78 µg/kg) did not contribute to a more valuable secretin stimulation test in diagnosing ZES. Therefore, we recommend the use of the low dose of secretin (0.26 µg/kg) in combination with a gastrin increase >100 ng/L as the optimal criterion for a positive secretin stimulation test to diagnose ZES.

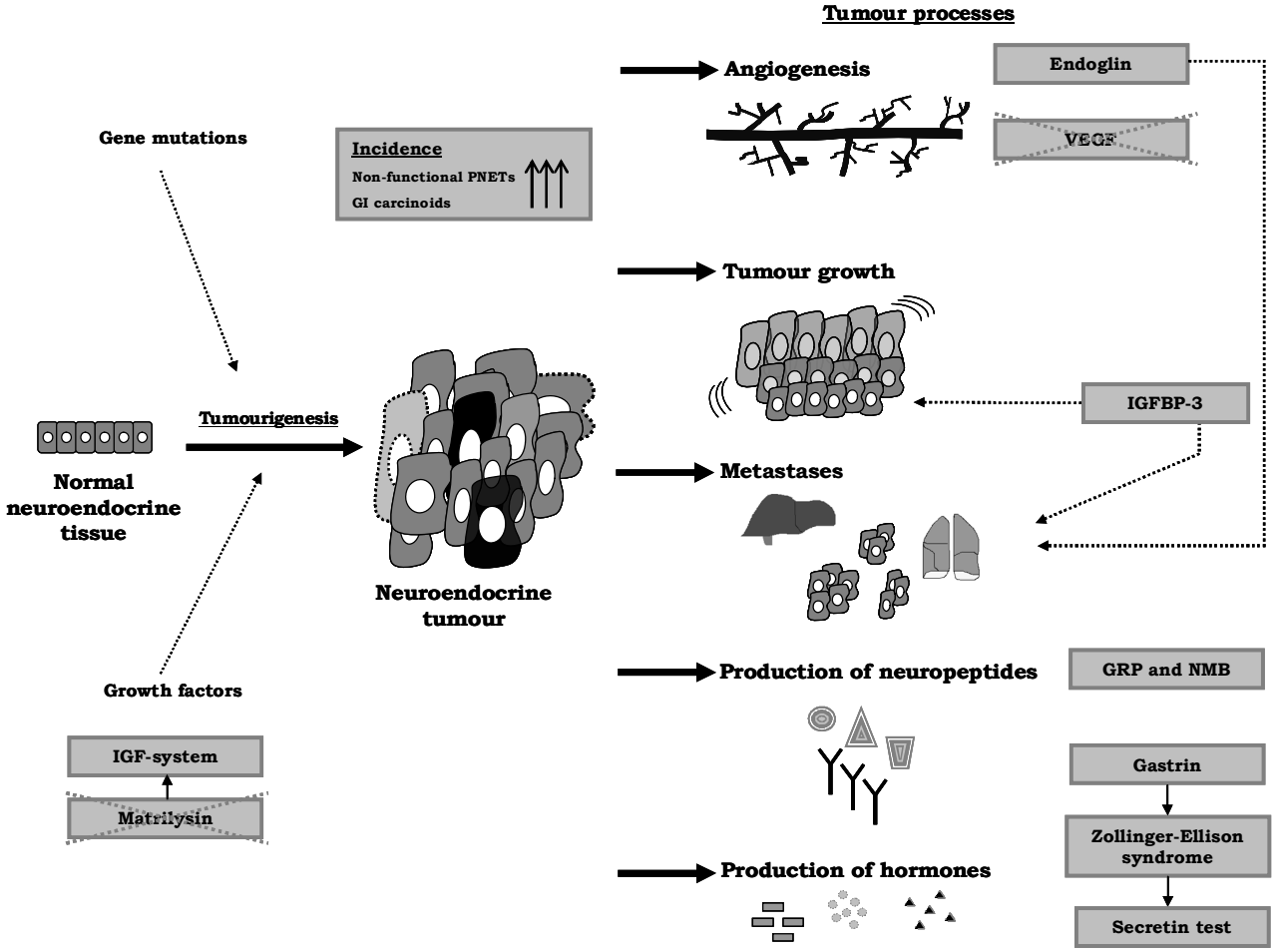


Figure 1. Summary of the results obtained in the studies as described in this thesis.

**Heterogeneity and tumour markers in GEP-NETs**

Neuroendocrine tumours of the gastroenteropancreatic tract are a group of diverse, heterogeneous tumours. Although gastrointestinal carcinoids and pancreatic neuroendocrine tumours share their common origin of neuroendocrine cells of the digestive tract, these tumours show variable histopathological characteristics and behaviour, making it hard to predict outcomes and prognosis on basis of these features[23]. Therefore, we aimed to identify tumour parameters which might have clinical implications in these tumours.

