Modern Surgical Treatment and Genomic Profiling of Pancreatic Neuroendocrine Neoplasms

- from the Operating Theater to the Gene Lab

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This work is dedicated to *CarciNor*, the Norwegian patient advocacy association for neuroendocrine cancer, for its invaluable efforts in raising awareness about and support for patients and caregivers affected by neuroendocrine cancer.

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Sven-Petter Haugvik

Abbreviations

AJCC – American Joint Cancer Committee
ANCA – average number of copy aberrations
CGH – comparative genomic hybridization
CI – confidence interval
ENETS – European Neuroendocrine Tumor Society
FC – fold change
GEP-NEC – gastro-entero-pancreatic neuroendocrine carcinoma
GEP-NEN – gastro-entero-pancreatic neuroendocrine neoplasm
HR – hazard ratio
Ki67 – antigen Ki67 encoded by the *MKI67* gene
LDP – laparoscopic distal pancreatectomy
LE – laparoscopic enucleation
MEN-1 syndrome – multiple endocrine neoplasia type 1 syndrome
mTOR – mammalian target of rapamycin
NF-1 – neurofibromatosis type 1
NNTG – Nordic Neuroendocrine Tumor Group
PCR – polymerase chain reaction
PDAC – pancreatic ductal adenocarcinoma
PNEC – high-grade pancreatic neuroendocrine carcinoma, G3
NANETS – North American Neuroendocrine Tumor Society
NEC – neuroendocrine carcinoma
NEN – neuroendocrine neoplasm
NET – neuroendocrine tumor
PNEN – pancreatic neuroendocrine neoplasm
PNET – pancreatic neuroendocrine tumor, G1 and G2
POPF – postoperative pancreatic fistula
PS – performance status
RAMPS – radical antegrade modular pancreatosplenectomy
RNA-seq – RNA sequencing
SEER – Surveillance, Epidemiology, and End Results Program
TNM – tumor-node-metastasis
TSC – tuberous sclerosis complex
UICC – International Union for Cancer Control
VHL syndrome – von Hippel-Lindau syndrome
WHO – World Health Organization

Publications included

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Long-term outcome of laparoscopic surgery for pancreatic neuroendocrine tumors.
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II. Haugvik SP, Labori KJ, Waage A, Line PD, Mathisen Ø, Gladhaug IP.
Pancreatic surgery with vascular reconstruction in patients with locally advanced pancreatic neuroendocrine tumors.
J Gastrointest Surg. 2013 Jul; 17(7):1224-32.

III. Haugvik SP, Janson ET, Österlund P, Langer SW, Falk RS, Labori KJ, Vestermark LW, Grønbæk H, Gladhaug IP, Sorbye H.
Surgical treatment as a principle for patients with high-grade pancreatic neuroendocrine carcinoma: a Nordic multicenter comparative study.
Ann Surg Oncol. 2016 May; 23(5):1721-8.

IV. Haugvik SP, Gorunova L, Haugom L, Eibak AM, Gladhaug IP, Heim S, Micci F.
Loss of 11p11 is a frequent and early event in sporadic nonfunctioning pancreatic neuroendocrine neoplasms.
Oncol Rep. 2014 Sep; 32(3):906-12.

V. Haugvik SP, Vodák D, Haugom L, Hovig E, Gladhaug IP, Heim S, Micci F.
Transcriptomic profiling of tumor aggressiveness in sporadic nonfunctioning pancreatic neuroendocrine neoplasms.
Pancreas. 2016 Feb. Epub ahead of print.

Introduction

Historical notes

In November 1902, the first report of an endocrine pancreatic tumor was published by the Canadian pathologist Albert George Nicholls (1870-1946)[1]. In September 1907, the German pathologist Siegfried Oberndorfer (1876-1944) presented his observations on the nature of a morphologically distinct class of tumors which he referred to as *carcinoids*[2, 3], *i.e.*, carcinoma-like neoplasms behaving like benign neoplasms. He thereby became the first to characterize neuroendocrine neoplasms (NENs). In May 1927, the American physician Russell M. Wilder (1885-1959) described the first case of insulinoma and was the first to report a surgical attempt on removal of a pancreatic neuroendocrine neoplasm (PNEN), which was undertaken by the surgeon William James Mayo (1861-1939)[4]. Two years later, the Canadian physician Goldwin Howland (1875-1950) described the first curative operation for a PNEN[5]. Some years later, in 1938, the Austrian pathologist Friedrich Feyrter (1895-1973) published a paper where he proposed that neuroendocrine neoplasms are derived from cells of the diffuse endocrine system[6]. In 1963, the British pathologist Merton Sandler (1926-2014) was the first to classify neuroendocrine neoplasms according to the embryonic divisions of the digestive tract, *i.e.*, foregut, midgut and hindgut[7]. In 1966, the British pathologist Anthony G.E. Pearse (1916-2003) recognized the uptake of 5-hydroxytryptophan (5-HTP) and its decarboxylation to 5-HT as a common cytochemical characteristic in a distinct population of endocrine cells[8]. These cells did not only include cells of the diffuse endocrine system, but also cells of several endocrine organs. He defined them as amine precursor uptake and decarboxylation (APUD) cells and thereby became the first to classify neuroendocrine cells[9]. All these contributions, made over a time span of seven decades, represent the early era of neuroendocrine oncology and surgical treatment of PNENs, and form the basis of our understanding of how neuroendocrine neoplasms develop and behave.

Pancreatic neuroendocrine neoplasms (PNENs)

Definition, clinical presentation, and epidemiology

PNENs arise from the endocrine cells of the pancreas, which are part of the diffuse endocrine system[10]. They represent a heterogeneous group of diseases and comprise about five percent of all pancreatic neoplasms[11, 12] (**Figure 1**). Multiple terms for the same group of diseases were suggested, *e.g.*, pancreatic carcinoid, islet cell tumor, pancreatic endocrine tumor, pancreatic neuroendocrine tumor, pancreatic neuroendocrine neoplasm[13]. For reasons of clarity, the general term "pancreatic neuroendocrine neoplasm" or "PNEN" is used in this thesis.

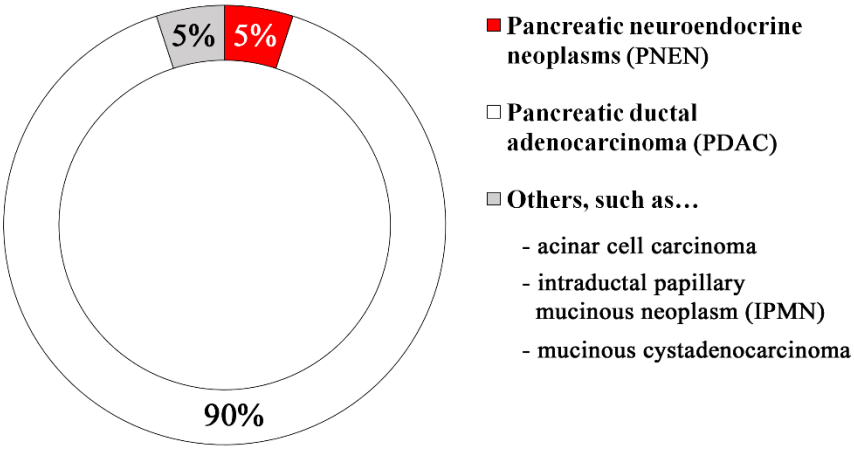

Figure 1. *Relative frequency of pancreatic neoplasms in humans*

PNENs are clinically diverse and are divided into functioning and nonfunctioning disease, depending on their ability to give symptoms due to hormone production[14]. Sixty to 90% of all PNENs are nonfunctioning[11, 15, 16] as they do not cause hormone-dependent symptoms (**Figure 2**). Since nonfunctioning PNENs do not cause characteristic hormonal symptoms and generally exhibit slow growth, they are often detected incidentally or through symptoms related to mass effects resulting from local or distant tumor progression[17]. Common symptoms and signs of nonfunctioning PNENs are abdominal pain, nausea, fatigue, obstructive jaundice, and abdominal mass[18, 19]. Patients with functioning PNENs often present with characteristic symptoms dependent on the hormones produced, such as

hypoglycemia (insulin in insulinoma), heartburn (gastrin in gastrinoma), and watery diarrhea (vasoactive intestinal peptide in VIPoma). Functioning PNENs will not be discussed further in this thesis.

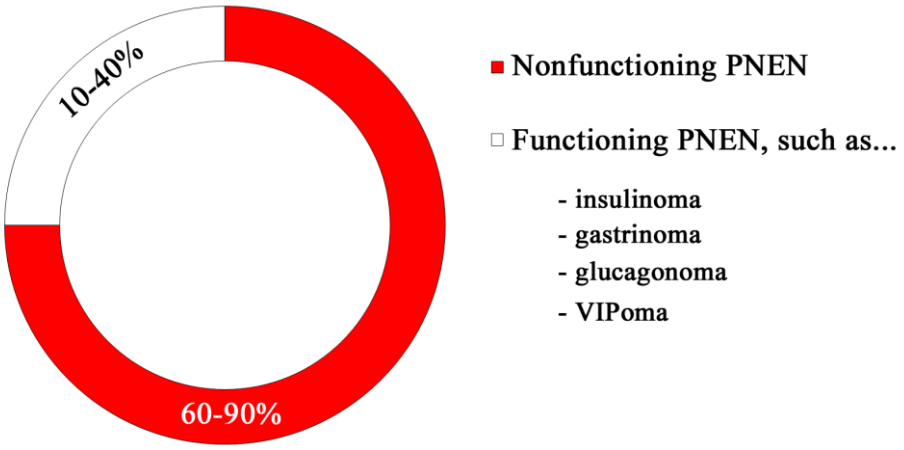

Figure 2. *Relative frequency of nonfunctioning and functioning pancreatic neuroendocrine neoplasms (PNENs)*

Most PNENs are sporadic, which means that they do not show any specific gene mutation resulting in their occurrence in specific families according to defined inheritance patterns. However, about 10-15% of all PNENs develop as part of familial syndromes associated with specific germline mutations, such as *multiple endocrine neoplasia type 1 syndrome* (MEN-1 syndrome, caused by mutation in the *MEN1* gene in chromosome subband 11q13.1), *von Hippel-Lindau syndrome* (VHL syndrome, caused by mutation in the *VHL* gene in chromosome subband 3p25.3), *neurofibromatosis type 1* (NF-1 or von Recklinghausen disease, caused by mutation in the *NF1* gene in chromosome subband 17q11.2), and *tuberous sclerosis complex* (TSC, caused by mutation in the *TSC1* or *TSC2* gene in chromosome subbands 9q34.13 and 16p13.3, respectively)[20]. The relative frequency of sporadic and familial PNENs is illustrated in **Figure 3**.

Most PNENs are solitary and located in the pancreatic head (35%), tail (30%), or body (10%). About 15% of all PNENs are multiple. The relative distribution of PNENs in the pancreatic gland is illustrated in **Figure 4**.

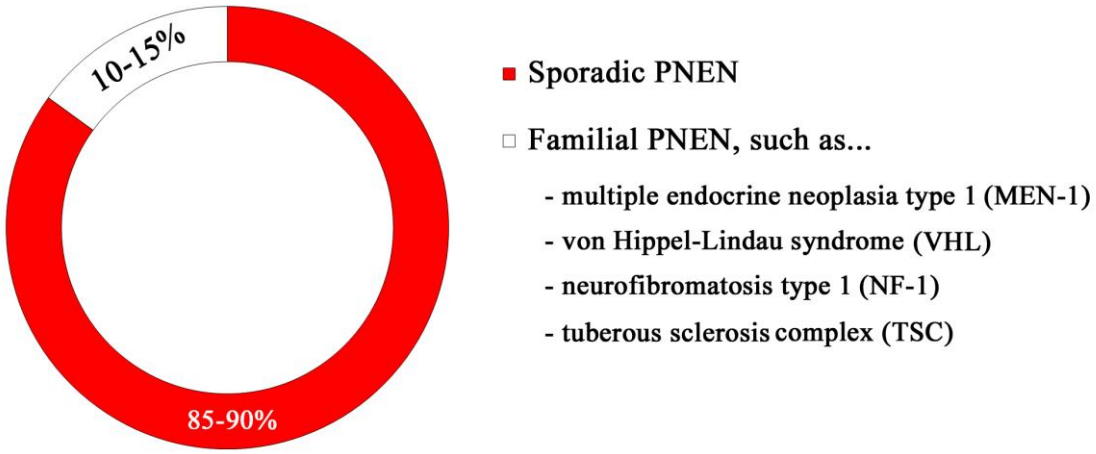

Figure 3. *The relative frequency of sporadic and familial pancreatic neuroendocrine neoplasms (PNENs)*

In Norway and in the USA, the median age at diagnosis of PNENs is about 60 years with a slight male predilection (55%) and with an observed increasing incidence rate throughout the last three decades[11, 16, 21-23]. While the current incidence rate for PNENs in Norway is 0.7 per 100,000 person-years, with an annual increase of about 7%[21], the current incidence rate in the USA is 0.3 per 100,000 person-years[11, 24]. The higher reported incidence in Norway is probably closer to the actual incidence as these data include PNENs classified with "uncertain behavior", which in similar studies have been excluded. Hence, the number of new cases of PNEN to be expected per year in Norway would now be 36.

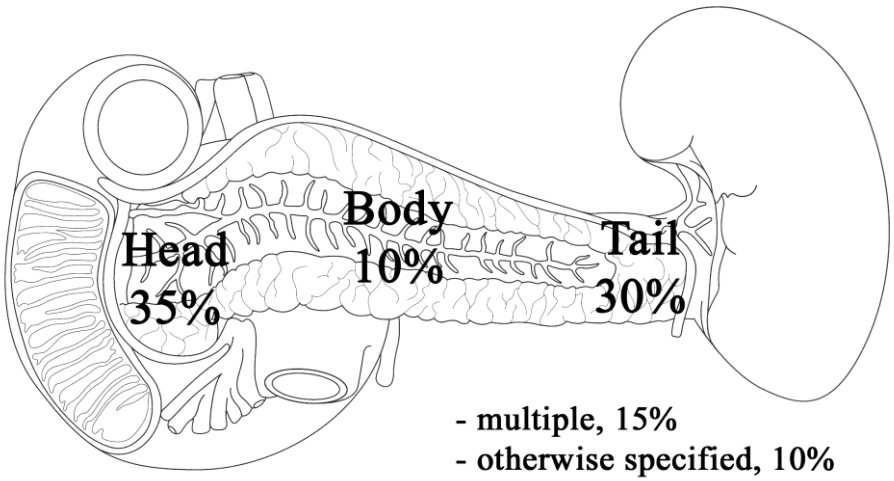

Figure 4. *Distribution of PNENs in the pancreas (numbers from Bilimoria et al.[25] and Fischer et al.[26]; illustration by Haugvik K)*

According to data from the Cancer Registry of Norway, most patients are diagnosed with distant metastatic disease (52%), followed by localized disease (29%) and regional disease (defined by tumor growth into a neighboring structure, including regional lymph nodes) (19%)[21], as illustrated in **Figure 5**. According to data from the Surveillance, Epidemiology, and End Results (SEER) program, most patients in the USA are diagnosed with distant metastatic disease (64%), followed by regional disease (22%) and localized disease (14%)[24]. It is important to notice that the SEER database excludes PNENs considered to be benign, causing overestimation of the frequencies of extrapancreatic disease, nodal metastasis, and metastatic disease[27]. Autopsy studies have shown that PNENs can be identified in as many as 10% of the population, suggesting that many people carry asymptomatic disease[28]. Whether the generally increasing use of cross-sectional imaging and ultrasound in the last three decades can explain the increase in the incidence of PNENs exclusively, remains unknown[22].

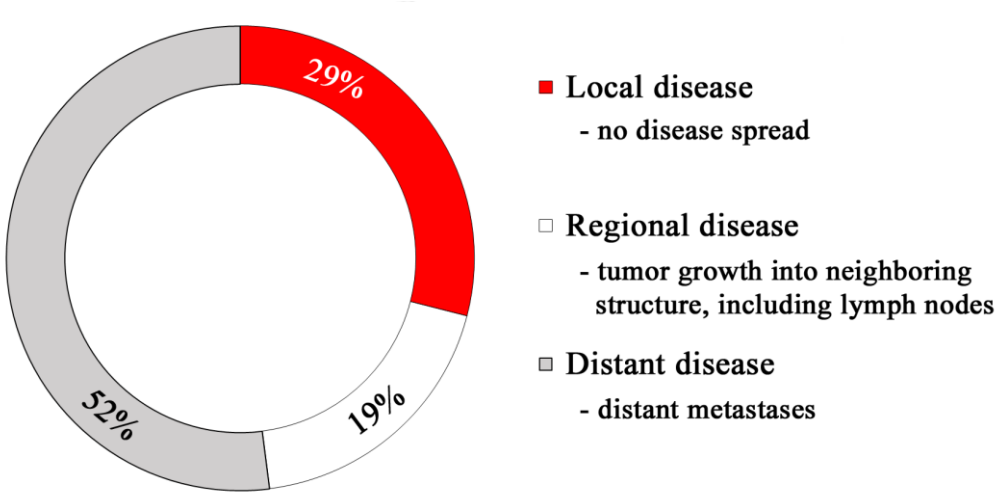

Figure 5. *The relative frequency of tumor stage of patients with PNEN in Norway from 1993 to 2010 (from Boyar Cetinkaya et al.[21])*

As this introductory chapter shows, most PNENs are nonfunctioning, sporadic tumors located in the pancreatic head, and with synchronous metastatic disease. In addition to classification according to hormonal activity and heredity, PNENs should be further classified in order to enable patient risk stratification and to improve clinical decision making[29]. Today, the prognosis of PNENs is largely

defined by the individual tumor's morphology, grading, and stage as determined by histopathology.

