Pancreatic Endocrine Tumors and GIST - Clinical Markers, Epidemiology and Treatment

SARA EKEBLAD
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Abstract

Pancreatic endocrine tumors and gastrointestinal stromal tumors are rare. Evidence regarding prognostic factors, and in the former also treatment, is scarce.

We evaluated the survival and prognostic factors in a consecutive series of 324 patients with pancreatic endocrine tumors treated at a single institution. Radical surgery, WHO classification, TNM stage, age and Ki67 ≥2% emerged as independent prognostic factors. Having a non-functioning tumor was not an independent prognostic marker, and neither was hereditary tumor disease. We present the first evaluation of the newly proposed TNM staging system for these patients. A separate analysis of well-differentiated neuroendocrine carcinomas is reported, suggesting tumor size ≥5cm and Ki67 ≥2% as negative prognostic markers in this group.

The first 36 patients with advanced neuroendocrine tumors treated with temozolomide at our clinic were evaluated. The median time to progression was seven months. Fourteen percent showed partial regression and 53% stabilization of disease. Side effects were generally mild. Investigation of O6-methylguanine DNA methyltransferase revealed a low expression in a subset of tumors. Four out of five patients responding to treatment had tumors with low expression.

Concomitant expression of the orexigen ghrelin and its receptor in pancreatic endocrine tumors is demonstrated. No significant difference in mean plasma ghrelin between patients and controls were found, but elevated plasma ghrelin was seen in five patients.

We provide the first report of expression of ghrelin and its receptor in gastrointestinal stromal tumors. Concomitant expression was frequent, indicating the presence of an autocrine loop. The tumors also expressed the neuroendocrine marker synaptic vesicle protein 2. Together, these findings are suggestive of neuroendocrine features.

Keywords: Pancreatic endocrine tumor, Gastrointestinal stromal tumor, Neuroendocrine, Multiple endocrine neoplasia type 1, Prognostic factors, Temozolomide, TNM staging, O6-methylguanine DNA methyltransferase, Growth hormone secretagogue receptor, Ghrelin

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Till mamma och pappa
List of papers

This thesis is based on the following papers, which will be referred to by their roman numerals.


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<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>5-HT3</td>
<td>5-hydroxytryptamine3</td>
</tr>
<tr>
<td>5-HP</td>
<td>5-hydroxytryptophan</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-Related Protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APUD</td>
<td>Amine Precursor Uptake and Decarboxylation</td>
</tr>
<tr>
<td>ASCO</td>
<td>American Society of Clinical Oncology</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CgA</td>
<td>Chromogranin A</td>
</tr>
<tr>
<td>CK19</td>
<td>Cytokeratin 19</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotropin Releasing Factor</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>EPT</td>
<td>Pancreatic Endocrine Tumor</td>
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<tr>
<td>FDG</td>
<td>Fluorodeoxyglucose</td>
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<tr>
<td>GHS-R</td>
<td>Growth Hormone Secretagogue Receptor</td>
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<tr>
<td>GIST</td>
<td>Gastrointestinal Stromal Tumor</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard Ratio</td>
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<tr>
<td>IAPP</td>
<td>Islet Amyloid Polypeptide</td>
</tr>
<tr>
<td>IGFII</td>
<td>Insulin-like Growth Factor 2</td>
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<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
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<tr>
<td>MEN1</td>
<td>Multiple Endocrine Neoplasia Type I</td>
</tr>
<tr>
<td>MGMT</td>
<td>O6-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MTIC</td>
<td>3-methyl-(triazene-1-yl)imidazole-4-carboxamide</td>
</tr>
<tr>
<td>NE</td>
<td>Neuroendocrine</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromatosis 1</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PDGFRA</td>
<td>Platelet-derived Growth Factor Receptor Alpha</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>PP</td>
<td>Pancreatic Polypeptide</td>
</tr>
<tr>
<td>PPI</td>
<td>Proton Pump Inhibitors</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PTHrp</td>
<td>Parathyroid Hormone-related Protein</td>
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<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SV2</td>
<td>Synaptic Vesicle Protein 2</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor-Node-Metastasis</td>
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<tr>
<td>UNL</td>
<td>Upper Normal Limit</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive Intestinal Peptide</td>
</tr>
<tr>
<td>WDHA</td>
<td>Watery Diarrhea Hypokalemia Achlorhydria</td>
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<td>WHO</td>
<td>World Health Organization</td>
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</table>
Introduction

Pancreatic endocrine tumors (EPT) and gastrointestinal stromal tumors (GIST) are rare. They affect and kill far fewer than do lung or breast cancer. Why then study these rare diseases? For the individual patient receiving a cancer diagnosis it does not matter whether the tumor is common or rare; it is every bit as big a tragedy either way. Less time and resources are spent on finding a cure for these rare tumors, and there is less guidance available for the clinician managing these patients. Research in this area is therefore highly important, to help improve the outlook for patients.

Endocrine cells in the gastrointestinal tract

Endocrine cells are scattered throughout the entire gastrointestinal tract, from the stomach to the rectum, as well as diffusely in the pancreas and clustered in the pancreatic islets of Langerhans. They share an amine precursor uptake and decarboxylation (APUD) capacity (Pearse, 1974) and have a common origin. They also share features with neural cells, such as expression of neuron-specific enolase, chromogranins and synaptophysin, and are called neuroendocrine. Recently, synaptic vesicle protein 2 (SV2) was suggested as a neuroendocrine cell marker (Portela-Gomes et al., 2000). The neuroendocrine cells of the gut produce hormones that have a variety of functions, ranging from glucose homeostasis to gut peristalsis. In the pancreas, the \( \alpha \) cell produces glucagon, the \( \beta \) cell insulin, the D cell somatostatin and the F cell pancreatic polypeptide (PP). Recently, ghrelin-producing cells were discovered in both the stomach and the pancreas (Kojima et al., 1999, Wierup et al., 2002). Most likely, this will not be the last discovery of new hormone-producing cells in the gastrointestinal tract.

Pancreatic endocrine tumors

EPTs occur in approximately 1 in 100000 of the population, representing 1-2% of all pancreatic neoplasms (Oberg & Eriksson, 2005). They are distinguished from the far more common exocrine pancreatic tumors by their endocrine phenotype, with expression of hormones and synaptic vesicle proteins, and by their often less aggressive clinical behavior. Lymph node me-
tastases occur in EPTs, as does local invasive growth. The liver is by far the most common site of distant metastases, but some patients experience lung or skeletal metastases. Brain metastases are exceedingly uncommon.

Functioning tumors

Some EPTs cause endocrine syndromes through excess hormone secretion; these tumors are called functioning. Tumor-associated endocrine syndromes are sometimes dramatic. The most common functioning tumor is insulinoma, which causes hypoglycemia by secreting inappropriate amounts of insulin. The patient suffers from symptoms of neuroglucopenia, such as double vision and confusion, as well as adrenergic symptoms such as agitation and tachycardia. In some cases inappropriate behavior has led to a false diagnosis of mental illness. Unconsciousness and subsequent brain damage can also be an effect of untreated hypoglycemia. The patient with insulinoma often has a long history of seeking medical attention. The diagnosis can be delayed due to the non-specific nature of the symptoms and lack of awareness among clinicians. Differential diagnoses include mesenchymal tumors producing IGF II, nesidioblastosis, abuse of insulin injections or oral antidiabetics, Addison’s disease, pituitary insufficiency and anorexia nervosa. Demonstration of low blood glucose and inappropriately high insulin levels after a prolonged fast (up to 72 h) settles the diagnosis. Second in frequency is gastrinoma (Zollinger & Ellison, 1955), causing multiple dyspeptic ulcers by secreting gastrin. Before the era of proton pump inhibitors (PPIs), gastrinoma patients died from bleeding ulcers. Now, symptoms can be effectively controlled. However, these potent drugs can sometimes mask the disease and prevent early diagnosis. Diagnosis is made by measurement of serum gastrin. Any PPIs should be withdrawn before testing, since they cause elevation of serum gastrin. The less common glucagonoma syndrome, caused by excess secretion of glucagon, is recognized by catabolism and hyperglycemia. These patients suffer massive muscle wasting, and are often severely cachetic upon presentation. They sometimes present with necrolytic migratory erythema, and it is not uncommon for the diagnosis to be made by a dermatologist. The even more uncommon tumor VIPoma causes watery diarrhea hypokalemia achlorhydria (WDHA), also known as Verner Morrison syndrome, by the secretion of vasoactive intestinal peptide (VIP) (Verner & Morrison, 1958). VIP causes massive diarrhea by binding to an adenylylate cyclase coupled receptor, just like the cholera toxin, and VIPoma syndrome is sometimes called pancreatic cholera. The patient can lose dangerous amounts of water and electrolytes, and intensive care unit treatment is often required. Rare somatostatinomas secrete somatostatin, an inhibitory hormone (Larsson L. I. et al., 1977). This causes more discrete symptoms, e.g., hyperglycemia. Other rare functioning tumors produce ACTH or CRF (causing Cushing’s syndrome) or PTHrp.
Non-functioning tumors

Tumors not responsible for any clinical syndrome are called non-functioning. This does not mean that they do not produce any hormones. They can either be producing a defective hormone that is not capable of inducing clinical effects, or a hormone with effects that are not yet fully known and thus not easily identified. These include PP, IAPP, calcitonin and the recently discovered ghrelin (see below). Patients with non-functioning tumors often present with symptoms related to tumor mass, such as pain or jaundice. The tumor can also be an incidental finding. Non-functioning tumors accounted for about 15-24% of tumors in the 1980s (Eriksson B. et al., 1989, Kent et al., 1981), but in recent reports the corresponding figure is about 60% (Hochwald et al., 2002, Tomassetti et al., 2005).

Survival and prognostic factors

EPTs are less aggressive than exocrine tumors of the pancreas, which carry a dismal prognosis. Some patients with EPT can live for years even with spread disease. In fact, sometimes patients with spread disease at presentation are initially diagnosed as exocrine, only to be re-diagnosed as endocrine years later, when the uncharacteristic, indolent course of their disease prompts further investigations. A median survival of 38-104 months from diagnosis (Chu et al., 2002, Eriksson B. et al., 1989, Hochwald et al., 2002, Solorzano et al., 2001, Tomassetti et al., 2005), and a five-year survival rate of 40-60% (Gullo et al., 2003, Kent et al., 1981, Panzuto et al., 2005, Pape et al., 2004, Tomassetti et al., 2005) has been reported.

EPTs exhibit a wide spectrum of clinical behavior, ranging from entirely benign tumors to very aggressive, undifferentiated cancers. Thus, it is of the utmost importance in each case to try to predict the clinical behavior of the tumor, in order to choose the right treatment approach. This is not always easy. Due to the rarity of these tumors, controlled studies of survival, treatment and prognostic factors are hard to execute. Some published studies have included only patients who have undergone surgery, creating a selection bias (Hochwald et al., 2002, La Rosa et al., 1996). Thus, there is still a lack of evidence-based guidelines for treatment. Furthermore, morphological signs of malignancy, such as nuclear atypia, pleomorphism and perineural growth, are not always present even in obviously malignant EPTs, i.e., metastatic tumors. Production of precursor hormones and/or ectopic hormone production are considered signs of malignancy in endocrine tumors, but are not always present in malignant EPTs. This lack of reliable signs of malignancy makes it difficult to predict the prognosis of the individual patient, and there is a need for better prognostic markers.