Carcinoids are predominantly found in the gastrointestinal (2/3rd) or pulmonary system (1/3rd). These tumours are able to secrete bioactive peptides, such as the bombesin-like peptides (BLPs) gastrin releasing peptide (GRP) and neuromedin B (NMB). In addition to stimulating a variety of physiological responses in the human body, BLPs are involved in the development and progression of several human cancers. By binding to their membrane-bound receptors on tumour cells, BLPs are able to activate autocrine loops, leading to growth of the tumour[24]. In small cell and non-small cell lung cancer, an autocrine loop involving BLPs has been suggested, whereas in colorectal cancer BLPs have been observed to act both as morphogens and mitogens[25-27]. We investigated the quantity and localization of bombesin receptors in gastrointestinal and pulmonary carcinoids, and revealed whether bombesin-like peptides and their receptors are of any value in distinguishing pulmonary carcinoids from carcinoids of intestinal origin (**Chapter 6**). Based on our results, we conclude that in both pulmonary and intestinal carcinoids, all three bombesin receptors are present, although the quantity and ligand binding affinities are diverse on carcinoids of different origin; apparently on pulmonary carcinoids, bombesin receptors have a low binding affinity for bombesin, while intestinal carcinoids possess predominantly receptors with a high ligand binding affinity. Therefore, overall bombesin receptor expression seems not to be a very useful marker to distinguish carcinoids based on tumour origin. The combined presence of bombesin and its receptors might suggest the presence of a paracrine or autocrine growth loop in carcinoids, although further research is required to confirm this hypothesis.

Angiogenesis plays an important role in tumour growth, progression and metastatic development[28]. Vascular endothelial growth factor (VEGF) and endoglin (CD105) are two key factors in angiogenesis. VEGF is a potent angiogenic growth factor stimulating endothelial cell proliferation, whereas endoglin, a TGF-beta co-receptor, is highly expressed on endothelial cells of newly formed blood vessels[29,30]. In **Chapter 7**, a study to evaluate the expression and potential prognostic role of VEGF and endoglin in GEP-NETs is described. Expression of endoglin and VEGF were two to four-fold higher on tumours compared to

associated normal tissue. This increased endoglin tissue expression in tumours was significantly related to the tumour's size, the presence of metastases and a more advanced tumour stage. These findings implicate that endoglin can serve as a marker to detect present and to predict future metastases in GEP-NETs. Assessment of endoglin tumour levels provides information on tumour aggressiveness, which might help to optimize the therapeutic approach in patients with these tumours. As several *in vivo* and *in vitro* studies using anti-endoglin antibodies for anti-cancer treatment show promising results, we suggest that endoglin might provide a new therapeutic vascular target in GEP-NETs as well[31]. Although VEGF tissue levels showed a similar pattern to endoglin, these were not significantly related to any clinicopathological parameter. Therefore, we assume that, although VEGF is most likely to be involved in the process of neoplastic blood vessel formation in GEP-NETs, this key mediator of angiogenesis is not the appropriate prognostic marker in these tumours.

The insulin-like growth factor (IGF) system, composed of the ligands IGF-1 and IGF-2, three receptors and six binding proteins (IGFBPs), plays an important role in cancer[32]. Several studies have shown that the expression of IGF-1 is up-regulated in various tumours, and related to tumour growth and malignant behaviour[33-35]. Matrix metalloproteinases (MMPs) are a family of endopeptidases which act to degrade the extracellular matrix and are essential for tissue remodelling[36].

Matrilysin (MMP-7) is exclusively produced by tumour cells and implicated to be involved in various tumour processes[37]. For example, in pancreatic and colorectal cancer, an increased expression of matrilysin was found to be related to invasion and the presence of metastases[38,39]. Recently, several studies have shown that MMPs, including matrilysin, can regulate the bioavailability of IGFs to tumour cells, thereby participating in IGF-induced growth activation in tumours[40].

We examined the expression of IGF-1, IGFBP-3 and matrilysin in GEP-NETs, in order to investigate their relation to the pathogenetic factors of these tumours (**Chapter 8)**. Tissue levels and expression of IGF-1 and IGFBP-3 were found to be up-regulated in GEP-NETs. In addition, higher IGFBP-3 expression was related to

a larger tumour size and the presence of metastases, which might be indicative for a more malignant tumour. The expression of matrilysin was down-regulated in tumours compared to associated normal tissue, and negatively correlated to the expression of IGFBP-3. These data suggest that IGFBP-3 plays a direct role in the etiopathogenesis of GEP-NETs, whereas matrilysin might indirectly be involved via regulation of this IGFBP-3 expression.

**Concluding remarks**

GEP-NETs comprise a group of heterogeneous tumours, with a wide and complex spectrum of clinical behaviour. They originate in a great diversity of tissues and are characterized by their ability to produce various hormonal peptides that cause distinct clinical syndromes. As incidence rates of both GI carcinoids and duodenopancreatic NETs are increasing over the past years in the Netherlands, these tumours might not be as uncommon as previously thought. This increasing incidence and large heterogeneity of GEP-NETs underlines the urgent need for better understanding of the underlying pathological mechanisms, in order to facilitate the development of new therapeutic strategies. In this thesis, several studies to reveal new markers in the pathogenesis of GEP-NETs are described. Foremost, we suggest endoglin as a novel marker to indicate the presence and potential development of metastases in GEP-NETs, of potential use in the post-resection approach in the therapy of these tumours. Next, preliminary evidence for a role of two autocrine growth systems, involving the bombesin-like peptides GRP and NMB, and the IGF-system and matrilysin, respectively, in the growth and development of these tumours, is provided. Although further research to reveal the exact mechanism of these autocrine growth systems in GEP-NETs is required, these studies might provide the basis for the development of new anti-cancer therapies in these tumours.

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# Chapter 10

## *Nederlandse Samenvatting voor Leken*

Inleiding

Doel

Resultaten

Conclusies

**Inleiding**

Gastroenteropancreatische neuroendocriene tumoren (GEP-NETs) vormen een zeldzame groep tumoren, die onderling sterk verschillen wat betreft hun biologische gedrag, tumorkenmerken en prognose. Ze bestaan uit neuroendocriene tumoren in de alvleesklier (pancreas) en neuroendocriene tumoren in het maag-darmkanaal, welke ook wel carcinoïden worden genoemd.