Pathology

The pathology of all PNENs is defined by tissue morphology, grading, and the tumor-node-metastasis (TNM) pattern.

Neuroendocrine cells are characterized by production of neurosecretory granules, containing proteins such as chromogranin A and synaptophysin, which can be detected by immunohistochemistry. The minimal immunohistochemical tests recommended for a diagnosis of PNENs, as for GEP-NENs in general, are: chromogranin A, synaptophysin, and Ki67. Chromogranin A and synaptophysin are the two most sensitive and specific general neuroendocrine markers and are used to confirm the diagnosis, whereas Ki67 is a marker of prognosis that also defines grading[13]. While the characterization of neuroendocrine cell morphology and evaluation of immunohistochemistry in PNENs remain the domain of pathologists[30] and as such will not be further discussed in this thesis, surgeons should have a thorough understanding of the grading and TNM staging of PNENs.

PNENs are classified according to their grading, defined by the World Health Organization (WHO) 2010 Classification[31]. The grading is based on the Ki67 index, defined as the ratio between the number of cells in a population positive for Ki67 to the total number of cells studied, or the mitotic index, defined as the ratio between the number of cells in a population undergoing mitosis to the number of all cells observed. Ki67 is a nuclear antigen and cell proliferation marker. The Ki67 index has become one of the most important indicators of tumor aggressiveness in GEP-NENs[32]. In PNENs, as for GEP-NENs in general, a mitotic rate of < 2 and/or Ki67 index of ≤ 2 corresponds to a neuroendocrine *tumor* (NET) G1. A mitotic rate of 2-20 and/or Ki67 index of 2.5-20 characterizes a NET G2, while a mitotic rate and/or Ki67 index of > 20 defines a neuroendocrine *carcinoma* (NEC) G3 (**Table 1**).

Table 1. *WHO 2010 grading system for pancreatic neuroendocrine neoplasms (modified from Bosman et al.[31]). [a] 10 HPP, high-power field = 2 mm^2, at least 40 fields (at x40 magnification) evaluated in areas of highest mitotic density. [b] MIB1 antibody, % of 2000 tumor cells in areas of highest nuclear labeling*

Grade	Mitotic count (10 HPF)[a]	Ki-67 index (%)[b]
G1	<2	≤2
G2	2–20	2.5–20
G3	>20	>20

High-grade PNECs (PNECs, G3) are defined as PNENs with poorly differentiated morphology and a higher proliferation rate than well-differentiated PNETs (G1 and G2). It is important to note that grading of a PNEN is determined by the highest mitotic rate or Ki67 index, irrespective of whether this is found in the primary tumor or a metastatic deposit. At diagnosis, most PNENs are graded as G1 (55%), followed by G2 (40%), and G3 (5%)[33], as shown in **Figure 6**.

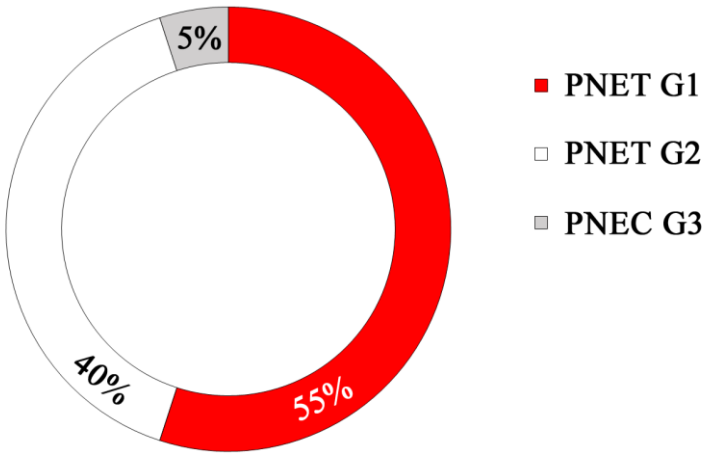

Figure 6. *The relative frequency of tumor grading, defined by the WHO 2010 Classification[31] of patients with pancreatic neuroendocrine neoplasms (PNENs). PNET, pancreatic neuroendocrine tumor; PNEC, pancreatic neuroendocrine carcinoma.*

Besides grading, PNENs are classified according to their TNM pattern, as defined by validated TNM staging systems. There are currently two TNM systems for staging of PNENs. The first classification was recommended by the European

Neuroendocrine Tumor Society (ENETS) in 2006[34] and is predominant in Europe. This was followed by the classification suggested by the American Joint Cancer Committee and International Union for Cancer Control (AJCC/UICC) in 2009[35], which is now widely used in the North American region. The ENETS and AJCC/UICC classification systems for PNENs differ in their definition of the T stage, as shown in **Table 2**. There is an ongoing debate as to which of the two staging systems is the more precise in terms of prognostic stratifications, with some studies demonstrating similar strength[36, 37] and others indicating superiority of the ENETS over the AJCC/UICC TNM system[38, 39]. According to the ENETS staging system, Stage I is defined by T1N0M0, stage IIA by T2N0M0, stage IIB by T3N0M0, stage IIIA by T4N0M0, stage IIIB by anyTN1M0, and stage IV by anyTanyNM1[34]. In several European cancer centers, PNENs are most often defined as stage I (28%) or IV (28%), followed by IIIB (19%), IIA (14%), IIB (7%), and IIIA (4%) at time of diagnosis[38]. In the work contained in this thesis, the ENETS TNM system was used. The prognosis of PNENs, following their ENETS stage and WHO grading, is illustrated in **Figure 7** and shows that grading and staging correlate directly with prognosis.

Table 2. *Comparison of the T category in the ENETS and AJCC/UICC TNM classifications of pancreatic neuroendocrine neoplasms. ENETS, European Neuroendocrine Tumor Society, AJCC/UICC, American Joint Cancer Committee And International Union for Cancer Control (from Rindi et al.[34] and Sobin et al.[35])*

	ENETS TNM	AJCC/UICC
T1	Confined to pancreas, <2 cm	Confined to pancreas, <2 cm
T2	Confined to pancreas, 2–4 cm	Confined to pancreas, >2 cm
T3	Confined to pancreas, >4 cm, or invasion of duodenum or bile duct	Extension beyond pancreas, but without involvement of celiac axis or superior mesenteric artery
T4	Invasion of adjacent organs or major vessels	Involvement of celiac axis or superior mesenteric artery

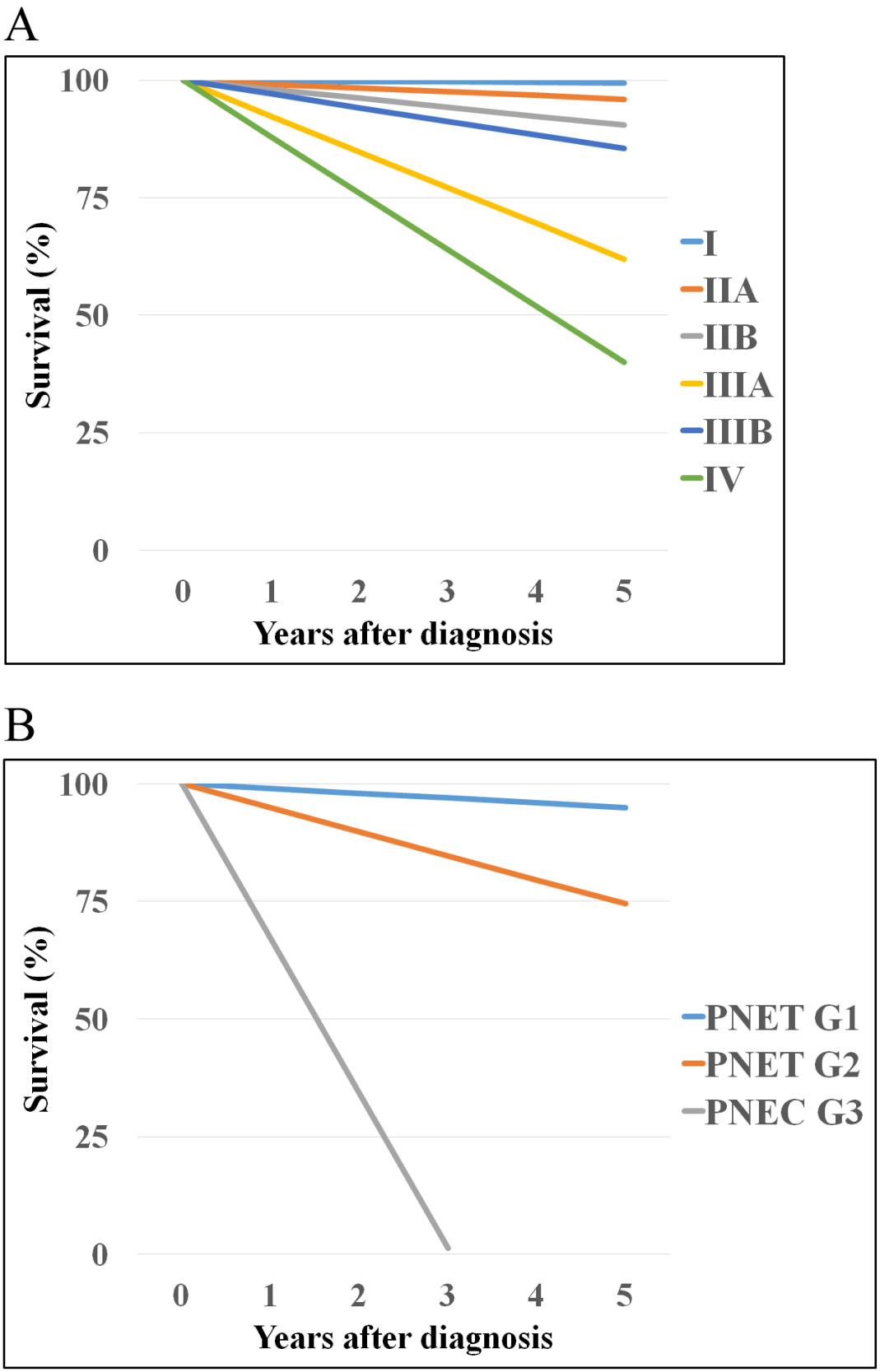

Figure 7. *Prognosis of PNENs according to the current* ***(A)*** *ENETS staging and* ***(B)*** *WHO grading (numbers from Rindi et al.*[38]*)*

As shown in this chapter, current classification systems for PNENs are defined by histopathology. The Ki67 index cut-off-values between the different grading classes have proven to correlate well with prognosis of NENs in different organs, including the pancreas[40-42]. However, a substantial fraction of PNENs do not show the prognosis predicted by their corresponding grading and stage. This implies the possibility of future revisions of current classification systems as new knowledge about the different subtypes of PNENs is acquired. In particular, this is to be expected in the group of PNET G2 and the rare group of PNEC, both of which have a wide Ki67 index range. This is exemplified by the recent discourse related to the optimal Ki67 index cut-off between PNET G1 and G2[43, 44], which is discussed in **Paper I** of this thesis. Another example is the discussion related to discordance of tissue morphology and grading in PNENs with well-differentiated morphology and a Ki67 index above 20%[45]. Beyond histopathology, there is also a need for development of platforms for molecular staging in patients with PNENs. In **Papers IV and V** of this thesis, initial steps toward a molecular staging in patients with sporadic nonfunctioning PNENs were taken using genomic profiling techniques.

Surgery for PNENs

Modern pancreatic surgery is characterized by both minimally-invasive and highly invasive procedures, which allow the surgeon to remove benign or malignant pancreatic disease at different stages. In the case of PNENs, the expression "modern surgical treatment" may be more relevant than for any other pancreatic neoplasm. Due to slow growth and frequently found small indolent lesions, parenchyma-sparing techniques are warranted[46]. On the other hand, slow growth of metastatic tissue allows surgery of the primary tumor only or of both the primary tumor and metastatic tissue, with evidence of prolonged survival compared to nonsurgical treatment[47-50]. This is especially important for patients with metastatic functioning disease[51, 52]. PNENs are generally associated with a favorable prognosis after surgery[24] as demonstrated by reports of an overall 10-year survival of up to 40%[25]. This is in sharp contrast to the more common and highly aggressive

pancreatic ductal adenocarcinoma (PDAC) with an expected median overall survival of around two years after surgery[53, 54]. Survival among patients with PNENs has improved over the last decades[11] and improvements in the field of surgery are likely to have contributed substantially to this. This becomes clear as surgical removal is the only curative treatment for patients with PNENs and improves survival compared to nonoperative treatment[55]. Hence, surgery has become a cornerstone treatment modality for patients with PNENs[18, 56-60] with increasing use over the last decades[16], as illustrated in **Figure 8**.

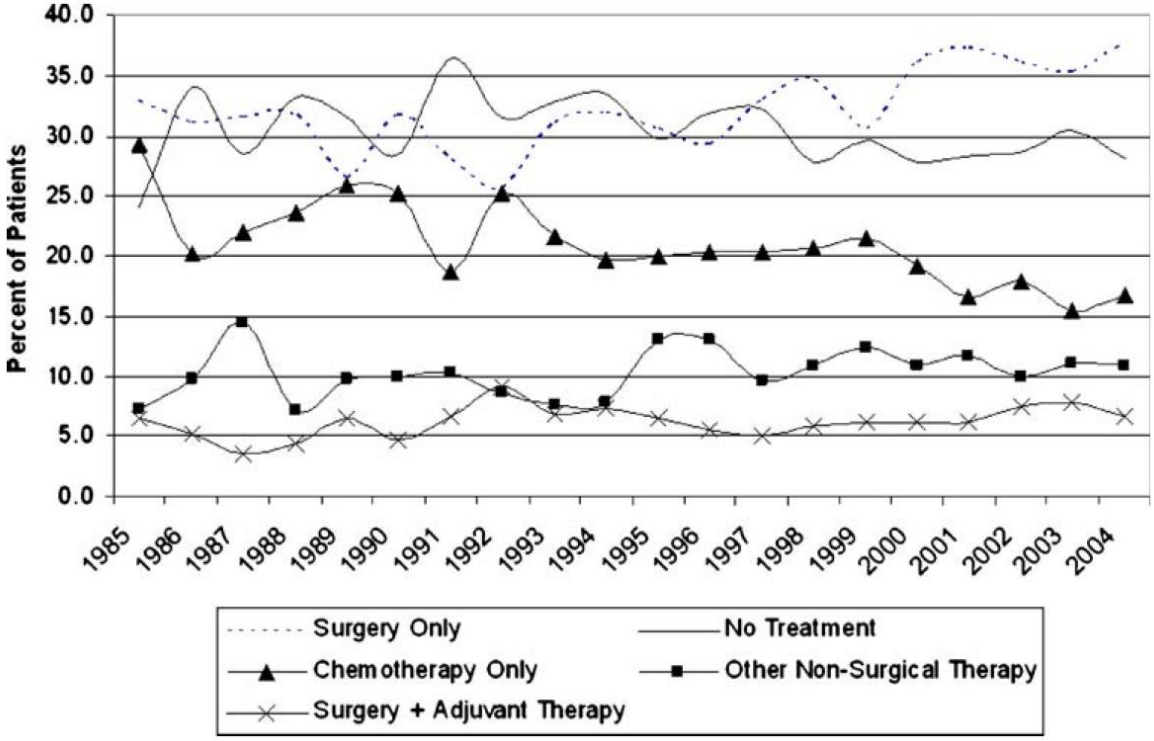

Figure 8. *Treatment trends for patients with PNENs from 1985-2004 in the USA (from Bilimoria et al.[16])*

The goals of surgical treatment for PNENs are cure, relief from hormonal symptoms caused by functioning tumors[51], or relief from nonfunctioning tumors causing symptoms related to mass effect (*e.g.*, biliary obstruction, gastric outlet obstruction, abdominal pain, or gastrointestinal hemorrhage). Resectability rates up to 60% have been reported among patients diagnosed with PNEN[55], and the resectability rate at our institution is about 50%[60]. The most common standard surgical procedures for PNENs include pancreatico-duodenectomy (Whipple

procedure), distal pancreatectomy, and enucleation[26, 61, 62]. Middle segment pancreatectomy is an alternative for lesions located in the pancreatic neck or body[63, 64], and total pancreatectomy is an alternative for lesions affecting all parts of the organ[65, 66]. Enucleation and middle segment pancreatectomy are examples of parenchyma-sparing procedures.

A general risk of standard pancreatic resections (pancreatico-duodenectomy and distal pancreatectomy) is functional impairment of the organ due to loss of parenchyma, resulting in exocrine and/or endocrine insufficiency. Pancreatic exocrine insufficiency is characterized by symptoms related to maldigestion such as steatorrhoea and weight loss due to deficiency of exocrine pancreatic enzymes, whereas pancreatic endocrine insufficiency is associated with development of diabetes mellitus secondary to loss of insulin-producing pancreatic tissue. Parenchyma-sparing procedures, such as enucleation and middle segment pancreatectomy, aim at reducing such side effects[46].

The first laparoscopic operation for a PNEN was performed in 1992 by the Canadian surgeon Michel Gagner[67, 68]. Since then, there has been a general trend towards minimally-invasive techniques in the management of PNENs, especially with laparoscopic procedures. As the laparoscopic approach in pancreatic surgery was proven feasible[69-72], the advantages of this minimally-invasive surgery slowly led to an increasing number of standard laparoscopic resections and parenchyma-sparing procedures of benign pancreatic lesions or lesions with low malignant potential, including PNENs[73-79]. Today, we know that the general advantages of the laparoscopic compared to the open approach in pancreatic surgery are less intraoperative bleeding[80], faster postoperative recovery[81], shorter hospital stay[74, 76], and improved cosmesis.

