Small Intestinal Neuroendocrine Tumors

Clinical Studies, Novel Serum Biomarkers and Sensitivity to Cytotoxic and Targeted Agents

KOSMAS DASKALAKIS
Dissertation presented at Uppsala University to be publicly examined in Rosénsalen, Akademiska sjukhuset, ing 95-96, Uppsala, Friday, 8 December 2017 at 09:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish. Faculty examiner: Associate professor Robert Brännström (Institutionen för molekylär medicin och kirurgi (MMK), Karolinska Institutet).

Abstract

Small Intestinal Neuroendocrine Tumors (SI-NETs) are indolent neoplasms with an increasing annual incidence of approximately 1/100 000 people. They are often diagnosed at a late stage, restricting treatment efficacy. The aim of this thesis was to investigate clinical aspects of patients with advanced and/or disseminated disease with regard to clinical signs and management of abdominal fibrosis, the role of locoregional surgery and liver transplantation, as well as the ex vivo sensitivity of tumor samples to cytotoxic and targeted agents. Additionally, novel serum biomarkers for the diagnosis and prognosis of SI-NETs were investigated. In Paper I, abdominal fibrosis induced by serotonin and other cytokines from tumor cells, was associated with clinically significant symptoms of intestinal ischemia and/or obstructive uropathy, and was linked to advanced disease. Prompt recognition and minimally invasive intervention with superior mesenteric vein stenting and/or percutaneous nephrostomy and J stent treatment were effective in disease palliation. Paper II challenged the role of prophylactic, upfront locoregional surgery in Stage IV, which conferred no survival advantage in asymptomatic SI-NET patients. The option of delayed surgery as needed seemed to be comparable in all the outcomes examined, whilst also offering the advantage of fewer re-operations for intestinal obstruction in patients with already disseminated disease. Paper III confirmed that most young patients (<65 years) with SI-NET and liver metastases had a favorable survival with standardized multimodality treatment and that survival figures reported after liver transplantation for NETs do not surpass these figures. In Paper IV, 145 biomarkers were analyzed in blood serum using two different multiplex proximity assays. Subsequent ELISA and immunohistochemical analyses identified DcR3, TFF3 and midkine as novel serum biomarkers for SI-NETs. In Paper V, SI-NET samples were profiled with respect to sensitivity ex vivo to a panel of standard chemotherapeutics and targeted agents using a short-term total cell kill assay. SI-NETs exhibited variable but generally intermediate sensitivity ex vivo compared with other cancer diagnoses, calling for individualized selection of therapy.

Keywords: SI-NET, fibrosis, locoregional surgery, liver transplantation, biomarkers, ex vivo sensitivity.

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urn:nbn:se:uu:diva-330554 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-330554)
“Primum non nocere”

(Above all, do no harm.)

Latin approximation of Hippocratic aphorism:

«ἀσκεῖν περὶ τὰ νοσήματα δύο, ὑφελεῖν ἢ μὴ βλάπτειν»

Hippocratis Epidemiarum librum I (400 B.C.)

To my wife, Julia
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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*Denotes equal contribution.
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Abbreviations

5-HT  5-hydroxytryptamine (serotonin)
CHD  Carcinoid Heart Disease
CLL  Chronic Lymphocytic Leukemia
CRC  Colorectal Cancer
DSS  Disease-Specific survival
EAM  Extra-abdominal Metastases
EC cell  Enterochromaffin Cell
ELISA  Enzyme-linked Immunosorbent Assay
ENETS  European Neuroendocrine Tumor Society
FMCA  Fluorometric Microculture Cytotoxicity Assay
GEP cell  Gastroenteropancreatic Cell
GEP-NEN  Gastroenteropancreatic Neuroendocrine Neoplasm
IC50  Half maximal Inhibitory Concentration
LM  Liver Metastases
LOS  Length of Hospital Stay
LRS  Locoregional Surgery
LTx  Liver Transplantation
mTOR  Mammalian Target Of Rapamycin
NCCN  National Comprehensive Cancer Network
NEN  Neuroendocrine Neoplasm
NET  Neuroendocrine Tumor
OS  Overall Survival
OU  Obstructive Uropathy
PC  Peritoneal Carcinomatosis
PEA  Extension Ligation Assay
PFS  Progression-free Survival
PLA  Proximity Ligation Assay
RFA  Radio-Frequency Ablation
PRRT  Peptide Receptor Radionuclide Therapy
RECIST  Response Evaluation Criteria In Solid Tumors
ROC  Receiver Operating Characteristic
S-CgA  Serum Chromogranin A
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEER</td>
<td>Survival Epidemiology and End Results</td>
</tr>
<tr>
<td>SI</td>
<td>Survival Index</td>
</tr>
<tr>
<td>SI-NET</td>
<td>Small Intestinal Neuroendocrine Tumor</td>
</tr>
<tr>
<td>SMV</td>
<td>Superior Mesenteric Vein</td>
</tr>
<tr>
<td>SSA</td>
<td>Somatostatin analogs</td>
</tr>
<tr>
<td>TACE</td>
<td>Transarterial Chemoembolization</td>
</tr>
<tr>
<td>TAE</td>
<td>Transarterial Embolization</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine Kinase Inhibitors</td>
</tr>
<tr>
<td>U-5HIAA</td>
<td>Urine-5-hydroxyindoleacetic Acid</td>
</tr>
<tr>
<td>UNOS</td>
<td>United Network for Organ Sharing</td>
</tr>
</tbody>
</table>
1. Introduction

This doctoral thesis will focus on Small Intestinal Neuroendocrine Tumors (SI-NETs), specifically on patients with advanced and disseminated disease with regards to clinical signs and management of abdominal fibrosis, on the role of locoregional surgery and liver transplantation, as well as the ex vivo sensitivity of tumor samples to standard cytotoxic drugs and recently introduced targeted agents. Additionally, novel serum biomarkers for diagnosis and prognosis of SI-NETs are investigated.
2. Background

2.1 History of SI-NETs

Neuroendocrine GEP cells were originally described by the pathologist Langerhans in his Doctorate of Medicine thesis in 1869, whilst working in Rudolf Virchow’s laboratory. EC cells in the intestinal mucosa were first identified by the Russian pathologist Nikolai Kultchisky in 1897. Around the same time that the GEP neuroendocrine system was being described, two different German pathologists, Theodor Langhans and Otto Lubarsch, published the first autopsy studies on SI-NETs. It was 40 years after Langhans’ and Lubarsch’s discovery of these peculiar tumors that Siegfried Oberndorfer, a German pathologist, introduced the term “carcinoid” in 1907 and first distinguished SI-NETs as less aggressive than most carcinomas. However, he amended his classification later in 1929 to include the possibility that SI-NETs could be malignant and also metastasize. Sadly, due to his Jewish origin, the brilliant career of “the father of carcinoid tumors” fell victim to the machinations of the Third Reich. In 1914, Gosset and Masson recognized that carcinoid tumors have endocrine features, containing silver-salt reducing granules, and proposed that they are derived from the EC cells of the small intestine.

2.2 TNM Classification, Staging and Grading

GEP-NENs were previously divided according to their embryological origin as foregut (lungs, esophagus, stomach, upper duodenum and pancreas), midgut (lower duodenum, jejunum, ileum and proximal colon) or hindgut (from the distal transverse colon to the anus). First published in 2010, the European Neuroendocrine Tumor Society (ENETS) proposed a tumor-node-metastases (TNM) staging classification system for small intestinal NETs (SI-NETs), describing the extent of tumor invasion and dissemination. The recently published 8th edition of the American Joint Committee on Cancer (AJCC) introduced updated GEP-NEN staging based on separation of staging of each organ, i.e. NENs of the pancreas, stomach, duodenum and ampulla of Vater, jejunum and ileum, appendix and colorectum. Additionally, the SI-NET classification system was also recently updated by the ENETS 2016 Consensus with some minor changes (Tables 1 and 2).
Table 1. TNM classification of SI-NETs.

<table>
<thead>
<tr>
<th><strong>TNM</strong></th>
<th><strong>Description</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T-primary tumor</strong></td>
<td></td>
</tr>
<tr>
<td>Tx</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor invades mucosa or submucosa and size ( \leq 1) cm</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor invades muscularis propria or size ( &gt;1) cm</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor invades subserosa</td>
</tr>
<tr>
<td>T4</td>
<td>Tumor invades peritoneum/other organs</td>
</tr>
<tr>
<td>For any T add (m) for multiple tumors</td>
<td></td>
</tr>
</tbody>
</table>

| **N-regional lymph nodes** | |
|NX | Regional lymph nodes cannot be assessed |
|N0 | No regional lymph node metastasis |
|N1 | Regional lymph node metastasis |

| **M-distant metastasis** | |
|MX | Distant metastasis |
|M0 | No distant metastases |
|M1 | Distant metastasis |

The WHO 2017 classification system divides GEP-NENs into three grades according to their proliferative activity (Table 3)\(^7\). The Grades 1 to 2 Ki-67 cut-off is changed from 2 to \(<3\) for clarification purposes, compared to the WHO 2010 grading system. Additionally, Grade 3 is sub-divided into two new entities: well-differentiated high-grade NETs and poorly-differentiated high-grade NECs. Both these entities (high grade NETs and NECs) have the same biopsy marker cut-offs but it is thought that at least for the pancreatic counterpart a cut-off of \(55\)% could influence the treatment regime. Generally, it has been increasingly apparent, and therefore incorporated in the WHO 2017 grading system, that the previously called NEC entity is heterogeneous, and that not all tumors are poorly differentiated\(^8\). Indeed, many well-differentiated GEP-NENs, particularly pancreatic NENs, previously fell into the NEC category due to having ki67 in the \(20\%-55\%\) range, but they are now classified as G3 NETs.

Table 2. Staging of SI-NETs.

<table>
<thead>
<tr>
<th><strong>Stage</strong></th>
<th><strong>TNM</strong></th>
<th><strong>Disease</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis</td>
<td>N0</td>
</tr>
<tr>
<td>Stage I</td>
<td>T1</td>
<td>N0</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T2</td>
<td>N0</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>T3</td>
<td>N0</td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>T4</td>
<td>N0</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>Any T</td>
<td>N1</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any T</td>
<td>Any N</td>
</tr>
</tbody>
</table>

The WHO 2017 classification system divides GEP-NENs into three grades according to their proliferative activity (Table 3)\(^7\). The Grades 1 to 2 Ki-67 cut-off is changed from 2 to \(<3\) for clarification purposes, compared to the WHO 2010 grading system. Additionally, Grade 3 is sub-divided into two new entities: well-differentiated high-grade NETs and poorly-differentiated high-grade NECs. Both these entities (high grade NETs and NECs) have the same biopsy marker cut-offs but it is thought that at least for the pancreatic counterpart a cut-off of \(55\)% could influence the treatment regime. Generally, it has been increasingly apparent, and therefore incorporated in the WHO 2017 grading system, that the previously called NEC entity is heterogeneous, and that not all tumors are poorly differentiated\(^8\). Indeed, many well-differentiated GEP-NENs, particularly pancreatic NENs, previously fell into the NEC category due to having ki67 in the \(20\%-55\%\) range, but they are now classified as G3 NETs.
Finally, the WHO 2017 classification introduced a change to the name of mixed cell tumors from Mixed Adeno-Neuroendocrine Carcinomas (MANECs) to Mixed Neuroendocrine Non-neuroendocrine Neoplasms (MiNENs)\(^7\).

It is important to note that, in all the articles contained in this thesis, we followed the WHO 2010 grading as well as the ENETS 2010 staging classification.

Table 3. Grading of GEP-NENs incorporating WHO 2017 changes.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Ki-67 index (%)</th>
<th>Mitotic index (mitoses/10 HPF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NET G1 (low grade)</td>
<td>&lt;3</td>
<td>&lt;2</td>
</tr>
<tr>
<td>NET G2 (intermediate grade)</td>
<td>3-20</td>
<td>2-20</td>
</tr>
<tr>
<td>NET G3 (well differentiated-high grade)</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>NEC G3 (poorly differentiated-high grade)</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Mixed Neuroendocrine Non-Neuroendocrine Neoplasm (MiNEN)</td>
<td>see text</td>
<td></td>
</tr>
</tbody>
</table>

2.3 Epidemiology

According to data from the SEER 18 database, the incidence of NENs has been rising steadily from 1.09 per 100 000 persons in 1973 to 6.98 per 100 000 persons in 2012. This is possibly due to improved detection of early-stage disease and stage migration\(^9\). SI-NETs have an incidence of 1.05 per 100 000 persons and account for 39%-42% of all GEP-NENs and for 27%-44% of all small bowel neoplasms in the western world\(^9\)-\(^11\). Reflecting the rising incidence and indolent nature of SI-NETs, the prevalence of the disease has also increased substantially in the last 20 years\(^9\). Interestingly, in eastern populations, SI-NETs account for less than 10% of NENs, leading to a virtual absence of the carcinoid syndrome\(^12\).

SI-NETs have been diagnosed with a slight male predominance and at a mean age of 65 years. They may be detected in up to 1/150 of routine autopsies, suggesting a silent, subclinical course throughout life in some cases. Most often, the primary sites occur in the distal ileum within 60-80cm of the ileocecal valve; less often in the proximal ileum or the jejunum, and may be multiple in up to 20%-30% of patients\(^13\).

2.4 Diagnosis

SI-NETs have an indolent clinical course and the disease is often diagnosed at a late stage. The majority of patients with SI-NETs present with
mesenteric lymph node metastases and also synchronous liver metastases at
diagnosis, whereas many patients develop metachronous liver metastases
during follow-up\textsuperscript{10,14}.