Neuroendocriene tumoren in de alvleesklier kunnen afkomstig zijn van verschillende cellen. Ze worden onderverdeeld in functionele en niet-functionele tumoren. Functionele tumoren kunnen hormonen als gastrine, insuline, glucagon, somatostatine en VIP (vasoactieve intestinaal peptide) uitscheiden. Elk hormoon geeft aanleiding tot een eigen klinisch beeld (syndroom). Functionele neuroendocriene tumoren in de alvleesklier worden vernoemd naar het voornaamste hormoon dat zij uitscheiden en kunnen dus gastrinomen, insulinomen, glucagonomen, somatostatinomen of VIPomen heten. Niet-functionele tumoren geven echter geen klachten op basis van hormoonuitscheiding en worden daarom vaak pas laat ontdekt, meestal wanneer de tumor zo groot is dat deze een obstructie veroorzaakt of uitzaaiingen (metastasen) heeft gevormd.

Neuroendocriene tumoren van de alvleesklier komen ook frequent voor op plaatsen buiten de alvleesklier, zoals in de twaalfvingerige darm (duodenum) of lymfeklieren. Minder vaak worden deze tumoren gezien op uitzonderlijke locaties als lever, eierstokken, schildklier en bijnieren.

Het aantal nieuwe gevallen per jaar (incidentie) van neuroendocriene tumoren in de alvleesklier en de twaalfvingerige darm is laag, en betreft ongeveer 1 per 1.000.000 personen per jaar. Onder de neuroendocriene tumoren in de alvleesklier worden de goedaardige insulinomen het vaakst gezien, terwijl gastrinomen de meest voorkomende soort kwaadaardige neuroendocriene tumoren in de alvleesklier zijn. Gastrinomen scheiden het hormoon gastrine uit. Normaal gesproken wordt gastrine in de maag geproduceerd door de G-cellen, waar het de uitscheiding van maagzuur stimuleert. Patiënten met gastrinomen hebben daarom klachten die gerelateerd zijn aan een verhoogde maagzuursecretie, zoals

oprispingen, buikpijn, diarree, misselijkheid en braken. Samen worden deze klachten het Zollinger-Ellison syndroom (ZES) genoemd. De diagnose gastrinoom kan in deze patiënten gesteld worden op basis van hun klachten en verhoogde waardes van het hormoon gastrine in het bloed. Echter door het gebruik van zuurremmende medicatie, waaronder bijvoorbeeld proton-pomp-remmers, wordt de diagnose vaak niet of pas laat gesteld. Verder zijn de bloedwaardes van gastrine soms niet zodanig verhoogd dat deze bewijzend zijn voor een gastrinoom. De secretine-stimulatietest biedt dan uitkomst. Injectie van het peptidehormoon secretine leidt bij patiënten met een gastrinoom tot een snelle stijging van de gastrinespiegels in het bloed, terwijl deze reactie veel minder is of uitblijft wanneer er geen tumor aanwezig is.

Neuroendocriene tumoren in het maag-darmkanaal zijn afkomstig van serotonine-producerende enterochromaffine cellen in de darm of histamine-uitscheidende enterochromaffine-achtige cellen in de maag. Opmerkelijk is dat deze tumoren ook in de longen kunnen voorkomen. Het aantal nieuwe gevallen per jaar van neuroendocriene tumoren in het maag-darmkanaal ligt op ongeveer 10 tot 20 personen per 1.000.000 per jaar, afhankelijk van de lokalisatie. Onder deze tumoren worden carcinoïden in het wormvormig aanhangsel van de dikke darm (appendix) het vaakst gezien. Verder komen deze tumoren voor in de maag, de dunne darm, de dikke darm, en de endeldarm. Neuroendocriene tumoren welke serotonine uitscheiden veroorzaken hiermee het klassieke 'carcinoïd syndroom' en worden daarom carcinoïden genoemd. Dit syndroom wordt gekenmerkt door ernstige diarree, opvliegers en ademhalingsstoornissen. Verhoogde spiegels van 5-HIAA, het afbraakproduct van serotonine, in het bloed, leiden tot de diagnose.

Neuroendocriene tumoren zijn naast hormonen ook in staat om neuropeptiden uit te scheiden. Bombesine is een neuropeptide, welke voor het eerst werd geïsoleerd uit de huid van de kikkersoort *Bombina bombina*. In mensen en zoogdieren zijn twee varianten van bombesine bekend, namelijk neuromedine B (NMB) en gastrin-releasing peptide (GRP). Een viertal receptoren waar bombesine en bombesine-achtige peptiden aan kunnen binden is geïdentificeerd: de neuromedine B receptor (BRS-1, NMBR), de gastrin-releasing peptide receptor

(GRPR, BRS-2), de bombesine receptor subtype 3 (BRS-3) en subtype 4 (BRS-4). Uit verschillende literatuurstudies blijkt dat GRP en NMB en hun receptoren een belangrijke rol spelen in kanker, waarbij zij onderdeel zijn van een zelfstimulerend (autocrien) systeem dat leidt tot tumorgroei.