Most studies describing laparoscopic pancreatic surgery have been focusing more on technical aspects and feasibility of the procedures rather than the underlying pancreatic disease. Hence, while we now have learned that laparoscopic pancreatic

surgery is feasible, knowledge of laparoscopic pancreatic surgery in patients with PNENs is limited. At the beginning of this thesis, only few large series of patients undergoing laparoscopic surgery for PNENs had been published[77, 82, 83]. In order to increase the knowledge about minimally-invasive surgery for this rare group of patients, we reported what at the time of publication was the largest single center series of patients undergoing laparoscopic surgery for PNENs (**Paper I** of this thesis).

Besides evolutions in surgical care, anesthesiology and intensive care medicine have developed rapidly over the last few years and now allow highly invasive approaches in pancreatic surgery without compromising perioperative patient survival. As some PNENs are large and infiltrate adjacent organs, *i.e*, show local advancement needing multivisceral resection and/or vascular reconstruction, highly invasive pancreatic surgery may be required.

There is no uniformly accepted definition of "locally advanced" disease for PNENs. Therefore, in this thesis, we defined locally advanced disease as a PNEN with an ENETS T3- (confined to pancreas, > 4 cm, or invasion of duodenum or bile duct) or T4-stage (invasion of adjacent organs or major vessels)[34]. Surgical treatment of locally advanced PNENs is controversial[84] and some regard vascular infiltration as a contraindication for resection[85]. There are only a few reports that include vascular reconstruction among patients with PNENs, and none of these discuss the role of vascular reconstruction as such[84, 86-96]. This is different from the more common and generally much more aggressive locally advanced PDAC, where the concept of vascular reconstruction has already been discussed widely[97-100] and has been associated with acceptable morbidity, mortality, and better overall survival as compared to unresected patients[101]. Hence, discussion on the role of vascular reconstruction in locally advanced PNEN seems to be warranted. In **Paper II** of this thesis, the role of vascular reconstruction in a small series of patients with locally advanced PNENs was assessed.

Based on what has been presented so far in the introductory chapters of this thesis, it is clear that PNENs constitute a rare, diverse, and medically challenging group of diseases that require multidisciplinary attention at specialized institutions in order to optimize patient treatment and outcome[85, 102-105]. While around half of all PNENs are resectable, as described earlier, the other half of patients will most probably need other or additional treatment modalities such as systemic chemotherapy, molecular therapy (*e.g.*, everolimus and sunitinib), biotherapy with long-acting somatostatin analogs, radiotherapy, peptide receptor radionuclide therapy, and/or locoregional interventional treatment of metastatic disease[15, 106]. For the latter group of patients, there are currently no evidence-based treatment sequences that involve surgery and attempts to develop such approaches are thus urgently needed. As surgery can be considered a treatment modality at all stages of PNENs, the surgeon plays an essential role in the multidisciplinary team. Although the clinical research included in this thesis belongs to the field of surgery, the results from each of the studies should be considered as matters for multidisciplinary discussions.

High-grade pancreatic neuroendocrine carcinoma (PNEC)

During the last two decades, notable progress has occurred in basic, translational, and clinical research on PNETs[107, 108]. At the same time, as described in the sections above, there has been a general trend towards both more minimally-invasive and highly invasive surgery of these patients[78, 85, 91, 109-111]. In contrast, patients with PNECs have not gained similar attention.

In Norway, the incidence of PNECs has remained stable through the past two decades with an incidence rate of approximately 0.04 per 100,000 person-years[21]. The tumors are most frequently diagnosed in patients around 60 years of age, with a male predilection (59%) and a predominance of tumors located in the pancreatic head (61%)[112]. The tumors are characterized by poorly differentiated morphology and a higher proliferation rate than well-differentiated PNETs.

In contrast to the indisputable importance of surgery as a treatment option for patients with PNETs, the role of surgery in the treatment of PNEC remains unclear. This may be explained by the common presence of synchronous metastatic disease and the rapid progression of PNECs, as illustrated in **Figure 7**, which traditionally has been seen as necessitating palliative systemic chemotherapy[113]. However, less than half of the PNEC patients respond to such treatment regimens[113] and alternative treatment options are urgently needed.

The current consensus guidelines of the ENETS for the surgical management of patients with gastro-entero-pancreatic NECs (GEP-NECs) refer to only three studies[114-116], out of which only one case report discussed surgery of PNEC as such[116]. The guidelines state that localized disease should be treated with surgery or radiotherapy and platinum-based chemotherapy, whereas surgical resection of metastasis is not recommended[106]. The North American Neuroendocrine Tumor Society (NANETS) guidelines state that the benefit of surgery among patients who have completed a course of chemoradiation is uncertain, but reference no studies on surgery for PNECs[117, 118]. Surgery is not even mentioned in the section on treatment for metastatic PNEC. Moreover, the European Society for Medical Oncology's (ESMO) guidelines state that there is general agreement not to operate on PNECs[119]. The current international consensus guidelines on surgical treatment for PNECs are based on expert opinions and very little evidence. This underscores the importance of defining the role of surgery in patients with PNEC by conducting clinical research[120]. In **Paper III** of this thesis, we have described the first comparative study on effect of combined surgical treatment and chemotherapy against chemotherapy alone, in patients with PNEC.

Genetics of PNENs

GEP-NENs share similar histological and morphological features. However, PNENs are characterized by a distinct genetic basis and corresponding biological behavior. As cancer in general, PNEN is the phenotypic result of the acquisition of one or more genomic changes taking place at the chromosomal and/or gene

level[121]. As mentioned earlier, some patients are diagnosed with PNEN in the context of familial syndromes caused by specific genetic alterations. These syndromes and their causal genetic patterns serve as reference models for the study of the much more common sporadic PNENs, as the same genes might be mutated in sporadic cases. Interestingly, most PNENs found in patients with familial syndromes are nonfunctioning[122].

Familial syndromes

As many as 10% of all PNENs occur as part of a *multiple endocrine neoplasia type 1 syndrome (MEN-1 syndrome)*, which is the most common familial syndrome related to PNENs. The MEN-1 syndrome is an autosomal dominant disorder clinically associated with predisposition to neoplasms of the parathyroid glands, anterior pituitary, and neuroendocrine pancreatic cells[123]. It is caused by inactivating mutations in the *MEN1* gene, which is a tumor suppressor gene in chromosome subband 11q13.1. *MEN1* encodes menin, a protein that inactivates transcription factors at the nuclear level, modulates cell cycle inhibitors, and interacts with the DNA repair process. These changes result in inhibition of the cell cycle. PNENs develop in up to 100% of patients with the MEN-1 syndrome.

The *von Hippel-Lindau syndrome (VHL syndrome)* is an autosomal dominant disorder characterized by at least one of the following: pheochromocytoma, renal cell carcinoma, retinal or cerebellar hemangioblastoma, and other less frequent neoplasms such as PNENs[124]. PNENS develop in up to 17% of patients with the VHL syndrome[125]. It is caused by inactivating mutations in the *VHL* gene, which is a tumor suppressor gene in chromosome subband 3p25.3. The *VHL* gene encodes for the protein VHL that inactivates angiogenesis via the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway.

Neurofibromatosis type 1 (NF-1) is one of the most common inherited disorders and shows an autosomal dominant inheritance pattern[126]. The syndrome is defined by multiple café-au-lait skin spots and neurofibromas, and carries a relatively high

risk of development of various malignant diseases, including PNENs. However, PNENs develop in very few patients with the NF-1 syndrome. It is caused by inactivating mutations of the *NF1* gene in 17q11.2, which codes for neurofibromin. Neurofibromin is a negative regulator of the Ras pathway, and in particular of mTOR function, which prevents overactivation of the mTOR pathway. Hence, cell proliferation is controlled.

The *tuberous sclerosis complex (TSC)* is an autosomal dominant disorder characterized by typical skin lesions, renal angiomyolipomas, hamartomas, mental retardation, and neurological disorders[127]. PNENs are very rarely associated with TSC. It is caused by mutations of the *TSC1* gene in chromosome subband 9q34.13 and the *TSC2* gene in 16p13.3 which encode hamartin and tuberin, respectively. Both proteins control cell proliferation through interaction with the PI3K/Akt/mTOR pathway and insulin receptor signaling.

Sporadic PNENs and altered signaling pathways

While 10-15% of all PNENs diagnosed are linked to a familial syndrome, most PNENs occur sporadically. Studies on sporadic PNENs have shown a relatively high frequency of genomic imbalances on chromosome arms 11q, 6q, 11p, 3p, 1p, 10q, 1q, 17q, 7q, 20q, 9p, 7p, and 9q[128]. Some of these chromosomal locations correspond to the gene loci of *MEN1* (11q), *VHL* (3p), and *NF1* (17q), suggesting a possible relationship to mutations seen in familial syndromes. This has been further investigated by means of high throughput DNA sequencing, which has shown that around 40% of sporadic PNENs show mutations in the *MEN1* gene, around 10% show mutations in the *TSC* gene, whereas mutations in the *VHL* gene rarely occur[129]. However, as genomic imbalances have also been detected at several other chromosomal locations, further genetic alterations in sporadic PNENs should be expected. DNA sequencing has shown that the most commonly mutated genes in sporadic PNENs encode proteins that are involved in chromatin remodeling, such as *MEN1*, *DAXX* (6p21.32), and *ATRX* (Xq21.1)[129]. As many as 45% of sporadic PNENs show mutations in either *DAXX* or *ATRX*.

Genetic research on PNENs has thus shown that there is a correlation between mutated genes and corresponding gene products in certain signaling pathways. These pathways include the *chromatin remodeling pathway*, *PI3K/Akt/mTOR pathway*, and the *TP53/Rb pathway*[122] as listed in **Table 3**. The *chromatin remodeling pathway* involves *DAXX*, *ATRX* and *MEN1*. As *DAXX* and *ATRX* encode proteins that are responsible for chromatin remodeling, mutations in these genes may lead to chromosomal instability resulting in further mutations and chromosomal abnormalities eventually promoting tumor progression[130]. *MEN1* mutations cause cell proliferation through altered signaling of different chromatin modification complexes.

Table 3. *Altered signaling pathways in pancreatic neuroendocrine neoplasms (PNENs) (from Shi et al.*[122]*)*

Signaling pathway	Genes or molecules	Frequency	Possible mechanisms	Clinical significance
Chromatin remodeling	*DAXX/ARTX*	45%	Loss of DAXX/ARTX→ALT→CIN	Prognosis
	MEN1	50%	(1) Loss of menin→decreased histone H3K4 methylation→decreased expression of CDKIs→increased cell proliferation (2) Loss of menin→ decreased histone deacetylation→decreased interaction with JunD→cell proliferation	Prognosis
PI3K/Akt/mTOR	*PIK3CA*, *PTEN*, and *TSC2*	15%	(1) Mutation in PIK3CA→activation of downstream Akt/mTOR	Therapeutic targets
	Growth factors Growth factor receptors	Majority	(2) Mutations in PTEN/TSC2→loss negative regulation of the PIK3/Akt/mTOR pathway (3) Overexpression of growth factors/growth factor receptors→increased activation of the pathway	
	MEN1	50%	Loss of menin →loss inhibitory effect on IGFBP2→activation of IGF-associated PI3K/Akt/mTOR activation	
	VHL	Small subset	Loss of *VHL*→increase HIF-1α (downstream of mTOR) activity→angiogenesis	
TP53/Rb	TP53	Mutation: common in PD-NECs Overexpression of MDM2/MDM4/WIP1: common in PanNETs	(1) Loss of TP53 →entry into cell cycle (cell proliferation) and decreased cell apoptosis (2) Loss of Rb→entry into cell cycle→cell proliferation	Potential therapeutic targets
	Rb	Mutation: common in PD-NECs Overexpression of Cdk4/Cdk6: common in PanNETs		

DAXX, death domain-associated protein gene; *ARTX*, alpha-thalassemia/mental retardation X-linked gene; ALT, alternative lengthening of telomere; CIN, chromosome instability; CDKIs, cyclin-dependent kinase inhibitor proteins; *MEN1*, multiple endocrine neoplasia type 1 gene; *PIK3CA*, phosphatidylinositol 4,5-bisphosphate 3-kinase, catalytic subunit alpha gene; *PTEN*, phosphatase and tensin homolog gene; *TSC2*, tuberous sclerosis complex 2 gene; mTOR, mammalian target of rapamycin; IGFBP2, insulin-like growth factor-binding protein 2; IGF, insulin-like growth factor; *VHL*, von Hippel–Lindau tumor suppressor gene; HIF-1α, hypoxia-induced factor 1α; PD-NECs, poorly differentiated neuroendocrine carcinoma; PanNET, pancreatic well-differentiated neuroendocrine tumor.

The *PI3K/Akt/mTOR pathway* is an intracellular signaling pathway that acts downstream of several receptors and regulates protein translation. It is activated in several types of cancer[131]. About 15% of sporadic PNENs show mutations in genes of the PI3K/Akt/mTOR pathway[129], which is also pathogenetically involved in the

MEN-1 syndrome, VHL syndrome, NF-1, and TSC (**Figure 9**). Growth factor receptors such as VEGFR and PDGFR normally stimulate the PI3K/Akt/mTOR pathway. In PNENs, these are frequently overexpressed[132]. Activation of this pathway may result in cell proliferation, invasion, or angiogenesis through downstream targets. TSC1/2 and PTEN are two negative regulators of the PI3K/Akt/mTOR pathway which are often downregulated in PNENs[133]. A third pathway often involved in PNENs is the *TP53/Rb pathway*, which involves the proteins p53 and Rb that are essential parts of tumor-suppressor pathways operative in other cancers. Mutations in *TP53* or *RB1* are not common in PNETs[129], but are often seen in PNECs[134]. Interestingly, PNECs do not show mutations in *DAXX* and *ATRX*. Taken together, these findings suggest that PNECs comprise a genetically distinct subgroup of PNENs.

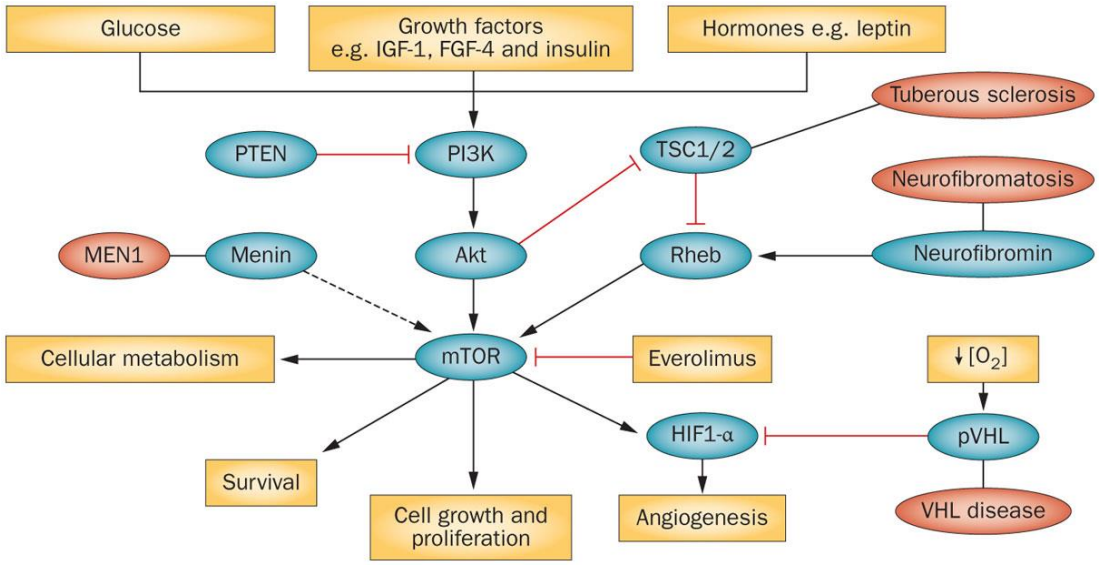

Figure 9. *The PI3K/Akt/mTOR pathway (from Oberg et al.[135]). Familial syndromes (red) are caused by mutations in these genes. MEN1, multiple endocrine neoplasia type 1 syndrome; VHL disease, von Hippel-Lindau disease (or syndrome).*

Cytogenetics and RNA sequencing

One way of gaining insight into the genetic mechanisms underlying PNENs is by detection of genomic alteration, both structural and numerical, through cytogenetic analyses. Cancer cytogenetics is concerned with the study of genomic alterations

in malignant disease at the level of chromosomes and/or chromosomal bands[121]. It represents a branch of genetics that involves methods such as karyotyping and comparative genomic hybridization (CGH). Screening of the whole tumor genome by cytogenetic methods is a natural starting point when trying to understand the pathogenetic mechanisms behind tumor development[121].

Karyotyping is the process of pairing and ordering all the chromosomes of an organism. In somatic cells, the chromosomes are usually studied at the metaphase stage of the cell cycle when chromatin is condensed and the morphology of the chromosomes is clear. In each chromosome, the short (p) and long (q) arms are divided into regions, which are further classified in bands and subbands. By staining techniques, such as G-banding, AT-rich sequences are distinguished from GC-rich sequences.