Patients with SI-NETs may present with distinct clinical symptoms and
signs due to hormonal excess, such as flushing and diarrhea, which
constitute the carcinoid syndrome, and/or local tumor-related symptoms due
to primary tumor and mesenteric lymph node metastases causing abdominal
pain, obstruction and/or impaired blood supply to the intestines. More
uncommon symptoms include carcinoid heart disease, bronchial constriction
and GI hemorrhage\textsuperscript{15}.

The hormones, peptides and biogenic amines, namely serotonin,
tachykinins, prostaglandins and Bradykinins, secreted by SI-NETs are not
only associated with the carcinoid syndrome, but can also induce
mesenteric and/or retroperitoneal fibrosis in a subset of patients\textsuperscript{16}.

Diagnosis is based on clinical signs and symptoms combined with
measurements of s-CgA and u-5HIAA levels, as well as cross-sectional (CT
and MRI) and functional imaging ($^{68}$Ga-DOTATOC PET). Histopathological
diagnosis is mandatory in all cases and usually obtained from ultrasonography-guided liver biopsy or surgical biopsy,
using hematoxylin-eosin staining and immunohistochemical staining
with CgA, synaptophysin and, optionally, serotonin\textsuperscript{6}.

\subsection*{2.5 Prognosis}

Survival for all NETs has improved over time, especially for stage IV GEP-
NENs according to recently published data from the SEER 18 database\textsuperscript{9,17},
possibly reflecting improvements in therapies. For SI-NETs specifically,
there is great diversity in the clinical course of the disease with unpredictable
and variable outcomes hidden in an overall favorable survival rate. Our own
series\textsuperscript{14} shows a 5-year OS of 68\% and a median OS of 8.4 years, which are
comparable to results from the Swedish National Cancer Registry, showing a
5-year OS of 56\%\textsuperscript{18}. The SEER 18 database reports median OS of 14 years
for localized disease, 11.7 years for regional disease and 5.8 years for distant
metastases\textsuperscript{9}. Interestingly, the 5-year OS for Stage IV SI-NETs was 69\% in
SEER 18 (2000 to 2012), whereas it was 57\% in the Uppsala database for
SI-NETs (1985 to 2010)\textsuperscript{9,14}. Studies based on the Swedish National Cancer
Registry have also demonstrated improved relative and cause-specific
survival over recent decades\textsuperscript{18,19}. However, we found no such trend in our
cohort prior to 2010, possibly due to a referral bias of patients with more
advanced disease or comorbidities to our tertiary center and even due to the
fact that any survival benefit from recently introduced novel multimodality
treatments has not yet been reached and/or assessed for patients with SI-
NETs in recent years in our database. On the other hand, the SEER
database is not complete and its information may be considered biased. Additionally, the improvement in OS for the gastrointestinal NET subgroup over the time intervals reported from the SEER 18 data may be contaminated by the inclusion of NETs of the GI-tract other than SI-NETs and even biased due to stage migration, as a result of the clinical application of modern imaging modalities over the last decade.\textsuperscript{9,20}

Age at diagnosis, carcinoid heart disease, WHO stage and grade, mesenteric lymph nodes, liver tumor load, peritoneal carcinomatosis, enlarged distant abdominal lymph nodes and extra-abdominal metastases have been identified as negative prognostic factors for OS.\textsuperscript{14}

The currently used biomarkers for SI-NETs are s-CgA and u-5HIAA. S-CgA is an independent prognostic factor for NETs, associated with tumor burden, recurrence and treatment response.\textsuperscript{21} However, it is more frequently elevated in well-differentiated tumors as compared to poorly differentiated ones, and treatment with SSA seems to reduce the correlation between s-CgA levels and tumor burden.\textsuperscript{21} In contrast, U-5HIAA levels demonstrate some correlation to OS only in patients with metastatic disease.\textsuperscript{22,23}

Contemporary translational research regarding disease prognosis has demonstrated a loss of chromosome 18 in 60\%-90\% of SI-NETs, but mutated genes on this chromosome have failed detection.\textsuperscript{24,25} Recently, a putative tumor suppressor role has been suggested for TCEB3C at 18q21, which may undergo epigenetic regression.\textsuperscript{26} CDKN1B has been identified as the sole recurrently muted gene in SI-NETs, but with a frequency as low as 8\%.\textsuperscript{25} Based on molecular profiling, SI-NETs are highly epigenetically dysregulated and in one recent study they could be classified into three groups, each demonstrating significantly different progression-free survival.\textsuperscript{27} The largest group was defined by loss of heterozygosity in chromosome 18 (chr18LOH), associated with the presence of cyclin-dependent kinase inhibitor 1B (CDKN1B) mutations, and CpG island methylator phenotype (CIMP) negativity. Patients classified within this subgroup had the most favorable PFS and an older age at diagnosis, suggesting a less aggressive phenotype. A second subgroup was characterized by the absence of arm-level copy number alterations (CNVs) and was associated with a high level of CIMP positivity and an intermediate PFS. The final subgroup comprised 26\% of tumors, characterized by the presence of multiple copy number variations (CNVs), a significantly poorer PFS and a younger age at onset, suggesting a more aggressive clinical phenotype.\textsuperscript{27}
2.6 Fibrosis

In SI-NETs, serotonin (5-hydroxytryptamine (5-HT)) and other cytokines released from tumor cells may induce fibrosis in cellular systems, leading to carcinoid heart disease and abdominal fibrotic reactions. Mesenteric lymph node metastases together with the accompanying desmoplastic reactions in the mesentery may encase the superior mesenteric vessels and lead to kinking of the bowel and obstruction of, or impaired blood supply to, the intestines. Another, more rare complication is diffuse retroperitoneal fibrosis, which can lead to obstruction of the urinary system. Occasionally, carcinoid syndrome may accompany retroperitoneal fibrosis, when tumor secretory products exceed the detoxifying capacity of the liver, or bypass it, draining directly into the systemic circulation through retroperitoneal lymphatic spread.

Plausible mechanisms for the induction of fibrosis in SI-NETs are stimulation of the 5-HT-2B receptor, which in turn increases TGF-beta 1 induced synthesis; and also connective tissue growth factor (CTGF) tachykinins, substance P and neurokinin A, all of which are known to stimulate fibroblasts in different ways.

Clinically, extensive procedures in patients with large mesenteric masses and extensive intra- and retroperitoneal fibrosis engaging the superior mesenteric vessels are highly complex and associated with postoperative morbidity. Palliative, minimally invasive measures, such as the insertion of self-expandable stents through the portal vein, have been reported in patients with SI-NETs and obstruction of the superior mesenteric vein due to mesenteric fibrosis. Obstructive uropathy (OU) due to retroperitoneal fibrosis may also be treated with stenting.

2.7 Treatment

Today, locoregional surgery (LRS), i.e. the removal of the primary tumor and regional metastases, is the only potential cure for patients in Stages I-III. However, the majority of radically perceived operated patients will still experience biochemical and/or radiological recurrence. Importantly, most SI-NET patients are diagnosed in Stage IV, and are then generally not considered curable, although in selected cases liver surgery or local ablative methods can be applied with a curative intent. Stage IV patients are often discovered in an emergency setting and will thus undergo LRS for intestinal obstruction, whereas a prophylactic surgical approach to these patients with no local tumor-related symptoms is still controversial. This matter is separately addressed in the next section (2.9).

Modern management of patients with Stage IV NETs takes place in centers of expertise and focuses on a multidisciplinary approach and
personalized treatment. A multimodal approach of systemic and targeted therapies is now available for patients with metastatic SI-NETs.

Treatment of liver metastases is mainly performed to palliate symptoms from the carcinoid syndrome, with liver surgery epitomizing the management of liver metastasis. Our own study series found no survival benefit after liver surgery or local ablative methods\textsuperscript{33}. Moreover, half the number of liver metastases from NETs are undetectable on preoperative imaging, and thus there is a high probability that undiagnosed disease can be left after surgery\textsuperscript{34}. Liver transplantation (LTx) in Stage IV SI-NET patients is debatable and this topic is also separately addressed in section 2.10.

Liver metastases may also be treated with a novel panel of different ablative techniques such as radio-frequency ablation (RFA), laser ablation, cryotherapy, transarterial embolization (TAE) and transarterial chemoembolization (TACE). These treatments generally have a palliative aim in patients with slow-growing functional tumors which are refractory to medical therapy\textsuperscript{35}.

Peptide Receptor Radionuclide Therapy (PRRT) with $^{177}$Lu-labeled DOTA–Tyr3-octreotate can be recommended in SI-NETs with evident high radiotracer expression in a somatostatin receptor imaging modality\textsuperscript{36}.

SSAs constitute the first line of treatment for Grades 1 and 2 SI-NETs with expression of somatostatin receptors. The indication for using SSAs as first-line therapy derives mainly from two studies: the PROMID and CLARINET trials\textsuperscript{37,38}. The PROMID study showed a trend for improved OS in patients with metastatic, well-differentiated SI-NETs and limited hepatic tumor load receiving octreotide LAR therapy. Additionally, in patients with hepatic tumor burden $>10\%$, time to progression in the octreotide-LAR arm was almost double that of the control arm\textsuperscript{38}. Unfortunately, this study did not clarify whether it is advantageous to wait until tumor progression or to treat at initial diagnosis. The CLARINET study instead confirmed an anti-proliferative effect of SSAs in well- or moderately differentiated GEP-NETs (Ki-67 $<10\%$)\textsuperscript{37}.

Interferon also has an anti-proliferative effect and may reduce tumor size, but its use alone or in combination with SSAs is rather limited due to side effects\textsuperscript{39,40}. Conversely, chemotherapy has no proven effect on low-proliferative SI-NETs. Aspects of chemotherapy and new systemic targeted agents are addressed separately in sections 2.12 and 2.13. Another promising agent is telotristat etiprate, a serotonin antagonist with considerable reductions of serotonin levels in Stage IV patients and improvement in their carcinoid symptoms\textsuperscript{41}.
2.8 Locoregional surgery

In patients in Stages I-III, LRS is indicated with a curative intent. However, in Stage IV patients, LRS is generally not considered curative, although sometimes liver surgery or local ablative methods are undertaken after or before radical LRS. However, even in the era of a broad panel of novel, targeted and systemic therapies for SI-NETs, recurrence after perceived radical liver resection is still very common and neither liver resection nor radiofrequency ablation of liver metastases has unequivocally been found to prolong survival. Therefore, even when achieving macroscopic radicality and cure is the intent, liver procedures for SI-NETs should generally be considered palliative in light of contemporary literature.

Many Stage IV patients may present with distinct clinical symptoms and signs due to hormonal excess and/or local tumor-related symptoms due to primary tumor, mesenteric lymph node metastases and associated fibrosis, causing abdominal pain, obstruction and/or impaired blood supply to the intestines. These patients with local tumor-related symptoms generally undergo LRS at the time of diagnosis. Some patients may be subjected to acute laparotomy due to intestinal obstruction of unknown diagnosis. Others will undergo palliative surgery for partial intestinal obstruction, bleeding, ischemic complications due to the tumor mass, or even for symptom relief in cases of hormonal syndrome refractory to medical therapy.

The extension of mesenteric lymph node metastases below or above the horizontal part of the duodenum is a crucial factor for treatment, as a number of patients will display mesenteric lymph node metastases in the root of the mesentery with associated fibrosis, encasing the superior mesenteric vessels. These patients are then usually considered inoperable. Generally, for tumors originating in the proximal ileum and the jejunum, segmental small intestine resection is performed. However, for primaries located near the ileocecal valve in the distal ileum, ileocecal resection or right hemicolecotomy is performed, with the latter possibly combined with improved clearance of regional lymph node metastases. A surgical approach with mobilization of cecum, terminal ileum and the mesenteric root by separation of retroperitoneal attachments, dissecting the lower aspect of the horizontal duodenum and the superior mesenteric vessels, allows the specimen to be lifted and approached from a posterior angle with proximal vascular control.

An arbitrary staging system of the mesenteric lymph node metastases has been developed in Uppsala to describe the extension of locoregional disease, as seen in Figure 1.
Figure 1. Uppsala staging system of mesenteric lymph node metastases in SI-NETs. Stage I: Lymph node metastases close to the intestine. Stage II: Lymph node metastases higher in the mesentery. Stage III: Lymph node metastases along, but not encasing the mesenteric vessels at the level of the horizontal duodenum. Stage IV: Lymph node metastases extending in the root of the mesentery, above the horizontal part of the duodenum, encasing the superior mesenteric vessels.

Palliative, minimally invasive measures such as stenting of the superior mesenteric vein have been applied for symptomatic patients with bulky mesenteric disease (Uppsala Stage IV), as LRS in these patients may be complicated and endanger circulation to substantial parts of the bowel.

In asymptomatic patients with distant metastases, prophylactic LRS has been advocated to avoid future intestinal obstruction, ischemia, perforation or bleeding. The survival rates after LRS in these patients, as reported in retrospective cohort studies, are probably largely influenced by both selection bias and immortal-time bias. Current ENETS guidelines, emphasize a possible effect of LRS in Stage IV SI-NETs with unresectable liver metastases, but these guidelines are based on information gathered from the above-mentioned cohort studies.