Over het algemeen hebben neuroendocriene tumoren van de alvleesklier en het maag-darmkanaal een goede bloedvoorziening (vascularisatie). Angiogenese, de vorming van nieuwe bloedvaatjes uit bestaande bloedvaten, is een belangrijk proces in de ontwikkeling en groei van tumoren. Wanneer tumoren ongeveer 1 tot 2 mm groot zijn worden ze afhankelijk van de vorming van nieuwe bloedvaten voor hun zuurstof- en voedingsstoffenvoorziening, maar ook voor hun verdere groei en de vorming van uitzaaiingen. Angiogenese wordt vaak gemeten als de microvessel density (MVD), het aantal vaatjes in een tumor. In vele tumoren, zoals borst-, blaas- en maagkanker, werd een associatie gevonden tussen een toename van de angiogenese en de ontwikkeling van uitzaaiingen, een slechte prognose en een verminderde overleving van patiënten. Vascular endothelial growth factor (VEGF) is een belangrijke speler in het proces van angiogenese, want het reguleert in de bloedvaten belangrijke celprocessen als uitrijping (differentiatie), verplaatsing (migratie) en celdeling (proliferatie). Endogline (CD105) is een co-receptor voor transforming growth factor beta (TGF-β), een belangrijke groeifactor die verscheidene tumorprocessen reguleert. Door de voornaamste expressie op bloedvatcellen van nieuw-gevormde bloedvaten, vormt endogline een belangrijke marker voor angiogenese.

Matrilysine, of matrix metalloproteïnase 7 (MMP-7), is onderdeel van de familie van matrix metalloproteïnases. Dit zijn enzymen die in staat zijn om het bindweefsel tussen cellen af te breken waardoor ze een belangrijke rol spelen bij zowel normale als ziektegerelateerde processen van weefselvernieuwing. Matrilysine wordt geproduceerd door tumorcellen en is in verschillende kankertypes bewezen betrokken te zijn bij tumoringroei en de vorming van uitzaaiingen.

Het insuline-achtige groei factor (IGF) systeem is een belangrijk signaleringssysteem, betrokken bij celprocessen als groei en ontwikkeling.

Daarnaast speelt het ook een belangrijke rol in de ontwikkeling van verschillende tumor types. Het IGF-systeem bestaat uit de eiwitten IGF-1 en IGF-2, hun receptoren IGF-1R en IGF-2R, en een zestal IGF-bindingseiwitten, de IGFBP-1 tot en met IGFBP-6.

Verschillende factoren zoals hierboven beschreven dragen bij het ontstaan, het ontwikkelen en het beloop (pathogenese) van de tumoren en de bijhorende ziekteprocessen. In de studies van dit proefschrift werd gekeken of verschillende tumormechanismen, bekend van andere kankers, ook een rol spelen bij neuroendocriene tumoren van de alvleesklier en het maag-darmkanaal. Omdat deze tumoren zeer gevarieerd zijn, is het waardevol om factoren te achterhalen die verantwoordelijk zijn voor deze diversiteit en de onderliggende mechanismen voor het ontstaan van deze tumoren, zodat in de kliniek hierop kan worden ingespeeld door verbeterde diagnostiek en aangepaste behandelmethoden bij deze patiënten. Om een goed beeld van deze tumoren te vormen, werden ook verschillende studies uitgevoerd om meer inzicht te krijgen in hun voorkomen en diagnostiek.

**Doel**

Het doel van de in dit proefschrift beschreven studies was inzicht te krijgen in het ontstaan, bestaan en beloop van neuroendocriene tumoren in het maag-darmkanaal en de alvleesklier.

**Resultaten**

Zoals eerder genoemd zijn gastroenteropancreatische neuroendocriene tumoren een verzameling van zeer diverse tumoren met onderling grote verschillen in het ontstaan, hun klinische symptomen en de prognose voor de patiënten.

**Hoofdstuk 2** geeft een overzicht van de huidige diagnostiek van deze tumoren. Aan de hand van de besproken veelvoorkomende problemen en nieuwe ontwikkelingen in de diagnostiek van neuroendocriene tumoren in het maag-darmkanaal en de alvleesklier, werd een algoritmische beslisboom opgesteld om de diagnostiek te standaardiseren. Gastroenteropancreatische neuroendocriene

tumoren staan bekend als zeldzame tumoren. Verschillende epidemiologische studies uit diverse landen rapporteerden echter een toename in de incidentie van deze tumoren over de afgelopen jaren. Daarom deden wij onderzoek naar het aantal nieuwe gevallen van neuroendocriene tumoren in de alvleesklier en de twaalfvingerige darm in Nederland per jaar, over de periode van 1991 tot 2009, zoals beschreven in **Hoofdstuk 3**. Als resultaat werd een toename in de incidentie van neuroendocriene tumoren van zowel de alvleesklier als twaalfvingerige darm over de bestudeerde onderzoeksperiode gevonden. Deze stijging was voornamelijk toe te schrijven aan een toename van het aantal nieuwe gevallen van niet-functionele tumoren, terwijl de incidentie van de functionele tumoren ongeveer constant bleef over de jaren. Wij denken dat de incidentiestijging van niet-functionele tumoren het resultaat is van een betere detectie, in plaats van een daadwerkelijke toename in het voorkomen van deze tumoren. Mogelijk hebben een verbeterde diagnostiek en beeldvormingtechnieken (zoals CT en MRI scans) en ook de introductie van de classificatie voor gastroenteropancreatische neuroendocriene tumoren volgens de World Health Organization in 2000 hieraan bijgedragen.