Factors suggested to have a prognostic impact in EPTs include primary tumor surgery, the presence of liver metastases, heredity, the presence of
endocrine symptoms, tumor necrosis, mitotic count, and proliferative index (Ki67) (Capella et al., 1995, Hochwald et al., 2002, La Rosa et al., 1996).

Ki67 is a protein expressed exclusively in proliferating cells (Gerdes et al., 1983). It is expressed in the nucleus in the G1, S, G2 and M phase of the cell cycle, but absent in resting (G0) cells. The percentage of nuclei expressing Ki67 is often used to estimate the rate of proliferation in tumors. Ki67 has been suggested to have prognostic value using a cut-off of two, five or ten percent in EPTs (Clarke et al., 1997, Deschamps et al., 2006, Hochwald et al., 2002, La Rosa et al., 1996, Panzuto et al., 2005, Pelosi et al., 1996). However, published studies are either small or include a mix of different tumor entities, making conclusions uncertain. A high nuclear expression of the apoptosis inhibitor survivin is associated with a poor prognosis in breast cancer (Span et al., 2004), and one study has suggested survivin as a prognostic factor in EPTs (Grabowski et al., 2005). A predictive value of CK19 in EPTs has also been suggested (Deshpande et al., 2004), as has an association between chromosomal alterations detected by comparative genomic hybridization and metastatic disease (Jonkers et al., 2005). Recently, a negative prognostic impact of elevated alkaline phosphatase was suggested (Clancy et al., 2006).

A WHO classification system (Rindi & Kloppel, 2004) is often used to divide tumors into three groups: well-differentiated NE tumors, well-differentiated NE carcinomas and poorly differentiated NE carcinomas. This classification is based on the number of mitoses, proliferative index (Ki67) and the presence or absence of gross invasion. Recently, a tumor-node-metastasis (TNM) staging system was proposed (Table I) (Rindi et al., 2006). The relevancy of this system for clinical use in the management of patients with EPTs has not yet been evaluated.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Disease stages</th>
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<tr>
<td>Stage I</td>
<td>Primary tumor only, &lt;2cm</td>
</tr>
<tr>
<td>Stage IIa</td>
<td>Primary tumor only, 2-4 cm</td>
</tr>
<tr>
<td>Stage IIb</td>
<td>Primary tumor only, &gt;4 cm or invading duodenum or bile duct</td>
</tr>
<tr>
<td>Stage IIIa</td>
<td>Tumor invading adjacent organs (stomach, spleen, colon, adrenal gland) or the wall of large vessels (celiac axis, superior mesenteric artery)</td>
</tr>
<tr>
<td>Stage IIIb</td>
<td>Lymph node metastases</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Distant metastases</td>
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</table>
Multiple endocrine neoplasia type I

EPTs arise either sporadically or as part of a hereditary tumor syndrome, most notably multiple endocrine neoplasia type 1 (MEN1) or the more uncommon von Hippel-Lindau disease. MEN1 is an autosomal dominant hereditary disease, initially recognized by Wermer (Wermer, 1954). In 1988, the \textit{MEN1} gene was characterized as a tumor suppressor gene and mapped to 11q13 (Larsson \textit{et al}., 1988), and in 1997 the gene was cloned (Chandrasekharappa \textit{et al}., 1997). Patients with MEN1 develop tumors in several endocrine glands, including the parathyroids, the endocrine pancreas and the anterior pituitary. The reason for the predominance of endocrine tumors is unknown. By definition, a person with no known affected relative is said to have the disease when he/she develops two of the above-mentioned lesions. For a person with an affected relative, only one lesion is needed for the diagnosis to be made.

Biochemical signs of EPT often occur in adolescence in MEN1 patients (Skogseid \textit{et al}., 1991), although the tumor might not clinically demonstrate until up to two decades later, when it is often metastatic at diagnosis (Skogseid \textit{et al}., 1996). Thus, early screening of these patients is imperative for the early detection of tumors. Today, it is possible to determine through genetic testing if a person has inherited a defective \textit{MEN1} gene or not. Thus, screening for tumors can be avoided in half of the relatives at risk. No convincing correlation between genotype and phenotype has yet been shown, i.e., it is not possible to predict the course of the disease based on the mutation found.

MEN1 patients often develop multiple EPTs. Biochemical screening enables detection while the tumors are very small and difficult to localize. Gastrinoma is the most common symptomatic EPT in MEN1 patients, but the majority of MEN1-related gastrinomas are duodenal (Jensen, 1998). EPTs in MEN1 patients often produce multiple hormones, in contrast to sporadic tumors that usually produce only one.

There is an ongoing debate over the management of MEN1 patients with EPTs, especially regarding early tumor surgery in asymptomatic patients. It could be important to remove any tumors as early as possible in order to prevent malignant transformation. But since surgery can itself carry significant morbidity, e.g., diabetes after pancreatic resection, it is also desirable to avoid unnecessary operations on tumors that might never have become malignant. Patients with EPT as part of the MEN1 syndrome often do better than patients with sporadic tumors, measured as survival from diagnosis. This fact is sometimes used as an argument for less aggressive treatment of these tumors. However, in one study, EPT was the number one cause of death for MEN1 patients, and the median age of death from pancreatic malignancy was only 46 years (Doherty \textit{et al}., 1998). Furthermore, a 64% 20-
year survival in MEN1 patients, compared to 81% in healthy age-matched controls, has been shown (Dean et al., 2000).

**Tumorigenesis**

The **MEN1** gene encodes the tumor suppressor menin, a protein with various functions. Menin is localized in the nucleus and is ubiquitously expressed throughout the body. In mouse models it is homozygous lethal. The exact function of menin has not yet been completely elucidated. It is known to interact with numerous proteins, such as transcription factors and proteins involved in cytoskeletal remodeling and cell cycle control, suggesting a role of menin in several biological pathways (Agarwal et al., 2005). The main molecular events behind EPTs in MEN1 and von Hippel-Lindau disease have been established: inactivation of a suppressor gene causes transformation. No known oncogenes are known to be involved in pancreatic endocrine tumorigenesis. There are a number of chromosomal alterations in sporadic EPTs. Loss of heterozygosity (LOH) on 11q, where the **MEN1** gene is located, is common, and homozygous somatic inactivation is seen in about one third of sporadic EPT (Hessman et al., 1998). Insulinomas, however, rarely show **MEN1** gene alterations (Jonkers et al., 2005).

**Plasma tumor markers**

Biochemical markers are helpful in the diagnosis and monitoring of EPTs. Important general tumor markers include chromogranin A (CgA) and pancreatic polypeptide (O'Connor et al., 1983, Oberg & Eriksson, 2005, Polak et al., 1976). Chromogranins are a family of water-soluble acidic glycoproteins that include chromogranin A, B and C. CgA is stored and released from dense-core secretory granules (Kim et al., 2001), and can be used as a marker for neuroendocrine tumors both in tissue analysis (O'Connot et al., 1983) and in blood (O'Connor & Deftos, 1986). Used as screening in the setting of a general practice, CgA in plasma has a low specificity. It can be elevated due to PPIs, kidney failure or diarrhea, and since these conditions are much more common than neuroendocrine tumors, the risk of a false positive test is high. However, if there is a clinical suspicion of a tumor, and other causes of elevation have been excluded, the specificity of a marked elevation is quite good. At diagnosis, the specific hormones often produced by tumors are also analyzed in blood. This is done both to help determine the functional status of the tumor (symptoms plus elevated hormone levels equals functioning tumor), but also to obtain a baseline value for future follow-up. Both CgA and any elevated hormones are used to monitor tumor progress and treatment effects. CgA is used also in the diagnosis and follow-up of midgut carcinoids. In these tumors, elevated CgA at diagnosis is related to a poor prog-
nosis (Janson et al., 1997). One study evaluating patients with EPT or carcinoid as one group found CgA elevation to be related to prognosis in univariate, but not multivariate, analysis (Clancy et al., 2006). In this study, CgA was not measured at diagnosis, but at some later point in the course of the disease. Not much is known regarding whether CgA elevation at diagnosis is related to prognosis in pancreatic endocrine tumors.

Diagnostic procedures

The diagnosis of EPT is not primarily based on radiology, but rather on histopathology and elevated markers in blood. The diagnosis of insulinoma is based on Whipple’s triad: symptoms likely to be caused by hypoglycemia, low blood glucose (<2.5 mmol/L) measured at the time of symptoms, and symptom relief when glucose is administered. Serum insulin and blood glucose are often measured during a prolonged fast (up to 72 h), to demonstrate a paradoxically high insulin level in spite of low blood glucose. The presence of multiple ulcers or ulcers in atypical locations leads to a suspicion of gastrinoma. This is verified by demonstrating elevated serum gastrin levels and a low pH in the stomach. High gastrin levels can also be caused by PPIs (which most patients with suspected gastrinoma are already on). If possible, PPIs should be withdrawn before testing. Other functioning tumors are diagnosed by demonstrating elevated hormone levels in plasma in combination with characteristic symptoms.

Radiology is, of course, important for tumor localization and staging. Computed tomography (CT) or magnetic resonance imaging (MRI) is the first choice for imaging. Tumors too small to localize with these techniques can be visualized with endoscopic, or even better, intra-operative ultrasound. The latter is especially important in MEN1 patients, where the decision to operate is often based on biochemical findings only, and tumors are routinely localized intra-operatively.

Somatostatin receptor scintigraphy is helpful in the diagnosis and follow-up of patients with tumors expressing somatostatin receptors (80-90% of neuroendocrine tumors) (Oberg & Eriksson, 2005). Positron emission tomography (PET) with (11)C-5-hydroxytryptophan (5-HTP) is useful in the diagnosis, and has been suggested to be more sensitive than CT or somatostatin receptor scintigraphy (Orlefors et al., 2005). Fluorine-18 fluorodeoxyglucose (FDG) PET, which is used for several other tumor types, is of limited value because it detects tumors with a low proliferation to a lesser extent (Adams et al., 1998). It might however be useful in detecting EPT with higher proliferation (Pasquali et al., 1998).
Treatment

Surgery
Surgery is the first choice of treatment for pancreatic endocrine tumors, as well as other neuroendocrine tumors. In the absence of metastatic disease, radical removal of the primary tumor is the obvious choice. Operating on the pancreas is complicated, and EPT surgery should preferably be performed by an experienced surgeon at a specialized center. Even so, morbidity can be substantial, e.g., pancreatitis, infections and diabetes. Depending on tumor size and localization, either enucleation, distal pancreatic resection or a Whipple procedure is chosen. Local lymph-node dissection should be performed. Intra-operative ultrasound is recommended, and is considered mandatory in MEN1 pancreatic surgery. There is an ongoing debate about laparoscopic surgery in EPT, and this is performed at a few centers (Berends et al., 2000). The role of this procedure in EPT has not yet been fully evaluated.