CGH is a molecular cytogenetic method that allows identification of genomic imbalances, *i.e.*, segments of the genome that are over- or underrepresented in neoplastic tissue[121]. Patterns of copy number alterations identified by CGH have helped classify tumors into biologically and clinically meaningful subtypes[136]. First, DNA is extracted from the tumor specimen and a normal reference sample. Tumor DNA and normal DNA are then amplified and labeled with fluorophores before they are mixed. This results in complementary target sequences with differences between the tumor and normal reference cells, which can be quantified by digital image analysis, as illustrated in **Figure 10**. Besides the traditional metaphase CGH, where the target sequences are normal chromosome spreads, array CGH is characterized by target sequences found as DNA fragments fixed in a matrix system. Array CGH enables a higher resolution than does metaphase CGH, but both techniques have limitations inasmuch as they cannot assess intercellular variability or balanced rearrangements such as inversions, insertions, and translocations[121]. The approximate resolution level is more than five megabases for metaphase CGH and more than fifty kilobases for array CGH. In **Paper IV** of this thesis, we applied metaphase CGH.

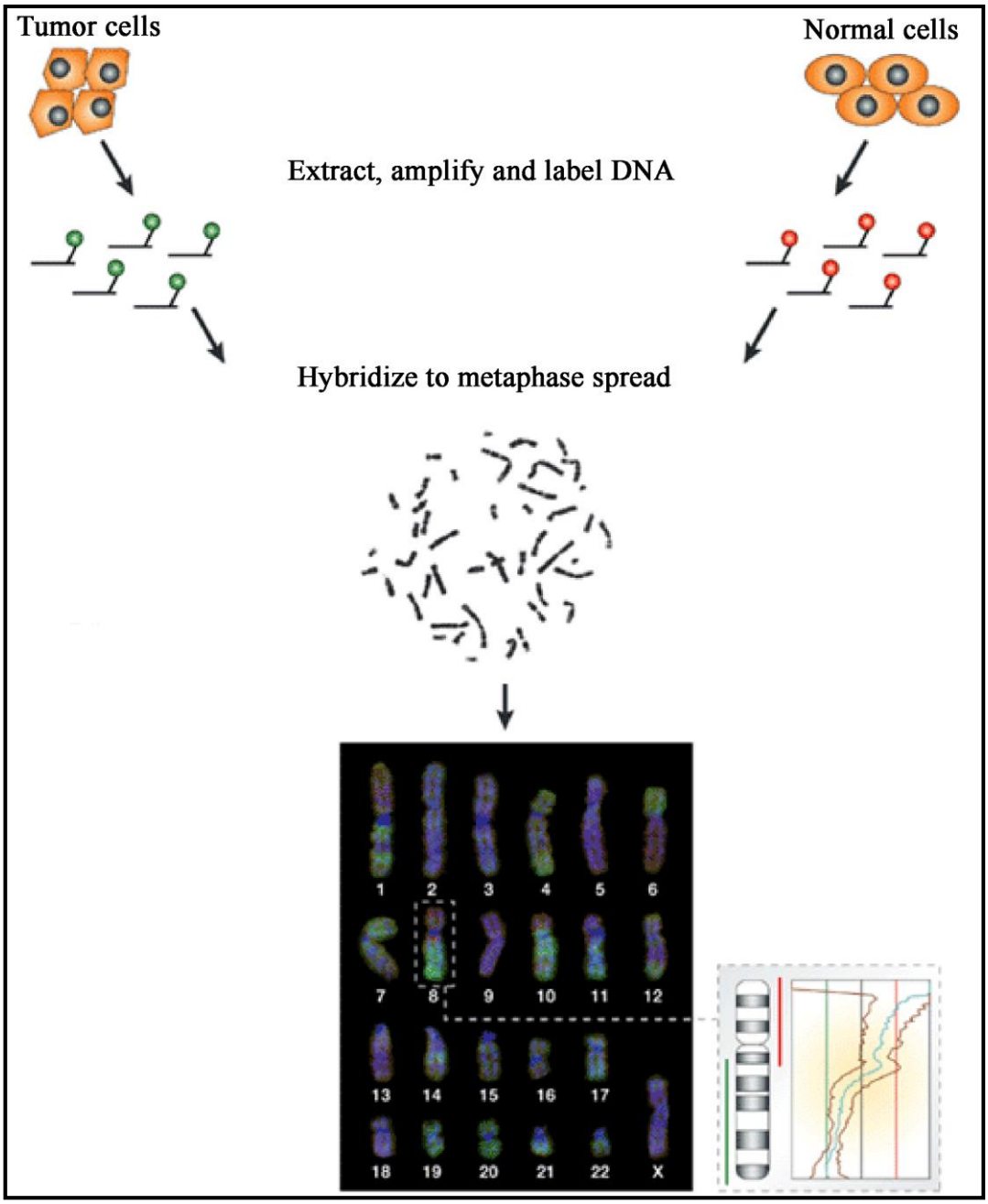

Figure 10. *The steps of conventional comparative genomic hybridization (after Chial et al.[137]). Tumor DNA is labeled with green fluorophore and normal DNA is labeled with red fluorophore. Chrosomal regions that were amplified in the tumor tissue appear green and regions that were deleted appear red on the metaphase spread on the bottom left panel*

Another way of increasing our knowledge about pathogenetically important genetic changes in PNENs is through genetic expression analysis by methods such as Northern blotting, fluorescent in situ hybridization (FISH), quantitative real-time polymerase chain reaction (PCR), DNA microarray, and high throughput sequencing of RNA. In high throughput RNA sequencing (RNA-seq), RNA is

converted to complementary DNA or RNA fragments with adaptors attached to one or both ends[138]. Each fragment is amplified by PCR and sequenced in a high throughput manner to gain short sequences from both ends, known as paired-end sequencing. The resulting reads are then aligned to a reference genome or reference transcripts, which allows quantification of the level of expression for each gene (**Figure 11**).

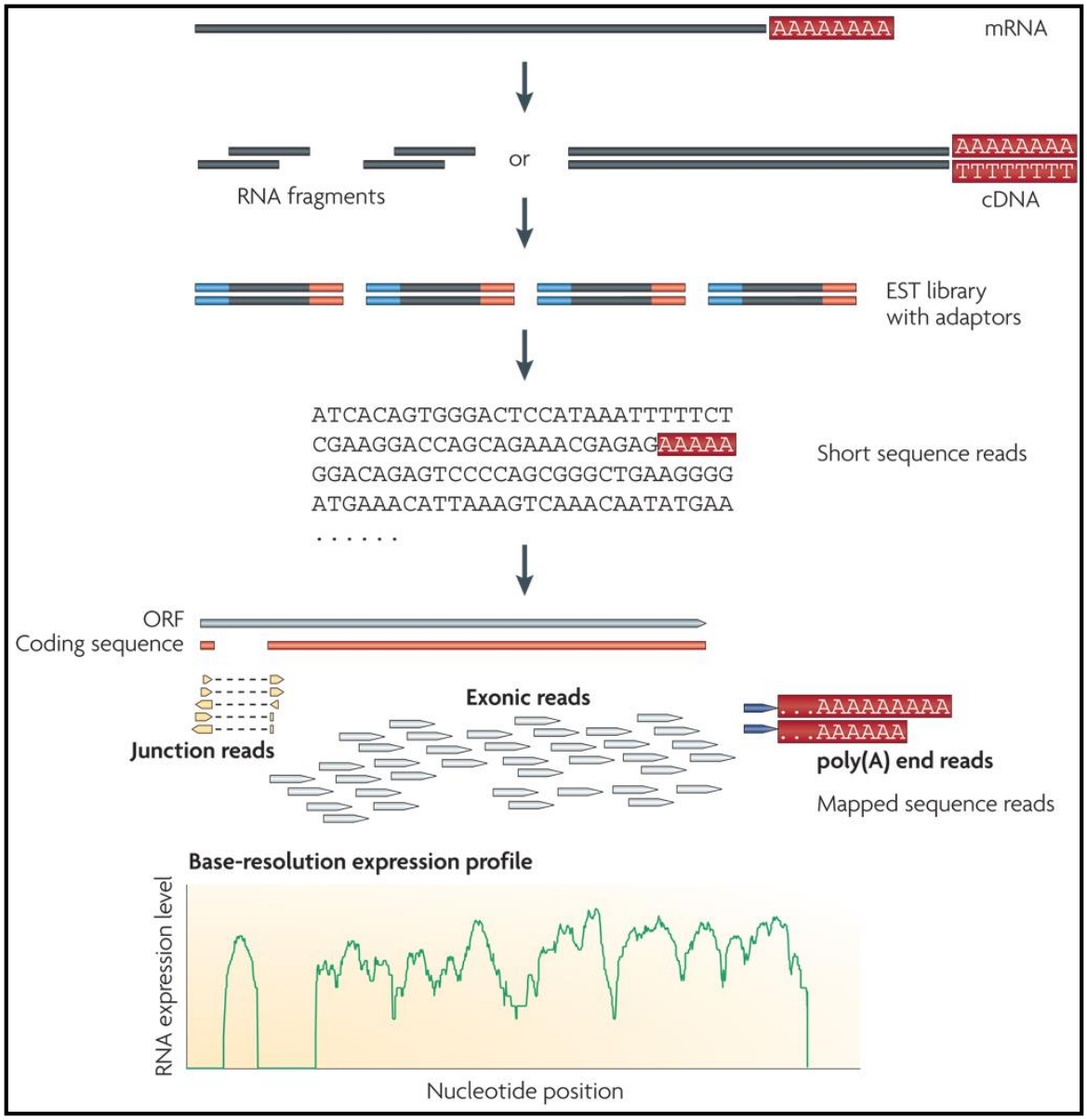

Figure 11. *High throughput RNA sequencing (from Wang et al.[138]). Sequencing adaptors (blue) are added to each fragment. The resulting sequence reads are classified as three types: exonic reads, junction reads, and poly(A) end-reads. mRNA, messenger RNA; cDNA, complementary DNA; EST, expressed sequence tag; ORF, open reading frame.*

At the beginning of this thesis, the Mitelman Database on Chromome Aberrations and Gene Fusions in Cancer reported seven PNENs with karyotypic aberrations[139], but no common chromosomal abnormalities[140-142]. Thus, knowledge regarding the chromosomal characteristics of this type of cancer was clearly insufficient. Information on genomic imbalances in nonfunctioning PNENs detected by CGH was limited to 54 cases[143-146], with common copy number gains of 7q, 17q, and 20q, and common copy number losses of 6q, 11p, and 11q. The available CGH data on PNENs had been obtained studying small and heterogeneous series of neoplasms and the findings were therefore difficult to generalize[128]. Furthermore, at the beginning of this thesis, there were only few studies that had investigated gene expression profiles in PNENs[133, 147-153], and no consistent patterns of upregulated or downregulated genes had been established[154]. Moreover, there were no published reports on high throughput RNA-seq of sporadic nonfunctioning PNENs.

At present, clinical management of patients with sporadic PNEN is largely based on grading and staging as defined by histopathology. However, as mentioned above, the malignant potential among sporadic PNENs of the same grade and stage may vary considerably. A more precise classification of PNENs, based on molecular characteristics might predict prognosis more precisely. Hence, further knowledge of the molecular pathology of these rare and still poorly understood neoplasms might serve as a starting point for development of such prognostic molecular markers. In **Paper IV** of this thesis, we performed karyotyping and CGH in a small series of sporadic nonfunctioning PNENs, in order to identify genomic imbalance patterns that might be important for molecular differentiation of tumor aggressiveness. In **Paper V**, we performed high throughput RNA-seq in the same series of PNENs in order to identify significant intertumor variations of transcripts of protein-coding genes that may reveal yet unknown molecular markers of prognosis.

Aims of the thesis

General aims:

- To investigate different aspects of modern surgical treatment for PNENs

- To identify genomic imbalance and genomic expression patterns that may be important for molecular differentiation of tumor aggressiveness in sporadic nonfunctioning PNENs

Specific aims:

- To describe the feasibility, outcome, and tumor characteristics in a PNEN patient cohort treated with laparoscopic surgery (**Paper I**)
- To evaluate the prognostic value of the WHO 2010 grading system and ENETS TNM system in a PNEN patient cohort treated with laparoscopic surgery (**Paper I**)
- To evaluate the feasibility and outcome of pancreatic surgery with vascular reconstruction in patients with locally advanced PNENs (**Paper II**)
- To compare the effect of combined surgical treatment and chemotherapy against chemotherapy alone, in patients with PNEC (**Paper III**)
- To identify potential prognostic factors for survival in patients with PNEC (**Paper III**)
- To identify genomic aberration patterns that may be important for molecular differentiation of tumor aggressiveness in sporadic nonfunctioning PNENs (**Paper IV**)
- To identify significant intertumor variations of transcripts of protein-coding genes in sporadic nonfunctioning PNENs (**Paper V**)

Summary of results

Paper I

Long-term outcome of laparoscopic surgery for pancreatic neuroendocrine tumors.

World J Surg. 2013 Mar; 37(3):582-90.

This paper reports the outcome of 72 patients at a university hospital in Norway between 1997 and 2011 (**Figure 12**). Sixty-five patients underwent laparoscopic removal of PNEN and their median follow-up was 51 (6-178) months. Overall morbidity was 42%, defined by the revised Accordion Classification, with a surgical morbidity rate of 21% and postoperative pancreatic fistula (POPF) formation of 21%. A higher rate of POPF was observed in patients undergoing laparoscopic enucleation compared with resection. Five-year disease-specific survival rate was 90%. Statistically significant prognostic factors included T stage, R stage, and Ki67 expression above the cut-off value of 5%.

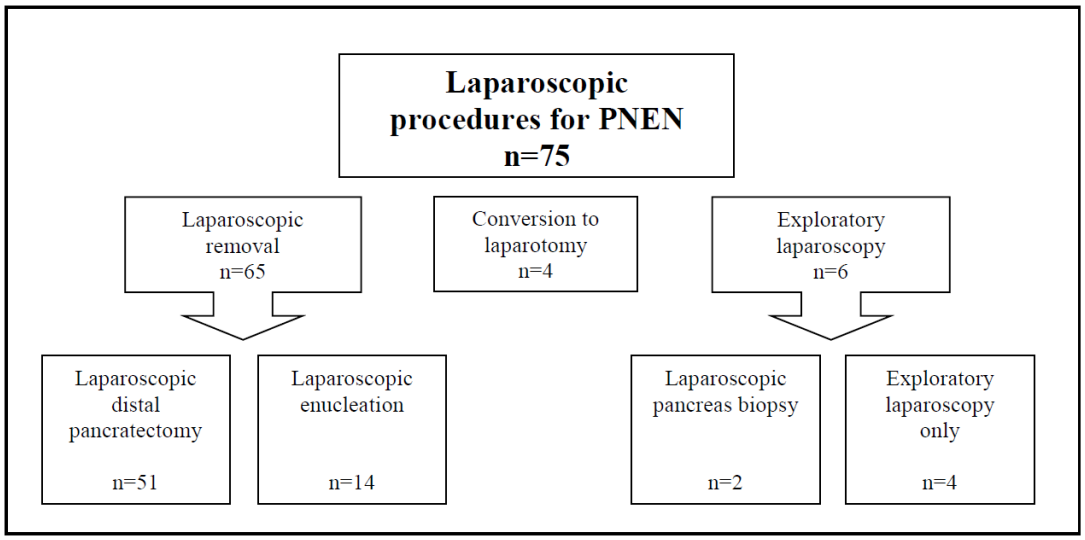

Figure 12. *Flowchart of the patients included in* ***Paper I****. Three patients had repetitive surgery: one patient with a small insulinoma underwent exploratory laparoscopy first and then laparoscopic pancreas biopsy in a subsequent procedure. One patient underwent exploratory laparoscopy first and then laparoscopic enucleation in a second procedure due to intraoperatively detected pancreatitis. One patient underwent a laparoscopic attempt to resect a PNEN in the pancreatic tail, which required cconversion to laparotomy. In the same patient, a laparoscopic attempt at resection of a local recurrence also required conversion to laparotomy.*

Paper II

Pancreatic surgery with vascular reconstruction in patients with locally advanced pancreatic neuroendocrine tumors.

J Gastrointest Surg. 2013 Jul; 17(7):1224-32.

This paper described seven patients with locally advanced PNEN who underwent pancreatic surgery with vascular reconstruction at a Norwegian university hospital. Four patients had metastatic disease at time of surgery. Four patients developed postoperative complications but there was no mortality associated with surgery. Median follow-up was 21 (3-58) months. One patient died 35 months after surgery, three patients had progressive disease 21, 9 and 4 months postoperatively, and three patients had disease in remission 58, 42 and 3 months postoperatively.

Paper III

Surgical treatment as a principle for patients with high-grade pancreatic neuroendocrine carcinoma: a Nordic multicenter comparative study.

Ann Surg Oncol. 2016 May; 23(5):1721-8.

In this paper, the effect of surgery on oncological outcome in patients with PNECs was described in a Nordic multicenter patient cohort. One hundred and nineteen patients were included (**Figure 13**). Median time from surgery for nonmetastatic disease to development of metastasis was 7 months. The median survival was 23 months from time of metastasis for patients undergoing initial resection of the primary tumor in nonmetastatic disease (SURG1), 29 months for patients undergoing resection of the primary tumor and synchronous metastatic liver disease (SURG2), and 13 months for patients with synchronous metastatic disease receiving systemic chemotherapy only (CT2). The following factors were found to be statistically significant independent factors for improved survival after occurrence of metastatic disease: resection of primary tumor, >4 courses of chemotherapy, Ki67 < 55%, and performance status 0.