On the other hand, the NCNN 2017 Guidelines on Carcinoid Tumors advocate against resection of a small asymptomatic, relatively stable primary tumor in the presence of unresectable metastatic disease. However, there are still very few good quality comparative studies regarding survival, postoperative morbidity and mortality, symptoms, re-operations and length of hospital stay, evaluating prophylactic upfront LRS and a more conservative approach with delayed surgery as clinically indicated.
2.9 Liver Transplantation

In patients with bi-lobar liver metastasis, slow disease progression and no extrahepatic disease, total tumor hepatectomy with liver transplantation (LTx) may be considered with curative intent, or in order to palliate from life-threatening hormonal disturbances. A meta-analysis of NET patients subjected to LTx demonstrated a varying 5-year survival rate of 24% to 48%, likely due to different selection criteria and primaries of different origin. The European Liver Transplant Surgery (ELTR) study of NETs, reported a 5-year OS after LTx of 52% and 3-month postoperative mortality of 10% as well as an overall LTx-related mortality of 17%. Additionally, three predictors of poor outcome were identified in this study, namely any amount of resection in addition to the LTx, age more than 45 years, and hepatomegaly.

Higher tumor grade, non-portal tumor drainage, extrahepatic metastases (with the exception of resectable perihilar lymph node metastases), and advanced carcinoid heart disease are generally accepted contraindications for LTx in NETs. Nevertheless some inclusion criteria for LTx are less controversial than others, such as the absence of extrahepatic disease, resected primary and well-differentiated Grades 1 and 2 tumors. The Milan group (Table 4) favors narrower patient selection in order to enhance outcomes.

Table 4. Milan criteria

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Milan criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&lt;55 years</td>
</tr>
<tr>
<td>Histology</td>
<td>Carcinoid</td>
</tr>
<tr>
<td>Primary tumor drainage</td>
<td>Portal system</td>
</tr>
<tr>
<td>Ki-67 index</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Extrahepatic disease</td>
<td>Not allowed</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>No (last 6 months)</td>
</tr>
<tr>
<td>Liver involvement</td>
<td>&lt;50%</td>
</tr>
</tbody>
</table>

The option of LTx has been considered especially for young patients (<50 years) with a WHO performance status of 0, non-resectable metastasis confined to the liver, Grades 1 and 2 SI-NETs and severe, uncontrolled endocrine symptoms. SI-NETs are generally tenacious and even when extrahepatic spread is excluded by sensitive imaging prior to LTx (68 Gallium-DOTATOC/PET/CT), the new liver will commonly become the site of new metastases with reported disease-free survival rates ranging from 11% to 77% at 5 years, with the latter being reported by the Milan group. To date there are no randomized trials available that compare LTx with standardized multimodality treatment.
2.10 Biomarkers

The presence of secretory products in serum can be exploited as tumor markers for SI-NETs in terms of their diagnostic, prognostic and predictive ability. Currently, the most commonly used biomarkers for SI-NETS are s-CgA and u-5HIAA.

General limitations of currently used biomarkers are that they are secretory biomarkers; are based on monoanalyte measurements; and lack sensitivity, specificity and predictive capacity.

S-CgA is a general marker for NETs, but also found elevated in various inflammatory conditions. It is a non-specific marker and additionally moderately sensitive, but has been proven to be an independent prognostic factor for SI-NETs, associated with tumor burden, recurrence and treatment response. However, it is more frequently elevated in well-differentiated tumors compared to poorly differentiated ones, and, in patients treated with SSAs, there is no correlation between s-CgA concentrations and tumor burden.

U-5HIAA is the breakdown product of 5-HT. It is measured in urine and found elevated late in the disease course, in patients with metastatic SI-NETs and carcinoid syndrome. There are several medications (including SSAs), types of food, and malabsorptive conditions that interfere with u-5HIAA levels.

The variability in SI-NET prognosis combined with delayed diagnosis and the absence of adequately sensitive and specific biomarkers obviates the need for novel markers that could be used for early diagnosis, prognostication and real-time monitoring of disease progression, as well as assessment of therapeutic efficacy.

2.11 Chemotherapy

Although NETs are generally more indolent than carcinomas, they have a widely variable clinical behavior and, on some occasions, a very aggressive clinical course. Except for pancreatic NETs and poorly differentiated neuroendocrine carcinomas (NEC, Grade 3), SI-NETs of Grades 1 and 2 have a rather low susceptibility to systemic chemotherapy with poor response rates of about 10%-30%. Therefore, available medical options for the systemic treatment of advanced and disseminated low-proliferative SI-NETs have been scant and of limited value for many decades. Low proliferation in SI-NETs as well as over-expression of the DNA repair enzyme methyl-guanine-methyltransferase (MGMT) may contribute to chemotherapy resistance. To date, there have unfortunately been few placebo-controlled randomized studies using RECIST criteria, with the bulk of the literature consisting of small Phase II studies. Thus, due to the absence
of high-level evidence, there are no guidelines in favor of the use of e.g. streptozocin- versus temozolomide- versus platinum-based therapies. However, even if SI-NETs seem not to benefit from chemotherapy, this treatment may still be considered in well-differentiated, pre-treated NET patients with progressive disease. Generally, in patients with low proliferative SI-NETs, non-cytotoxic drugs may be preferable in early lines of therapy, reserving chemotherapy for the salvage setting. G2 SI-NET patients could be potentially considered by a multidisciplinary team in order to identify those patients who might benefit from chemotherapy as a second line of treatment in selected cases\textsuperscript{35}. Validation of predictive factors/markers is, of course, imperative in order to match patients with optimal chemotherapy. However, such markers are lacking to date and the usefulness of Ki-67 in SI-NETs remains to be elucidated prospectively. Nevertheless, the roles of cytotoxic drugs in the treatment strategy for metastatic SI-NETs are still not well-defined.

2.12 Targeted agents

NET biology has been elucidated to some extent in recent years, paving the way for the development of new therapeutic agents. Aberrant activation of the mammalian target of rapamycin (mTOR) pathway as well as enhanced angiogenesis might be essential in NET pathogenesis and progression\textsuperscript{65,66}. The mTOR, a main protein kinase downstream to the phosphoinositide 3-kinase/Akt signalling pathway, is an important intracellular mediator involved in multiple cellular functions, such as proliferation, differentiation, apoptosis, angiogenesis and tumorigenesis. Alterations of mTOR itself and of mTOR-related kinases in this pathway have been identified in NETs, rendering the mTOR pathway as an attractive therapeutic target in these tumors. On the other hand, the most prominent trait of NETs is a paradoxically high vascularization in low proliferative tumors and a hypoxia-dependent angiogenesis in the higher grade ones, which is associated with the expression of pro-angiogenic molecules. Therefore, a number of anti-angiogenic compounds have been tested in NETs, including targeting the vascular endothelial target receptor (VEGFR) and platelet-derived growth factor (PDGFR) pathways.

Specifically, the mTOR inhibitor everolimus has been extensively studied in NETs\textsuperscript{67,68}, whereas angiogenesis inhibitors, such as sunitinib, are currently under intensive clinical investigation. In the RADIANT-2 study, everolimus plus octreotide LAR treatment seemed to demonstrate significant benefits and improve outcomes for patients with advanced SI-NETs and associated carcinoid syndrome. However, the survival benefit in this study did not reach the predefined level of significance\textsuperscript{69}. Everolimus, even as a single agent, has demonstrated robust anti-tumor activity with acceptable tolerability across a
broad range of neuroendocrine (non-pancreatic) tumors as seen in the RADIANT-4 study\textsuperscript{67}. Additionally, sunitinib and pazopanib hydrochloride, both multiple tyrosine kinase inhibitors, are currently evaluated separately in randomized Phase II trials, (NCT01731925 (the SUNLAND trial) and NCT00454363) in progressive advanced or metastatic SI-NETs. As well-differentiated NETs, particularly of non-pancreatic origin, are rather resistant to conventional chemotherapy, the recently demonstrated anti-tumoral activity of new targeted agents in GEP-NENs, has triggered great enthusiasm in the field. Additionally, the combination of these newly introduced therapies with chemotherapy may be an interesting option, since such combinations might prevent the development of escape or resistance mechanisms.
3. The rationale for the thesis

Paper I
- The complex clinical entity of extensive abdominal fibrosis in the root of the mesentery and/or the retroperitoneum in SI-NET patients has only been addressed in case series.

Paper II
- Despite the fact that prophylactic LRS in Stage IV asymptomatic SI-NET patients has been considered standard practice, it has not been evaluated in any randomized, controlled trials and the survival rates after LRS, reported in retrospective cohort studies, are largely influenced by selection and immortal-time bias.

Paper III
- There are a number of treatments for LM in clinical use for SI-NET patients with disseminated disease. No randomized or quasi-randomized trials are available that compare LTx of NETs with other treatments. Results are thus solely available from case series. Indications for the use of LTx in NET patients are subject to debate, with the Milan group favoring narrower selection criteria to enhance outcomes.

Paper IV
- New diagnostic, prognostic and predictive biomarkers for SI-NETs are urgently needed. Screening for multiple biomarkers in serum holds great promise for detecting novel markers.

Paper V
- Experimental and clinical experience regarding the sensitivity of SI-NETs to standard cytotoxic drugs and newly introduced targeted molecular agents is limited.
4. The aims of the thesis

**Paper I**
- To examine the prevalence and management of long-term, clinically significant complications as a result of extensive abdominal fibrosis in patients with SI-NETs.

**Paper II**
- To assess the outcome of prophylactic, upfront LRS in asymptomatic patients with Stage IV SI-NETs compared to delayed LRS as needed. The primary endpoint was OS. Secondary endpoints were 30-days’ postoperative mortality and morbidity, length of hospital stay, rates of re-operation and incisional hernia repair.

**Paper III**
- To examine outcomes between different selection criteria for LTx in Stage IV SI-NETs within our cohort, for patients undergoing only conventional multimodality treatment in order to produce a “non-transplantation” benchmark.

**Paper IV**
- To identify new diagnostic and prognostic biomarkers by screening serum from patients with SI-NETs and comparing the concentrations of biomarkers in patients with those in healthy controls.

**Paper V**
- To assess the sensitivity pattern *ex vivo* for standard cytotoxic drugs and targeted agents in SI-NETs, and to assess whether established prognostic factors for OS as well as the currently used biomarkers in this tumor type are associated with drug sensitivity.
5. Patients and Methods

5.1 Ethical considerations

Uppsala University’s Ethics Committee approved all studies.

5.2 Common Patients & Methods in Papers I, II and III

We included 824 patients from our prospective database of patients with SI-NETs, treated at the University Hospital in Uppsala between 1985 and 2015. In Paper I, four patients were from abroad, whereas in Papers I and III only Swedish residents were included, 820 and 672 (until 2012 only) respectively. Only patients with a histopathologically confirmed diagnosis of SI-NET from either liver metastasis biopsy or surgical specimens were included, whereas patients with NECs (according to the WHO 2010 grading classification) at baseline were not included in these studies.

Patients were followed until death or their last follow-up at the Department of Surgery or Oncologic Endocrinology (until December 2015 (Paper I), May 2016 (Paper II) or April 2013 (Paper III)).

Patient charts were scrutinized for the following parameters: age, gender, carcinoid symptoms, as well as local tumor-related symptoms (e.g. abdominal pain) at baseline, carcinoid heart disease diagnosed by echocardiography, presence of lymph node metastases, liver metastases and/or extra-abdominal metastases, proliferation according to the Ki-67 index, locoregional surgery, liver surgery as well as ablation of liver metastases, biotherapy with SSAs and/or interferon-alpha, PRRT with 177Lutetium-DOTA-Tyr3-octreotate, TAE or TACE, cytotoxic drugs and/or targeted agents (mTOR inhibitors and TKI). Subsequent treatments, reoperations, complications, length of hospital stay as well as disease progression and cause of death were documented prospectively during follow-up. In Paper II, follow-up data regarding length of hospital stay, reoperative procedures and postoperative morbidity and mortality were also extracted from the National Patient Register, which covers approximately 99% of all health-care in Sweden.
5.3 Additional methods in Papers IV and V

Proximity Ligation Assay (PLA)

PLA, performed at Olink Bioscience, was used in Paper IV. This is a sensitive method for detecting proteins by converting them to DNA molecules for subsequent quantification as described in Figure 2. One µl serum was required, and positive, negative and four spikes in controls were included. Every sample was mixed with pairs of proximity probes, each composed of an antibody linked to an oligonucleotide. Upon binding of the probes to a common antigen, these pairs of probes come into close proximity and are ligated using a connector, forming a unique amplicon, representing each target protein. The ligated products are then pre-amplified and analyzed in quadruplicates by real-time PCR. Ct values were linearized, and the samples were normalized against their corresponding GFP value.

![Figure 2. Schematic description of PLA](image)

1. The antibodies used in this assay are equipped with an oligonucleotide, which is called a PLA probe. The samples are incubated with 23 pairs of PLA probes in a 1 µl sample.  
2. Upon binding of the probes to a common antigen, these pairs of probes come into close proximity and are ligated using a connector.  
3. The ligated products are then pre-amplified.  
4. Finally, quantification of each biomarker takes place by real-time PCR.
**Proximity Extension Assay (PEA)**

The PEA is also a nucleic acid proximity-based method, based on pairs of antibodies that are linked to oligonucleotides having a slight affinity for one another (PEA probes). Upon target binding, the probes are brought into proximity, and the 2 oligonucleotides are extended by a DNA polymerase, forming a new sequence that now acts as a unique surrogate marker for the specific antigen. Related PEAs use DNA polymerization rather than DNA ligation to create the amplifiable DNA reporter strands. This sequence is quantified by qPCR, where the number of PCR templates formed is proportional to the initial concentration of antigen in the sample\(^71\).

The PEA design, which requires recognition by specific combinations of affinity reagents in order to generate detection signals, has the ability to measure large numbers of proteins in parallel (up to 96 analytes) while decreasing the cross-reactivity between antibodies and the background noise, thus resulting in higher specificity and sensitivity\(^72\). A schematic description of PLA is presented in Figure 3.