Even in the presence of metastases, the removal of tumor burden can be of value to decrease hormone secretion and perhaps also improve the prognosis (Musunuru et al., 2006, Yao et al., 2001). This can be accomplished either by surgical resection of liver metastases, or with newer techniques for debulking.

Debulking procedures
Debulking of liver metastases can be accomplished with radiofrequency ablation or embolization. Radiofrequency ablation can be done either intra-operatively or percutaneously, and metastases are selectively destroyed though targeted heating. This technique is particularly suitable for patients with only a small number of liver metastases. Tumor volume reduction is seen in a majority of patients, mortality is low and morbidity acceptable (Hellman et al., 2002, Siperstein & Berber, 2001, Siperstein et al., 1997). The procedure can be performed repeatedly.

Hepatic artery embolization can be performed to reduce tumor mass in patients with multiple liver metastases (Clouse et al., 1983). Metastases receive their nutrition from the systemic circulation while the normal liver tissue can function on portal circulation alone. Embolization can thus selectively cut off tumor blood supply. Objective responses are achieved with this treatment (Eriksson B et al., 1998, Granberg et al., 2007), but a prolongation of survival has not yet been shown. Monitoring liver enzymes is imperative after embolization in order to detect rare but severe side effects such as liver necrosis or cholecystitis. In some instances, debulking can also be done using cryotherapy.

Patients with pancreatic endocrine tumors often have multiple liver metastases. Radiation would need to be directed at the entire liver, and is thus
not a feasible strategy. It can however be successfully used in skeletal metastases in these patients.

**Systemic treatments**

For patients with metastases or locally advanced disease, there are a number of systemic treatment options available. This reflects the lack of curative treatments, as well as the lack of controlled studies regarding which regimen is superior.

Chemotherapy with the alkylating agent streptozotocin in combination with 5-fluorouracil (5-FU) produces significant tumor regression in about 20-63% and symptomatic improvement in about 50% of patients with metastatic EPT (Eriksson B. *et al.*, 1990, Moertel *et al.*, 1980). Streptozotocin plus doxorubicin has been suggested as a superior combination, with a reported response rate of 69% (Moertel *et al.*, 1992). Side effects include nausea and vomiting, and dose-related nephrotoxicity. These combinations are mainly used in well-differentiated tumors and carcinomas.


Historically, it was often impossible to control symptoms caused by tumor hormone secretion. Inoperable patients suffered, and often died, from their endocrine syndromes (insulinoma, gastrinoma, glucagonoma or VIP-oma syndrome). This changed radically with the introduction of somatostatin analogs in the 1980s (Long *et al.*, 1979). These bind to somatostatin receptors, which are expressed on most EPTs (Reubi *et al.*, 1987), inhibit hormone secretion into the bloodstream and thus ameliorate the symptoms caused by endocrine overproduction. Native somatostatin has a very short half-life and is not clinically useful. The long-acting analogs and slow-release preparations most commonly used today can be injected two to three times daily or once a month. Side effects include nausea and diarrhea. Somatostatin analogs are mostly given to patients with functioning tumors. Benign insulinomas frequently lack somatostatin receptor expression (Lamberts *et al.*, 1991). Somatostatin can relieve symptoms in patients with insulinoma syndrome, but sometimes hypoglycemia is aggravated, perhaps due to greater suppression of growth hormone and glucagon secretion compared to insulin (Maton *et al.*, 1989). Patients with spread or inoperable insulinomas where glucose control is a problem are sometimes helped by diazoxide or glucocorticoids. Gastrinomas, VIPomas and glucagonomas frequently express somatostatin receptors, and somatostatin analogs can often dramatically improve symptoms for these patients. In addition, PPIs have radically changed the outlook for patients with gastrinoma. While many gastrinoma
patients previously died from bleeding ulcers, ulcers can now be prevented, greatly improving survival (Jensen, 2004).

A possible antiproliferative effect of somatostatin has been debated. Stabilization of tumor growth has been demonstrated in patients with EPT (Aparicio et al., 2001). It has been suggested that an antiproliferative effect could be dose-related, and treatment with high-dose somatostatin analogs can sometimes lead to tumor cell necrosis (Eriksson B. et al., 1997, Imam et al., 1997).

Interferons are involved in the immune system, and recombinant interferon is used in the treatment of several diseases. There are three types of interferon: α, β and γ. Interferon α is used in the treatment of neuroendocrine tumors, and may control symptoms but also lead to tumor regression. Side effects include flu-like symptoms, bone-marrow suppression and occasional autoimmune reactions. In Uppsala, interferon α is often used as second-line treatment after progressive disease upon treatment with streptozotocin and 5-FU in well-differentiated EPT, and may lead to tumor regression in some patients (Bajetta et al., 1993, Eriksson B. & Oberg, 1993). Internationally, interferon α is, however, not widely used in the treatment of EPT. Recently, an antiproliferative effect of a novel group of interferons, interferon lambdas, on neuroendocrine cells was suggested (Zitzmann et al., 2006).

Radiolabeled somatostatin analogs
Somatostatin analogs labeled with radioactive indium, yttrium or lutetium are increasingly being used to selectively target tumor tissue. One study of treatment with 177Lu-labeled somatostatin analogs showed a 28% response rate in 131 patients with endocrine gastroenteropancreatic tumors (43 of these were pancreatic tumors). A time to progression of more than 36 months was reported in patients with at least stable disease as their best response (Kwekkeboom et al., 2005). Side effects include nausea and abdominal pain. To be suitable candidates for treatment with radiolabeled somatostatin analogs, tumors must have a density of somatostatin receptors higher than normal tissue.

Treatment of other neuroendocrine tumors
Thymic carcinoids are almost exclusively found in men. The few women with thymic carcinoids have the tumor as part of their MEN1 syndrome. Radical surgery is the only treatment of proven substantial value. Recurrences in spite of seemingly radical surgery are common, and thymic carcinoid carries a poorer prognosis than many other neuroendocrine tumors. There are no standard recommendations for chemotherapy, and experiences have mainly been negative, with the treatment having little or no effect (Gal et al., 2001, Spaggiari & Pastorino, 2001, Wang et al., 1994). One report,
however, demonstrated effectiveness of neoadjuvant radiotherapy and chemotherapy with cisplatin and etoposide (Filosso et al., 2004). Radiation therapy can be considered post-operatively, and in the case of threatening vena cava superior syndrome.

Bronchial carcinoids are separated into two clinical entities: typical carcinoids with a relatively good prognosis, and atypical carcinoids which are more malignant. The overall ten-year survival rate for patients with bronchial carcinoids has been estimated at 77%-87% (Chughtai et al., 1997, Godwin, 1975, McCaughan et al., 1985). Results from systemic treatment have generally been discouraging. Stabilization of disease has been shown after treatment with streptozotocin plus doxorubicin, and short-lived responses after treatment with cisplatin plus etoposide (Granberg et al., 2001). Recently, promising results from a study with $^{177}$Lu-labeled somatostatin analogs were published: partial remission in five out of nine patients, minor response in one and stabilization of disease in two patients (van Essen et al., 2007).

### Temozolomide

Temozolomide is an alkylating agent. It is spontaneously converted to its active metabolite, MTIC, which it shares with dacarbazine. Dacarbazine is used in the treatment of metastatic neuroendocrine tumors, and response rates of 25-30% have been reported in a group of mixed neuroendocrine tumors (Bajetta et al., 1998, Bajetta et al., 2002). The fact that the two drugs share the same metabolite suggests that temozolomide could also be of value in the treatment of neuroendocrine tumors.

Temozolomide has shown promising activity in melanoma (Bleehen et al., 1995), although superiority to dacarbazine could not be demonstrated in a large phase III trial (Middleton et al., 2000b). It penetrates the blood-brain-barrier, thus opening up the possibility of treating brain tumors and brain metastases. Temozolomide has become established as a first-line treatment for high-grade glioma, and has shown promising results in the treatment of brain metastases from solid tumors (Christodoulou et al., 2001).

A few studies of treatment with temozolomide in neuroendocrine tumors have recently been presented. They have all evaluated temozolomide as part of a combination therapy. Temozolomide plus thalidomide, an angiogenesis inhibitor, rendered an overall radiologic response rate of 25%, with a median duration of 13.5 months, in a recent phase II study including patients with metastatic neuroendocrine tumors of different kinds (Kulke et al., 2006b). In a retrospective study of treatment of EPT with a combination of temozolomide and capécitabine (a 5-FU prodrug) presented as an abstract at the 2006 American Society of Clinical Oncology (ASCO) meeting, 6% of patients had a complete response and 53% had a partial response, with a me-
median duration of 9.5 months (Isacoff et al., 2006). At the same meeting, data from a phase II study of temozolomide in combination with bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor and thus inhibiting angiogenesis, were presented. An overall response rate of 14% was seen (Kulke et al., 2006a). There are no published data on the effect of temozolomide as monotherapy in neuroendocrine tumors.

Unlike dacarbazine, which is an intravenous drug, temozolomide is taken orally. Reported side effects include myelosuppression, fatigue, nausea and headache (Middleton et al., 2000b). Temozolomide is better tolerated than dacarbazine, and thus possibly associated with a higher quality of life (Kiebert et al., 2003, Yung et al., 2000). This makes it an attractive candidate for palliative treatment, where quality of life is an especially important objective.

Since 1999, temozolomide has been given to selected patients at our clinic, as salvage treatment upon progression on standard treatments.

O6-methylguanine DNA methyltransferase

Temozolomide methylates DNA at the O6-position on guanine. This leads to incorrect pairing during DNA replication, activation of the mismatch repair system, and subsequent apoptotic cell death (D'Atri et al., 1998).

O6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme. It is expressed in several tissues, and is an important part of the body’s normal defense against cellular damage. It is also an important cause of cancer cell resistance to temozolomide. The MGMT molecule accepts the methyl group that temozolomide has placed on the DNA in an irreversible reaction, restoring the DNA to its original function. The cancer cell avoids the apoptosis that DNA methylation otherwise would have caused. Accepting the methyl group inactivates the MGMT molecule, which is then ubiquinated and undergoes proteolysis. New MGMT has to be synthesized de novo, which can take up to 72 hours (Payne et al., 2005).

Expression of MGMT in tumors is variable: sometimes lower but often higher than in normal tissue (Chen et al., 1992, Zaidi et al., 1996). Silencing of the MGMT gene by promoter methylation, resulting in a lower protein expression, is associated with a response to temozolomide in glioblastoma (Hegi et al., 2005). Low MGMT protein expression correlates with a response to temozolomide in oligodendrogliomas, and MGMT immunostaining has been suggested as a marker for predicting tumor chemosensitivity (Levin et al., 2006). MGMT promoter hypermethylation has been observed in small groups of EPTs and carcinoids (Chan et al., 2003, House et al., 2003). There are no immunohistochemical data on MGMT expression in neuroendocrine tumors, and neither are there any data regarding a possible
association between MGMT expression and response to temozolomide in these tumors.