Gastrinomen zijn de meest voorkomende soort kwaadaardige neuroendocriene tumor, welke meestal voorkomen in de alvleesklier, de twaalfvingerige darm en de daaromheen liggende lymfeklieren. Deze gastrinomen komen echter in zeldzame gevallen ook op andere plaatsen in het lichaam voor, zoals de eierstokken, het darmscheil (mesenterium), de maag, de bijschildklieren, nieren, galwegen, en de lever. In **Hoofdstuk 4** beschreven wij een patiënt met een terugkerend (recidiverend) gastrinoom in de lever, waarbij het bestaan van primaire lever gastrinomen bediscussieerd wordt aan de hand van een uitvoerige literatuurstudie.

Door hun gastrineproductie geven gastrinomen aanleiding tot het Zolliner-Ellison syndroom (ZES). De secretine-stimulatietest is een veelgebruikte test voor de diagnose van ZES. Echter, in de literatuur staat de dosis van secretine en de daarbij behorende grenswaarde voor een positieve test ter discussie. Aan de hand van een patiëntenstudie onderzochten wij of een hogere dosis secretine meer

effectief was in het diagnosticeren van ZES, in vergelijking met een lagere dosis, en welk criterium bijdroeg aan de hoogste gevoeligheid (sensitiviteit) en specificiteit van de test voor het diagnosticeren van ZES (**Hoofdstuk 5**). Wij vonden dat het gebruik van een lage dosis secretine resulteert in een sensitieve en specifieke secretine-stimulatietest, wanneer als criterium voor een positieve test een gastrinestijging van meer dan 100 ng/L wordt gebruikt.

Gastroenteropancreatische neuroendocriene tumoren produceren naast hormonen ook neuropeptiden, zoals bombesine. Gastrin-releasing peptide en Neuromedine B behoren tot de 'bombesin-achtige peptides' (BLPs). In de studie beschreven in **Hoofdstuk 6** onderzochten we of we op basis van deze bombesine-achtige peptides in staat waren om carcinoïden van verschillende afkomst te onderscheiden. De expressie van verschillende bombesine receptoren bleek niet afhankelijk van de lokalisatie van de tumor. Daarom achten wij bombesine niet bruikbaar als specifieke marker carcinoïden, hoewel carcinoïden in de darmen en longen wel diverse bindingsaffiniteiten voor deze receptoren en verschillende bombesine peptide waardes vertoonden. Wij suggereren daarnaast dat de gelijktijdige expressie (co-expressie) van de bombesine-achtige peptides en hun receptoren op tumorcellen mogelijk de aanwezigheid van een autocrien groeisysteem in carcinoïden illustreert, al zal verder onderzoek nodig zijn om dit te kunnen bewijzen.

Angiogenese speelt ook in het ontstaan en ontwikkeling van neuroendocriene tumoren een belangrijke rol. Het onderzoek naar de expressie en weefselwaardes van endogline en VEGF in gastroenteropancreatische neuroendocriene tumoren staat beschreven in **Hoofdstuk 7**. Hierbij werd gevonden dat endogline een potentiële marker is om de aanwezigheid van metastasen te detecteren en de mogelijke ontwikkeling van metastasen te voorspellen. Hoewel de expressie van VEGF verhoogd was in tumoren ten opzichte van geassocieerd normaal weefsel in patiënten met gastroenteropancreatische neuroendocriene tumoren, vonden wij geen relatie met tumorkenmerken, zodat wij VEGF niet aanbevelen als mogelijke marker in deze tumoren. Echter, verschillende studies onderzoeken het gebruik van antilichamen tegen endogline. Op basis van onze resultaten suggereren wij

dat mogelijk ook in neuroendocriene tumoren van de alvleesklier en het maag-darmkanaal een behandeling met anti-endogline effectief zou kunnen zijn om angiogenese en daarmee verdere groei en ontwikkeling van de tumor tegen te gaan.

Het IGF-systeem speelt een belangrijke rol in kanker. Verschillende studies hebben aangetoond dat de expressie van IGF-1 verhoogd is in diverse tumoren, gerelateerd aan de groei en agressiviteit van de tumor. Ook blijkt de expressie van matrilysine in verschillende kankers, waaronder maag-, slokdarm- en alvleesklierkanker te zijn toegenomen en geassocieerd met een kwaadaardig gedrag van de tumor. Recent is aangetoond dat tumorgroei wordt gestimuleerd door IGF-1, nadat matrilysine IGF-1 heeft losgeknipt van IGFBP-3. In **Hoofdstuk 8** beschrijven wij een studie naar de expressie van IGF-1, IGFBP-3 en matrilysine in neuroendocriene tumoren van de alvleesklier en het maag-darmkanaal, om de rol van het IGF-matrilysine groeinetwerk in het ontstaan van deze tumoren te onderzoeken. Wij vonden dat matrilysine geen directe invloed heeft op de tumorgroei of andere tumorprocessen in gastroenteropancreatische neuroendocriene tumoren. Echter vonden wij wel voorlopig bewijs dat ook in neuroendocriene tumoren van de alvleesklier en het maag-darmkanaal een IGF-matrilysine netwerk aanwezig is. Verder bleek in deze tumoren een hogere expressie van IGFBP-3 gerelateerd te zijn aan een grotere tumor en de aanwezigheid van metastasen.