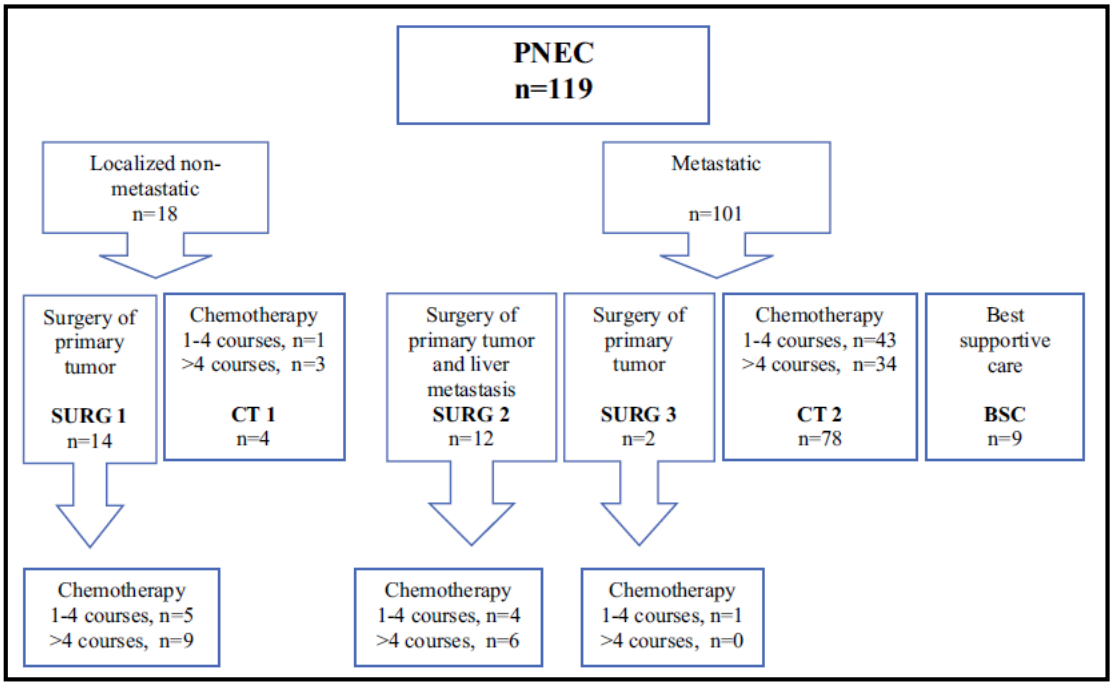

Figure 13. *Flowchart of the patients and treatment groups in* ***Paper III****. Patient data on the number of chemotherapy courses were missing for three patients*

Paper IV

Loss of 11p11 is a frequent and early event in sporadic nonfunctioning pancreatic neuroendocrine neoplasms.

Oncol Rep. 2014 Sep; 32(3):906-12.

In this paper, screening of genomic imbalances in a series of 16 surgical specimens from 15 patients with sporadic PNEN was performed. G-band karyotyping and metaphase comparative genomic hybridization (CGH) were performed. G-banding revealed abnormal karyotypes in 2 of 10 tumor samples analyzed. DNA copy number changes were detected in 13 samples, whereas three tumors showed a balanced genome. In general, gains were more frequent than losses. Common gains were scored at 5p12-13, 4q13-24, 5p15, 5q11-31, and 9q21-22, whereas common losses were found at 11p11, 11p14-15, 11q23, 11p12-13, and 11q22. The average number of copy aberrations (ANCA index) was 12 for 13 nonfunctioning primary tumors, 4.8 for the nonfunctioning tumors with low Ki67 (≥ 5%), 21.2 for the tumors with high Ki67 (< 5%), 2.5 for small tumors (< 3.5 cm), and 17.8 for large tumors (≥ 3.5 cm). There was a statistically significant difference in the ANCA index between the groups defined by Ki67 and tumor size. Nonmetastatic nonfunctioning pancreatic neuroendocrine tumors with low Ki67 (< 5%) and small size (< 3.5 cm) had few aberrations detected by CGH, but frequent loss of material from chromosomal band 11p11.

Paper V

Transcriptomic profiling of tumor aggressiveness in sporadic nonfunctioning pancreatic neuroendocrine neoplasms.

Pancreas. 2016 Feb. Epub ahead of print.

This is an experimental study where high throughput RNA-seq was performed on eleven samples of sporadic nonfunctioning PNEN, grouped in mild disease (n=7; Ki67 < 5% and nonmetastatic disease) and aggressive disease (n=4; Ki67 ≥ 5% and metastatic disease), on Illumina's Genome Analyzer II platform. A set of 309 genes were statistically significantly differentially expressed between the two groups, out of which 143 were over- and 166 under-expressed in the aggressive disease group. Amongst the top protein-coding over-expressed genes, we found genes encoding proteins involved in DNA packaging (*HIST1H2AL*, logFC=-4.1, P-adj=0.03; *HIST1H2BF*, logFC=-3.8, P-adj=6.9e-04), chromosome structuring (*TRIP13*, logFC=-3.7; P-adj=1.0e-06), cytoskeleton structuring (*ADD2*, logFC=-3.5; P-adj=8.5e-04), cell-cell-signaling (*WNT3*, logFC=-3.6; P-adj=1.7e-08; *ITPKA*, logFC=-3.6; P-adj=5.9e-06), and ability to taste (*TAS2R38*, logFC=-3.7; P-adj=0.03). Amongst the top protein-coding under-expressed genes, we found genes encoding proteins involved in neuronal differentiation (*MYT1L*, logFC=5.1; P-adj=8.9e-09), cytoskeleton structuring (*KRT27*, logFC=3.8; P-adj=2.1e-03), cell-cell-signaling (*GABRP*, logFC=3.8; P-adj=2.2e-03), and the immune system (*CTSE*, logFC=3.7; P-adj=0.003).

Methodological considerations

Patient selection and ethics

The patients included in the studies of this thesis underwent treatment for PNEN in the period between 1997 and 2013. Patients included in **Paper I** (n=72), **II** (n=7), **IV** (n=15), **and V** (n=11) all underwent surgery at the Department of Hepato-Pancreato-Biliary Surgery at Oslo University Hospital, Oslo, Norway. Patients included in **Paper III** (n=119) underwent treatment for PNEC at one of the following Nordic university hospitals: Oslo University Hospital (n=14; 7 with surgery), Uppsala University Hospital (Sweden, n=28; 4 with surgery), Copenhagen University Hospital (Denmark, n=25; 1 with surgery), Karolinska University Hospital (Sweden, n=2; no surgery), Helsinki University Hospital (Finland, n=13; 8 with surgery), Haukeland University Hospital (Norway, n=10; 3 with surgery), Trondheim University Hospital (Norway, n=2; no surgery), Stavanger University Hospital (Norway, n=3; no surgery), Aarhus University Hospital (Denmark, n=12; 2 with surgery), and Odense University Hospital (Denmark, n=10; 3 with surgery).

As PNENs are rare and clinically diverse, prospective studies on homogenous cohorts of patients with PNENs are hard to conduct. This is reflected by the fact that there are as yet no published randomized controlled trials involving surgery in this group of patients. The clinical studies contained in this thesis were of retrospective design. This may have led to missed cases of relevant PNEN patients in the study period. The Department of Hepato-Pancreato-Biliary Surgery, Oslo University Hospital, was the only institution performing laparoscopic pancreatic surgery and pancreatic surgery with vascular reconstruction in the South-Eastern Norway Regional Health Authority in the study period. This health authority serves about 2.7 million, is the nation's largest, and includes more than half of Norway's inhabitants. The patient cohorts in **Papers I and II** should sufficiently represent the corresponding health region in the period 1997-2011 for **Paper I**, and 2007-2012 for **Paper II**. Due to the highly aggressive nature and low incidence of PNECs, one could assume that many patients with PNEC may have died before

being diagnosed or referred to a university hospital. This represents an important selection bias in **Paper III**. Because the data were acquired from several institutions in different countries, there may also have been a selection bias associated with divergent diagnostic and treatment strategies.

There was no overlap among the patient cohorts of **Papers I, II and III**. Tumor tissue obtained from one patient in **Paper I** was used in **Papers IV and V**, among tissue samples from other patients. Likewise, tumor tissue obtained from one patient in **Paper II** was used in **Papers IV and V**, among tissues from other patients. Tumor tissues obtained from 11 patients in **Paper IV** were used in **Paper V**. In total, findings from 213 unique patients with PNEN were included in this thesis.

Tissue samples examined in the studies of **Papers IV and V** were collected from the Institutional Biobank for neuroendocrine neoplasms at Oslo University Hospital, established in 2011. In **Paper IV**, patients with sporadic nonfunctioning PNENs were divided in groups according to the Ki67 index of the primary tumor, size of the primary tumor, and whether or not metastatic disease was present at time of surgery. Intertumor copy number variation between the groups, quantified by CGH, was compared. In **Paper V**, the intertumor variation of transcripts of protein-coding genes, *i.e.*, differential expression, was described by means of high-throughput RNA-seq of tissue samples from sporadic nonfunctioning PNENs. Tumor samples were compared according to "aggressive" or "mild" tumor behavior, defined by the primary tumor's Ki67 index and patient's metastatic status. Genetic screening for familial neuroendocrine syndromes was not performed routinely upon diagnosis of a PNEN among the patients included in this thesis. There may therefore have been cases of unrecognized familial PNEN among the patients included.

Papers I and II are classified as clinical audits and necessary permissions were obtained from the hospital review board. **Papers III, IV and V** are classified as

research and were approved by the Regional Committee for Medical and Health Research Ethics (project number: 2012/490 and 2011/1945D), respecting the Helsinki Declaration[155]. The Biobank for neuroendocrine neoplasms at Oslo University Hospital is approved by the Regional Committee for Medical and Health Research Ethics (project number: 2011/497A).

In **Papers I and II**, the revised Accordion Classification was used for definition of surgical morbidity[156] and the International Study Group Definition of Pancreatic Fistula (ISGPF) was used for definition of POPF[157].

In **Paper V**, PNEN tissue was among other variables categorized according to the Ki67 index. Of the 11 samples examined, seven had a Ki67 index of 1-2% while the other four had a Ki67 index ≥ 12%. We believe this was a good design in the sense that there was not an intermediate range of Ki67 values. The potential for identifying differentially expressed genes based on the Ki67 index was thus maximized.

Statistical analysis

In **Paper I**, continuous data were presented as median (range) and analyzed using the non-parametric Kruskal-Wallis test for independent samples. Median was chosen over mean in order to minimize unwanted effects of extreme outliers in the relatively small patient cohorts. A normal distribution was not assumed, as the low sample size in each group did not necessarily indicate such distribution. The Kruskal-Wallis test was applied in order to compare group differences in four independent groups for both continuous data (age, body mass index (BMI), operative time, intraoperative bleeding, and hospital stay) and nominal data (surgical morbidity and POPF). In retrospect, the Chi-squared test should have been used instead of the Kruskal-Wallis test to compare nominal data in **Paper I**. This has later been done and results in a statistically significant group difference for POPF (p=0.029), but no statistically significant group difference for overall

surgical morbidity (p=0.439), which is consonant with the results already presented and discussed in **Paper I**.

In **Paper I**, post-hoc analysis with Bonferroni correction of multiple comparisons and Tukey's test were suggested following rejection of the Kruskal-Wallis test. As the Kruskal-Wallis test was only rejected in one case of group comparisons of nominal data (POPF), such post-hoc analysis was not possible to perform. Instead, a post-hoc analysis of the Chi-squared test results for POPF could have been performed in this case. This has later been done with contingency table analysis, as described by Beasley et al.[158]. First, a contingency table analysis was performed on the chi-square analysis. Then, adjusted standardized residuals (Z-values) for each cell were calculated before they were transformed to chi square values and then to p-values. Finally, the p-values were compared against the Bonferroni-corrected p-value. This resulted in a statistically significant correlation between laparoscopic enucleation of PNENs in the pancreatic head and the development of POPF (p=0.015). This conclusion was not included in the published paper.

One weakness of the statistical model used in **Paper I** was the suggested use of a post hoc test (Tukey's test) meant for parametric data on the assumption of non-parametric data. A more appropriate test would be the Mann-Whitney test for group comparisons after correction for multiple comparisons by the Bonferroni method or Dunn's test. As no statistically significant group differences for continuous data were found with the Kruskal-Wallis test in **Paper I**, there was no need to run an adjusted post-hoc analysis. In **Paper I**, disease-specific survival was estimated using Kaplan-Meier curves and the log-rank test was used to compare differences in survival among patient subgroups.

Only descriptive statistics was performed in **Paper II** due to the low sample size (7 patients).

In **Paper III**, descriptive statistics were presented as frequencies, medians, ranges, and proportions. Overall survival was constructed using Kaplan-Meier curves with accompanying risk tables. Cox-proportional hazard models (uni- and multivariate) were fitted for evaluation of the effect of factors potentially influencing survival. Due to the limited number of patients included in **Paper III** (n=119), a statistical model with five variables was constructed. Each of the chosen variables was tested for clinical relevance (resection of primary tumor, courses of chemotherapy, Ki67 index, small cell morphology, and performance status (PS)) and independence before they were included in the Cox-analysis. The statistical analysis included in **Paper III** was planned and performed in close cooperation with a statistician.

In **Paper IV**, the ANCA index was used to define the prevalence of genomic imbalances in each tissue group. The Mann-Whitney U test was used to compare median for two independent samples without the assumption of a normal distribution.

In **Paper V**, we applied the DESeq2 for differential gene expression analysis of RNA-seq data, using the Wald test[159]. The selected method uses shrinkage estimation for dispersions and fold changes (FCs) to improve stability and interpretability of the estimates. Functional annotation analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID)[160], which allowed identification of overrepresented functional categories among the genes that were differentially expressed.

Pathology

Preoperative cytology or biopsy was generally not performed in the patients included in this thesis. For the patients operated at Oslo University Hospital, preoperative percutaneous biopsy of PNENs was generally avoided because of the theoretical risk of tumor dissemination[161], despite limited evidence for the occurrence of this phenomenon in the case of PNENs. In some cases, preoperative endoscopic ultrasound-guided fine needle aspiration cytology was performed. As

the presence of PNENs is largely detected by cross-sectional (CT and MRI) and nuclear imaging, exact grading by quantification of the mitotic rate and/or Ki67 index is typically possible only after tissue sampling. The possibility of preoperative evaluation of grading of PNENs is a matter of debate, as current techniques for tissue sampling have limitations[162]. The lack of information on tumor grading preoperatively may have influenced the surgeon's choice of procedure and therefore represents a bias.

All surgical specimens associated with **Papers I, II, IV and V** were assessed by pathologists at the Department of Pathology, Oslo University Hospital. During the study period of **Paper I**, the WHO Classification for NENs changed[31, 163]. The Ki67 index then became essential for classification purposes. PNENs assessed before the introduction of the current WHO 2010 Classification without quantification of the Ki67 index were re-assessed in order to allow for re-classification. The remaining specimens were not re-assessed. A re-evaluation of all surgical specimens by two or three independent pathologists would have improved validity of histopathological data in all papers of this thesis. In **Paper I**, a resection status of R2 was defined as residual *metastatic* disease and not residual *local* disease.

Paper I revealed a relatively high fraction of surgical specimens with an Nx status, indicating that lymph nodes were not found by the pathologist. Most of the patients included in **Paper I** underwent distal pancreatectomy, with or without concomitant splenectomy. The total number of lymph nodes found in three of the seven patients included in **Paper II** was remarkably low with two nodes found in a Whipple specimen, one node found in a distal pancreatectomy specimen, and no lymph nodes found in another distal pancreatectomy specimen. This raises questions about suboptimal surgical technique or issues related to suboptimal pathological assessment of the distal pancreatectomy specimens. In the study period of this thesis, pathology assistants performed the gross examination of pancreas specimens at our institution. Peripancreatic lymph nodes were routinely searched

for by palpation and sight, and, if found, dissected from the main specimen with surrounding adipose tissue and sent for histological assessment. Thus, small lymph nodes could have been overlooked. Suboptimal lymph node sampling in PNEN specimens has been reported by others[164]. The method of gross examination of the PNEN specimens at our institution may represent a bias toward underreporting of the actual number of peripancreatic lymph nodes present. One measure of improvement could be to identify, dissect and embed standardized peripancreatic lymph node regions regardless of macroscopic findings.

As PNECs are morphologically and biologically heterogeneous[165], thorough and standardized histopathological reporting is of great importance for treatment planning and prognostic evaluation of patients. Due to the low incidence of PNEC and the ensuing risk of misdiagnosis, cases should be reviewed by pathologists with expertise in the evaluation of GEP-NENs[112]. In **Paper III**, pathologists at each of the participating ten institutions assessed surgical specimens and biopsies. However, there was a lack of a centralized pathology re-evaluation. This is one of the major weaknesses of the study, as it is known that well differentiated neuroendocrine neoplasms and acinar cell carcinoma can be misdiagnosed as PNECs[112].