Ghrelin

Synthetic growth hormone secretagogues have long been used to stimulate growth hormone release in situations of deficiency, e.g., short stature in children. They act via the growth hormone secretagogue receptor (GHS-R). Until recently, there was no known endogenous ligand for this receptor. This changed with the discovery of ghrelin in 1999. Ghrelin is a growth hormone-releasing and orexigenic peptide hormone (Kojima et al., 1999), and the name ghrelin is derived from “ghre”, meaning grow.

Ghrelin is produced mainly in X/A-like cells in the gastric mucosa (Date et al., 2000), but also in a vast array of other tissues, e.g., the pancreas, intestine, and kidney (Date et al., 2002b, Gnanapavan et al., 2002). There is some controversy over which cell type is responsible for pancreatic ghrelin production. Suggestions have included α cells, β cells and the newly identified epsilon (ε) cells (Date et al., 2002b, Prado et al., 2004, Wierup et al., 2002). In the fetus, gastric ghrelin production is low and ghrelin is produced mainly in the pancreas. After birth, the number of ghrelin-producing cells in the pancreas decreases. Unlike gastric ghrelin, pancreatic ghrelin expression is not affected by fasting/feeding (Kojima & Kangawa, 2005).

Two splice variants of the ghrelin receptor, GHS-R, have been identified: 1a and 1b. The 1a receptor is the functional receptor through which ghrelin exerts its effects. The 1b receptor has been widely believed to be inactive, but there have been suggestions of it having a function (Barzon et al., 2005). The existence of another, yet unidentified, ghrelin receptor has also been suggested (Baldanzi et al., 2002, Zhang et al., 2004).

The ghrelin receptor is expressed in high amounts in the hypothalamus (Guan et al., 1997), which is responsible for appetite control (Schwartz et al., 2000). Injection of ghrelin into the cerebral ventricles in rats, as well as intravenous or subcutaneous injections, stimulates food intake and decreases energy expenditure, resulting in body weight gain (Nakazato et al., 2001). Ghrelin from the gastrointestinal organs signals the central nervous system to stimulate feeding by stimulating production and secretion of the appetite stimulants NPY and AgRP in the arcuate nucleus of the hypothalamus (Nakazato et al., 2001). There is some evidence that peripheral ghrelin signals are transmitted to the central nervous system via the vagus nerve (Date et al., 2002a).

Plasma ghrelin levels rise upon fasting and fall dramatically within one hour after eating (Cummings et al., 2002, Tschop et al., 2001b). It is generally believed that ghrelin functions as an initiation signal for food intake. The extent of postprandial suppression is related to the amount of ingested
calories (Callahan et al., 2004). Individuals with low BMI have high fasting plasma ghrelin, and the obese have low levels (Tschop et al., 2001a). Low plasma ghrelin is associated with high insulin levels and diabetes type II, independently of BMI (Poykko et al., 2003). After gastric bypass surgery, performed to reduce body weight in severely obese individuals, total ghrelin secretion decreases by more than 50% before any weight loss takes place (Cummings et al., 2002). The lower ghrelin levels may be contributing to the good weight-loss results often achieved with this method. Ghrelin also influences gastrointestinal functions. It dose-dependently increases gastric acid secretion and stimulates gastric motility in rats (Masuda et al., 2000).

A large number of studies are now investigating the possible clinical applications of ghrelin. Its physiological properties suggest ghrelin could be of value in preventing or treating cardiac and cancer cachexia. Blocking its actions could prove a new approach to achieving weight loss in the obese. Increased proliferation after exposure to ghrelin has been shown in a pancreatic carcinoma cell line expressing the ghrelin receptor (Duxbury et al., 2003) and in prostate cancer cell lines (Jeffery et al., 2002), suggesting malignancies can be ghrelin-responsive. However, an inhibitory effect of ghrelin on cell proliferation in thyroid carcinoma cell lines has been suggested (Volante et al., 2003). Various tumors of endocrine origin express both ghrelin and its receptor (Papotti et al., 2001, Volante et al., 2002), and an autocrine/paracrine loop driving proliferation has been suggested (Jeffery et al., 2003). A stimulatory effect of ghrelin on tumor cell proliferation could of course prove a major obstacle to treatment of cancer cachexia with ghrelin.

Ghrelin immunoreactivity has been shown in a few series of EPTs. Between 25% and 40% of tumors showed ghrelin immunoreactivity (6 of 15, 11 of 28, and 3 of 12, respectively) (Raffel et al., 2005, Rindi et al., 2002, Volante et al., 2002). Expression of ghrelin receptor mRNA has been shown, but there are no immunohistochemical data. Analysis of serum ghrelin levels in a series of 24 patients with EPT found one patient with advanced stage cancer with extremely high serum ghrelin levels (Corbetta et al., 2003). No clinical syndrome attributable to the ghrelin overproduction has been found.

Patients with EPT seldom suffer from the cancer cachexia commonly seen in other types of cancer, even in advanced stages of the disease (House & Schulick, 2006). Perhaps in a subset of patients hypersecretion of the potent appetite-stimulant ghrelin could contribute to this relative well-being.

Gastrointestinal stromal tumors

For a long time, the origin of GISTs was uncertain (Connolly et al., 2003). They were thought to be related to smooth muscle tumors. Studies showed, however, that this group of tumors lacks muscle differentiation, and gradu-
ally the term gastrointestinal stromal tumor was adopted. Eventually, expression of the KIT tyrosine kinase receptor was demonstrated in GIST and a close relationship with the interstitial cells of Cajal was established (Corless et al., 2004, Kindblom et al., 1998, Sarlomo-Rikala et al., 1998). The interstitial cells of Cajal, originally identified by Santiago Ramón y Cajal in the 19th century, are a network of cells located between the muscle layers in the gastrointestinal tract. They are responsible for pacemaking and regulation of intestinal motility (Barajas-Lopez et al., 1989, Thuneberg, 1982), and are involved in neurotransmission (Ward & Sanders, 2006). Although originating from cells with neural characteristics, GISTs are sometimes called sarcomas. The term stromal tumor poorly reflects the origin of these tumors.

Clinically, the behavior of GISTs ranges from indolent to very aggressive tumor disease. It is not obvious which tumors will behave in a certain way, but according to a commonly used risk score, the risk of malignancy can be estimated based on tumor size and mitotic count (Fletcher et al., 2002). Histopathologically, GISTs have three different growth patterns: epithelioid, spindled or mixed. Mutations in the tyrosine kinase receptor KIT are seen in a majority of GISTs (Hirota et al., 1998), most commonly in exon 9 or 11. The mutation leads to constitutional activation of the receptor, driving proliferation. Other GISTs have activating mutations in the KIT-related kinase gene PDGFRA (Corless et al., 2004).

Surgery is the treatment of choice for all GISTs. Chemotherapy has little or no effect (Plaat et al., 2000), and until recently no meaningful treatment was available for patients with spread disease. A median survival of 129 months was reported from a large population-based study of GIST (Nilsson et al., 2005). This study included patients with tumors behaving in a benign fashion as well as malignant tumors, and the survival of patients with spread disease was considerably shorter. The outlook for patients with metastatic GIST changed radically with the introduction of selective tyrosine kinase inhibitors. The use of Imatinib mesylate in GISTs is the first example of successful treatment with a selective agent in solid tumors (Joensuu et al., 2001). Imatinib selectively targets the KIT receptor tyrosine kinase, inhibiting phosphorylation, and thus stops the downstream signaling that drives proliferation. It is now the first-line treatment for metastatic GIST, and is also used in an adjuvant setting (Bumming et al., 2003). FDG-PET is the radiology method of choice for evaluating response (Stroobants et al., 2003). For patients with tumors refractory to imatinib, sunitinib, another receptor tyrosine kinase inhibitor, is the recommended second-line treatment (Goodman et al., 2007).

There is an interesting association between GISTs, hereditary cancer syndromes and neuroendocrine tumors. Patients with neurofibromatosis 1 (NF1) have an increased risk of developing GISTs. Mutations of NF2 have been reported in single cases of GISTs (Fukasawa et al., 2000). GISTs can also occur with paraganglioma and pulmonary chondroma in the setting of Car-
ney triad (Bumming et al., 2006, Carney et al., 1977). The interstitial cells of Cajal express cholecystokinin, tachykinin and somatostatin receptor subtypes (Lecci et al., 1999, Patterson et al., 2001, Sternini et al., 1997) as well as 5-HT3 receptors (Glatzle et al., 2002). Expression of other peptide hormone receptors, e.g. bombesin subtype 2 and vasoactive intestinal peptide subtype 2 receptors, has been demonstrated in GISTs (Reubi et al., 2004). Expression of SV2, a neuroendocrine marker, has been reported in eight cases of GIST (Jakobsen et al., 2002).
Aims of the study

The specific aims of the study were:

- to evaluate survival and potential prognostic factors, and describe patient and tumor characteristics in a large group of patients with pancreatic endocrine tumors, treated at a single institution.

- to evaluate the efficacy and toxicity of temozolomide in metastatic or inoperable malignant neuroendocrine tumors, and examine tumor expression of O6-methylguanine DNA methyltransferase.

- to study the expression of the orexigen ghrelin and its receptor in pancreatic endocrine tumors, and to evaluate a possible association between ghrelin expression and survival or BMI.

- to study potential neuroendocrine features, such as the expression of ghrelin and its receptor, in gastrointestinal stromal tumors, as well as to evaluate a possible association with BMI or signs of malignancy.
Materials and methods

Patients and tumors

Paper I
The medical records for all patients treated for EPTs at the Clinic of Endocrine Oncology, diagnosed between 1967 and 2005 were examined. Nine patients were excluded due to incomplete data. Three-hundred-and-twenty-four patients with confirmed EPTs, consecutively treated at our clinic, were retrospectively evaluated. Data regarding survival and potential prognostic factors were retrieved from medical records. Survival was defined as the time from diagnosis to the last date of follow-up or death from any cause. Tumors were classified into three groups (well-differentiated NE tumor, well-differentiated NE carcinoma and poorly differentiated NE carcinoma) according to the number of mitoses, percentage of Ki67 positive cells, and the presence of gross invasion. TNM staging was based on information in medical records.

Paper II
Patients with histologically confirmed metastatic or inoperable malignant neuroendocrine tumors treated with temozolomide between October 1999 and January 2006 (n=36) were retrospectively evaluated. Tumor tissue for immunohistochemistry was retrieved from the department of pathology. Seven patients had thymic carcinoid, 13 bronchial carcinoid, 1 gastric carcinoid, 12 EPT, 1 NE foregut tumor, 1 paraganglioma and 1 NE cecal cancer. Six patients had brain metastases. In most cases, temozolomide was given after progression on standard treatments. Patients had received a mean of 2.4 previous systemic treatment regimens.

