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Small Intestinal neuroendocrine tumours Grade 2

Studies of tumour biology and treatment

DIMITRIOS PAPANTONIOU



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Abstract

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Grade 2 small intestinal neuroendocrine tumours (G2 Si-NET) have higher proliferation index (PI) Ki-67 (3-20%) and more aggressive clinical course than more indolent G1 tumours. However they have not been studied separately. The aim of this thesis was to evaluate the efficiency of standard treatments and to explore prognostic markers in this population.

In the first paper we showed that baseline chromogranin A (CgA) was associated with cancer-specific survival (CSS) irrespective of treatment, and with progression-free survival (PFS) after peptide receptor radionuclide therapy (PRRT). Early CgA and 5-hydroxyindoleacetic acid (5-HIAA) reductions were prognostic of longer PFS after somatostatin analogues (SSA), but not after PRRT. In the second paper we found that treatment with SSA is effective in G2 Si-NET (median PFS 12.4m, similar to PFS for G1 patients in the PROMID trial). Dose intensification had modest effect. Importantly, in subgroups with lower (3-5%), intermediate (5-10%) and higher Ki-67 (10-20%), PFS for SSA declined with increasing Ki-67 (31, 18 and 10m), whereas it was stable for PRRT (29, 25 and 25m). In the third paper, we evaluated an alternative estimation method of PI (phospho-histone H3, PHH3). Both Ki-67 and PHH3 separated groups of longer and shorter CSS (128 vs 95 and 149 vs 88m, HR: 1.18 and 1.16, respectively). PHH3 but not Ki-67-based PI was associated with PFS. A cut-off of >2 PHH3-estimated mitoses per 10 high-performance fields seemed to provide better discrimination. We finally investigated the prognostic value of inflammation scores after treatment with PRRT. We found that parameters based on CRP and albumin, but not derived neutrophil to lymphocyte ratio, were associated with overall survival. After adding inflammation markers to a model of standard prognostic factors, the model based on hypoalbuminemia had better prognostic power.

Collectively, these studies confirm the efficacy of standard medical treatments in G2 Si-NET, but underline that SSA might be less effective in the higher Ki-67 subgroup. Additionally, they investigate the prognostic value of tumour markers, inflammation and proliferation parameters, which can be used for patient counseling and as stratification factors in future clinical trials.

Keywords: Small intestinal neuroendocrine tumours, Si-NET, Chromogranin A, CgA, 5-HIAA, Ki-67, somatostatin analogues, peptide receptor radionuclide therapy, PRRT, hypoalbuminemia, inflammatory markers, phospho-histone H3, PHH3

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*To my parents
Vassili and Vassiliki*

Generative AI was used for language proofing and for the formulation of the summary in Swedish. I reviewed, edited and refined the text to my own liking and retain full responsibility for the content presented in this publication.

List of Papers

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

- I. Papantoniou, D.; Grönberg, M.; Landerholm, K.; Welin, S.; Ziolkowska, B.; Nordvall, D.; Janson, E.T. Assessment of Hormonal Levels as Prognostic Markers and of Their Optimal Cut-Offs in Small Intestinal Neuroendocrine Tumours Grade 2. *Endocrine* 2021, 72, 893–904, doi:10.1007/s12020-020-02534-8.
- II. Papantoniou, D.; Grönberg, M.; Thiis-Evensen, E.; Sorbye, H.; Landerholm, K.; Welin, S.; Tiensuu Janson, E. Treatment Efficacy in a Metastatic Small Intestinal Neuroendocrine Tumour Grade 2 Cohort. *Endocr Relat Cancer* 2023, 30, e220316, doi:10.1530/ERC-22-0316.
- III. Papantoniou D, Grönberg M, Tiensuu Janson E, Phosphohistone H3 and Ki-67 as prognostic markers in metastatic small intestinal neuroendocrine tumours: A comparative, retrospective, cohort study. Submitted
- IV. Papantoniou, D.; Fröss-Baron, K.; Garske-Román, U.; Sundin, A.; Thiis-Evensen, E.; Grönberg, M.; Welin, S.; Tiensuu Janson, E. Hypoalbuminemia, but Not Derived Neutrophil to Lymphocyte Ratio (dNLR), Predicts Overall Survival in Neuroendocrine Tumours Undergoing Peptide Receptor Radionuclide Therapy: A Retrospective, Cohort Study of 557 Patients. *J Neuroendocrinol* 2025, 37, e13379, doi:10.1111/jne.13379.