**Conclusies**

De studies beschreven in dit proefschrift geven inzicht in de epidemiologie, de diagnostiek, het ontstaan en het beloop van gastroenteropancreatische neuroendocriene tumoren. De diverse studies naar het aantal nieuwe gevallen per jaar van deze tumoren laten zien dat neuroendocriene tumoren van de alvleesklier en het maag-darmkanaal, ook in Nederland, in de afgelopen jaren in incidentie zijn toegenomen. Mogelijk zijn deze tumoren dus niet zo zeldzaam als lange tijd werd gedacht. Mede daarom achten wij het een noodzaak dat de ontstaanswijze en ontwikkeling van deze tumoren duidelijk(er) worden. In de studies welke staan

beschreven in dit proefschrift, hebben wij onderzoek gedaan naar factoren die een rol spelen in de pathogenese van neuroendocriene tumoren in de alvleesklier en het maagdarm-kanaal. Zo vonden wij dat endogline een potentiële marker is om aanwezige en toekomstige metastasen te detecteren en te voorspellen. Het meten van endogline spiegels in tumor weefsels geeft informatie over de mate van kwaadaardigheid van de tumor, wat zeer bruikbaar kan zijn in het bepalen van de behandelingsstrategie en follow-up na operatie van patiënten met gastroenteropancreatische neuroendocriene tumoren. Daarnaast vonden wij aanwijzingen voor de aanwezigheid van twee groeisystemen in deze tumoren. Allereerst doet de gelijktijdige expressie van bombesine-achtige peptides en hun receptoren in carcinoïden in de longen en darmen het bestaan van een autocrien groeisysteem in deze tumoren sterk vermoeden. Daarnaast lijkt matrilysine samen met IGF-1 en IGFBP-3 in neuroendocriene tumoren van de alvleesklier en het maag-darmkanaal een netwerk te vormen resulterend in tumorgroei. Aanvullende studies naar de exacte werking van deze autocriene groeisystemen in gastroenteropancreatische neuroendocriene tumoren zullen moeten uitwijzen of bombesine-achtige peptides, matrilysine en het IGF-systeem mogelijk ook gebruikt kunnen worden als doel voor anti-kanker behandelingsstrategieën.

Mogelijk dragen de studies beschreven in dit proefschrift bij aan de ontwikkeling van meer doelgerichte behandelingen, waardoor de prognose en overleving van de patiënten met neuroendocriene tumoren van de alvleesklier en het maag-darmkanaal in de toekomst verbeterd kunnen worden.

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Kuiper P, Biemond I, Verspaget HW, Lamers CB. A case of recurrent gastrinoma in the liver with a review of "primary" hepatic gastrinomas. *BMJ Case Reports* [published online 11 June 2009].

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# Curriculum Vitae

De auteur van dit proefschrift, Patricia Kuiper, werd op 2 augustus 1986 geboren in ’s Gravenhage. Zij behaalde in 2004 haar VWO diploma aan het Segbroek College in ’s Gravenhage. In dit jaar begon zij met de opleiding Geneeskunde aan de Universiteit Leiden. Tijdens haar studie heeft zij haar wetenschapsstage van 24 weken gelopen in het laboratorium van de afdeling Maag-, Darm- en Leverziekten in het Leids Universitair Medisch Centrum, Leiden, onder supervisie van Dr. L.J.A.C. Hawinkels, Dr. C.F.M. Sier en Dr. ir. H.W. Verspaget, waar zij onderzoek deed naar de rol van endoglin in colorectaal kanker. In 2008 werd in de Geneeskunde het doctoraal diploma behaald. In april 2008 begon zij onder verantwoordelijkheid van Dr. ir. I. Biemond, Dr. ir. H.W. Verspaget en Prof. dr. C.B.H.W. Lamers het onderzoek naar karakteristieken en groeimechanismen van neuroendocriene tumoren van de tractus digestivus en het pancreas aan het Leids Universitair Medisch Centrum, wat geresulteerd heeft in een aantal publicaties en de totstandkoming van dit proefschrift. Op dit moment is Patricia bezig met haar co-schappen en het vervolg van haar studie Geneeskunde, om haar diploma voor basisarts te behalen.

# Nawoord

Dit proefschrift is tot stand gekomen met de hulp van velen. Ik wil de wetenschappelijk medewerkers en het analytisch personeel van de afdeling Maag-, Darm-, en Leverziekten (Hein, Izäk, Eveline, Wim, Johan, Annie, Marij, Bert-Jan, Marjolijn, Auke, Christine, Rutger, Lianne, Vanesa, Liudmilla, Jarom, Sanne, Manon, Niké, Luuk, Pim) bedanken voor hun steun en inzet. Daarnaast ben ik de afdeling Pathologie zeer erkentelijk voor het beschikbaar stellen van resectieweefsel. Voorts bedank ik de afdelingen Pathologie van het Antoni van Leeuwenhoek Ziekenhuis en het Amsterdam Medisch Centrum, Willem en Henk-Jan van het Medisch Centrum Alkmaar, en Lucy van het Pathologisch Anatomisch Landelijk Geautomatiseerd Archief voor de zeer waardevolle samenwerking. Tenslotte gaat mijn dank uit naar mijn familie (papa, mama, Dennis, Menno en Joury), schoonfamilie en vrienden voor hun interesse en aanmoedigingen. Meeste dank gaat natuurlijk naar mijn partner Wouter voor zijn grenzeloze geduld en steun gedurende het onderzoek en de afronding van dit proefschrift.