Paper III
Thirty-one patients with verified EPTs were identified, and frozen tumor tissue was retrieved from the Endocrine Oncology Unit’s tumor bank. Nine
patients had MEN1-related EPTs and 22 had sporadic tumors. Twenty-seven tumors were well-differentiated NE carcinomas, two were poorly differentiated NE carcinomas, and two were well-differentiated NE tumors.

Paper IV
Twenty-two patients with verified GISTs were included. Due to the limited availability of tumor material, both paraffin-embedded blocks (n=12) and fresh frozen surgical specimens (n=10) were used.

Temozolomide

Treatment
Patients were treated with temozolomide, in most cases after standard treatment options had failed. The drug was administered orally for five consecutive days every 28 days. The first cycle consisted of 100 or 150 mg/m²/day. In subsequent cycles, the dose was escalated to the recommended 200 mg/m²/day, unless there was bone marrow suppression from previous chemotherapy. In the absence of disease progression and unacceptable toxicity, cycles were repeated every four weeks on an outpatient basis. Tropisetron was routinely given as an antiemetic.

Recording of toxicity
Hemoglobin, leukocytes and thrombocytes were measured at least at nadir, which is day 21 of each cycle. Data regarding toxicities and adverse events were gathered from medical records, and toxicity grading was based on National Cancer Institute Common Toxicity Criteria.

Evaluation of treatment
Radiologic evaluation of response was done after every third treatment cycle, with CT or MRI. In two patients, response was followed using ultrasonography. Radiologic tumor response was classified according to the Response Evaluation Criteria in Solid Tumors (Tsuchida & Therasse, 2001). Complete response was defined as the disappearance of all lesions, and partial response as a decrease of 30% or more in the sum of the greatest diameters. Progressive disease was defined as either the appearance of new lesions or an increase of 20% or more compared to the minimum sum of the greatest diameters recorded since the start of treatment. A sum increasing less than 20% or decreasing less than 30% and without the appearance of new lesions
was considered stable disease. Response was confirmed by a repeat measurement four weeks or more after the criteria for response were first met. Time to progression was defined as the time between the first administration of temozolomide and the recording of progressive disease. Biochemical response was assessed by analysis of the neuroendocrine marker CgA in plasma.

Immunohistochemistry

Paper II

MGMT protein expression was investigated in 23 tumors. Paraffin-embedded sections of four µm were used for immunohistochemistry. For antigen retrieval, sections were subjected to pretreatment with 45 minutes of pressure boiling in a citrate buffer (pH 6.0). Immunohistochemistry was performed using an autostainer (DakoCytomation, Carpinteria, CA). Sections were incubated with a mouse monoclonal MGMT antibody (MAB16200, Chemicon, 1:500), diluted in Antibody Diluent (DakoCytomation), at room temperature for 60 min. The reaction product was revealed using Dako kit 50087 (DakoCytomation). Sections were counterstained with Mayer’s hematoxylin. Initial experiments were performed with the omission of the primary antibody. All sections were scored by two individuals, blinded for outcome, according to the fraction of nuclear staining, as high (≥50%), intermediate (10-49%) or low (<10%).

Papers III and IV

Frozen tumor tissue and paraffin-embedded blocks were used for immunohistochemistry. Frozen sections (6 µm) were fixed in acetone, and incubated overnight with rabbit anti-ghrelin (H-031-30, Phoenix Pharmaceuticals, Belmont, CA; 1:2400), or rabbit anti-GHS-R (H-80, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200), diluted in PBS with 1% BSA. The reaction product was visualized using a biotinylated secondary rabbit antibody, VECTASTAIN® Elite ABC (Vector, Burlingame, CA) and the chromogen 3-amino-9-ethylcarbazol. For antigen retrieval, the paraffin-embedded sections were subjected to pretreatment (2 x 5 min microwave heating at 900W in TRIS-EDTA at pH 9). Sections were incubated with rabbit anti-human ghrelin (GHS-11-A, Alpha Diagnostics; San Antonio, TX, 1:50), diluted in Antibody Diluent (DakoCytomation, Glostrup, Denmark), or goat anti-GHS-R (F-16, Santa Cruz Biotechnology, 1:200) diluted in PBS with 1% BSA at room temperature for two hours. The reaction product was revealed using the Envision+ System with DAB as chromogen (DakoCytomation). All sec-
tions were counterstained with Mayer’s hematoxylin. Initial experiments with each antibody were performed with or without the inclusion of the primary antibody. All sections were examined by a pathologist. Ghrelin immunoreactivity was graded as negative, 1-25% positive cells, 26-75% positive cells or more than 75% positive cells. GHS-R immunoreactivity was graded as negative or positive.

Real-time quantitative PCR

Papers III and IV
Total RNA from frozen tumors was isolated using Trizol (Invitrogen, Carlsbad, CA). Consecutive slides were stained with Mayer’s hematoxylin and studied with light microscopy to ensure adequate tumor sampling and avoid the inclusion of normal tissue. The quantity of RNA was assessed with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the quality with a BioAnalyzer (Agilent Technologies, Palo Alto, CA) (Paper III). Complementary DNA (cDNA) was synthesized with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Commercially available primer and probe sets were used (Hs00175082_m1, Hs00372069_m1, Hs0026978_s1 and Hs01026313_m1, Applied Biosystems, Foster City, CA) and measured against standard curves generated from dilution series of pancreatic endocrine tumor cDNA, human pooled cDNA or human fetal brain cDNA from Human Total RNA Master Panel II (636643, BD Biosciences, San Jose, CA). Reactions were performed and analyzed using an Applied Biosystems PRISM 7700 Sequence Detector. Standard cycling conditions were used (Heid et al., 1996). The gene-specific signals were normalized to that of the β-actin, 18S or HPRT housekeeping genes to correct for differences in RNA quantity. As a negative control, cDNA was omitted and replaced with water. This was done in all qPCR experiments. All TaqMan assay reagents were obtained from Perkin-Elmer Applied Biosystems.

Radioimmunoassay

Paper III
Fasting plasma levels of total ghrelin were analyzed in 26 patients. At the time of treatment at our clinic, blood had been drawn into chilled tubes containing sodium heparin and 400 units/mL kallikrein inhibitory aprotinin,
centrifuged at 4°C, and frozen within one hour. Samples had been stored for up to 19 years at -70°C. A commercial radioimmunoassay kit (Linco Research Inc., St. Charles, MO) was used for analyses. Fresh plasma from five healthy individuals was used as a control. Elevated levels were defined as the control group mean +2 SD. Plasma ghrelin response to a standardized carbohydrate-rich meal test (560 kcal), with measurements of plasma ghrelin at -5, +0, +10, +20, +30, +45 and +60 min (Skogseid et al., 1987), was analyzed in three patients as well as the control subjects.

Statistics

Paper I
Kaplan-Meier methodology was used to estimate survival, and the log-rank test was used to test differences in survival. Multiple Cox regression models were used to explore the independent effects of several prognostic factors. The proportional hazards assumption was assessed using a graphical approach. p < 0.05 was considered significant.

Paper II
Survival and time to progression estimates were calculated using Kaplan-Meier methodology. The log-rank test was used to test the null hypothesis that time to progression was equal in patients with bronchial carcinoids, thymic carcinoids and pancreatic endocrine tumors. Differences in response rates between tumors with a high or low expression of MGMT were tested using the $\chi^2$ test. p < 0.05 was considered significant.

Papers III and IV
The $\chi^2$ test was used to test the null hypothesis that there was no difference in the frequency of immunoreactivity between groups. Differences in mRNA expression, plasma ghrelin levels and BMI were tested with the Mann-Whitney, one-way ANOVA and Kruskal-Wallis tests. Survival was estimated using Kaplan-Meier methodology, and the log-rank test was used to test differences in survival. p < 0.05 was considered significant.

Ethical approval
All included studies were approved by the appropriate ethical review boards.
Results

Paper I

Patient and tumor characteristics
A small majority of patients, 55%, were men. Eighty-four percent of tumors were sporadic, 15% MEN1-related and 1% part of von Hippel-Lindau disease. Non-functioning tumors were the most common (59%) followed by insulinoma (17%), gastrinoma (13%), glucagonoma (6%) and VIPoma (5%). One percent of tumors produced a Cushing syndrome. Sixty percent of tumors had distant metastases at presentation. Forty-two percent of the patients came from our uptake area and 58% were external referrals. The median size of the primary tumor was 4 cm (range, 0.3-17). The median age at diagnosis was 53 years (range, 12-86), and the median BMI was 24.0 (range, 15-43).

Survival
The median overall survival in patients with EPT treated at the Endocrine Oncology Unit was 99 months (95% CI 82-116). The 5- and 10-year survival rates were 64% and 44%, respectively. Patients with well-differentiated NE tumors had a 5-year survival of 93% and a 10-year survival of 93%. Patients with well-differentiated NE carcinomas had a 5-year survival rate of 62% and a 10-year survival rate of 54%, and patients with poorly differentiated NE carcinomas a 5-year survival rate of 17% and a 10-year survival rate of 6%. There was no significant difference in survival between patients diagnosed before or after 1995.

Factors of prognostic value in univariate analysis
Several variables had a significant impact on survival in univariate analysis (Table II, Fig. 1).
Fig. 1. Overall survival (months), comparison of TNM stages.

### Table II. Prognostic factors significant in univariate analysis.

<table>
<thead>
<tr>
<th>Factor</th>
<th>HR  (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>4.8 (3.3-7.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Well diff. carcinoma</td>
<td>4.2 (2.3-7.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Poorly diff. carcinoma</td>
<td>14.6 (6.9-30.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-functioning tumor</td>
<td>1.6 (1.2-2.2)</td>
<td>0.004</td>
</tr>
<tr>
<td>Ki67 ≥ 2%</td>
<td>6.0 (2.7-13.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CgA ≥ 3xUNL</td>
<td>2.6 (1.6-4.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI &lt; 20</td>
<td>2.5 (1.3-4.7)</td>
<td>0.007</td>
</tr>
<tr>
<td>Primary referral</td>
<td>1.4 (1.0-1.9)</td>
<td>0.023</td>
</tr>
<tr>
<td>Sporadic tumor</td>
<td>2.7 (1.7-4.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stage IIIa</td>
<td>4.6 (1.8-12.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Stage IIb</td>
<td>2.6 (1.2-5.5)</td>
<td>0.011</td>
</tr>
<tr>
<td>Stage IV</td>
<td>6.5 (3.5-12.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Size 3-3.9 cm</td>
<td>3.1 (1.7-5.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Size 4-4.9 cm</td>
<td>2.2 (1.2-4.2)</td>
<td>&lt;0.014</td>
</tr>
<tr>
<td>Size 5-9.9 cm</td>
<td>3.6 (2.3-5.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Size ≥ 10 cm</td>
<td>3.8 (1.9-7.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age ≥ 60 yrs</td>
<td>1.7 (1.2-2.3)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

HR, hazard ratio; diff., differentiated; UNL, upper normal limit.
Multivariate analysis

Five multiple Cox models were used. The first included the variables evaluated in univariate analysis, except Ki67, CgA and BMI, where there were far fewer observations (115, 139 and 155, respectively). In this analysis, five variables retained their significant prognostic impact (Table III).