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Additional Publications by the author

- Asowed, M.; Elander, N.O.; Pettersson, L.; Ekholm, M.; Papantoniou, D. Activity and Safety of KEES - an Oral Multi-Drug Chemo-Hormonal Metronomic Combination Regimen in Metastatic Castration-Resistant Prostate Cancer. *BMC Cancer* 2023, 23, 309, doi:10.1186/s12885-023-10780-y.
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Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
AJCC/UICC	American Joint Committee on Cancer/ Union Internationale Contre le Cancer
AIC	Akaike's information criterion
AST	Aspartate transaminase
CgA	Chromogranin A
CHD	Carcinoid heart disease
CI	Confidence intervals
CRP	C-reactive protein
CSS	Cancer-specific survival
CT	Computed tomography
dNLR	Derived neutrophil to lymphocyte ratio
DFS	Disease-free survival
ECOG PS	Eastern cooperative group performance status
ENETS	European Neuroendocrine Tumor Society
ESR	Erythrocyte sedimentation rate
¹⁸ FDG	¹⁸ Fluorodeoxyglucose
¹⁸ F-DOPA	¹⁸ Fluoro-dihydroxyphenylalanine
FFPE	Formalin-fixed, paraffin-embedded
G1/2/3	Grade 1/2/3
GBq	Gigabecquerel
GEP-NET	Gastroenteropancreatic neuroendocrine tumour
GPS	Glasgow Prognostic Score
HPF	High power field
HR	Hazard ratio
H&E	Haematoxylin-eosin
IFN α	Interferon-alpha
IHC	Immunohistochemical
IQR	Interquartile range
LDH	Lactate dehydrogenase
MC	Mitotic count
MRI	Magnetic resonance imaging

NEC	Neuroendocrine carcinomas
NEN	Neuroendocrine neoplasms
NET	Neuroendocrine tumour
NKA	Neurokinin A
NLR	Neutrophil to lymphocyte ratio
NPV	Negative predictive value
NSE	Neuron-specific enolase
NT-ProBNP	N-terminal pro-brain-natriuretic peptide
OS	Overall survival
PD	Progressive disease
PET	Positron emission tomography
PHH3	Phosphohistone H3
PLR	Platelet to lymphocyte ratio
PFS	Progression-free survival
PPV	Positive predictive value
PR	Partial response
PRRT	Peptide receptor radionuclide therapy
SD	Stable disease
SEER	Surveillance Epidemiology and End Results
Si-NET	Small intestinal neuroendocrine tumours
SIRT	Selective internal radiation therapy
SRI	Somatostatin receptor imaging
SRS	Somatostatin receptor scintigraphy
SSA	Somatostatin analogues
SSTR	Somatostatin receptor
TAE	Transarterial liver embolization
ULN	Upper limit of normal
WHO	World Health Organisation

Introduction

Neuroendocrine neoplasms (NEN) are a group of tumours deriving from cells of the neural crest. They can manifest in various organs throughout the body, but have been traditionally studied as a homogeneous entity, based on their clinicopathological properties, namely that of containing secretory granules and producing biogenic amines and polypeptide hormones, which may give rise to hypersecretion syndromes. They are classified according to The American Joint Committee on Cancer/Union Internationale Contre le Cancer (AJCC/UICC) system in stage I-II (local disease), stage III (regional disease) and stage IV (metastatic) disease [1]. In an alternative classification system proposed by the World Health Organisation (WHO)/European Neuroendocrine Tumour Society (ENETS), NEN are described as Grade 1 (G1, indolent, slowly proliferating tumours), G2 (intermediate) and G3 neoplasms (higher proliferation rate). The last category incorporates both well-differentiated, G3 tumours, and poorly differentiated neuroendocrine carcinomas (NEC) [2].