![Figure 3. Design and description of 96-plex PEA\(^71\). (A) 94 pairs of specific antibodies are equipped with oligonucleotides (PEA probes) and mixed with an antigen-containing sample. (B) Upon sample incubation, all proximity probe pairs bind to their specific antigens, bringing the probe oligonucleotides into close proximity to hybridize. The oligonucleotides have unique annealing sites that allow pair-wise binding of matching probes. The addition of a DNA polymerase leads to an extension and joining of the two oligonucleotides and the formation of a PCR template. (C) Universal primers are utilized to pre-amplify all 96 different DNA templates in parallel. (D) Uracil-DNA glycosylase partly digests the DNA templates and removes all unbound primers. (E) Finally, each individual DNA sequence is detected and quantified using specific primers in a microfluidic qPCR.](image-url)
Enzyme-linked immunosorbent assay (ELISA)
ELISA is a rapid immunochemical test that involves an enzyme used for measuring a wide variety of body fluid tests. ELISA tests detect substances that have antigenic properties, primarily proteins.

In Paper IV, a commercial ELISA kit was used. A standard curve, blank controls and duplicated samples were run according to the manufacturer’s instructions. Absorbances were measured at 450nm. The following ELISA kits were used: Human Spondin 2, human soluble DcR3, human trefoil factor 3, midkine, Colony Stimulating Factor 1, C-X-C motif chemokine 9 and C-X-C motif chemokine 10.

Immunohistochemistry
In Paper IV, immunohistochemical staining was performed for the selected proteins in both primary tumors and metastases. Paraffin-embedded tumor tissue sections (5μm) were passed through descending alcohol concentrations and distilled water. Background staining was blocked with 3% hydrogen peroxide and heated in citrate buffer. The tissues were treated with normal serum from rabbit polyclonal antibodies. Anti-Spondin 2, anti-DcR3, anti-TFF3, anti-midkine, anti-CXCL9, anti-CXCL10 and anti-MSCF were used. A biotinylated secondary antibody was added to the tissues and then treated with ABC complex. Visualization was then done with DAB color reagent.

Ex vivo chemotherapy testing
A way to develop and test drug candidates in a preclinical or clinical setting is the ex vivo chemosensitivity testing for tumors. Clonogenic and cell proliferation assays are two methods of measuring the proliferative activity of cancer cells\textsuperscript{73}. A fully automated, fluorometric microculture cytotoxicity assay (FMCA) has been developed and adapted to robotics at the Uppsala University Hospital. FMCA measures the esterase activity of cells with intact plasma membranes by estimating the fluorescence generated when fluorescein diacetate is hydrolyzed. Tumor samples of non-NET origin have been analyzed previously using the FMCA method for determining chemosensitivity to novel chemotherapeutic agents\textsuperscript{74}.

5.4 Statistics
In all the studies, tests for normality were conducted with the Shapiro-Wilk and Kolmogorov-Smirnov tests in all relevant calculations, to investigate eventually different distributions from normality. Variables are presented as mean values $\pm$ SE or median with ranges, as appropriate. Survival analyses
were performed with the Kaplan-Meier method, and crude analysis of OS was computed with the log-rank test. Cox regression was used for multivariate analysis. P<0.05 was considered significant. In Papers I and III, statistical analysis was performed with SPSS v20 (IBM SPSS Statistics for Windows, Version 20.0, Armonk, NY: IBM Corp.), and in Papers II and IV, with SPSS v23 (IBM SPSS Statistics for Windows, Version 23.0, Armonk, NY: IBM Corp.). In Paper V, the statistics software used throughout was GraphPad Prism version 7.00 for Macintosh (GraphPad Software, San Diego, CA, USA).

In Paper II, differences between groups were assessed using the Mann-Whitney U-test, Chi-square test and Student’s t-test for unmatched data or the Wilcoxon signed-rank test and McNemar’s test for matched data, as appropriate. Hazard ratios were calculated with a stratified Cox regression model. Power analysis was performed for the definition of sample size, along with a propensity score match to reduce bias between groups. The sample size calculation was based on the primary outcome (OS between matched groups) and a match ratio of 1:1 was utilized. The relative hazards ratio used in the sample size calculation between the matched groups was chosen to be greater than 2 (or less than 0.50). The probability of failure (death) within the cohort was projected to be 55% during follow-up, based on previous data from this cohort. A 1:1 nearest neighbor propensity score match with a caliper width of 0.1 was performed between the LRS and control group, using known and presumed confounding covariates at baseline. Standardized differences were used to examine variances in baseline characteristics before and after matching, with a standardized difference of <10 considered as an adequate balance between groups.

In Paper IV, uni- and multivariate analysis was performed for multiplex PLA and PEA results. Multivariate classifiers were designed to identify samples of the different types (healthy, Stage III, and Stage IV) based on their expression. Their performance was evaluated using the repeated holdout method with 100 replicates and test sets containing 20% random selected samples.

Two different classification methods (decision trees) were then used: nearest shrunken centroids (NSC, capturing simple linear patterns) and random forest (RF, capturing non-linear complex patterns). Due to the difficulty of these methods to separate Stage III and Stage IV samples, further analyses of PLA and PEA markers were focused on the differences between healthy and cancer samples regardless of stage.

For the statistical analysis of the ELISA results in Paper IV, Student’s t-test and the Mann-Whitney test were used as appropriate to compare serum concentrations for the investigated proteins between healthy controls and patients. Dichotomous and quartile division of protein serum levels in
patients was used for survival analyses. Additionally, ROC analysis was performed for sensitivity and specificity analyses.

In Paper V, concentration-response SI data formed the basis for calculation of the 50% inhibitory concentrations (IC$_{50}$), using a non-linear regression to a standard sigmoidal dose–response model. For subgroup analysis of samples from SI-NETs, data on the presence of PC and EAM, tumor grade, disease stage, age as well as pre-treatment s-CgA and u-5HIAA concentrations were collected from the patient charts within the subset of SI-NETs. For comparisons of in vitro sensitivity between different cancer diagnoses, statistical inference was calculated with 1-way ANOVA with Dunnett’s post-test and the SI-NET samples as reference. For comparisons of in vitro sensitivity between different clinicopathological factors as well as biomarker concentrations within the subset of SI-NETs, statistical significance was calculated with the Mann-Whitney test due to the small sized subgroups, which demonstrated distributions other than the normal.
6. Results

6.1 Paper I: Clinical signs of fibrosis in small intestinal neuroendocrine tumors.

A total of 824 patients were diagnosed with SI-NETs in the study period, of whom 538 had Stage IV disease. Significant clinical abdominal signs and symptoms of extensive fibrosis occurred in 36 patients (4.4% of all 824 patients; 6.7% with Stage IV disease). Of these, 19 had symptomatic occlusion of mesenteric vessels and 16 had clinically significant obstructive uropathy with hydronephrosis. One patient had both conditions, but was included only in the mesenteric vessel occlusion group for analysis.

All 36 patients had liver metastases. The majority had diffuse peritoneal carcinomatosis and all patients with retroperitoneal fibrosis had para-aortic lymph node metastases. CT images and available pathology reports for the eight patients with central mesenteric fibrosis who underwent locoregional resection confirmed fibrotic reactions in close proximity to mesenteric and/or para-aortic lymph node metastases.

Median survival among the 20 patients with mesenteric vessel occlusion was 90 (range 1–239) months. This was comparable to that of 260 patients with Stage IV disease, who had undergone primary surgery (85 (range 1–454) months, \( P = 0.80 \)). Similarly, median survival of 16 patients with clinically significant obstructive uropathy (96 (range 37–261) months) did not differ from that of the 260 patients who had undergone primary surgery \( (P = 0.87) \). Among the 260 patients with Stage IV disease who underwent LRS, signs of perioperative intestinal ischemia were confirmed in 29 patients and/or small intestinal obstruction in 94 patients. Thus, 49 (9.1%) of all 538 patients with Stage IV disease had some degree of bowel ischemia.

Univariable analysis demonstrated that ischemia \( (P < 0.001) \), peritoneal carcinomatosis \( (P < 0.001) \) and extrahepatic metastases \( (P < 0.001) \) affected survival. Adjusted multivariable Cox regression analysis confirmed that ischemia and peritoneal carcinomatosis \( (P < 0.001) \), but not extrahepatic metastases \( (P = 0.213) \), were independent prognostic factors for survival in patients with Stage IV disease.

No significant differences in overall survival and mortality after intervention were observed in patients treated with a stent versus laparotomy for mesenteric fibrosis. However, there was less morbidity after stenting \( (P = 0.036) \) and the hospital stay was shorter \( (P = 0.005) \). In the stent group
(12 patients), regression of ascites occurred in all three patients with this condition. Normalization of flow in the SMV after stenting was verified in eight of the patients who had radiological follow-up. Subjective symptom alleviation was observed in both the stenting (4 of 12) and laparotomy (5 of 8) groups.

As extensive fibrosis was accompanied by diffuse PC in 22 of 36 patients, OS for these 22 patients was compared with that of a subgroup of 32 patients with Stage IV SI-NETs with diffuse PC. This analysis confirmed that the patients with both extensive fibrosis and PC, subjected to minimally invasive intervention or surgery, survived longer than patients in Stage IV with a similar tumor burden ($P = 0.007$, Figure 4).

Figure 4. Kaplan–Meier survival analysis of patients with diffuse peritoneal carcinomatosis and those with extensive fibrosis and similar tumor burden. ($P = 0.007$ log-rank test).

Hydronephrosis due to retroperitoneal fibrosis was right-sided (7 patients), left-sided (3) or bilateral (6). Subgroup analyses showed no differences in OS within the obstructive uropathy group between those who underwent intervention and patients who received conservative treatment. No morbidity or 30-day mortality was reported. Median OS in the intervention group was 96 months after diagnosis, and 16 months after intervention.

In 21 of the 36 patients, considerable relief of fibrosis symptoms was achieved by extensive mesenteric surgery (before 2005), stenting of the SMV (after 2005) or insertion of a J stent in the affected ureter.
6.2 Paper II: Association of a Prophylactic surgical approach to Stage IV Small Intestinal Neuroendocrine Tumors with Survival.

From a total of 820 patients with SI-NETs (Swedish residents only), we included 363 asymptomatic patients with Stage IV disease (173 females; mean age at diagnosis with standard deviation 62.4±11 years). Median overall follow-up was 5.4 years (range 0-29.6).

Unmatched groups

Baseline variables of the unmatched prophylactic upfront surgery combined with oncological treatment group (LRS group, n=161) and the unmatched delayed surgery as needed combined with oncological treatment group (delayed LRS group, n=202) are presented in Table 5. In the unmatched delayed LRS group, the patients were older with more advanced disease in terms of LTL, EAM, and CHD, and exhibited higher u-5HIAA levels compared with the unmatched LRS group.
Table 5. Baseline characteristics for the LRS group and the delayed LRS group before propensity score matching.

<table>
<thead>
<tr>
<th></th>
<th>LRS group (n=161)</th>
<th>Delayed LRS group (n=202)</th>
<th>P-valuea</th>
<th>Standardized difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propensity score</td>
<td>0.62(0.1-0.92)</td>
<td>0.28(0-0.84)</td>
<td>&lt;0·001f</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>77(47.8)</td>
<td>96(47.5)</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>84(52.2)</td>
<td>106(52.5)</td>
<td>-0.6</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>60.1(10)</td>
<td>64.2(11.2)</td>
<td>&lt;0·001f</td>
<td></td>
</tr>
<tr>
<td>≤44</td>
<td>14(8.7)</td>
<td>14(6.9)</td>
<td>6.7</td>
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<tr>
<td>45-54</td>
<td>34(21.1)</td>
<td>26(12.9)</td>
<td>22</td>
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<td>53(45.7)</td>
<td>55(27.2)</td>
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</tr>
<tr>
<td>65-74</td>
<td>53(45.7)</td>
<td>66(32.7)</td>
<td>26.9</td>
<td></td>
</tr>
<tr>
<td>≥75</td>
<td>6(3.7)</td>
<td>41(20.3)</td>
<td>-52.8</td>
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</tr>
<tr>
<td>Calendar year</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1985-1995</td>
<td>41(25.5)</td>
<td>62(30.7)</td>
<td>-11.6</td>
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<tr>
<td>1996-2005</td>
<td>71(44.1)</td>
<td>74(36.6)</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>2006-2015</td>
<td>49(30.4)</td>
<td>66(32.7)</td>
<td>-5</td>
<td></td>
</tr>
<tr>
<td>Carcinoid Heart Disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extra-abdominal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastases Liver Tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Load</td>
<td>95(59)</td>
<td>145(71.8)</td>
<td>0.011</td>
<td>-27.2</td>
</tr>
<tr>
<td>No liver metastases</td>
<td>18(11.2)</td>
<td>12(5.9)</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>&lt;5, unilobar liver metastases</td>
<td>39(24.2)</td>
<td>26(12.9)</td>
<td>29.4</td>
<td></td>
</tr>
<tr>
<td>5-10 and/or bilobar liver metastases</td>
<td>44(27.3)</td>
<td>45(22.3)</td>
<td>11.6</td>
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Values in parentheses are percentages unless indicated otherwise;  
a. values are median (range)  
b. Pearson χ² test, unless otherwise stated.  
d. Student's t-test for independent samples

In the unmatched, delayed LRS group, a total of 89 patients underwent surgery while 72 patients did not. Thirty-two patients in the unmatched, delayed LRS group underwent LRS due to abdominal pain, obstruction and/or acute ischemia, whereas 57 patients underwent delayed elective LRS
with no obvious locoregional symptom or indication stated in the patient chart.