Karyotyping and comparative genomic hybridization (CGH)

In **Paper IV**, karyotyping and metaphase CGH were performed on samples of PNEN. The aim of the study was to compare intertumor copy number variation between different groups of samples. Tumor samples were disaggregated mechanically and enzymatically and the resulting cells and cell clumps were cultured for 7-10 days. Abnormal karyotypes were only present in two of 10 analyzed tumor samples. As karyotyping of tumors requires culturing of neoplastic parenchyma cells *in vitro*, the low yield of abnormal karyotypes could indicate poor division of neoplastic parenchymal cells in the cell cultures. In our study, we applied a standardized cell culture protocol previously used in our lab with satisfactory results for solid neoplasms[166]. Systematic measures to modify the cell

culture medium and protocol during the study period were not undertaken. It appeared to us that pancreatic neuroendocrine tumor cells do not divide well under laboratory conditions. This may account for the severely limited cytogenetic information of PNENs hitherto reported in the literature with only seven karyotypical abnormal cases in three studies[140-142]. The difficulty in growing pancreatic neuroendocrine cells may be explained by the fact that most PNENs are highly differentiated with a relatively low proliferating rate *in vivo*, as was the case for eight of the 15 patients (Ki67 index < 5%) in our study. Other possible reasons for the low number of clonal aberrations detected by karyotyping could be overgrowth of stromal fibroblasts or contamination with bacteria or yeast, which are known threats to cultures of neuroendocrine cells[167]. When comparing the cell culture protocol applied in our study with protocols applied in studies of successful culturing of pancreatic neuroendocrine cells[168-170], no clear reasons for our failure in establishing effective cell cultures appeared. One measure of improvement in our study could be to implement control of the purity of the neuroendocrine cell preparations. This could have been done by immunostaining with antibodies against specific neuroendocrine cell antigens, such as chromogranin A and synaptophysin[168]. When cytogenetic analysis is performed on cultured cells, it is important to consider whether the results are representative of the *in vivo* situation[121]. Two main types of heterogeneity can be seen in cytogenetic analysis of tumor samples: heterogeneity between neoplastic and non-neoplastic cells, and heterogeneity among various neoplastic cells[171]. Short-term cultures, such as in our study, help minimize such heterogeneity and should therefore be preferred.

In **Paper IV**, CGH was performed on isolated DNA from representative fresh-frozen PNEN tissue. The presence of neuroendocrine tissue in each specimen used in **Papers IV and V** was confirmed by histopathology. Whereas metaphase CGH allows investigation from a chromosomal band level under the microscope, array CGH allows investigation of individual genes[172]. As mentioned above, one limitation of both CGH techniques is that they reflect a theoretical average of tumor samples so that intercellular variability is impossible to assess[121]. Another

limitation is the failure to detect balanced rearrangements such as inversions, insertions, and translocations. As the aim of **Paper IV** was to screen the tumor genome of PNENs for genetic imbalances, without identification of individual genes, metaphase CGH seemed appropriate.

RNA sequencing and bioinformatics approach

In **Paper V**, high-throughput RNA-seq was performed on PNEN tissue and the resulting sequencing data were further analyzed with bioinformatics tools. RNA-seq was performed on isolated RNA from representative fresh-frozen PNEN samples stored at -80º C. Before sequencing, the RNA quality of all tumor samples was evaluated. Insufficient RNA quality was found in four samples out of 15 primary PNENs and these were excluded from further analysis. High-throughput paired-end RNA-seq was performed on a HiSeq 2500 (Illumina, San Diego, CA, USA) platform at the Norwegian Sequencing Centre in Oslo, Norway. Validation of the findings with gene expression profiles of other PNEN series or public datasets was unsuccessful in that Ki67 and clinical data were inconsistently defined in such series, and were not identified in gene expression omnibus and similar databases. This is an important limitation of this study. The lack of validation of the RNA-seq results with other methods, such as real-time PCR or immunohistochemistry in the same patient cohort or larger independent cohorts of sporadic nonfunctioning PNEN, is another important limitation of this study.

General discussion

In this thesis, we sought to investigate different aspects of modern surgical treatment for PNENs (**Papers I, II and III**). Based on surgical specimens from sporadic nonfunctioning PNENs, we then wanted to identify gene expression patterns that may be important for molecular differentiation of tumor aggressiveness (**Papers IV and V**). Below follows a discussion of the main findings.

Laparoscopic surgery for PNENs

At the beginning of the study period for this thesis, there were only few published large series describing laparoscopic surgery of PNENs, with cohorts of less than 50 patients[82, 83]. These studies did not include information on grading according to the current WHO 2010 classification or information on long-term survival related to the laparoscopic approach alone.

In **Paper I**, we reported the, at the time of publication, largest single center series of patients undergoing laparoscopic surgery for PNENs. In a cohort of 72 patients, we demonstrated that laparoscopic surgery with enucleation or distal pancreatectomy, with or without splenectomy, is feasible in patients with PNENs. Feasibility was based on tumor size, operative time, and hospital stay. The operated tumors had a relatively small median diameter of 2.2 cm, but even smaller (0.5 cm) and much larger lesions (9.5 cm) were successfully removed. Whereas laparoscopic distal pancreatectomy (LDP) was performed on both small and large tumors, laparoscopic enucleation (LE) was only performed on relatively small lesions (0.8-2.8 cm). Our findings indicate that LDP is not limited by the size of the primary tumor in patients with PNENs, which had been shown previously and also been confirmed in similar studies, where resections of PNENs with diameters up to 13 cm had been reported[83, 173]. The median operative time for LDP in our study ranged from 175 to 190 minutes, depending on whether concomitant splenectomy was performed or not (shorter with splenectomy). This is similar to earlier reported operative times for LDP (157-230 min)[72, 73, 174] and open distal

pancreatectomy (130-216 min)[73, 175] with and without concomitant splenectomy. The marginally longer operative time for LDP, compared with open distal pancreatectomy, could be explained by inclusion of cases performed when surgeons were still on the early learning curve for the laparoscopic approach. This is supported by evidence of shorter operative times when experience with LDP increases[174]. The duration of LE in our study was shorter than for LDP, especially for PNENs in the pancreatic body or tail (head, 167 min versus body/tail, 111 min). This is coherent with the operative time for LE (120 min)[72, 176] and for open enucleation (140-162 min)[175, 177] reported by others. Operative times do not seem to differ significantly between laparoscopic and open distal pancreatectomy/enucleation in patients undergoing such surgery for PNENs.

Patients undergoing pancreatic surgery are generally at high risk for development of postoperative morbidity and require optimal surgical and postoperative care. However, as surgical techniques and perioperative monitoring and management have improved, durations of hospital stays have become shorter. One important advantage of laparoscopic pancreatic surgery is reduced hospital stay compared with open pancreatic surgery[178]. The median hospital stay in our study was 7 days, which is similar to observations in other studies of LDP and/or LE (6-11 days)[73, 179] and open distal pancreatectomy and/or pancreatic enucleation (8-9 days)[175]. In conclusion, **Paper I** shows that laparoscopic pancreatic surgery, exemplified by LDP and LE, is feasible in the treatment of PNENs. These findings are in agreement with results of comparable studies[67, 69-71].

As mentioned above, pancreatic surgery is generally associated with a high risk of postoperative morbidity. In particular, development of pancreatic fistula is a feared complication besides other common morbidities such as septic complications following intra-abdominal abscess formation and abdominal hemorrhage. Developments in pancreatic surgical techniques aim at minimizing the risk of morbidity and mortality, and improvements have been noted throughout the last three decades[180].

In **Paper I**, we found an overall postoperative complication rate of about 42% with no surgery-related mortality in patients undergoing LDP and LE for PNENs. This is comparable to overall complication rates in similar studies of patients undergoing pancreatic enucleation and resection by laparotomy (45%)[181] and previous studies of LDP and LE for PNENs (31%)[83]. In our study, POPF was the most frequent overall complication and also the most frequent severe complication, occurring in about 21% of the patients. This observation is also in accordance with previously described complication rates in comparable laparoscopic procedures (22%)[83]. Other complications occurred primarily after LPD. These were mild or moderate, such as transient fever treated successfully with antibiotics or a temporary fall in Hemoglobin, which was managed by blood transfusion. An interesting and important finding in our study was the relatively high rate of POPF after LE compared with LDP (50% versus 14%). A higher rate of POPF after enucleation has also been observed in open surgery when compared with resection (38% versus 15%)[181]. This might indicate a higher risk of intraoperatively unrecognized damage to the main pancreatic duct when performing enucleations. Hence, preoperative planning with identification of the main pancreatic duct and estimation of the distance to the tumor margin on cross-sectional imaging is essential to minimize intraoperative damage to the duct and subsequent pancreatic leakage. Based on the high risk of POPF after LE compared with LDP observed in our study, we now routinely perform LDP for PNENs located in the pancreatic body and tail.

Whereas LDP represents a feasible minimally-invasive alternative to LE for PNENs in the pancreatic body and tail, the surgical alternatives to enucleation for removal of lesions located in the pancreatic head are more invasive (*e.g.*, pancreatico-duodenectomy or duodenum-preserving pancreatic head resection[182]). Open enucleation for patients with PNENs in the pancreatic head has been associated with decreased blood loss, shorter operative time, and shorter hospital stay compared to open pancreatico-duodenectomy[175, 181, 183]. Based on this, we

recommend that noninvasive and small PNENs in the pancreatic head be considered for LE rather than more invasive procedures.

In conclusion, **Paper I** demonstrates that feasibility and postoperative morbidity is acceptable after LDP for PNENs in the pancreatic body and tail and acceptable after LE for PNENs in the pancreatic head. The latter conclusion is supported by other studies which have shown that enucleation of pancreatic neoplasms is associated with long-term survival, despite a relatively high risk of pancreatic fistula formation[77, 177]. However, bearing the high risk of POPF and associated complications in mind, we believe that decisions regarding LE for lesions located in the pancreatic head should be evaluated for each individual patient at hepato-pancreato-biliary centers with experience in advanced laparoscopy.

The direct comparison of the results of this study with the findings in other studies should be done with caution as different classification systems for surgical morbidity exist and are not uniformly applied. An obvious weakness of our study is the retrospective design. Thirty-day postoperative morbidity was not assessed systematically for all patients, and occurrence of complications after discharge may therefore have been underreported in our study. Another limitation is that data on postoperative exocrine and endocrine insufficiency were not included. This could potentially have given additional information about the benefits of parenchyma-sparing procedures as the development of exocrine and endocrine insufficiency in theory should be higher in resections compared with enucleations[183].

In **Paper I**, we also evaluated the prognostic value of the WHO 2010 grading system[31] and showed that the suggested cut-off between a PNET G1 and a PNET G2 tumor, defined by a Ki67 index of 2%, did not predict survival in a cohort of patients undergoing laparoscopic surgery for PNENs. Based on similar observations by others, we then tested a Ki67 cut-off value of 5% and could show that this was a significant predictor of prognosis. This is coherent with the results

of comparable studies[38, 43, 44, 85, 108] and may indicate the need for a change in the grading classification for PNET G1 and PNET G2 in the WHO system.

In our study, we also demonstrated a favorable oncological long-term outcome in patients undergoing laparoscopic surgery for PNENs, with an overall 5-year survival of 90%. Based on the Kaplan-Meier estimates, prognosis seemed to correlate with the size of the primary tumor (T stage), something that has been shown previously[38, 184]. The excellent long-term prognosis after surgery for PNENs in our study, with lesions with a median diameter of 2.2 cm, may question the liberal use of surgery for small indolent nonfunctioning PNENs as pancreatic surgery in general is associated with high risk of postoperative morbidity. There is an ongoing discussion about whether small indolent PNENs should be observed or operated. In our study, nonfunctioning PNENs with a diameter as small as 0.5 cm underwent surgery. This is in contrast to other reports where indolent nonfunctioning PNENs < 2 cm generally seem to be observed rather than removed[15, 26, 83]. In our department, the general opinion has been that surgery should be attempted in any case of a suspected localized PNEN as long as the patient's PF is satisfactory. This is supported by the fact that even small PNETs < 2 cm can metastasize, and cases of small PNECs do occur, as exemplified by the patient cohort in **Paper III** of this thesis and other studies[185, 186].

In **Paper I**, histopathology showed that most patients undergoing a resection or enucleation of a PNEN, were classified as ENETS T1 (47%), Nx (69%), and G1 (69%). Most of the patients did not have metastatic disease at time of surgery (83%). An unexpected finding in our study was the high number of specimens without any observed lymph nodes (Nx status) while noticing a positive lymph node status in a third of the patients where lymph nodes were actually found. In specimens after both open and laparoscopic distal pancreatectomy, an average lymph node sampling rate of 12-14 has been reported, with no significant difference between open and laparoscopic surgery[76]. However, there are also reports showing a lower sampling rate in LDP compared with open surgery[74]. On

the other hand, enucleations are associated with a low lymph node sampling rate compared with standard resections (such as distal pancreatectomy)[164]. An important observation in our study, which was not addressed in the published paper, was that nearly all spleen-preserving LDP specimens were classified as Nx (22 of 23 specimens), whereas this was the case in only a third of the LDP specimens with spleen (9 of 28 specimens). Hence, LDP with concomitant splenectomy seems to increase the lymph node sampling rate in PNEN specimens. Furthermore, all of the 14 LE specimens were classified as Nx. The high number of specimens with an Nx status in our study raises questions about suboptimal surgical technique, as it is known that surgical technique in patients undergoing distal pancreatectomy influences the lymph node sampling rate[187]. Low lymph node yield after distal pancreatectomy has led to development of techniques such as the radical antegrade modular pancreatosplenectomy (RAMPS)[187, 188]. This procedure is also possible to perform laparoscopically[189]. We have not found obvious surgical reasons for the generally low lymph node yield in our study. Therefore, in cooperation with the pathologists at our institution, we have now initiated a protocol for standardized pathological assessment of LDP specimens in order to find ways of improving the lymph node sampling rate.

The role of lymph node sampling in PNEN is not yet fully understood, but there are several studies suggesting lymph node status as a prognostic factor[44, 190-196]. It is also known that the risk of peripancreatic lymph node metastasis in patients with PNENs correlates with increasing tumor size and tumor grading[193]. However, only about 40% of patients with PNENs who undergo surgery have lymph node metastasis[26], which could indicate that lymphadenectomy should not be performed in all patients. Thus, there is an ongoing debate as to whether or not peripancreatic lymphadenectomy should be performed routinely in patients with PNEN, regardless of the type of surgical procedure[190-192, 197-199].

It is of great importance to know the prognostic implications of positive lymph nodes in patients with PNENs and to know to what extent laparoscopic pancreatic

surgery can provide sufficient lymph node sampling to achieve optimal oncological outcome. Taking recent observations from other studies into consideration and knowing that issues related to lymph node sampling in patients with PNENs have not been investigated in a randomized manner, we believe that lymph node sampling should be performed routinely when performing laparoscopic removal of PNENs, to avoid understaging[78, 200].

Since the initiation of the work contained in this thesis, LDP has now become an established procedure at several institutions worldwide[72, 73, 75, 79, 174, 178, 201-203]. The procedure provides similar short- and long-term oncological outcomes as open distal pancreatectomy[76] and seems to be a cost-efficient alternative to open distal pancreatectomy[80]. Besides LDP for PNENs in the pancreatic body and tail, laparoscopic enucleation of nonfunctioning PNENs in the pancreatic head[204] and laparoscopic pylorus-preserving pancreatico-duodenectomy[205] (laparoscopically assisted or total laparoscopic) are advanced, but feasible procedures that can be considered in selected cases. A single-incision approach could also be performed, with comparable feasibility measures[206]. Attempts to compare laparoscopic and open surgery for PNENs have been made, but the results are inconclusive since different procedures are included[82, 207]. The first comparative study of laparoscopic and open distal pancreatectomy for patients with PNENs was recently published[173] showing a significant reduction in postoperative morbidity without compromising oncological outcomes and survival. In our department, initiatives have been taken to conduct a retrospective international multicenter comparative study between laparoscopic and open distal pancreatectomy.

In conclusion, **Paper I** demonstrates that laparoscopic surgery of PNENs is feasible with acceptable surgical morbidity and a good overall disease-specific long-term prognosis. This is supported by the current ENETS consensus guidelines, where **Paper I** has been cited[15]. However, laparoscopic surgery for PNENs is still a barely investigated field of surgery. Prospective surgical trials are difficult to conduct due to the low incidence and significant clinical heterogeneity

of the disease. This is reflected by the fact that there are no trials on surgery for PNEN registered in ClinicalTrials.gov[208] (a registry of clinical studies maintained by the American National Library of Medicine). As the number of comparative studies on laparoscopic surgery for PNENs is very limited and no randomized trials exist to date, surgeons should focus on conducting large retrospective multicenter comparative studies and consider initiating randomized trials[209].

Vascular reconstruction in patients with PNENs

The increasing use of laparoscopic pancreatic surgery in the treatment of PNENs in recent years could indicate a parallel gradual decline in the use of open pancreatic surgery for this group of patients. However, we have observed that as many PNENs are nonfunctioning and slow-growing, a substantial proportion of these present with locally advanced disease, which may not be tackled by minimally-invasive procedures. In this thesis, locally advanced disease was defined as a PNEN with an ENETS T3- (confined to pancreas, > 4 cm, or invasion of duodenum or bile duct) or T4-stage (invasion of adjacent organs or major vessels).

Surgical treatment of locally advanced PNENs, and T4 tumors in particular, remains controversial[84]. There are previous reports which discuss vascular reconstruction among patients with locally advanced PNENs[84, 86-96, 210], but none of these discuss the role of vascular reconstruction as such. This is remarkable, as the concept of vascular reconstruction has already been discussed widely over years in the treatment of locally advanced PDAC[97, 98, 211], generally a much more aggressive disease. Among patients with PDAC, combined portal vein resection and reconstruction provides acceptable morbidity and mortality, compared with nonsurgical treatment[101]. Therefore, in **Paper II**, we wanted to evaluate the feasibility and surgical morbidity of pancreatic surgery in all patients with locally advanced PNEN who underwent pancreatic surgery with vascular reconstruction in the period between 2007 and 2012. The study is among the largest addressing the issue of pancreatic resection with vascular reconstruction for locally advanced

PNENs and shows that this is feasible with acceptable postoperative morbidity and no mortality.