# Full-colour illustrations

*Chapter 6*

**Figure 1. Immunostaining on carcinoids**

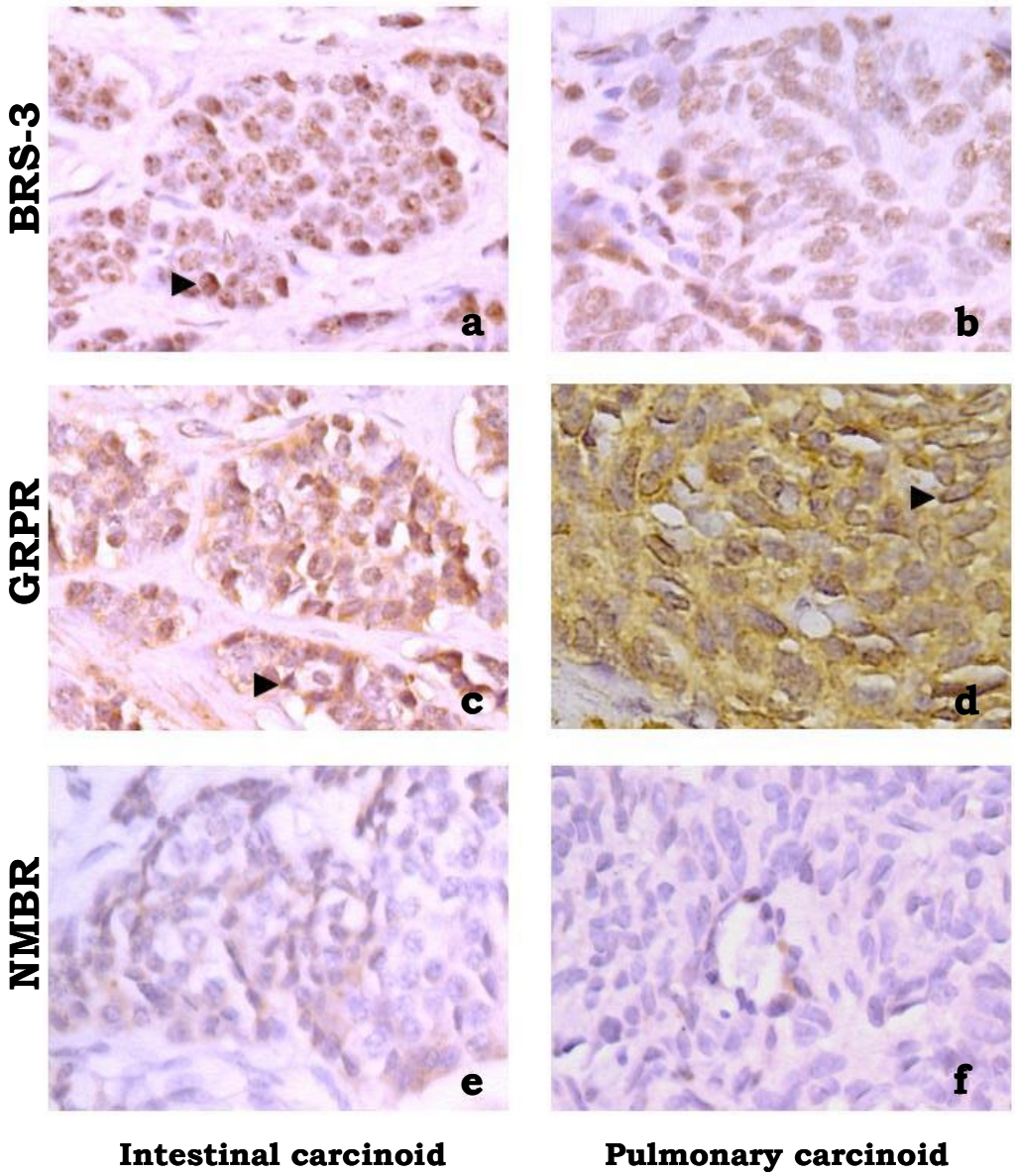


Figure 1. Immunostaining for the three bombesin receptors on an intestinal and a pulmonary carcinoid. Magnification x400. The carcinoids are stained with antibodies for BRS-3 (a,b), GRPR (c,d) and NMBR (e,f). Incidental membrane-bound staining is indicated with ►. Immunohistochemical scores were 3 for BRS-3 (a), 5 for GRPR (c) and 4 for NMBR (e) in the intestinal carcinoid, and 4 for BRS-3 (b), 8 for GRPR (d) and 3 for NMBR (f) in the pulmonary carcinoid.