For patients in the unmatched, delayed LRS group subjected to surgery, 30-day postoperative morbidity was 1.0% and mortality was 0.5% compared with 30-day morbidity and mortality in the unmatched LRS group of 4.3% and 0%, respectively. The median OS of the unmatched LRS group was longer than the unmatched, delayed LRS group (9.5 years [7.5-11.6] versus 5.3 years [4.1-6.6], log-rank p=0.01, Fig 6).

**Propensity score matched groups**
Propensity score matching resulted in two isonumerical groups with similar baseline variables (Table 6).
Table 6. Baseline characteristics for the LRS group and the delayed LRS group after propensity score matching.

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Values in parentheses are percentages unless indicated otherwise.
a. values are median (range).

In the matched LRS group, 53 out of 91 patients underwent LRS after more than 6 months, whereas 38 patients never underwent LRS. The median time until surgery for these 53 patients was 18 months (range 6-168) and the indication for surgery was due to symptomatic disease in 20 patients: abdominal pain (n=14), acute bowel obstruction (n=5) or perforation (n=1). In the remaining 33 patients subjected to delayed LRS, surgery was elective, and locoregional symptoms were not stated in the patient charts. The matched LRS and delayed LRS groups were comparable in OS (HR 0.92,
95%CI 0.59-1.46), median 7.9 years (5.1-10.7) and 7.6 years (5.8-9.5) respectively (log-rank p=0.93) (Fig 5). Similarly, there was no difference in postoperative morbidity in the matched groups at 30 days (LRS 2.2% and delayed LRS 1.1%, p>0.99). There was no 30-day mortality in either matched group.

![Figure 5. Kaplan-Meier survival analyses of the unmatched LRS group (n=161; light blue line) and the delayed LRS group (n=202; dark blue line), as well as the matched LRS group (n=91) and delayed LRS group (n=91).](image)

No difference was found in LOS due to SI-NET disease between the matched LRS group (median 73 days [2-270]) and the matched delayed LRS group (median 76 days [0-339], p=0.635). Additionally, there was no difference between groups in LOS, specifically due to local tumor-related symptoms later in the disease course (LRS group median 7 days [0-90], delayed LRS group median 11.5 days [0-69], p=0.806)). The number of re-operations due to bowel obstruction (adhesiolysis, re-resection and/or intestinal bypass) was higher in the LRS group (n=13 in LRS group, n=3 in the delayed LRS group (p<0.001)), whereas the number of incisional hernia repairs was the same (n=4) in both matched groups (p>0.99).

**Multimodal treatment between matched groups**

Liver surgery, at baseline and during follow-up, was performed more frequently in the matched LRS group, compared to the matched delayed LRS group (p<0.001). No difference was seen between the matched groups regarding the use of RFA of liver metastases, TAE and TACE, PRRT with $^{177}$Lu-labeled DOTA-Tyr3-octreotate, SSA, interferon-alpha or other antitumoral agents.
6.3 Paper III: Indication for liver transplantation in young patients with small intestinal NETs is rare.

All patients <65 years
A total of 78 patients were included in our cohort. Fifty-three patients underwent liver-specific multimodality treatment according to clinical routine in addition to commonly administered SSA (n = 74) and interferon alpha (n = 70). Most patients (54%) underwent either liver resection or ablation (RFA/microwave ablation, or ethanol injections) of LM, although only 19% of such procedures were performed with curative intent. Thus, 81% displayed remaining LM after liver resection and/or RFA. Two subgroups were assessed for survival: (1) all patients and (2) patients not treated with resection and/or ablation of LM. The 5-year OS rates (±95% CI) of these two subgroups in all patients <65 years of age (Group 1) were 1A: 84% (±8%) and 1B: 70 (±15%) as shown in Figure 6. The corresponding 5-year DSS rates were 1A: 86% (±8%) and 1B: 73% (±15%). Finally, the 10-year OS rates for these two subgroups were 65 (±12%) and 55 (±18%), respectively.

Figure 6. Overall survival of patients <65 years of age (n = 78), divided into either all patients (A) or those in whom no liver surgery or ablation with either radiofrequency or microwave were performed (B).
All patients <55 years
Half of the patients in our cohort (n = 39) were <55 years of age at baseline (Group 2). The baseline characteristics of these patients were no different from those of the entire cohort. These patients were divided into two groups: (2A) all patients and (2B) patients not treated with resection and/or ablation of LM. The 5-year OS rates (±95% CI) of the two subgroups were as follows: (2A) 92% (±9%); (2B) 75% (±25%) (Figure 7). The corresponding 5-year DSS rates were: (2A) 94% (±8%) and (2B) 82% (±23%).

Figure 7. Overall survival of patients <55 years of age (n = 39), divided into either all patients (A) or those in whom no liver surgery or ablation with either radiofrequency or microwave were performed (B).

Patients fulfilling the Milan criteria
Thirty-three patients fulfilled the Milan criteria (Group 3, Table 2). The 5-year OS and DSS (±95% CI) rates of the two groups, (3A) all patients and (3B) no liver resection/RFA, were 97% (±6%) and 89% (±21%), respectively (Figure 8).
Figure 8. Overall survival of patients fulfilling the Milan criteria (n = 33), divided into either all patients (A) or those in whom no liver surgery or ablation with either radiofrequency or microwave were performed (B).

Survival after liver transplantation of SI-NET patients according to the literature
During our search of the literature, we found 26 SI-NET patients subjected to LTx where sufficient data to generate a survival curve were available. The age of these patients ranged from 22 to 64 years, the primary tumor was resected in all cases, and no patients exhibited extrahepatic disease at the time of LTx. The 5-year OS (±95% CI) for this group was 76% (±23%).
6.4 Paper IV: DcR3, TFF3 and Midkine are Novel Serum Biomarkers in Small Intestinal Neuroendocrine Tumors.

**Novel Biomarkers for SI-NETs**

Using a multiplex PLA, serum from 23 SI-NETs, classified as Stage IV disease, and from 23 healthy controls was screened for 69 cancer biomarkers. Nineteen biomarkers had significantly different concentrations when comparing patients to controls. Seventeen markers were up-regulated in patient serum compared to that of healthy controls, and two proteins, EGFR and MMP9, displayed lower concentrations in patient serum.

Using the PEA, an additional 76 biomarkers were screened for, and this resulted in detection of another 13 biomarkers with higher serum concentrations in patients with SI-NETs than in healthy controls. Four biomarkers (MMP3, GM-CSF, SCF, and EGFR) were found to be decreased in patients compared to controls. Sixteen biomarkers were included in both the PLA and the PEA analyses, and 15 of these revealed the same result. The serum concentrations of 12 biomarkers were the same for SI-NETs and healthy controls: 1 biomarker, EGFR, was significantly lower in patients, and 2 biomarkers, EpCAM and PRL (prolactin), were increased in Stage IV tumors compared to healthy controls. IL8 was differentially expressed in PLA but not in PEA analysis.

The nearest shrunken centroid classification using the multiplex PLA concentrations achieved a mean accuracy of 79.8% across the holdout replicates. Mindin and MMP9 were scored as the most important biomarkers. Twenty-two additional markers were also selected by the models, including DcR3/TNFRSF6B and TFF3. In the PEA analyses, midkine was identified as a good biomarker for SI-NETs by both univariate testing and multivariate classification.

**SI-NETs Express DcR3, TFF3, and Midkine**

DcR3, TFF3, mindin (analyzed by PLA), and midkine (analyzed by PEA) were chosen for further analysis due to the great differences seen in serum concentrations between patients and controls; the fact that they were considered interesting markers due to their cellular functions and involvement in tumorigenesis, and also because multivariate classification suggested they would be good markers. Immunohistochemistry showed that mindin was expressed by tumor cells but the ELISA analysis for mindin did not run well, and further experiments with mindin were not performed in this study. Immunohistochemistry was used to analyze protein expression in tumor tissue. Positive and negative control staining was performed for all 3 antibodies.
DcR3 protein expression was analyzed in 20 primary tumors, 19 lymph node metastases, and 5 liver metastases. A majority of the tumors (37/44) displayed smaller cell clusters with very strong positive staining (Figure 9a). In the remaining tumor areas of these 37 tumors, positive tumor cells were detected in 9 cases, weakly positive tumor cells in 16 cases, and negative staining in 12 cases. Four tumors displayed larger areas with positive staining, while 2 samples demonstrated strong staining and 1 sample showed weak staining in all tumor cells.

Figure 9. Immunohistochemical analysis. (a) DcR3 protein expression in a lymph node metastasis. Strong staining in a cell cluster and weak staining around it. ×20. (b) TFF3 protein expression in a primary tumor, with strong staining in the cells surrounding the insular areas. ×20. (c) Midkine expression in the primary tumor. ×10.

Protein expression of TFF3 was analyzed in 19 primary tumors, 21 lymph node metastases, and 5 liver metastases (total n = 45). Thirty-two tumors displayed strong or very strong staining (Figure 9b). Eleven tumors showed a variable pattern with weak staining or positive staining in few cells. Two tumors did not express TFF3 protein. Twenty-seven tumors displayed an insular growth pattern, and in 20 of these, strong staining was detected in the cells surrounding the insular areas.

Midkine expression was analyzed in 12 primary tumors, 9 lymph node metastases, 2 liver metastases, and 1 ovarian metastasis. Midkine was strongly expressed in both primary tumors and metastases (Figure 9c).

_Elevated Serum Concentrations of DcR3, TFF3 and Midkine_

The protein concentrations of DcR3, TFF3, midkine, and CgA were analyzed in serum in the original cohort by ELISA for validation of the PLA (n = 23 for DcR3, TFF3, and CgA) and the PEA (n = 68 for midkine). The protein concentrations of DcR3, TFF3, and CgA were also analyzed by ELISA in an independent cohort (n = 70 for DcR3; n = 71 for TFF3; and n = 69 for CgA).

For DcR3, 37 samples from patients with TNM Stage III disease and 54 samples from Stage IV patients (including 21 patients from the PLA analysis) as well as all healthy controls (n = 23) were included in the ELISA
analysis. Independent replicates were used, and the coefficient of variation (CV) between 3 runs was 6.7%. There was a difference in concentrations \(p < 0.01\) between controls and Stage IV patients, and also between patients with Stage III and those with Stage IV disease \(p < 0.01\) (Figure 10a). No difference \(p = 0.4\) was seen between healthy individuals and patients with Stage III disease. Survival analysis revealed that after dichotomization of the material, high serum concentrations of DcR3 correlated with poor survival \(p < 0.001\). To analyze the specificity and sensitivity of DcR3 as a prognostic marker, a ROC curve was made, demonstrating that it is a fairly good biomarker candidate for prognosis \(AUC = 0.74\) when comparing concentrations from patients with TNM Stage III to those from patients with TNM Stage IV disease (Figure 10c).

![Figure 10. Analysis of DcR3.](image)

TFF3 protein concentrations in serum were analyzed in 39 patients displaying TNM Stage III and in 53 patients with Stage IV disease (including 21 patients and all healthy controls from the PLA analysis). One healthy control was excluded due to an extreme value of unknown cause. The CV between 3 runs was 8.3%. As shown in Figure 11a, there was a significant \(p < 0.05\) difference in concentration between healthy controls and patients with TNM Stage III disease, and also patients classified as TNM Stage IV \(p < 0.01\). Survival analysis showed that, after dichotomization, high concentrations of TFF3 were related to poor survival \(p < 0.01\). A ROC curve analysis showed that TFF3 is a candidate for being a fairly good diagnostic biomarker \(AUC = 0.72\) for separating healthy controls from SI-NET patients (Figure 11c).
Figure 11. Analysis of TFF3. (a) ELISA analysis with boxplots demonstrating increased concentrations in patients with TNM stages III ($p < 0.05$) and IV ($p < 0.01$) disease compared to healthy controls. For No. 50, 57, and 71, the value was set to 12 due to unmeasurable, high absorbance. (b) Survival analysis. Group 0 displays a low concentration and Group 1 displays a high concentration (dichotomous division). Kaplan-Meier analysis shows shorter survival for patients displaying high concentrations of TFF3 ($p < 0.01$). (c) ROC curve for the analysis between patients with SI-NETs and healthy controls (AUC = 0.72).

For midkine, altogether 27 samples from patients with TNM Stage III disease and 28 samples from Stage IV patients as well as all healthy controls ($n = 23$) were included in the ELISA analysis. The CV between 2 runs was 3%. ELISA analysis displayed increased midkine levels ($p < 0.01$) in patient serum from the original cohort compared to controls. Midkine was found to be elevated in TNM Stages III ($p < 0.05$) and IV disease ($p < 0.01$) compared to healthy controls (Figure 12a). In the survival analysis, after dichotomous division of the material, based on the serum concentrations of midkine, there was no difference in OS (Figure 12b). ROC analysis for midkine comparing patients with healthy controls displayed an AUC of 0.71 (Figure 12c), indicating that midkine is a fairly good diagnostic biomarker for SI-NETs. Moreover, ROC analysis demonstrated an AUC = 0.71 for the ability of midkine to discriminate between patients in Stage IV disease and the controls.
Figure 12. Analysis of midkine. (a) ELISA analysis with boxplots demonstrating increased concentrations in patients with TNM Stages III ($p < 0.05$) and IV ($p < 0.01$) disease compared to healthy controls. (b) Survival analysis. Group 1 displays a high concentration and Group 2 displays a low concentration (dichotomous division). Kaplan-Meier analysis shows no difference in survival for patients displaying high versus low concentrations of midkine ($p = 0.55$). (c) ROC curve for the analysis between patients with SI-NETs and healthy controls (AUC = 0.71).