Table III. Independent prognostic factors.

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>3.3 (1.6-7.2)</td>
<td>p=0.002</td>
</tr>
<tr>
<td>Poorly diff carcinoma</td>
<td>12.3 (3.5-43)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Primary referral</td>
<td>1.9 (1.2-3.1)</td>
<td>p=0.007</td>
</tr>
<tr>
<td>Stage IV</td>
<td>7.9 (1.6-38)</td>
<td>p=0.010</td>
</tr>
<tr>
<td>Age ≥ 60</td>
<td>2.9 (1.7-4.8)</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

A second multiple model (n=93) was used to evaluate the prognostic value of Ki67. Here, only Ki67 ≥2% was a significant negative prognostic variable (HR 5.2, p=0.002). However, the absence of surgery had a higher hazard ratio (8.4) and was borderline significant (p=0.057). The material did not permit inclusion of WHO classification and stage in this analysis.

The negative prognostic impact of a chromogranin A three times the upper normal limit or more remained when corrected for age and sex (HR 2.2, 95% confidence interval, 1.3-3.8, p=0.004). The negative prognostic impact of being underweight (BMI <20) also remained when corrected for age and sex in multivariate analysis (HR 2.4, 95% confidence interval, 1.3-4.7, p=0.008).

In a multiple model including only well-differentiated NE carcinomas, Ki67 ≥2% (HR 3.7, p=0.009) and size ≥5 cm (HR 4.9, p=0.011) were negative prognostic factors, corrected for age, sex, presence of endocrine syndrome and heredity. The material did not permit the inclusion of TNM staging in this analysis.
Efficacy of temozolomide

Radiologic response
Five patients (14%) had a partial response (4 bronchial carcinoids, 1 EPT). Nineteen patients (53%) had stable disease as the best response, and 13 of these were still stable at the end of the clinical follow-up reviewed in this study. Twelve patients had progressive disease as the best response to therapy. Of these, three patients had died before the first radiologic evaluation and one did not complete the first cycle of treatment. Median time to progression was seven months (95% confidence interval, 3-10). There was no significant difference in the time to progression between patients with bronchial carcinoid, thymic carcinoid and EPT. Only one of the five patients with a radiologic response had progressed at follow-up after 8.5 months. The remaining four did not have tumor progression during follow-up, and were still stable after a median of 7.3 months (range, 2.5-13).

Biochemical response
Results from repeated measurements of plasma CgA were available in 27 patients with elevated baseline CgA. Five patients (19%) had a biochemical response, i.e., more than 50% decrease in CgA, 10 (37%) had stable levels and 12 (44%) had biochemical progression as the best response.

Survival
At the end of the clinical follow-up for this study, 13 of 36 patients had died. The median overall survival, as estimated by Kaplan-Meier methodology, was 16 months (95% confidence interval, 11-22). Considering the limited follow-up, this might be an overly pessimistic estimate.

Toxicity
Patients received a median of 4.5 cycles of temozolomide (range, 0-17). Medical records were available for the assessment of toxicity in 35 patients. There were no fatal side effects. Toxicity was mainly hematologic, with 32% experiencing anemia, 37% thrombocytopenia, 15% leucopenia and 6% neutropenia. Dose reduction due to hematologic toxicity was needed in four patients. Three patients required blood transfusions, and one patient required platelet transfusions. No patient had febrile neutropenia, opportunistic infections or bleeding due to low platelets. Forty-one percent experienced fatigue and 29% nausea, but both were mild in the majority of patients.
One patient with a VIP-producing EPT suffered severe diarrhea and dehydration, and required treatment in an intensive care unit due to excessive VIP secretion from the tumor after only one temozolomide tablet. Two patients experienced seizures and one suffered mycoplasma pneumonia.

Expression of O\(^6\)-methylguanine-DNA methyltransferase
Nine of the 23 analyzed tumors had a high percentage of MGMT immunoreactive nuclei. Of these nine patients, one had a radiologic response to treatment with temozolomide, five had stable disease, and three had progressive disease as the best response. Four tumors had medium MGMT immunoreactivity. All patients with such tumors had stable disease as the best response to temozolomide. Ten tumors had a low percentage of MGMT immunoreactive nuclei. Of these patients, four had radiologic responses, five stable disease and one progressive disease as the best response to therapy. However, no significant differences in response rates could be shown.

Paper III
Immunohistochemistry
Twenty-one of 31 examined EPTs expressed ghrelin. Expression was mainly cytoplasmic. There was no significant difference in the frequency of immunoreactivity between MEN1-related and sporadic tumors. Neither was there any difference between functioning and non-functioning tumors.

Expression of the ghrelin receptor was seen in 21/30 analyzed tumors. There was no significant difference in the frequency of expression between MEN1-related and sporadic tumors. Neither was there any difference between functioning and non-functioning tumors. Fifty percent of the tumors in our material had concomitant expression of ghrelin and the receptor.

qPCR
Ghrelin mRNA was detected in 19/20 analyzed tumors. There was no significant difference between MEN1-related and sporadic tumors, or between functioning and non-functioning tumors.

Total GHS-R (1a + b) mRNA was detected in all 20 analyzed tumors. The relative levels among the tumors varied substantially, with a factor of close to 400. Insulinomas, however, had a higher expression of total receptor mRNA (mean, 12.5 times higher; median, 9.6) compared to non-insulinomas (p<0.05). GHS-R 1a mRNA was detected in 13/20 tumors. Insulinomas had a higher expression than non-insulinomas (mean, 45 times higher; median,
There was no difference in expression of either total receptor or receptor 1a between MEN1-related and sporadic tumors.

**Plasma ghrelin**

Our control group had a mean plasma ghrelin of 952 ± 164 ng/l (median, 865; range, 823-1188), which corresponds well to levels reported in the literature (Kojima & Kangawa, 2005, Morinigo et al., 2004, Otto et al., 2005). No significant difference was found between patients and controls. The former had a mean plasma ghrelin of 908 ± 569 ng/l (median, 756; range, 121-2306). Neither was there any difference between patients with immunohistochemically positive or negative tumors, nor between patients with functioning or non-functioning tumors. Patients with MEN1-related tumors had significantly higher plasma ghrelin levels compared to patients with sporadic tumors.

Five patients (two sporadic, three MEN1) had elevated plasma ghrelin (defined as >2 SD above our control group mean; thus levels exceeding 1280 ng/l were considered elevated). These patients had a mean BMI of 25.6 ± 6.4 (median, 23.3; range, 20.9-34.9), and tumor masses varying from only biochemical signs of recurrent disease to multiple liver metastases, at the time of plasma ghrelin sampling. Three of these tumors were ghrelin immunoreactive.

No difference was seen between patients and controls in the plasma ghrelin response to a standardized carbohydrate-rich meal.

**BMI**

Mean BMI among patients was 24.3 ± 3.9 (median, 24.1; range, 16.3-32.8) Patients with ghrelin immunoreactive tumors had a mean BMI of 24.5 ± 4.4, compared to a mean BMI of 23.9 ± 2.7 in patients with tumors without ghrelin immunoreactivity. Patients with non-functioning tumors had a mean BMI of 23.1 ± 3.8, compared to patients with functioning tumors; mean 25.3 ± 3.8. MEN1 patients had a mean BMI of 23.9 ± 4.4 whereas patients with sporadic tumors had a BMI of 24.5 ± 3.7. None of these numeric differences were statistically significant.

**Survival**

Mean survival from diagnosis was 193 months (median, 276; 95% confidence interval 150-273). There was no difference in survival between patients with ghrelin immunoreactive and non-immunoreactive tumors. Neither was there any difference between patients with tumors coexpressing ghrelin and the receptor and tumors with no coexpression.
Paper IV

Immunohistochemistry

Seventeen out of 22 GISTs expressed ghrelin protein, as determined by immunohistochemistry (Fig. 2). Expression was cytoplasmic and showed a granular pattern. No association was seen between tumor morphology, location, size or KIT genotype and ghrelin expression. Neither was there any association between expression and mitotic rate, Ki67, or risk score.

The ghrelin receptor was expressed in five out of ten analyzed tumors, and all five had concomitant ghrelin expression. No association was seen between receptor expression and risk score, Ki67, mitotic rate, tumor location, size, or morphological type.

Fig. 2. Light microscopy of a GIST tumor with a strong immunoreactivity for ghrelin (frozen tissue, x400).

qPCR

The mean ghrelin mRNA level in GISTs was similar to those in EPTs used as controls, as was the mean ghrelin receptor level. SV2 mRNA expression was found in all six analyzed GISTs. The mean SV2 level in GISTs was also similar to that seen in EPTs.
BMI

Mean preoperative BMI, assessed in 14 patients, was 25.1 ± 3.1 (range, 20.8-31.1; median, 25.2). Thus, this patient group was slightly overweight according to international definitions. There was no difference in BMI between patients with tumors expressing ghrelin protein or not. More than half of the patients were overweight (BMI >25), and two patients were obese (BMI >30). Both obese patients had ghrelin immunoreactive tumors. No patient was underweight (BMI <20).
Discussion

Paper I

We report survival data and evaluate prognostic factors in 324 patients with EPT, treated at a single center. Considering the unusual nature of this disease, this constitutes a very large patient material. Over the years, the Endocrine Oncology Unit in Uppsala has acquired a vast patient base, enabling this kind of comprehensive study.

Median overall survival was 99 months. This is similar to the figure reported from our department in 1989 (104 months), and no significant difference in survival before and after 1995 was seen. Not much seems to have happened regarding survival in the 18 years that have passed since the last study. Does this mean that treatment, and thus survival, has not improved at all? Not necessarily. In the last few years, more patients with poorly differentiated endocrine carcinomas, highly aggressive tumors, have been diagnosed at our clinic or referred to us. We believe an improvement in diagnostic tools and an increased awareness of this tumor entity among clinicians has led to an increased frequency of correct diagnosis of these patients, previously often misdiagnosed as exocrine pancreatic cancer. With this increase in more malignant endocrine carcinomas being treated at our clinic, it would be reasonable to expect that survival would have diminished significantly compared to the previous study from 1989. However, this is not the case. This might be a reflection of improved treatment, but our data cannot answer this question.

Reported frequencies of non-functioning tumors from other institutions have increased from 15-24% in the 1980s (Eriksson B. et al., 1989, Kent et al., 1981), to over 60% in recent reports (Hochwald et al., 2002, Tomassetti et al., 2005), and a similar increase has been observed at our clinic. In fact, after 2000, the frequency is 74%. We believe this is partly due to a change in the use of the terms functioning and non-functioning. Tumors not causing any endocrine syndrome, today correctly defined as non-functioning, were previously sometimes classified as functioning merely on the basis of immunoreactivity or raised plasma levels of a certain hormone. Another reason is the increased recognition of poorly differentiated tumors as neuroendocrine, mentioned above. The prognostic significance of a tumor-associated endo-
Crine syndrome has been debated. Clinically, it has been widely accepted that non-functioning tumors are more aggressive than their functioning counterparts. In univariate analysis, having a non-functioning tumor was indeed a significant negative prognostic variable. In multivariate analysis however, when corrected for the effect of a number of other variables, non-functioning tumors did not carry a worse prognosis. It appears that the shorter survival of patients with non-functioning tumors is better explained by other factors than the functional status of the tumor per se. Prediction of the malignant potential of an EPT thus should not be influenced by the presence or absence of hormonal symptoms, but rather be guided by other prognostic factors, such as stage, the possibility of radical surgery, WHO classification, tumor Ki67, and patient age.