*Chapter 7*

**Figure 2. Immunostaining of gastroenteropancreatic neuroendocrine tumours**

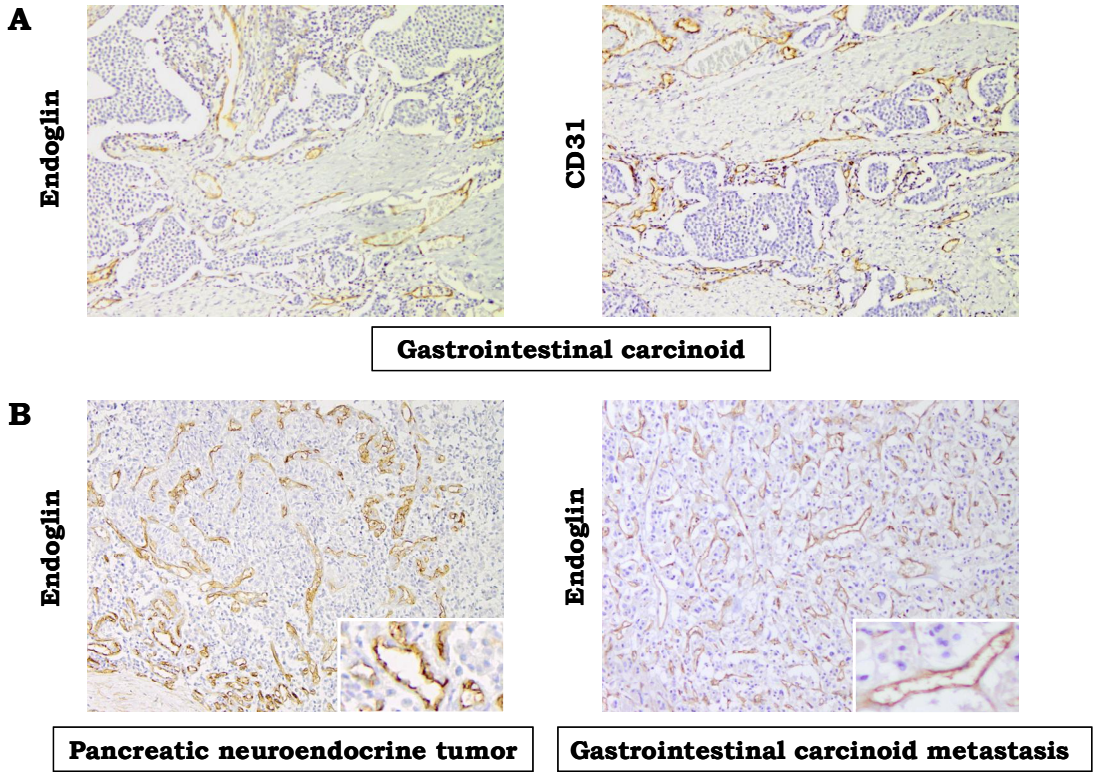


Figure 2. Immunostaining of endoglin and CD31 on peritumoural and intratumoural vessels in GEP-NETs. A) Endoglin staining is limited to angiogenic vessels, whereas CD31 stains both old and new blood vessels in tumour tissue. Magnification 100x. B) Representative endoglin staining in a pancreatic neuroendocrine tumour and a gastrointestinal carcinoid metastasis (mesenterium of small bowel). Magnification 100x. Inserts show a higher magnification at 200x.

*Chapter 8*

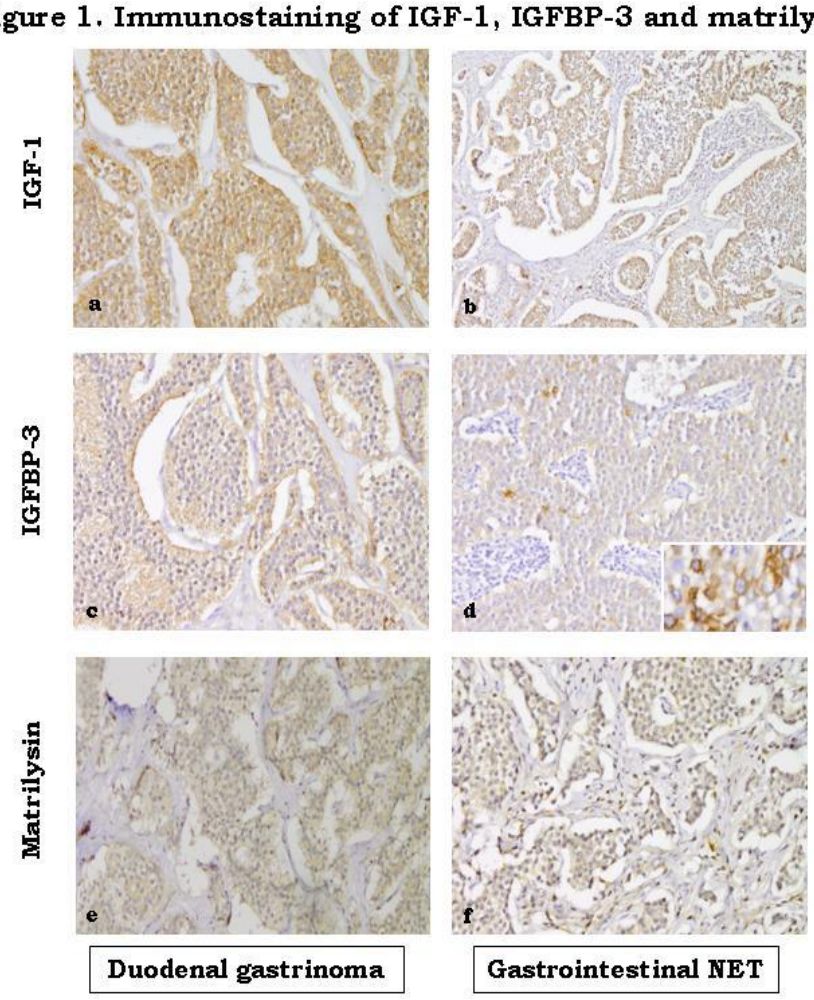


Figure 1. Immunohistochemical staining for a,b) IGF-1, c,d) IGFBP-3 and e,f) matrilysin on tumour cells of a duodenopancreatic gastrinoma (a, c, d) and gastrointestinal NET (carcinoid) (b, c, e). Magnification x100. In the insert of d) membrane staining of IGFBP-3 is shown at a higher magnification (x200).