For CgA, 38 patients with Stage III disease, 45 with Stage IV disease, and all 23 healthy controls were analyzed. The CV between 3 runs was 5.9%. There was no significant difference ($p = 0.14$) between healthy controls and patients with Stage III disease, but a significant difference ($p < 0.001$) was detected between healthy controls and patients with Stage IV disease (Figure 13a). Survival analysis showed that high concentrations of CgA were associated with poor survival ($p < 0.01$; Figure 13b). A ROC curve analysis showed that CgA is a fairly good diagnostic biomarker (AUC = 0.73) for discerning healthy controls from SI-NET patients (both Stages III and IV) (Figure 13c).

Figure 13. Analysis of CgA. (a) ELISA analysis with boxplots showing increased concentrations in patients with TNM Stage IV disease ($p < 0.001$) compared to healthy controls. (b) Survival analysis. Group 0 displays a low concentration and Group 1 displays a high concentration (dichotomous division). Kaplan-Meier analysis shows shorter survival for patients displaying high concentrations of CgA ($p < 0.01$). (c) ROC curve for the analysis of patients with SI-NETs versus all healthy controls (both Stages III and IV) (AUC = 0.73).
For DcR3, TFF3, and midkine there were no correlations of serum concentrations with gender and the presence of flushing and/or diarrhea at diagnosis. There was a tendency ($p = 0.06$) towards higher concentrations of TFF3 in patients with G2 tumors. DcR3 concentrations were not subject to any change based on the age of the patients. However, older patients demonstrated higher concentrations of TFF3 and midkine. No age-dependent relationship with any of these markers was observed in the healthy controls.
6.5 Paper V: *Ex vivo* activity of cytotoxic drugs and targeted agents in small intestinal NETs.

Drug sensitivity *ex vivo*

Samples from SI-NETs had lower IC$_{50}$ values to standard cytotoxic drugs than samples from renal cancer, with the exception of mitomycin C and irinotecan (Figure 14). Additionally, for half of the protein kinase inhibitors studied, IC$_{50}$ values in SI-NETs were lower than those in renal cancer (Figure 15).

*Figure 14.* IC$_{50}$ values for standard drugs indicated in all tumor samples. Results are presented as means values + SE. Statistical inference was calculated with 1-way ANOVA with Dunnett’s post-test and with the SI-NET samples as reference.

*, ** and *** denote P < 0.05, 0.001 and 0.0001 versus SI-NET samples, respectively. Absence of asterisks means that no statistical differences were observed compared to SI-NET samples.
Figure 15. IC₅₀ values for targeted drugs indicated in all tumor samples. Results are presented as means values ± SE. Statistical inference was calculated with 1-way ANOVA with Dunnett’s post-test and with the SI-NET samples as reference.

*, ** and *** denotes P < 0.05, 0.001 and 0.0001 versus SI-NET samples, respectively. Absence of asterisks means that no statistical differences were observed compared to SI-NET samples.

SI-NET samples compared to CRC demonstrated lower IC₅₀ values for half of the cytotoxic agents studied (Figure 13), whereas for the majority of protein kinase inhibitors studied (7/12), IC₅₀ values in SI-NETs were lower (Figure 14). The CLL and ovarian cancer samples demonstrated IC₅₀ values similar to or lower than those of the SI-NETs to both cytotoxic drugs and protein kinase inhibitors (Figure 13 and 14). Notably, SI-NET samples were
relatively sensitive to 5-FU, gemcitabine, gefitinib, nintedanib, ruxolitinib and sirolimus, whereas irinotecan is not expected to be active in SI-NET. Of particular interest in SI-NETs, sirolimus was relatively active, with marginal statistical significance ($p=0.054$) in comparison with CRC (Figure 14 and 16).

Figure 16. Tumor cell sensitivity, expressed as survival index percentages (SI%) of the SI-NET samples for the indicated standard cytotoxic drugs. The curves represent non-linear regression lines calculated for all individual samples included and demonstrate great variability in drug sensitivity between individual samples.

SI-NET samples demonstrated great variability in drug sensitivity. Some samples were essentially unaffected by the highest drug concentrations tested, whereas other samples exhibited decreased viability even at the lowest concentration tested (Figure 16 and 17). Octreotide demonstrated very low activity (Figure 17).
Figure 17. Tumor cell sensitivity, expressed as survival index percentage (SI%) of the SI-NET samples for the indicated targeted agents. The curves represent non-linear regression lines calculated for all individual samples included and demonstrated variability in drug sensitivity between individual samples.

Cross-resistance between the different drugs and targeted agents investigated, varied greatly. It was high between protein kinase inhibitors that were mechanistically related and considerably lower for the chemotherapeutic drugs tested, except for 5-FU and gemcitabine (Figure 18).
Figure 18. Correlations between the cytotoxic activities (SI%) for the indicated pairs of standard cytotoxic drugs and targeted agents at concentrations selected to provide optimal activity variation. The correlations are based on all SI-NET samples investigated. The $r$ denotes the correlation coefficient and $p$ the level of statistical significance.

Drug sensitivity in subset of SI-NETs.
We estimated the median IC$_{50}$ values for each drug according to different clinicopathological categories and biomarker concentrations in the SI-NET subgroup. Age, stage and grade at diagnosis, as well as the presence of PC and EAM, were not obviously associated with sensitivity to the standard cytotoxic drugs or the protein kinase inhibitors tested. Likewise s-CgA and u-5HIAA concentrations at diagnosis did not consistently correlate to drug sensitivity.
7. General Discussion

7.1 Abdominal fibrosis in SI-NETs

SI-NETs are commonly associated with the presence of mesenteric lymph node metastases\textsuperscript{76}, with regional and distant-abdominal/para-aortal nodal disease occurring at diagnosis in 88% and 18% of cases, respectively. LM occur in more than 60% and PC in 17% of patients\textsuperscript{14}.

When present, bulky metastatic disease in the root of the mesentery may cause chronic vascular compromise of the intestine, which involves both arterial and venous branches. However, arteries are not commonly occluded, despite being involved more often. Veins, on the other hand, are easily compressed, since they have thinner walls. Pressure phenomena on the root of the mesentery are caused by the nodal spread, but also by the presence of postoperative adhesions, as well as chronic, extensive fibrosis attributed to serotonin over-secretion\textsuperscript{31,77,78}.

The diagnosis of fibrosis may be made during laparotomy or on a CT scan, where the typical spoke-wheel appearance indicates the presence of radiating fibrotic bands (Figure 2A). Some of these desmoplastic reactions are also calcified\textsuperscript{79}.

Aggressive surgical resection is the optimal solution in fibrotic cases, with resection of the primary tumor as well as extensive lymph node dissection, regardless of the presence of metastatic disease. Retrospective studies have shown that locoregional resection in symptomatic patients is beneficial even when liver metastases are present, as it is often performed on vital indication\textsuperscript{80}. Furthermore, this is supported by the results of our study, which show that surgical intervention was successful in the subgroup of patients with occlusion in the SMV treated before 2005. However, this has to be balanced with the dissection of the root of the mesentery, which may be complex and lead to devastating complications.

Modern medical treatment has a stabilizing effect on the growth of the metastases, creating opportunities for a more conservative approach in some cases in recent years. Established therapies, such as SSAs have demonstrated efficiency in relief from symptoms\textsuperscript{44}, and antiproliferative effects have been demonstrated in up to 66.7% of cases by Rinke et al.\textsuperscript{38} However, maximal response was observed in patients with low burden and excision of the primary. Additionally, there is a lack of clinical data to support the hypothesis that there is a “downsizing” effect produced by systemic therapy.
in these cases. This was clearly evident in our SMV occlusion cohort, where stenting of the SMV was considered after the patients had already been on systemic therapy without response concerning node size and, consequently, developed locoregional symptoms and occlusion of the SMV.

Serotonin produced by the tumor cells induces fibrosis in cellular systems and 5HT2B receptor antagonists may be a possible route to reducing the fibrotic reactions. This has also been demonstrated for inhibition of right cardiac ventricular fibrosis. Another promising drug is telotristat etiprate, which interacts in the synthesis pathway of 5-hydroxytryptamine with potentially dramatic reductions in serotonin levels secondary to lowering the risk of the development of fibrosis. Interacting in TGF-beta may be possible, since serotonin seems to induce upregulation of TGF-beta 1, which in turns induces fibrosis. Other plausible mechanisms for the induction of fibrosis in SI-NETs are through connective tissue growth factor (CTGF), tachykinins, substance P, neurokinin A or TGF-beta 1 itself, which are known to stimulate fibroblasts in different ways. Indeed, during the last two decades, lowering hormone levels, especially 5-hydroxytryptamine, has been associated with a marked reduction in the number of patients with CHD, implying that abdominal fibrotic complications also may develop far more rarely in the future.

Our analysis demonstrated that mesenteric disease causing intestinal vascular obstruction is an independent prognostic factor for survival even in the elective setting (p<0.001). However, in advanced SI-NETs with extensive fibrosis, large inoperable masses in the mesenteric root and established ischemic symptoms of the intestine, stent placement in the SMV is beneficial in terms of palliation.

The efficacy of transhepatic stent placement in the portomesenteric axis to treat occlusion or portal hypertension has been documented in the context of palliation for inoperable hepatobiliary and pancreatic cancers, and the use of self-expandable metallic stents has also been reported after transplantation and hepatobiliary surgery, as well as a means of treatment for post-traumatic arteriovenous fistulas. From a technical point of view, the facilitation of mesenteric dissection by the presence of a metallic stent in the SMV may be of paramount importance.

In our cohort, patients treated successfully with either surgery or stenting of the SMV did not present again with clinical signs of stasis. Overall survival analyses demonstrated a significantly higher survival rate after intervention as compared to those with PC and a similar Stage IV tumor burden. This implies that intervention is meaningful and should also be undertaken, when feasible, in patients with PC.

OU in the setting of idiopathic retroperitoneal fibrosis has been addressed in the literature demonstrating that intra-ureteral J stent and percutaneous nephrostomy are good alternatives for drainage and decompression, with the
latter being easier in the acute setting\textsuperscript{87}. Our statistical analysis shows that median survival in the intervention group was 110 months after diagnosis, but survival post-intervention was 16 months, indicating that symptomatic hydronephrosis in need of intervention occurs late in the progression of the disease and is combined with excessive tumor burden.

In conclusion, it seems that clinical signs of extensive long-term fibrotic reactions affect survival, but are also amenable to treatment, and these patients possibly have an increased survival as compared to patients with similar tumor burden. OU seems to occur late in the disease, but intervention in cases, where this is needed, offers significant symptom relief. The small number of patients expressing these complications makes any prospective or randomized trial impossible, especially given the fact that patients demonstrating clinical complications of fibrosis often have intractable symptoms, which must be taken care of in a patient-tailored way.

Apart from palliation with reduced morbidity and probable benefit in survival, attention should also be given to the potential use of these techniques as bridging therapy and as a means to operate on cases previously deemed inoperable. Lastly, the development of a medical treatment which would reduce fibrosis and might alleviate associated symptoms, is probably the most important future task if we are to prevent the development of intestinal ischemia and OU in patients with SI-NETs.

### 7.2 Locoregional surgery in asymptomatic Stage IV patients

This study assessed outcomes of asymptomatic patients with SI-NETs and distant metastases following prophylactic upfront LRS. No survival benefit was found after propensity score matching for these patients compared to matched controls, with initial non-surgical management. In addition, the 30-day postoperative morbidity and mortality after upfront LRS was no different from delayed LRS. There was no difference in length of hospital stay (LOS) due to SI-NET disease between the two matched groups, even when locoregional symptoms were specifically addressed. Interestingly, a higher rate of re-operation due to bowel obstruction was associated with prophylactic upfront LRS, presumably due to the development of postoperative adhesions along with a lack of locoregional macro-radicality and accompanying fibrotic manifestations in the mesentery of patients with tenacious Stage IV disease.

Despite the fact that prophylactic LRS in Stage IV asymptomatic SI-NET patients has been considered standard practice, it has not been evaluated in
any randomized controlled trials and the survival rates after LRS, reported in retrospective cohort studies, are likely influenced by selection and immortal-time bias\textsuperscript{14,88-92}. This is supported by our study, where the unmatched groups varied in baseline variables and the use of propensity score matching eliminated these confounding factors. The results challenge current European Neuroendocrine Tumor Society (ENETS) guidelines\textsuperscript{6,50}, which suggest that prophylactic LRS in Stage IV SI-NET patients offers a possible benefit. However, these guidelines are based on results from retrospective studies with no attempt to correct for confounding factors. On the other hand, the benefit from resection of asymptomatic primaries in patients with advanced and non-resectable metastatic disease is being questioned in current literature and the National Comprehensive Cancer Network (NCCN) recommendations\textsuperscript{51,93}.

Despite the fact that surgery may alleviate carcinoid syndrome symptoms, flushing and diarrhea may also be efficiently controlled by anti-tumoral agents. SSA treatments have been shown to control time to progression and stabilize the growth of metastases\textsuperscript{38,94}. These medical options provide an avenue for control symptoms while avoiding surgery and its associated risks. PRRT has also been added to the therapeutic armamentarium for SI-NETs in Stage IV and may also potentially alter the role of surgery\textsuperscript{36}.

Limitations of the present study were the lack of mesenteric lymph node staging plus the fact that macro-radicality in the mesentery was not assessed in patients who underwent LRS. However, the majority of operations were performed at Uppsala University Hospital, where a standardized mesenteric dissection was undertaken as described by Ohrvall et al.\textsuperscript{45} Although the propensity score method may exacerbate imbalance in unmeasured covariates, the propensity score match in this study controlled extensively for variables that were known or presumed confounders. However, critical variables may have been overlooked and thus, instead, increased hidden heterogeneity between groups. For example, neither pathologic T tumor categories, extent of mesenteric lymph node metastases, nor PC, were included in the propensity score match as not all patients were operated upon and these variables could not be assessed in the same manner. Lymph node staging was impossible due to lack of data and PC was only assessable in the group that had LRS at baseline. Although anti-tumoral treatments were not controlled in the propensity score model, there were no differences in the use of SSA, interferon alpha, PRRT or liver embolization between matched patients in our study. Only three matched patients received an mTOR inhibitor and only one matched patient was treated with a tyrosine kinase inhibitor, making it implausible that these recently approved drugs influenced study outcome. The LRS group was more likely to undergo liver surgery, which may have influenced the results, although a previous
propensity score matched study showed that whether a patient had liver surgery or not did not influence survival in locoregionally resected SI-NETs\textsuperscript{33}.