Patients with EPT as part of their MEN1 syndrome often do better than patients with sporadic tumors. That is, they have a longer survival from the date of diagnosis. This is sometimes used as an argument for less aggressive treatment of these tumors (Norton et al., 2006). But since MEN1 patients are often diagnosed with EPTs early in life, a longer survival from diagnosis does not automatically translate into a longer life overall. MEN1 patients have a significantly shortened life expectancy compared to age-matched healthy controls (Dean et al., 2000), and EPT is a common cause of death among these patients (Doherty et al., 1998). Furthermore, in multivariate analysis of our material, hereditary disease was not a significant positive prognostic factor. This implies that MEN1-related EPT should be managed similarly to sporadic tumors, and should not be considered inherently less malignant simply on the basis of heredity alone. It also supports the notion that early screening and surgery on pancreatic lesions is crucial.

We found a Ki67 of $\geq 2\%$ to be a significant negative prognostic factor, in both univariate and multivariate analysis, providing confirmation in a larger material of previous reports suggesting this cut-off to be of clinical value (La Rosa et al., 1996, Panzuto et al., 2005). The material did not permit the inclusion of WHO classification and stage in our multivariate analysis evaluating Ki67, due to the high correlation between these variables. Since Ki67 evaluation was only widely performed at our clinic from 1998, the analysis of Ki67 includes mostly patients with a more recent diagnosis. In this cohort, there were slightly more men, slightly more patients with poorly differentiated NE carcinomas, and more patients presenting with a stage III or higher tumor compared to the entire patient material. The number of patients with non-functioning tumors in this group was 80%.

A TNM classification was recently suggested for EPT. No clinical evaluation has yet been published. In univariate analysis, we found stage IIIa, IIIb and IV to be significant negative prognostic factors when compared to stage I. Stage IV (distant metastases) was still highly significant when corrected for other factors in multivariate analysis, and there was a tendency towards stages IIb, IIIa and IIIb having a negative impact on prognosis, even though
this did not quite achieve statistical significance. The proposed staging system appears to be of clinical value. We found a tendency towards stage IIIa meaning a worse prognosis than IIIb. The invasion of large vessels (IIIa) seems to be a more ominous sign than mere lymph node metastases (IIIb). If this finding is confirmed in future studies, perhaps an adjustment of the definitions of TNM staging for EPTs should be considered. The prognostic significance of stage IV appears unambiguous, but investigation of an even larger patient material is needed to further evaluate the prognostic value of stages II and III.

It is hardly surprising that patients who have undergone macroscopically radical surgery have a better prognosis than patients who have not. This is of course due to the beneficial effects of surgery, but also represents the fact that the patients who undergo primary surgery are those with a limited tumor burden. The negative prognostic effect of the absence of macroscopically radical surgery remained valid after correction for the effects of tumor stage.

The WHO classification divides EPTs into three groups. In univariate analysis, both well-differentiated and poorly differentiated carcinoma had a significantly worse prognosis compared to the most benign group. In multivariate analysis, only poorly differentiated carcinoma was a significant risk factor. In fact, this prognostic factor had the highest hazard ratio of all evaluated variables. The WHO classification is indeed helpful in selecting patients with a good or very bad prognosis. However, in our material the vast majority (72%) of patients had well-differentiated NE carcinomas. This large group of patients is very heterogeneous, and the WHO classification is of little help to the clinician when advising the individual patient with a well-differentiated NE carcinoma about his or her prognosis. Prognostic markers identifying patients with a better or poorer prognosis within this group are sorely needed. A separate multivariate analysis including only patients with well-differentiated NE carcinomas showed a prognostic value of Ki67 ≥2% and tumor size ≥5cm. These variables should be considered when assessing the prognosis of patients with well-differentiated NE carcinoma.

Chromogranin A elevated three times the upper normal limit or more was a negative prognostic factor when corrected for age and sex. A prognostic value of elevated chromogranin A at diagnosis in midgut carcinoids is known (Janson et al., 1997), but a potential prognostic value in EPTs has not previously been satisfactorily evaluated.

The median BMI at diagnosis was 24.0, illustrating that patients with EPTs are generally not cachectic at presentation. Being underweight was a significant risk factor in both univariate and multivariate analysis. This is no surprise: cancer cachexia is of course not a good sign. However, a prognostic relevance of BMI in EPT has not previously been reported.

An unexpected finding was that patients from our primary uptake area had significantly shorter survival compared to externally referred patients, both in univariate analysis and when corrected for stage, WHO classifica-
tion, age and tumor size for example. When tumor Ki67 was entered in to the Cox model, the hazard ratio was 1.7, even though this was no longer significant. We believe that this difference in survival represents a selection bias; patients with an apparently poor prognosis are less often referred to a tertiary referral center, due to the increased cost and effort for the patient and the referring center.

Paper II

In this paper, we report the results of a retrospective evaluation of treatment with temozolomide in 36 patients with advanced inoperable neuroendocrine tumors.

Treatment with temozolomide rendered an overall objective radiologic response rate of 14%, and stabilization of tumor growth in another 53%. There are, of course, limitations to a retrospective study design. Most notably, there may have been a selection in the choice of which patients would receive temozolomide. However, these results are still promising. Patients were heavily pretreated, having received a mean of 2.4 previous palliative antitumoral medical regimens. Temozolomide was in most cases given as a last option when standard treatments had failed, and for a total of 67% of the patients to benefit from treatment at this stage is a very good result indeed.

Patients with bronchial and thymic carcinoids showed clinical benefit (response or stabilization) in 62% and 71% of cases, respectively. This was similar to patients with EPTs. However, the treatments currently available for these two unusual tumor types are fewer than for EPTs. Most studies have shown chemotherapy to have little or no effect in thymic carcinoids and there are no established treatments for bronchial carcinoids with any impressive results. The addition of temozolomide as a potential new treatment for these tumor types could prove to be of great value. Promising results from a study with 177Lu-labeled somatostatin analogs in bronchial carcinoid were recently published. The respective roles of temozolomide and radiolabeled somatostatin analogs in the treatment of bronchial carcinoids remain to be established.

In three recently published reports, combinations of temozolomide and thalidomide, bevacizumab or capecitabine rendered response rates of 14-59% (Isacoff et al., 2006, Kulke et al., 2006a, Kulke et al., 2006b). It is hard to compare the different studies and assess the impact of combination therapies, since the studies differ in a number of important respects. Firstly, the studies use different schedules of temozolomide administration. In both studies, Kulke et al. used a more dose-intense temozolomide schedule, with 150 mg/m² for seven days every other week, while we used the standard schedule of 200 mg/m² for five days every four weeks. It has been suggested that a more compressed, dose-intense schedule might be preferable, perhaps be-
cause it causes a more complete MGMT depletion (Middleton et al., 2000a). However, this remains to be established, and so far 200 mg/m\(^2\) for five days every four weeks is the recommended regimen. Secondly, the different studies include variable patient material. The study of temozolomide plus thalidomide included patients with carcinoids, EPTs and pheochromocytoma. Only one carcinoid patient (7\%) had a response, while we found patients with bronchial carcinoids to be frequent responders, with 31\% demonstrating an objective response. These differences could be due to different patient material; the former study does not say what types of carcinoids were included. Thirdly, the number of treatments previously received by patients differs. In the temozolomide plus thalidomide study, patients had previously received a mean of 0.8 chemotherapy regimens, while in our study the corresponding figure was 2.4. The possibility of achieving better results with different combination therapies or a variation of the schedule for temozolomide administration is interesting, and further studies in this area will surely be conducted.

In general, treatment with temozolomide was well tolerated. Mainly mild hematologic toxicity was seen. Fatigue and nausea were acceptable in the majority of patients. It is our experience that for a large majority of patients, treatment with temozolomide led to improved well-being. This being a retrospective study, no quality of life evaluations could be made. However, superior quality of life on treatment with temozolomide compared to dacarbazine has been demonstrated in patients with other tumors (Kiebert et al., 2003, Yung et al., 2000). The improved well-being in our patients was due to a combination of tumor regression, fewer side effects compared to previous chemotherapy, and not being subjected to repeated hospitalization. In palliative cancer treatment, good quality of life is of the utmost importance. The possibility of taking an oral medication in one’s own home instead of receiving intravenous treatment in a clinical setting is likely to contribute to this.

The two patients experiencing seizures proved to have brain metastases. However, seizures have been reported as a side effect of temozolomide treatment (Chamberlain et al., 2004), and the possibility that temozolomide triggered the seizures in these patients cannot be ruled out. In addition we have recently experienced severely impaired hearing in two patients treated with temozolomide after the end of the defined observation period of this study.

Tumors with a low expression of MGMT, as measured either with methylation-specific PCR or with immunohistochemistry, respond better to temozolomide than tumors with abundant expression (Hegi et al., 2004, Hegi et al., 2005, Levin et al., 2006). Methylation-specific PCR for MGMT has been more extensively evaluated. However, immunohistochemistry is more relevant for use in a clinical setting, since formalin-fixed tissue is easier to obtain than frozen tissue in routine clinical practice. Immunohistochemistry also eliminates the risk of contamination from surrounding normal tissue.
We therefore chose the latter method to evaluate MGMT expression. We found a low percentage of MGMT positive tumor cell nuclei in 43% of tumors. Of patients with a radiologic response to temozolomide, 80% had tumors with low MGMT expression. It seems that endocrine tumors follow the pattern seen in gliomas, and respond better to temozolomide if they have a low MGMT expression. However, this was not quite significant in this small material. Most likely, further investigation of a larger patient cohort would reveal a significant association between MGMT expression and response to temozolomide. If so, immunohistochemistry could become an important tool in guiding the clinician in decisions regarding whether or not the patient might benefit from treatment with temozolomide, and perhaps in choosing different dosages for different patients.

**Paper III**

We here report the first immunohistochemical data on the expression of GHS-R in EPT. GHS-R protein was present in 70% of the analyzed tumors, and coexpression of ghrelin was common. This suggests that there is an autocrine loop, possibly driving proliferation. There was, however, no difference in survival between patients with tumors coexpressing ghrelin and its receptor and tumors lacking coexpression in our material, as would have been expected if ghrelin had a stimulating effect on tumor growth. It is possible that such a difference might be revealed in a larger patient material, but we cannot draw any conclusions regarding a possible proliferatory effect. The question of whether ghrelin increases tumor cell proliferation is an important one. One trial has shown an increase in energy intake after ghrelin infusion in cancer patients (Neary et al., 2004), and there are hopes that ghrelin will prove to be of value in treating cancer cachexia. Obviously, if ghrelin had a stimulatory effect of ghrelin on tumor proliferation, it would jeopardize this potential application.