In **Paper II**, we reported vascular reconstruction in seven patients with locally advanced PNEN. Vascular reconstruction was performed on the portal vein, common hepatic artery, left hepatic artery, and/or left gastric artery. In addition to vascular surgery, extensive visceral resections were required in five patients. Relatively high levels of intraoperative bleeding (500-4750 ml) and long operative times (232-718) were observed. Severe complications developed in two patients, with upper gastrointestinal bleeding secondary to ischemic necrosis of parts of the stomach in one patient (successfully treated with transfusion) and development of a liver abscess and pseudoaneurysm of the pancreatico-duodenal artery (successfully treated with percutaneous transluminal embolization). The complexity of the procedures led to a relatively long overall median hospital stay (median 25 days).

Previous studies have shown that resection for locally advanced PNENs is feasible and can result in favorable disease-free survival and overall survival in selected patients[212], despite risk of recurrence[84]. This is supported by evidence that resection of the primary tumor in patients with PNEN is associated with improved survival across all stages of disease[55]. When not operated on, patients with locally advanced disease may suffer from complications related to local mass effect and infiltrative growth, including gastrointestinal bleeding, vascular/intestinal/biliary obstruction, and occlusion of the superior mesenteric (SMV) or portal vein (PV)[48, 86].

Today, surgery for locally advanced PNENs with vascular involvement remains controversial, which is probably reflected by the low number of studies published on this topic. Our study has certain limitations besides the obviously low number of samples. It is of retrospective design and includes some patients who underwent concomitant resection of metastatic liver disease. This may have biased feasibility

and surgical morbidity. However, taken these limitations into consideration and based on the findings in our study, which are coherent with findings from previous studies on locally advanced PNENs, we would recommend that surgery is always considered in these patients, even in cases where vascular encasement or infiltration is suspected. This is also in line with the current ENETS consensus guidelines, where **Paper II** has been included as one of the references[15]. Important to remember is that in most cases where preoperative radiologic evaluation suggests vascular involvement, or the intraoperative findings of a partial encasement of a large vessel, the tumor can be removed with careful dissection without the need of vascular reconstruction[91].

Surgery for PNECs

PNEC is a highly malignant disease that typically invades adjacent structures or has metastasized at diagnosis[213]. At the beginning of the study period for this thesis, some surgeons[120], oncologists[113] and pathologists[45] had shown interest in this rare and highly aggressive malignancy. The largest cohort of patients with advanced GEP-NECs was published by the Nordic Neuroendocrine Tumor Group (NNTG)[113] in 2013. The cohort included 305 patients, out of whom 71 had a PNEC, which was associated with a median survival of only 15 months. Interestingly, 15% (n=11) of the PNEC patients had a resection of the primary tumor performed. However, the role of surgery was not assessed further. Surgical resection is an established treatment method for PDAC[214], a much more common cancer than PNEC with an incidence ratio of 11-14 per 100,000 person-years[215, 216] (versus < 0.1 per 100,000 person-years for PNECs), but with comparable malignant potential. As improvement of treatment options and treatment sequences in patients with PNEC is urgently needed, we wanted to investigate the effect of surgery on survival and to identify potential prognostic factors for the survival in patients with metastatic PNEC.

In **Paper III**, we described the first comparative study on the effect of combined surgical treatment and chemotherapy versus chemotherapy alone, in patients with

PNEC. In a cohort of 119 patients, 28 patients underwent combined surgery, 82 patients received only chemotherapy, whereas 9 patients received best supportive care. During a median follow-up period of 13 months (0-165 months), 92 patients (77%) died of disease. In this study, we showed, for the first time, that surgical treatment combined with chemotherapy may improve survival in patients with metastatic PNEC compared with chemotherapy alone.

One of the main findings in our study is that resection of the primary tumor was an independent prognostic factor of improved survival for patients at different disease stages (HR 1.28, 95% CI 1.4-5.7). The study demonstrated that surgery and chemotherapy of nonmetastatic disease improved survival, despite recurrent disease after a median of 7 months postoperatively, compared with chemotherapy alone (median survival 23 versus 13 months). In a recent comparable study, patients with nonmetastatic PNEC $\leq$ 2 cm who underwent resection, had a median survival of 29 months, while patients who were left to best supportive care had a median survival of 5 months (versus 2 months in our study)[217]. The three-year survival from time of metastasis after resection of nonmetastatic PNEC in our study was surprisingly high (45%). This is consistent with a similar current report of a 5-year survival of 43% after surgical resection of nonmetastatic PNEC[26]. An important observation, and essential limitation, is that the case number of patients with nonmetastatic PNEC undergoing surgery in these studies, including our paper, was generally low ranging from 20 to 26.

According to the current ENETS consensus guidelines for GEP-NECs, combination of postoperative platinum-based chemotherapy with local treatment consisting of surgery, radiotherapy, or both probably offers the greatest likelihood of long-term survival for patients with nonmetastatic disease, irrespective of the exact site of the primary[106]. As most of the patients in our study were treated with platinum-based chemotherapy regimens (81%), our data support this recommendation for nonmetastatic PNEC and suggests surgery and adjuvant systemic platinum-based chemotherapy.

The impact of surgery on the prognosis of patients with locally advanced PNEC, defined as ENETS T-stages T3 or T4, has not been evaluated yet. However, the results of **Paper III**, which includes 19 patients with a T3 or T4 PNEC, indicate that surgery combined with chemotherapy may improve survival in locally advanced disease when compared with chemotherapy alone. The current NANETS consensus guidelines provide only an expert opinion on this matter, which supports surgery, if the risk of morbidity is low and the risk of intestinal obstruction is high[118]. The results of our study are in accordance with this recommendation. Furthermore, we would suggest surgery for resectable locally advanced nonmetastatic PNEC for selected patients, despite a higher risk for a margin-positive resection. This is supported by an observed improved survival after R0/R1 resections of PNEC, compared with R2 resections[218].

Most patients with PNEC develop distant metastases, which are often present already at diagnosis[219]. This reduces the prospects for long-term survival. Eighty-five percent (n=101) of the patients in our study had synchronous metastatic disease. Surgery for metastatic disease in patients with PNEC is not recommended in the current ENETS and NANETS guidelines[106, 117, 118]. This is also supported by a recent international consensus conference on the treatment of neuroendocrine liver metastasis[220]. Of the patients with synchronous metastatic disease in **Paper III**, 14 underwent surgical treatment for the primary tumor alone (n=2) or the primary tumor and metastatic liver disease (n=12). In spite of the limitations of our study, the reported three-year survival rate of 69% among 12 patients with surgical treatment of all metastatic disease questions the very rigid current international guideline recommendations. Another important finding in our study is that surgical treatment of both the primary tumor and metastatic liver disease combined with systemic chemotherapy may improve the survival of patients with metastatic PNEC compared with chemotherapy alone (median survival 29 versus 13 months). This is coherent with a comparable study where overall survival after surgery of metastatic PNEC was 24 months with a 5-year survival of 21%, among 13 patients who underwent surgery for metastatic liver disease[221].

One important limitation of the study reported in **Paper III** is that predefined criteria for surgical treatment were lacking. When considering the retrospective nature of the study and the fact that the patients included were from hospitals in different countries, this bias seemed difficult to eliminate. Variations in surgical treatment protocols for PNEC patients among the participating hospitals should be assumed. One observation suggesting this was that surgical activity on PNEC patients differed among the hospitals.

In **Paper III**, we observed that most nonmetastatic PNECs recurred or metastasized within one year after resection (in 13 out of 14 patients). This might suggest the presence of occult metastases at diagnosis. Thus, postoperative platinum-based chemotherapy should probably always be considered for patients with PNEC, regardless of the stage of the disease and provided the treatment is tolerated[106]. The multivariate regression analysis in **Paper III** showed that > 4 courses of postoperative chemotherapy was a significant factor of improved survival compared with 1-4 courses (≤ 4 versus > 4 courses; HR 3.1, CI 1.9-5.2). This finding is influenced by a selection bias as some patients will die or show clinical deterioration before receiving more than four courses of chemotherapy. However, based on these findings and being aware of the limited clinical evidence available, we recommend upfront surgery of nonmetastatic disease with postoperative systemic chemotherapy of more than four courses. This is also supported by expert opinion reported in the current NANETS guidelines[118].

In **Paper III**, we found the Ki67 index to be another significant predictor of survival in patients with PNEC (≥ 55% versus < 55%; HR 2.2, CI 1.3-3.6). As prognosis of GEP-NECs correlates with the Ki67 index, tumors with a very high Ki67 are more aggressive than tumors with a Ki67 just above 20%[113, 222]. At the same time, GEP-NECs with a high Ki67 (≥ 55%) do respond better on systemic platinum-based chemotherapy than GEP-NECs with a lower Ki67 (< 55%)[113]. Based on these observations it could be argued that upfront surgery should be performed for PNECs with a Ki67 < 55% and neoadjuvant systemic platinum-

based chemotherapy for PNECs with a Ki67 ≥ 55%. There are, so far, no systematic studies that have assessed the use of neoadjuvant chemotherapy in patients with apparently nonmetastatic PNEC. However, given the early manifestation of recurrent metastatic disease after surgery for apparently nonmetastatic PNEC in our study, measures to elucidate the effect of neoadjuvant chemotherapy on survival in these patients seem warranted. Based on what we currently know about GEP-NECs in general and PNECs in particular, we recommend neoadjuvant systemic platinum-based chemotherapy for PNECs with a Ki67 ≥ 55%. However, this should be considered as nothing more than an expert opinion.

A discrepancy in grading defined by mitotic rate and Ki67 has been observed in up to 44% of PNENs[223]. PNENs with a mitotic rate within the G2 range and a Ki67 index corresponding to G3 have been described[165, 224, 225]. Such "grade-discordant" PNENs were found to have better prognosis compared with true PNECs (median survival 54 versus 11 months), but a worse outcome compared with "grade-concordant" PNENs (median survival 54 versus 68 months)[165]. A further recent observation that exemplifies the heterogeneity of PNECs is the difference in response rate to first-line platinum-based systemic chemotherapy among patients with a GEP-NEC depending on whether they had a Ki67 above or below 55%. Interestingly, the response rate correlated with the Ki67 index (response rate 42% with Ki67 above 55% versus 14% with Ki67 below 55%)[113]. Grade concordancy, as described in this section, was not included in our study. However, based on the results of other aforementioned studies, the existence of a "grade-discordant" group of PNEC with unique clinical features and the association between the Ki67 index and effect of platinum-based chemotherapy of PNEC, imply the need for modification of the current WHO 2010 grading system for PNENs[31].

Coming back to the issue raised earlier about lymph node sampling in patients with PNENs, it is unknown if peripancreatic lymphadenectomy improves survival in patients with PNEC since this has not been evaluated in clinical trials. However,

as lymph node stage predicts prognosis, peripancreatic lymphadenectomy should probably be performed routinely in patients undergoing surgery for PNEC.

Considering the aggressive nature of PNEC, one can assume that the performance status (PS) of patients suffering from PNEC deteriorates rapidly. This assumption is in line with our study, where 20% of the patients presented with a WHO PS equal to or above 2. We also found the grade of PS to be a significant variable of survival (PS 1 versus PS 0; HR 1.9, CI 1.0-3.3; PS ≥2 versus PS 0; HR 7.5, CI 3.4-16.6).

As mentioned before, there are important limitations to our study other than the small sample size, especially for the patients who underwent surgical treatment, and the retrospective design, which both explain a selection bias of the study. The lack of a centralized histopathology reevaluation of the tissues from the enrolled patients has led to comparison of results from different pathologists over many years, which may diverge in terms of tissue staining techniques and Ki67 index assessment procedures. The lack of Ki67 values from both primary tumor and metastatic tissue was another limitation. For seven patients, all of whom underwent surgery, Ki67 was determined from both the primary and metastatic tissue. Metastatic tissue generally had a higher Ki67 than primary tumor tissue, consistent with other reports of other GEP-NENs[226, 227]. However, in our study, the mean Ki67 value for those who did not undergo surgery was 48 ± 26%, whereas the mean Ki67 value of the tissues from the surgically treated patients was 47 ± 26%. Based on these data, the two groups seemed comparable in terms of tumor biology as defined by Ki67. Another limitation of the study was absence of data on the total hepatic tumor burden for patients with metastatic liver disease as well as heterogeneity of the chemotherapy regimens that were administered. However, given the very low incidence of PNECs and the current shortage of evidence concerning surgical treatment of this patient group, we believe that **Paper III** gives important novel knowledge about multimodal management of PNEC. It raises important questions that should be further investigated in future studies.

In conclusion, the findings of our study suggest that resection of the primary tumor should be considered, and additionally, that patients with resectable PNEC and resectable synchronous metastatic disease should be considered for surgery of both the primary tumor and the metastases. This is supported by recent reports of the effect of surgery on survival for PNENs across all stages of disease, including PNECs[49, 109]. Since work began on this thesis, a novel interest in surgical treatment of PNEC has been noted[26, 217, 218], although the underlying clinical evidence for a surgical approach is still scarce and prospective trials are lacking. There is a need for raised awareness of this evolving field in pancreatic surgery. Further efforts should be made to increase the attention of surgeons to surgery of PNEC as a part of a multimodal management of patients with PNEC aiming at improved survival. The therapeutic approach for nonmetastatic PNEC is at present neither consistent nor uniform[228]. Future studies on surgical treatment of PNEC should focus on the establishment of standardized sequences of treatment, especially the combined use of platinum-based chemotherapy pre- and postoperatively[229]. PNECs should be studied as a separate entity with precise reporting of their characteristics in future trials[230]. Moreover, initiatives should be taken to plan and conduct prospective multicenter studies. We propose the need for a prospective trial on surgical treatment combined with platinum-based chemotherapy of resectable metastatic disease versus platinum-based chemotherapy alone. As the current guidelines are exclusively based on expert opinions, and considering the latest clinical data on this matter that diverge from the recommendations, we could expect that the expert opinions may be modified in the near future as more clinical data on surgery of PNEC become available.

Genomic imbalance profiling in PNENs

As mentioned earlier, most PNENs are sporadic and nonfunctioning[11, 15, 16]. In the patient cohorts included in the clinical part of this thesis, around half of the patients underwent removal of a sporadic nonfunctioning PNEN (**Papers I and II**).

In **Paper IV**, we performed karyotyping and CGH on a small series of sporadic nonfunctioning PNENs. The aim was to describe genomic imbalances in the whole cohort and to stratify these by known factors of prognosis. In doing so, cytogenetic data were compared between tumor samples that had been stratified by three known prognostic factors: the Ki67 index, metastatic status, and size of the primary tumor[184]. In **Paper I**, we showed that a Ki67 cut-off value of 5% is a prognostic factor of survival after laparoscopic surgery for PNENs, something that has also been seen by others[44]. We therefore chose a Ki67 cut-off value of 5%. Our study was the first to correlate data obtained by CGH with karyotyping and cell proliferation (Ki67 index) in patients with PNENs.

We found copy number changes to be common in sporadic nonfunctioning PNENs, with an ANCA index of 12. Common gains were scored at 5p12-13, 4q13-24, 5p15, 5q11-31, and 9q21-22, whereas common losses were scored at 11p11, 11p14-15, 11q23, 11p12-13, and 11q22. After subdivision of the results according to Ki67, metastatic status, and tumor size, we found a higher ANCA index in samples from patients with high Ki67, metastatic disease, and a large-sized tumors (≥ 3.5 cm), which implicates acquired genomic imbalances in sporadic nonfunctioning PNEN progression. The most frequent genomic imbalance in our series was loss of 11p11, which was not only seen in tumor samples of more "aggressive" behavior but occurred frequently also in samples with low Ki67, non-metastatic disease, and small tumor size. We interpreted the loss of 11p11 as a potential early event in PNEN tumorigenesis and proposed the chromosomal band 11p11 as a possible location of relevant hitherto unrecognized tumor suppressor genes in sporadic nonfunctioning PNENs.

Previous studies of genomic imbalances in nonfunctioning PNENs detected by CGH have examined, to the best of our knowledge, 54 cases[143, 144, 146, 231]. Common gains were described in 7q, 17q, and 20q, whereas common losses were seen in 6q, 11p, and 11q. The *MEN1* gene, located in chromosomal band 11q13.1, is associated with development of some PNENs[129]. The presence of copy number losses at 11q might indicate *MEN1* mutations. In our study, loss of chromosome band 11q13 was not particularly common as it was seen in only four sporadic nonfunctioning PNENs (3 mild and 1 aggressive), which is consistent with the presence of mutations in the *MEN1* gene in less than half of all patients with PNENs[129]. Other associations between the genomic imbalances found in our study and genes associated with PNEN tumorigenesis are suggested in **Table 4**.

Out of ten samples that were karyotyped, we found one abnormal karyotype with an extra chromosome 12 as the only clonal aberration, a change that was confirmed by CGH. Previous studies of genomic imbalances in PNENs have reported seven cases with karyotypic aberrations, but no clearly nonrandom, let alone specific, chromosomal abnormalities[140-142].

In summary, **Paper IV** indicates the existence of distinct cytogenetic patterns in sporadic nonfunctioning PNENs, depending on their Ki67 index (cell proliferation), metastatic status, and size. In particular, loss of chromosomal band 11p11 might indicate the site of a primary, or at least early, pathogenetic event in these neoplasms. Our study represents a step towards molecular pathological classification of sporadic nonfunctioning PNENs, despite important limitations such as the small sample size and the lack of validation of the findings in larger series. Future investigations should focus on potential primary events of tumorigenesis on chromosome band 11p11.