Moreover, incomplete matching occurred in around 50% of the total cohort and this was accepted, because including more patients in the matching would have led to severe imbalance remaining in baseline covariates. Incomplete matching may limit the generalizability of the results. Therefore, the results of this study may not apply to patients who were not matched due to extreme propensity scores, e.g. very young patients, mostly found in the LRS group, or patients with high comorbidity, overrepresented in the delayed LRS group. Furthermore, although our \textit{a priori} calculated sample size was met, the study may have been underpowered to detect a smaller but still clinically significant difference in OS. However, the striking lack of difference of the survival estimates between the groups fuels the idea that the LRS and delayed LRS approaches are no different with regards to OS.

In the delayed LRS group, 53 of the 91 patients eventually underwent surgery, and certainly, a proportion of these patients (20/53) would have benefited from prophylactic surgery, since they developed symptoms over time. However, 33 patients may have had prophylactic surgery while they were without symptoms. We can only speculate as to the reasons for this approach, including differences in clinical expert opinion, patient preference and physician’s delay. Importantly, all patients included in this study received multimodality treatment including SSAs, interferon alpha, liver-directed treatment and/or PRRT as indicated. Allowing for a 6-month interval between diagnosis and surgery, the potential exists to establish tumor biology and possible treatment effects prior to invasive therapy. For example, it is possible that patients in the control group with growing metastases were selected for surgery during follow-up. Finally, the fact that the patient population studied includes only referrals to our tertiary center may include a certain referral bias.

LRS retains its value in the treatment of patients with SI-NETs when radical resection is feasible or when symptomatic disease is present, regardless of disease stage. However, our results challenge the traditional view for extensive locoregional surgery in patients with distant metastases in the absence of local tumor-related symptoms. No benefit in survival could be demonstrated and patients were burdened with more re-operations for intestinal obstruction. On the contrary, a more conservative approach with delayed LRS as clinically indicated seems to yield comparable survival without the risks in terms of increased morbidity and mortality or prolonged hospital stay.
This approach therefore seems reasonable for this subgroup of SI-NET patients and may complete the armamentarium of systemic and liver-directed treatments, as indicated for each patient. In the era of personalized treatment, it seems that maximalistic surgery should be reconsidered and replaced by a comprehensive multidisciplinary approach for the optimal treatment of patients with SI-NETs. Finally, despite the sophisticated methodology followed and the design of this study, it is clear that a prospective randomized controlled trial is needed to further elucidate the value of prophylactic LRS and strengthen current recommendations. In light of these results, a composite including re-operation due to growing metastases or symptoms may be more feasible than OS as an endpoint in a future randomized controlled trial.

7.3 Liver transplantation for SI-NET liver metastases

This study reports that survival of SI-NET patients with liver metastases, but no extrahepatic metastases, is at the same level as survival after LTx as reported in case series in the literature.

In other words, we show that prime results can be achieved with multimodality treatment in these patients, without the need for LTx. SI-NET patients <65 years suitable for LTx had an excellent 5-year survival (84 ± 8%), much higher than the often reported 20% – 35% in other historical series of NETs of all types. This outstanding 5-year survival probably originates in the selection of SI-NET patients who lack two of the strongest known negative prognostic factors, namely advanced age and remaining extrahepatic metastases after surgery. However, it should be noted that the positive effects of multimodality treatment of LM by means of liver resection, ablation, and hepatic arterial embolization, as well as drug therapy with SSAs and interferon-alpha may also play important roles.

Many series of LTx for NET with LM that report 5-year survival are limited by the number of patients included, and the survival figures retrieved from these are thus insecure. The Kaplan–Meier analysis of data from our literature review of survival of SI-NET patients after LTx yielded a 5-year survival of 76 ± 23%, no different from the 5-year survival in our cohort. Also, one multicenter study reports a 5-year survival after LTx for SI-NETs of 62%, which might even be lower than the 84 ± 8% survival of all patients in our cohort, although it is similar to the 70 ± 15% 5-year survival of the patients in our cohort who were not selected for resection/ablation of LM (commonly because of massive liver involvement).

Long-term survival (10-year) after LTx of NET patients is not reported in either of the two multicenter reports described above, although it may be extracted from a report from the United Network of Organ Sharing (UNOS)
database from 2011, where it was approximately 30%. However, this figure is statistically imprecise because of the very few patients remaining in the survival analysis. Nevertheless, 10-year OS after LTx of different benign diagnoses such as Budd-Chiari syndrome, Wilson’s disease, and cirrhosis caused by hepatitis, range from 64% to 84%, which may be compared with the 65 ± 12% (all patients) or 55 ± 18% (those not treated with resection/ablation of LM) in our series. Thus, even in the hypothetical absence of any tumor-related death after LTx in SI-NET patients, the survival would just barely exceed the expected survival of non-transplanted SI-NET patients. However, this best-case scenario is probably very unlikely because of the high rate of recurrence seen after LTx in NETs.

Two major multicenter studies of LTx in NETs report an improvement of survival over time, owing to narrower selection criteria of patients, and, hopefully, also better surgical care. Several authors have advocated the use of narrower selection criteria for LTx to optimize outcome. However, this endeavor may be problematic in different ways. One is easily shown in our study where the 5-year survival is higher in the group fulfilling the Milan criteria (97 ± 6%) than in all patients (84 ± 8%) merely due to selection bias. Thus the “improved outcome” shown in the Milan series may be due to selection and not to treatment effect. This is further supported by the fact that the Milan group’s 5-year survival (90%) is lower or similar to the studied cohort’s 5-year survival (97 ± 6%), even when compared with the subgroup that was never treated by resection/ablation (89 ± 21%). Despite the impressive survival figures in our study, they may nevertheless be underestimated in comparison with the Milan series, as patients with >50% liver tumor involvement were not excluded in our study. The Milan group recently reported a survival benefit for patients undergoing LTx with 5- and 10-year OS of 97.2% (even higher than the previously 90% reported from this center) and 88% respectively, comparable with the figures in our study for patients fulfilling the Milan criteria. However, the initiation date of the time-line calculations and the handling of patients on the waiting list for LTx, but who never received a transplant, were unclear. Patients included were not age-matched, and the LTx-related morbidity and mortality were not addressed. Another problematic issue of narrower patient selection criteria is that without the use of a control group some patients who would actually benefit from LTx may be excluded.

In conclusion, SI-NET patients with similar inclusion criteria to those selected for LTx at other centers may have excellent long-term survival with multimodality treatment, undoubtedly much higher than reported in historical NET study series. As most patients in our cohort who did not undergo LTx were still alive after 5 years, very few would have benefited in terms of 5-year OS from LTx. Indeed, long-term results (10-year OS) from more transplantation series, other than the impressive figures reported from the Milan group, are needed for further comparison. Although survival rates
between cohorts are often difficult to compare owing to differences in study populations, we found no evidence that indicated any increased survival after LTx in comparison with the multimodality treatment used at our center. There are two ways to finally elucidate the possible survival benefit of LTx in SI-NET patients. One would be to perform a randomized study, and the other would be to improve the outcome after LTx beyond doubt without the use of narrower selection criteria. Neither of these courses is easy to achieve due to the scarcity of cases and the indolent course of the disease. However, because evidence regarding increased survival after LTx in SI-NET cases is lacking, we believe that it is very important to carefully inform patients being considered for LTx of this. The centers that perform LTx in SI-NET patients with the aim of increasing OS should collect data, preferably within a prospective trial, for future treatment improvements. Of course, indications other than increased survival, such as uncontrollable hormonal symptoms, may still qualify SI-NET patients for LTx, and such indications were not studied in the present report. However, in the majority of cases, the multimodality treatments used today do control these symptoms.

7.4 Novel biomarkers

Our study on biomarkers revealed several interesting markers involved in SI-NET tumorigenesis, and further analysis was performed on DcR3, TFF3 and midkine due to their cellular functions and involvement in immune response and tumorigenesis. Further investigation of, for example, mindin, prolactin, serpin E1, CSF-1, CXCL9 and CXCL10, is of interest and could also potentially be of importance for diagnosis and prognosis.

DcR3 is also known as tumor necrosis factor receptor superfamily member 6b (TNFRSF6B) and the gene is located on chromosome 20 (20q13.3). DcR3 has pleiotropic effects and functions as an immunomodulator, acting as a secreted soluble decoy receptor for FasL, LIGHT and TL1A, and neutralizing the effects of these cytokines. These effects are favorable for tumor growth and inhibition of apoptotic stimuli.\textsuperscript{110} Multiple tumor types express elevated concentrations of DcR3 and the expression is correlated with poor prognosis and/or resistance to treatment.\textsuperscript{111,112} DcR3 has been demonstrated to be a potential ovarian cancer biomarker and may improve early diagnostics.\textsuperscript{113} High expression is correlated to metastasis and poorer OS in different cancer types.\textsuperscript{114-116} In this study, we confirmed that SI-NETs express DcR3 and patients with liver metastases display elevated serum concentrations compared to controls. Interestingly, patients with Stage III disease did not have elevated concentrations, indicating that DcR3 may be a marker for bulky disease in SI-NETs. It may also be a prognostic marker since high concentrations were correlated with worse survival. DcR3 also induces NF-kB mediated
expression of ICAM-1, VCAM-1 and IL-8 in monocytes\textsuperscript{117}. Serum concentrations of IL-8 and VCAM-1 were also elevated in SI-NET patients, suggesting that the NF-kB signaling pathway is regulating the immune response in the tumor microenvironment of SI-NETs. Knock-down of DcR3 \textit{in vivo} (in mice xenografts) inhibited pancreatic cancer tumor growth, displaying the importance of this protein in cancer progression, and suggests DcR3 to be a potential therapeutic target\textsuperscript{118}. Different treatments have been shown to reduce high concentrations of DcR3 in pancreatic adenocarcinoma cells (a tyrosine kinase and a PI3K inhibitor, an NF-kB inhibitor and an insulin growth factor-1 inhibitor)\textsuperscript{119}, indicating potential new treatment options for SI-NETs too. The specificity and sensitivity of DcR3 as a prognostic biomarker was good according to the ROC curve analysis, and further analyses in a larger cohort should be performed to confirm its clinical potential as a biomarker for bulky disease and prognosis.

TFF3 is mostly produced in the mucus-secreting goblet cells in the small intestine and colon, and its main function is to protect and repair epithelial surfaces\textsuperscript{120}. Elevated expression is seen in a variety of solid tumors and is associated with an aggressive phenotype and poor prognosis\textsuperscript{121-124}. TFF3 enhances migration, is anti-apoptotic and induces angiogenesis. It is also suggested that it is a marker for circulating tumor cells (CTCs)\textsuperscript{125}. SI-NET patients showed elevated serum concentrations of TFF3 compared to healthy controls, suggesting that it could be a novel marker for diagnosis. High serum concentrations were correlated to a reduced survival, indicating that TFF3 also could act as a prognostic marker. According to ROC curve analysis, TFF3 is a good biomarker.

Midkine is a multifunctional protein with anti-apoptotic, migration-promoting, neurogenic and angiogenic activities\textsuperscript{126}. Midkine is overexpressed in many malignant tumors, contributing to their invasive phenotypes as well as their resistance to chemotherapy\textsuperscript{127,128}. Overexpression of midkine in different tumor types resulted in elevated circulating midkine concentration\textsuperscript{126}. Midkine was elevated in all cases of metastatic colorectal cancer, and strongly outperformed CEA at detecting colorectal cancer at all disease stages\textsuperscript{129}. Importantly, midkine outperformed the currently used blood biomarker test in multiple tumor types. These include the hepatocellular carcinoma marker, α-fetoprotein (AFP)\textsuperscript{130,131}, the esophageal squamous cell carcinoma markers CEA and cytokeratin 19 fragment (CYFRA21-1)\textsuperscript{132}, and the differentiated thyroid cancer marker, thyroglobulin\textsuperscript{133}. This study demonstrates that midkine is elevated in SI-NET patients’ serum and is thus potentially a novel biomarker for diagnosis. Midkine also has predictive ability for adjuvant chemotherapy response in multiple cancers\textsuperscript{134,135}. There have been several successful attempts to develop novel cancer treatments targeting the midkine gene in tumors overexpressing midkine, with methods including molecular engineering\textsuperscript{136-142}. In our study, all the SI-NETs analysed expressed midkine and targeting
this gene might potentially result in novel treatment for SI-NET patients. However, as midkine seems to be overexpressed in many other malignancies, it could be potentially utilized in combination with other markers, namely as part of a plasma multianalyte measurement.

The novel biomarkers identified here may provide the foundation for a new set of markers which, if used together, may form the basis for a novel assay which can provide more reliable information concerning prognosis in the future. However, this potential use will have to be tested in larger, unselected cohorts of SI-NET patients and its specificity has to be tested against other NETs, for example, in the pancreas and lung.