We found a higher frequency of ghrelin protein expression in EPTs, as well as a higher intra-tumoral percentage of ghrelin immunoreactive cells compared to the only earlier report of comparable size (Volante et al., 2002). In the previous study, the same antibody was used, but also signal amplification. Our patient material, representing a tertiary referral center selection, could include an overrepresentation of more malignant EPTs. If ghrelin were more often expressed in more malignant tumors, this could be an explanation for the higher frequency in our material. However, the previous study found no correlation between ghrelin production and tumor stage, and neither could we demonstrate any association between ghrelin expression and survival. Also, the group of patients included in this study had a longer survival and a lower frequency of liver metastases compared to the larger patient material.
analyzed in Paper I, suggesting these tumors were, if anything, less malignant.

More tumors expressed total GHS-R mRNA compared to receptor 1a mRNA. This could mean that some tumors express only GHS-R 1b, consistent with a previous study, where expression of GHS-R 1b mRNA was shown in insulinomas (Volante et al., 2002). GHS-R 1b is a truncated form and generally believed to be inactive, although it has been suggested to have functions separate from GHS-R 1a (Barzon et al., 2005).

In our material, insulinomas had significantly higher relative GHS-R mRNA levels (in total and for 1a) compared to other functioning and non-functioning tumors. Expression of the GHS-R on INS-1 cells (a cell-line that secretes insulin in response to glucose) has been shown and ghrelin has been suggested to inhibit insulin secretion via a direct effect on the β-cell (Wierup et al., 2002). Expression of the GHS-R in β-cells could explain why insulinomas would have higher receptor mRNA levels.

EPTs in MEN1 patients often produce multiple hormones, in contrast to sporadic tumors that usually produce only one. It would not be surprising therefore if MEN1-related EPT were to express ghrelin more often than their sporadic counterparts. In our small material, we could not see any difference in the frequency of ghrelin expression between MEN1-related and sporadic tumors. However, we did find significantly higher plasma ghrelin levels in MEN1 patients compared to patients with sporadic tumors, despite similar BMI. This is an intriguing finding: why would MEN1 patients have higher ghrelin levels? We have not investigated plasma ghrelin levels in MEN1 patients with no signs of EPTs, and cannot know if the high levels found in this study were related to the tumor, or to some unknown characteristic of this disease. Also, it is difficult to draw any firm conclusions from such a small number of observations. Further investigation of plasma ghrelin in MEN1 patients would be useful.

There was no significant difference in mean plasma ghrelin in patients and controls, a result that supports the previous study suggesting that ghrelin in plasma is not a useful general marker for EPTs (Corbetta et al., 2003). No clear autonomous post-prandial ghrelin production could be seen after a standardized carbohydrate-rich meal. We used patient plasma that had been stored for up to 19 years and control plasma that had not been stored. It is possible that ghrelin degradation in patient samples might have masked actual differences.

Mean BMI at diagnosis was 24.3. Though this is not above the normal range, these patients were certainly not cachexic. Considering the significant tumor mass, in many cases multiple liver metastases, this is quite good. It is tempting to speculate that secretion of ghrelin from the tumors could play a part in preventing cancer-associated cachexia in these patients; however there was no significant difference in BMI between patients with tumors expressing ghrelin protein and those not. Thus we found no support for the
hypothesis that tumor ghrelin production prevents these patients from losing weight. However, there were some interesting observations. One patient with a ghrelin-producing tumor had plasma ghrelin close to the upper normal limit. After radical pancreatic surgery (with no gastrectomy), ghrelin levels fell 42%, and BMI 20%, suggesting that at least in this case, tumor ghrelin may have been influencing body weight.

In Paper I, BMI was found to be of prognostic value; being underweight was associated with a significantly shorter survival, independently of age and sex. One might speculate on a number of possible explanations for this. People with a low BMI generally have higher circulating levels of ghrelin. If EPT were a ghrelin-responsive malignancy, higher levels of ghrelin in the blood could cause more aggressive tumor growth. Alternatively, tumor ghrelin production could prevent cancer cachexia in EPT patients. The association between underweight and poor prognosis could be due to lower ghrelin secretion from less differentiated and more malignant tumors. This is of course mere speculation. Associations between variables can not tell us which is the cause and which is the effect. In this case, the cachexia most probably resulted from having an aggressive tumor or a tumor in an advanced stage. This would be a satisfactory explanation for the poor prognosis for these patients. Just as anticipated, the significance of underweight as a negative prognostic factor in Paper I disappeared when corrected for tumor stage.

**Paper IV**

In this paper, we describe the first data on ghrelin expression in GISTs. We demonstrate expression of ghrelin and receptor mRNA in all the analyzed tumors, and protein in the majority of tumors. Levels of ghrelin and receptor mRNA were comparable to, or higher than, mean levels in pancreatic endocrine tumors. We also found high levels of SV2 mRNA, an established marker for neuroendocrine cells, in all tumor samples. For a long time, the origin of GISTs was unclear. When expression of the KIT receptor was shown, their connection to the interstitial cells of Cajal was clarified. Expression of the neuroendocrine marker SV2 and the peptide hormone ghrelin in GISTs raises questions about neuroendocrine features in these tumors. The interstitial cells of Cajal are crucial in the regulation of gut peristalsis and ghrelin stimulates gastric motility. For the interstitial cells of Cajal to express the ghrelin receptor, and ghrelin to play a role in regulating the actions of these cells, would thus be logical. Expression of the ghrelin receptor in GISTs, the tumors originating from the interstitial cells of Cajal, is therefore not surprising. Expression of ghrelin itself, however, is more unexpected. We did not have access to isolated interstitial cells of Cajal and have not been able to either confirm or exclude expression of ghrelin in these
cells. Alternatively, the ability to produce ghrelin could be acquired somewhere along the line of transformation into malignant tumor cells. It would be interesting to examine expression of SV2, as well as other neuroendocrine markers, in interstitial cells of Cajal. Investigation of the expression of other hormones in GISTs would also be very interesting.

Ghrelin and its receptor were often expressed concomitantly in GISTs. This indicates a possible autocrine/paracrine loop. Does ghrelin promote aggressive behavior in GISTs? If so, one would expect to find an association between ghrelin expression and factors related to malignancy. In our material, we could not find any such correlation between the expression of ghrelin and its receptor and malignant features (e.g. tumor size, high risk score, \textit{KIT} exon 11 mutations or disseminated disease). Because this is a very small patient material, any conclusions regarding a potential association between ghrelin and malignancy would be inappropriate. It would be very interesting to study a potential association between ghrelin expression and malignancy in a larger group of GIST patients.

Patients with GISTs seldom suffer from cancer cachexia, even in advanced disease. This is in sharp contrast to patients with other smooth muscle tumors, sarcomas, where cachexia is often a prominent feature of the disease. Our GIST patients had a mean pre-operative BMI of 25.1 (25.4 in patients with ghrelin-immunoreactive tumors). Thus they did not suffer from cancer cachexia, but were instead bordering on being overweight, just like the Swedish population in general (Sundquist \textit{et al.}, 2004). The majority of patients presented with large tumors (>10 cm; 8/14), high risk score indicating malignancy (12/14), and distant metastases (7/14). It is intriguing that GIST patients seldom lose weight as their disease progresses. Endogenous secretion of the potent orexigen ghrelin from the tumor could potentially contribute to their relative well-being. However, we were not able to perform any analyses of circulating ghrelin levels in the present retrospective study, and thus unfortunately we cannot draw any conclusions regarding the role of ghrelin in the prevention of cancer cachexia in these patients. It would be very interesting to see a more comprehensive metabolic evaluation of GIST patients in a prospective study.
Concluding remarks

In a large series of 324 patients with EPT treated at a single institution, surgery, WHO classification, TNM stage, age and Ki67 ≥2% were independent prognostic variables. Contrary to widespread belief, having a non-functioning tumor was not an independent marker of poor prognosis, and neither was having a sporadic tumor. These findings imply tumors should not be regarded as less malignant solely on the basis of heredity or automatically more malignant because they are non-functioning. A prognostic value of low BMI, as well as elevated CgA, independent of age and sex, is shown for the first time.

Among well-differentiated NE carcinomas, the largest group by far, Ki67 and size were predictors of prognosis. Both of these variables should be considered when trying to predict the prognosis of a patient with well-differentiated carcinoma. However, further effort to identify better prognostic markers to help identify tumors with a higher risk of aggressive behavior among well-differentiated neuroendocrine tumors and carcinomas is needed.

We provide the first clinical evaluation of the recently proposed TNM staging system for EPT. This system appears to be of value, but further investigation is needed to evaluate and perhaps fine-tune the definitions of the different stages.

We report for the first time that temozolomide as monotherapy is effective in advanced malignant neuroendocrine tumors, with acceptable toxicity. Two thirds of our patients, with advanced stage disease, benefited from treatment, results suggesting temozolomide could become a valuable addition to the palliative treatments available today. This could prove especially important for patients with bronchial or thymic carcinoid, where there are very few effective treatments available. Temozolomide allows treatment on an outpatient basis and has a favorable toxicity profile, making it suitable for palliative treatment. Prospective studies, evaluating temozolomide as monotherapy or in combination with other compounds in the treatment of neuroendocrine tumors, as well studies of different schedules of temozolomide administration, are warranted.

We also demonstrate a low expression of MGMT in four out of five tumors with a radiologic response to treatment. Although not quite significant in this small material, this finding suggests a value of MGMT immunohistochemistry in predicting response to treatment with temozolomide in neuro-
endocrine tumors. This could prove quite useful in clinical practice, and further studies are warranted.

Concomitant expression of ghrelin and its receptor was frequently seen in pancreatic endocrine tumors, suggesting an autocrine loop. However, no association was found between expression and survival. There was no association between ghrelin expression and patient BMI, but we report a median BMI of 24.1, showing that these patients are generally not cachexic at diagnosis.

We provide the first report of expression of the hormone ghrelin as well as its receptor in gastrointestinal stromal tumors. Concomitant expression was frequent, suggesting the presence of an autocrine loop. However, no association with tumor characteristics suggestive of aggressive behavior was found. High levels of mRNA coding for synaptic vesicle protein 2 were also found, and this together with expression of a hormone suggests neuroendocrine features in these tumors.

The studies included in this thesis have provided additional and updated information on the characteristics and survival of patients with EPT, as well as prognostic factors. The first clinical evaluation of the newly proposed TNM staging system has been reported, and a new promising treatment for patients with EPT and other neuroendocrine tumors has been evaluated. Expression of a hormone and a neuroendocrine marker, suggesting the presence of neuroendocrine features, has been demonstrated in gastrointestinal stromal tumors. Some of the findings presented will prove immediately clinically useful, while for others no obvious clinical application comes to mind. Together, hopefully, they can make some small contribution towards improving the outlook for future patients with these rare tumors.
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