Genomic expression profiling in PNENs

In **Paper V**, we performed high-throughput sequencing and bioinformatics analysis of a series of sporadic nonfunctioning PNENs. The aim was to identify

expression patterns that could be important for molecular differentiation of tumor aggressiveness in a small series of sporadic nonfunctioning PNENs. To our knowledge, the study is the first published report on high-throughput transcriptome sequencing of sporadic nonfunctioning PNENs.

Tumor aggressiveness was dichotomized into “mild” and “aggressive” disease, based on three parameters. These parameters were: the degree of cell proliferation as indicated by the Ki67 index (< or ≥ 5%), the presence of metastatic disease at time of diagnosis, and the size of the primary tumor (< or ≥ 3.5 cm). "Aggressive tumors" were defined by a Ki67 index ≥ 5%, metastatic disease at time of surgery, and tumor size ≥ 3.5 cm.

We found a set of 309 protein-coding genes that were significantly differentially expressed according to tumor aggressiveness, of which 166 were upregulated and 143 downregulated in the aggressive disease group. Some of the most upregulated genes were involved in DNA packaging (*HIST1H2AL*, *HIST1H2BF*), ability to taste (*TAS2R8*), chromosome structuring (*TRIP13*), cytoskeleton structuring (*ADD2*), and cell-cell signaling (*WNT3*, *ITPKA* and *GDF15*). Among the most downregulated genes were genes involved in neuronal differentiation (*MYT1L*), cytoskeleton structuring (*KRT27*), cell-cell signaling (*GABRP*), and immune reactions (*CTSE*). Although some of these genes have already been associated with malignancies, others have never before been linked to cancer.

Four of the most upregulated genes were of particular interest: *HIST1H2AL*, *HIST1H2BF*, *ADD2*, and *WNT3*. *HIST1H2AL* and *HIST1H2BF*, also known as histone cluster 1 H2al and histone cluster 1 H2bf, express two of the four core histones that are essential for packaging of DNA in nucleosomes. Histones play an important part in the process of gene expression[232]. Our data suggest, for the first time, a correlation between aggressiveness of sporadic nonfunctioning PNENs and expression of histones encoded by *HIST1H2AL* and *HIST1H2BF*. An interesting

observation was the presence of copy number gain on 6p22 in one patients with aggressive disease in **Paper IV**. This band covers the gene locus of *HIST1H2AL* and *HIST1H2BF* (6p22.1) (**Table 4**). Correlation between grade of pulmonary neuroendocrine tumors and expression of histone H1.5 has been reported[232]. Expression of *HIST1H2AL* and *HIST1H2BF* might similarly be of use as an immunohistochemical marker of tumor aggressiveness in sporadic nonfunctioning PNENs. This could be further investigated in future studies using commercially available antibodies. In our study, *ADD2* was significantly overexpressed in tumors associated with high aggressiveness. Overexpression of *ADD2 (adducin 2)* in NENs has not been reported previously. However, expression of adducin genes correlates with grade of proliferation and Ki67 expression in basal cell carcinoma and oral/cutaneous squamous cell carcinoma[233], and may therefore represent another yet undescribed marker of tumor aggressiveness in sporadic nonfunctioning PNENs.

One of the main findings in **Paper V** was the upregulation of *WNT3* (17q21) in sporadic nonfunctioning PNENs with aggressive behavior. This was in line with the findings of copy number gains on 17q21 in 3 of the 13 analyzed tumor samples, all of which showed aggressive behavior. These findings suggest a relationship between the level of *WNT3* expression and the grade of tumor aggressiveness in sporadic nonfunctioning PNENs. *WNT3* encodes a secreted signaling protein associated with regulation of cell fate and patterning during embryogenesis through activation of the Wnt signaling pathway, as illustrated in **Figure 14**. Cell proliferation is stimulated through Wnt signaling, which increases nuclear and cytoplasmatic levels of beta-catenin[234]. Beta-catenin leads to increased expression of proteins such as cyclin D1 and MYC that both control the G1 to S phase transition in the cell cycle[235]. Previous studies have shown a relationship between the expression of *WNT3* and development of colorectal cancer[236], malignant mesothelioma[237], non-small cell lung cancer[238], breast cancer[239], and cholangiocarcinoma[240]. Moreover, evidence exists that *WNT3* expression stimulates pancreatic islet beta-cell proliferation[241]. Aberrant Wnt signaling has

also been correlated with decreased expression of Wnt inhibitors in a pancreatic neuroendocrine cell line[242, 243]. There is also evidence suggesting that menin (encoded by *MEN1*) activates the Wnt signaling pathway resulting in inhibition of islet tumor cell proliferation in a mouse model[244]. Further, it was recently been demonstrated that neurotensin is a target of the Wnt signaling pathway and promotes growth of pancreatic neuroendocrine cells[245]. These observations strengthen the idea of the Wnt signaling pathway as a potential target for molecular markers of prognosis in patients with sporadic nonfunctioning PNEN.

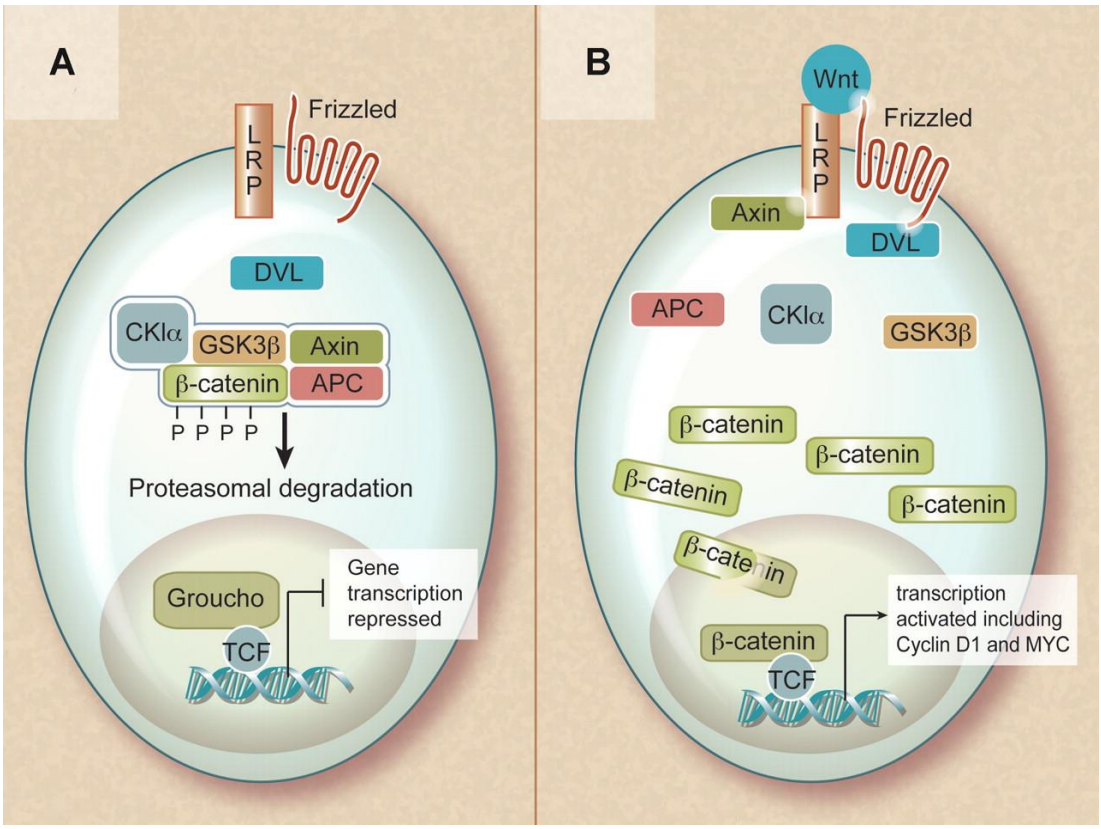

Figure 14. *The Wnt signaling pathway (from Ansell[246]).* ***A****, in the absence of a Wnt signal.* ***B****, in the presence of a Wnt signal*

The association between PI3K/Akt/mTOR pathway genes, novel genes associated with PNENs, genomic imbalance profiling results of **Paper IV**, and genomic expression profiling results of **Paper V** is summarized in **Table 4**.

Table 4. *Association between PI3K/Akt/mTOR pathway genes, novel genes associated with PNENs, from the genomic imbalance profiling results of* ***Paper IV****, and the genomic expression profiling results of* ***Paper V****. "Aggressive" is defined as metastatic sporadic nonfunctioning PNEN with Ki67≥5%. "Mild" is defined as nonmetastatic sporadic nonfunctioning PNEN with Ki67<5%. *Upregulated gene in aggressive versus mild disease*

Gene	Cytogenetic band	Copy number gain (Paper IV)	Copy number loss (Paper IV)	Statistically significant differential expression (yes/no) (Paper V)*
PI3K/Akt/mTOR pathway genes (PNENs in general)				
MEN1	11q13.1	No	11q13 in 3 mild and 1 aggressive	No
DAXX	6p21.32	6p21 in 1 aggressive	6p21 in 1 aggressive	No
ATRX	Xq21.1	No	No	No
PTEN	10q23.31	10q23 in 1 mild and 1 aggressive	No	No
TSC2	16p13.3	16p13 in 1 aggressive	16p13 in 2 aggressive and 1 mild	No
PIK3CA	3q26.32	No	3q26 in 1 mild	No
TP53	17p13.1	17p13 in 2 aggressive	No	No
Novel genes (sporadic nonfunctioning PNENs)				
HIST1H2AL	6p22.1	6p22 in 1 aggressive	No	Yes, upregulated
HIST1H2BF	6p22.1	6p22 in 1 aggressive	No	Yes, upregulated
ADD2	2p13.3	2p13 in 1 aggressive	2p13 in 1 aggressive	Yes, upregulated
WNT3	17q21	17q21 in 3 aggressive	No	Yes, upregulated
WNT signaling target genes (sporadic nonfunctioning PNENs)				
BIRC5 (survivin)	17q25.3	17q25 in 3 aggressive	No	Yes, upregulated

After publication of **Paper V**, we have examined different Wnt target genes among the 309 protein-coding genes that were found to be significantly differentially expressed. One interesting finding was the significant upregulation of the *BIRC5* gene (17q25.3) in aggressive disease (logFC -2.1). *BIRC5* encodes survivin, which is an apoptosis inhibitor normally present in fetal tissue but absent from terminally differentiated tissue. It has previously been reported to be upregulated in PNENs

and has been associated with poor survival[247, 248]. An interesting link between the results of **Papers IV and V** is the copy number loss of 17q21 and 17q25 in three samples categorized as "aggressive disease" (**Paper IV**) and the identification of significantly upregulated genes in sporadic nonfunctioning PNENs with aggressive behavior on the same chromosomal bands (*WNT3* on 17q21 and *BIRC5* on 17q25.3) (**Paper V**). As mentioned above, previous studies have also shown gains of 17q in nonfunctioning PNENs.

In summary, **Paper V** reveals novel patterns of correlation between tumor aggressiveness and genomic expression in sporadic nonfunctioning PNENs. We identified upregulation of genes involved in DNA packaging, cytoskeleton structuring, and cell-cell-signaling in aggressive disease, which could indicate yet undescribed molecular targets for biomarkers and drugs in histones and/or the Wnt signaling pathway. Our study has important limitations and the results should be interpreted with care. One limitation is the small sample size, only 11 tumors, which makes the results difficult to generalize. Further, we did not validate our findings by means of real-time PCR and/or immunohistochemistry in the same set of tumor samples. Another way of validating the results could have been to perform real-time PCR on a large independent cohort of sporadic nonfunctioning PNENs. However, despite the clear limitations of the study, its preliminary results give important and new knowledge about potentially important biomarkers for sporadic nonfunctioning PNENs. In particular, more investigation is needed to clarify the role of the Wnt signaling pathway in the tumorigenesis of sporadic nonfunctioning PNENs.

Conclusions

Paper I

- Laparoscopic surgery for PNENs is feasible with acceptable overall surgical morbidity and a good long-term prognosis
- In laparoscopic surgery for PNENs, a Ki67 index cut-off value of 5% is a significant prognostic factor

Paper II

- In patients with locally advanced PNET, pancreatic surgery with vascular reconstruction is feasible with acceptable surgical morbidity and short-term prognosis

Paper III

- In patients with PNEC, combined surgical treatment and chemotherapy improves survival compared with chemotherapy alone
- In patients with PNEC, resection of the primary tumor is an independent factor of improved survival

Paper IV

- In sporadic nonfunctioning PNENs, the number of genomic imbalances seems to correlate directly with cell proliferation, tumor size and metastatic status. Loss of genomic material from chromosomal band 11p11 might represent a primary pathogenetic event

Paper V

- In sporadic nonfunctioning PNENs, a higher level of tumor aggressiveness seems to be associated with upregulation of genes involved in regulation of the cell cycle and cell division, such as genes of the Wnt signaling pathway

*Based on the clinical studies of this thesis and the discussed evidence on surgical management of patients with PNENs, we finally propose two algorithms (in the **Appendix**) that should help surgeons choose the optimal surgical treatment.*

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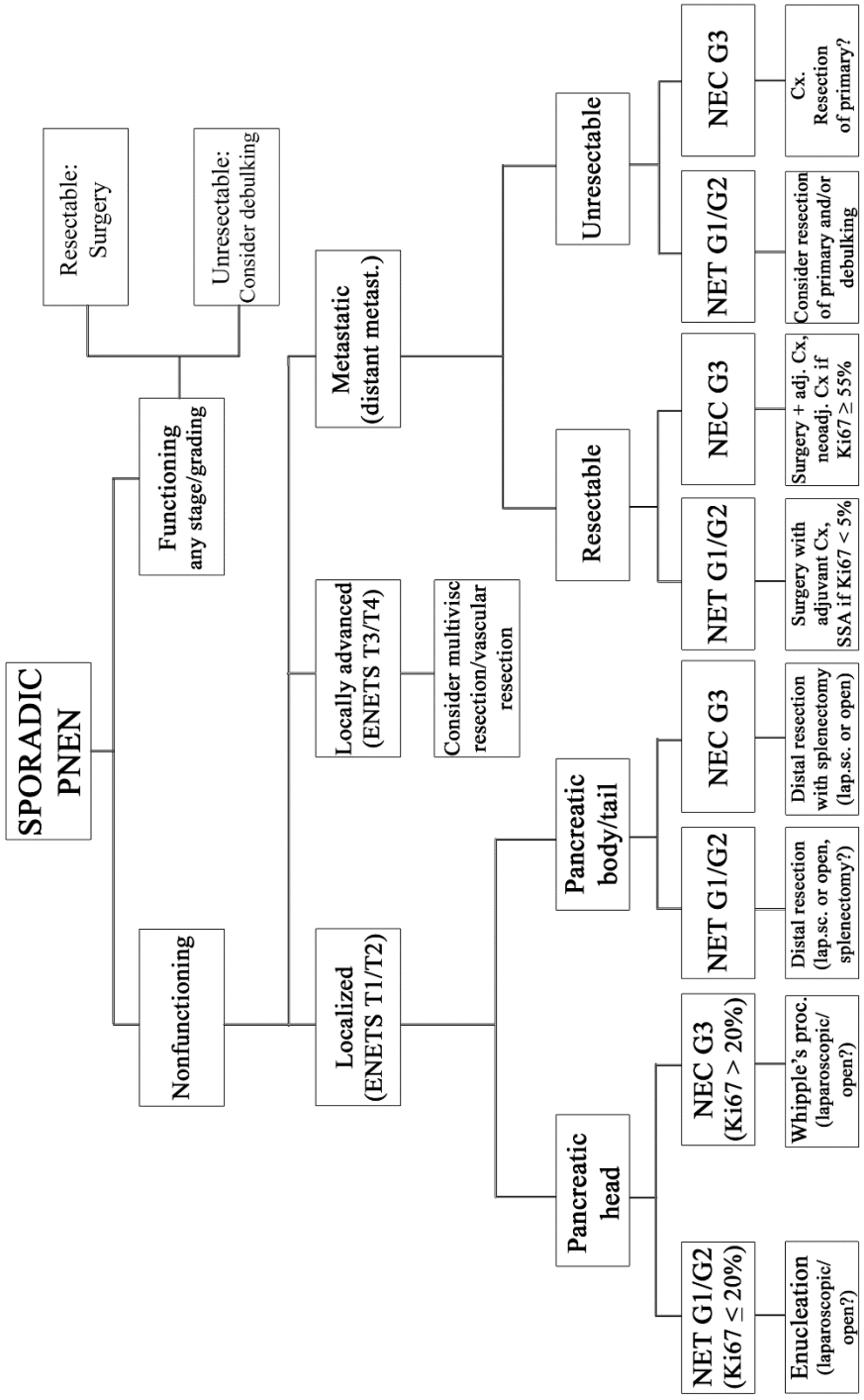
Appendix

Surgical treatment algorithm for sporadic PNENs



Algorithm for the surgical treatment of patients with sporadic pancreatic neuroendocrine neoplasms. The treatment depends on disease stage (European Neuroendocrine Tumor Society (ENETS) T-stage) and grading (G), defined by the proliferation index (Ki67). PNEN, pancreatic neuroendocrine neoplasm; NET, neuroendocrine tumor; NEC, neuroendocrine carcinoma; Cx, chemotherapy; SSA, somatostatin analog.

Surgical treatment algorithm for PNECs

Algorithm for the treatment of patients with pancreatic neuroendocrine carcinoma. The treatment depends on performance status, metastatic status and the Ki67 index. PNEC, pancreatic neuroendocrine carcinoma; Cx, chemotherapy; PS, ferfomrance status; BSC, best supportive care. PNEC as defined by the WHO 2010 Classification (Ki67>20%).