7.5 Ex vivo activity of cytotoxic and targeted agents in SI-NET samples

Treatment of metastatic SI-NETs does not include cytotoxic drugs and/or systemic targeted agents as a first line of therapy, as these agents are generally considered to have limited activity in this tumor type. Additionally, secretory symptoms are often well controlled with SSAs and the tumor itself may have an indolent course without need for anti-cancer drugs. However, some well-differentiated SI-NETs may differ in their proliferative activity between the primary tumor and metastasis, thus making the start of tumor-controlling therapy a higher priority and the choice of therapy complicated. With the exception of peptide receptor radionuclide therapy (PRRT) as a standard second line of treatment for metastatic SI-NETs, the mTOR inhibitor everolimus has emerged as the treatment of choice to achieve tumor control if local treatment strategies fail. Studies have observed the activity of cytotoxic drugs like 5-FU/capecitabine with or without oxaliplatin, dacarbazime and temozolomide, as well as with tyrosine kinase inhibitors like sunitinib and sorafenib. However, the roles of these drugs in the treatment strategy for metastatic SI-NETs are still not well defined.

In this study, SI-NETs were generally more sensitive to standard cytotoxic drugs and targeted agents ex vivo compared to renal cancer, but less sensitive compared to ovarian cancer and CLL. Compared to CRC, SI-NETs were found to be more sensitive to protein kinase inhibitors and some cytotoxic drugs, e.g. 5-FU, cisplatin and doxorubicin. This pattern of drug activity would arbitrarily label SI-NETs intermediately drug sensitive, similar for example to CRC, a tumor type in which several cytotoxic and targeted drugs are now well established to provide benefit in the advanced setting. Thus, there is seemingly a discrepancy between the clinical notion that a SI-NET is a drug resistant tumor type and our observations of often
high, yet variable, activity of anti-cancer drugs against the tumor cells from SI-NETs.

The reasons for this discrepancy are probably multifaceted. First, most clinical studies on the systemic treatment of SI-NET are small, with the exception of the recent trials with everolimus. Secondly, these trials recruited patients with poor prognostic features that might have obscured potentially more beneficial effects in less advanced patients. Of course, drug activity observed ex vivo using the FMCA might not necessarily transmit to drug activity in vivo, although the FMCA has been shown to adequately reflect the clinical activity of cytotoxic drugs with considerable inter-individual variability in a broad spectrum of other cancer diagnoses. However, the tumor microenvironment of SI-NETS might be different to that of other cancer types with yet unknown interactions between tumor cells, tumor stroma and/or immune cells that preclude drugs from exerting their effects in the patient.

Finally, to date, there is either very scarce or no clinical experience for many of the drugs indicated in the FMCA being potentially active in SI-NETs, notably gemcitabine, gefitinib, nintedanib and ruxolitinib, making it too early to draw conclusions on the ex vivo – clinical drug activity relationship. In this context, it could be noted that drugs that have shown some effect in the clinic, i.e. 5-FU, oxaliplatin, gemcitabine and sunitinib, were all among drugs that were relatively active in the FMCA. Based on this ex vivo assessment of drug activity in SI-NETs, nintedanib and ruxolitinib appear to be especially promising drugs for this tumor type.

Sirolimus was only tested in five SI-NET samples but the IC$_{50}$ indicated from these was relatively low compared with the other diagnoses, which is also in line with the observed clinical benefit from everolimus in SI-NETs.

Somatostatin analogs are the mainstay treatment for controlling hormonal excess symptoms in patients with metastatic SI-NETs. Apart from anti-secretory activity, somatostatin analogs have demonstrated a stabilizing effect in gastroenteropancreatic NETs. This is consistent with the very modest ex vivo cytotoxicity of octreotide seen in this study and in agreement with the direct and indirect mechanisms of somatostatin analogs’ anti-proliferative action.

In the absence of definitive pretreatment markers as predictive factors for drug response, therapeutic decisions currently rely mostly on clinicopathological criteria. Importantly, the development of novel targeted agents has indeed necessitated the implementation of predictive markers. Mutational status in the PIK3CA/Akt/mTOR pathway has failed to predict response to everolimus treatment in a clinical setting, probably due to errors related to epigenetic changes. As protein/mRNA markers in this pathway are lacking, ex vivo chemosensitivity testing may be utilized in order to select patients for treatment with mTOR inhibitors. Regarding other
targeted novel therapies, interleukin-8, sVEGFR-3, and SDF-1α were recently identified as predictors of response to the multiple tyrosine kinase inhibitor sunitinib in a Phase II study\textsuperscript{156}. However, protein kinase inhibitors exhibit a wide spectrum of activities in the cell, and available markers do not test for these. Thus \textit{in vitro} chemosensitivity testing may also have a place in individualizing treatment with protein kinase inhibitors.

Interestingly, in the SI-NETs in this study, clinicopathological factors linked to worse prognosis, such as advanced age and stage at diagnosis, tumor grade and the presence of PC and EAM, as well as the currently used biomarkers s-CgA and u-5HIAA, were not clearly associated with \textit{ex vivo} sensitivity, either for standard cytotoxic or targeted drugs. These findings indicate that the traditionally used clinicopathological criteria as well as clinically available SI-NET biomarkers do not reflect tumor cell sensitivity to cytotoxic drugs and targeted agents and thus are not immediately predictive for the clinical effect of systemic treatment of SI-NETs. Moreover, the aforementioned markers are probably more indirectly related to treatment outcome, e.g. patient tolerance to treatment and tumor burden, than to tumor cell drug sensitivity \textit{per se}.

The cross-resistance between the different standard cytotoxic drugs and targeted agents investigated varied greatly, with reasonably high correlations between kinase inhibitors that are known to at least partly share their mechanisms of action. In clinical practice this means that resistance to one of these kinase inhibitors drug would also imply resistance to the other. SI-NET samples in this study were naïve to chemotherapy and/or systemic targeted agents, as the patients included had not previously treated with any of these drugs, other than SSAs in some cases. Nevertheless, individual SI-NET samples could be clearly resistant to one drug but sensitive to another, thus supporting the combined or sequential use of drugs, preferably based on a predictive test like the FMCA. Furthermore, the very conspicuous inter-individual variability between the SI-NET samples in sensitivity to most drugs also emphasizes the need for a predictive test for the selection of patients with tumor cells that might be affected by drugs. For tumor cells essentially unaffected by high concentrations of anti-cancer drugs, the chance of clinical benefit is extremely low and another treatment strategy should be selected.

The strengths of this study are the assessment of sensitivity to a broad panel of established and recently introduced anti-cancer drugs in a rare tumor type using an assay reported to reflect clinical drug activity. An important limitation of the \textit{ex vivo} chemosensitivity assay used, however, is that only drugs that act directly on the tumor cells can be assayed. Drugs that target stroma, e.g. angiogenesis inhibitors, or which modulate the immune
response, will require development of novel assays that can account for complex interactions between different cell types. Another obvious limitation is the rather low number of tumor samples available for analysis within a reasonable study period. This, of course, considerably limits the power of statistical analysis on, for example, the relationship between drug activity and various clinical biomarkers.

SI-NETs exhibited intermediate, yet variable sensitivity \textit{ex vivo} to established cytotoxic and new targeted drugs, calling for an individualized choice of therapy. Clinicopathological factors and currently used biomarkers were not associated with \textit{ex vivo} sensitivity, challenging these criteria in clinical practice. Taken together, in the attempt to develop personalized cancer medicine, \textit{ex vivo} sensitivity testing might have a place for the identification of appropriate systemic cytotoxic drugs and targeted agents for a subset of SI-NET patients who might benefit from them.
8. Conclusions

- Extensive abdominal fibrosis associated with clinically significant symptoms of intestinal ischemia and/or obstructive uropathy is linked to advanced disease in patients with SI-NETs. Prompt recognition and minimally invasive intervention may be effective in disease palliation.

- Prophylactic upfront locoregional surgery confers no survival advantage in asymptomatic Stage IV SI-NET patients. Delayed surgery as needed seems to be comparable in all examined outcomes, whilst offering the advantage of fewer re-operations for intestinal obstruction. Thus, the value of a priori locoregional surgery in the presence of distant metastases is challenged.

- Young patients (<65 years) with SI-NETs and LM generally have a favorable survival with standardized multimodality treatment. Most survival figures reported after LTx of NETs do not surpass these figures.

- Three novel serum biomarkers DcR3, TFF3, and midkine are recognized for SI-NETs. DcR3 seems to be a marker for liver metastases, while TFF3 and midkine may be new diagnostic markers for SI-NETs. Both DcR3 and TFF3 are associated with poor survival.

- SI-NETs exhibit variable but generally intermediate sensitivity ex vivo to established cytotoxic and novel targeted agents compared with other tumor diagnoses. Clinicopathological factors and currently used biomarkers are not clearly associated with ex vivo sensitivity, challenging these criteria for treatment decisions in SI-NETs and calling for individualized selection of therapy.
9. Future implications

Based on the findings of this thesis, additional clinical, genetic and laboratory studies are warranted in SI-NET patients with advanced and/or disseminated disease. Randomized trials regarding locoregional surgery, liver surgery and ablative modalities are needed. Due to the scarcity of the disease and in light of our current results, these studies may be difficult to conduct, having OS as the primary endpoint. However, it may be interesting to conduct a nationwide or international randomized controlled study to better address the question of prophylactic surgery in Stage IV SI-NETs in asymptomatic patients, possibly with a softer composite endpoint including re-operation due to growing metastases and/or the development of symptoms. Parameters such as pathological findings from locoregional surgery, staging of the lymph node metastases and assessment of fibrotic reactions in the mesentery, may also be prospectively evaluated.

The new biomarkers identified here may provide the foundation for a new set of biomarkers which, if used together, may form the basis for a new assay which can provide more reliable information concerning prognosis in the future. However, this potential use will have to be tested in larger, unselected cohorts of SI-NET patients and the specificity will have to be tested against other NETs, e.g. in the pancreas and lung.

*Ex vivo* sensitivity testing might have a place for the identification of appropriate systemic cytotoxic drugs and targeted agents for selected SI-NET patients with treatment failure and progressive disease. In the era of personalized medicine and in a clinical setting, the response rate and PFS of such patients treated with the aforementioned agents may be prospectively evaluated in connection with the results of *ex vivo* sensitivity testing.
10. Swedish Summary of the Thesis

Neuroendokrina Tunntarmstumörer (SI-NETs) är indolenta neoplasier med en årlig incidens kring 1 / 100,000 personer. De är ofta diagnoscerade senare i sjukdomsförloppet, vilket begränsar behandlingens effektivitet. Syftet med denna avhandlingen är att undersöka pre-kliniska och kliniska aspekter av patienter med avancerad och / eller spridd sjukdom, med speciellt fokus på hantering av intra-abdominell fibros, rollen av lokoregional kirurgi och levertransplantation, samt ex vivo sensitivitet av cytotoxiska substanser på tumörprover. Dessutom undersöktes nya serum biomarkörer för diagnos och prognos av SI-NETs.

Bakgrunden i delarbete I var att serotonin och andra cytokiner, som utsöndras från SI-NET tumörcells kan orsaka fibros, vilket leder till karcinoid hjärtsjukdom och fibrotiska reaktioner intraabdominellt. Syftet med arbetet var att studera förekomsten, kliniska komplikationer och hantering av den fibrotiska reaktionen i buken. Trettiosex patienter identifierades med signifikanta radiologiska tecken och symptomer på fibros. Av dessa hade 20 patienter central mesenteriell fibros, som orsakade tarmischemi och 16 hade retroperitoneal fibros, som orsakade obstruktiv uropati med hydronefros. Palliativa ingrepp i form av stentinläggning i Vena mesenterica superior eller resektion av centrala mesenteriella metastaser och/eller perkutan nefrostomi och J-stent behandling var fördelaktiga hos majoriteten av patienterna. Slutsatsen från detta delarbetet var att tidig diagnos och minimalinvasiva åtgärder var effektiva för sjukdomspalliation.

I delabete II undersöks rollen av lokoregional kirurgi vid diagnos hos asymptomatiska patienter med SI-NET och fjärrmetastaser. Data erhölls från vår Uppsala databas för SI-NET och det Nationella Patientregistret. Trehundrasextiotre patienter med SI-NET utan lokala tumörrelaterade symptom vid diagnos uppdelades i två grupper, varav en grupp som opererades inom sex månader från diagnos och en kontrollgrupp, som antingen behandlades icke-kirurgiskt eller opererades senare i sjukdomsförloppet. ”Propensity score” matchning utfördes för att reducera risken för selektions-bias. De matchade grupperna visade ingen skillnad i total överlevnad, 30-dagars postoperativ mortalitet och morbiditet samt vårdtid vid sjukhus. Däremot visar studien att gruppen som opererades vid diagnos genomgick flera re-operationer pga tarmobstruktion. Slutsatsen är
att man generellt inte kan rekommendera lokoregional kirurgi i ett profylaktiskt syfte vid diagnos hos asymptomatiska SI-NET-patienter vid stadium IV.

I delarbete III undersöktes rollen av levertransplantation (LTx) hos patienter med levermetastaserade SI-NET. Från Uppsaladatabasen för SI-NET identifierades 78 patienter, som uppfyllde befintliga LTx kriterier: <65 år, radikalt genomförd lokoregional kirurgi och avsaknad av extra-hepatisk sjukdom. Patienterna genomgick behandling enligt de kliniska protokollen vid vårt centrum, och under denna tidsperiod utfördes inte några LTx. Resultaten i detta delarbete visar att de flesta unga patienter (<65 år) med SI-NET och levermetastaser har en lång överlevnad med standardiserad multimodal behandling, dvs utan LTx, och att de flesta överlevnadssiffror som rapporteras efter LTx för NET i andra centra inte överträffar dessa siffror.


innebär att det sannolikt finns utrymme för individuellt anpassad behandling där fler faktorer inklusive ex vivo sensitiviteten vägs in.
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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)