Small intestinal neuroendocrine tumours (Si-NET) are the third largest subgroup of NET and represent 25-50% of all gastroenteropancreatic NEN [3]. They arise from the enterochromaffin cells of the small bowel and have a distinct clinical history compared to NEN from other sites. They often present at an advanced stage and feature characteristically lower proliferation indexes (Ki-67), with the vast majority belonging to the G1 and G2 groups.

Epidemiology

Si-NET are a relatively rare group of tumours. Incidence of Si-NET has been reported to be 1.12/100,000 in Sweden [4], 0.78/100,000 in Iceland [5], 0.81/100,000 in Norway, 1.46/100,000 in the UK [6] and 1.05/100,000 in the US [7], and it appears to be rising steadily in most countries [6,8], with an almost five-fold absolute increase of age-standardised incidence between 1995-2018 in one case [6]. Whether this increase might be wholly attributed to the routine use of cross-sectional and somatostatin-receptor imaging, or as-of-yet-unknown environmental factors might contribute to this rising incidence, remains to be seen [8]. The incidence post-mortem has been reported to be much higher, up to 1.22/100 [9].

Pathology

A pathological diagnosis requires examination of material from a biopsy or surgical specimen. Tumour morphology is examined with standard haematoxylin-eosin (H&E) slides; immunohistochemical (IHC) staining for Chromogranin A (CgA) and synaptophysin are used to confirm diagnosis, and additional markers (such as serotonin and CDX2) may indicate a primary tumour originating in the small bowel. Of note, negative staining for CgA might predict for more aggressive tumours, although this is unusual in Si-NET.

Classification of all tumours according to grade and staging is mandatory. Estimation of the proliferation index Ki-67 in hot-spots or of the mitotic count (MC) is required, as these form the basis of the grading system (Table 1).

Table 1. Classification of Si-NET according to WHO 2019 and AJCC/UICC

Grade	Differentiation	Ki-67 (%)	Mitotic count (per 10 HPF)
G1 NET	Well-differentiated	<3	<2
G2 NET	Well-differentiated	3-20	2-20
G3 NET	Well-differentiated	>20	>20
NEC	Poorly differentiated	>20	>20
Stage	Extension		
T – primary tumour			
Tx	Cannot be assessed		
T0	No evidence of primary tumour		
T1	Invades lamina propria or submucosa and size ≤1 cm		
T2	Invades muscularis propria or size >1 cm		
T3	Invades subserosa		
T4	Invades visceral peritoneum or other organs		
N – Regional metastases			
Nx	Cannot be assessed		
N0	No regional lymph nodes		
N1	<12 lymph node metastases		
N2	≥12 lymph node metastases, or mesenteric metastases >2 cm		
M – Distant metastases			
M0	No distance metastasis		
M1	Distant metastasis a: liver, b: extrahepatic sites, c: liver + extrahepatic sites		
Stage grouping			
Stage I	T1N0M0, Local disease		
Stage II	T2-3N0M0, Local disease		
Stage III	T4, any N, M0 or any T, N1-2M0, Regional disease		
Stage IV	Any T, any N, M1, Metastatic disease		

WHO: World health organization, AJCC/UICC: American Joint Committee on Cancer/Union Internationale Contre le Cancer, HPF: high power fields. Si-NET: small intestinal neuroendocrine tumour. G3 NET or NEC are exceedingly rare.

Imaging

Three-phase contrast-enhanced computed tomography (CT) is usually the initial imaging modality used to visualize the primary tumour, lymph node and distant metastases. In these protocols, unenhanced images are acquired, followed by images in the late arterial (portal-venous inflow) and venous phases. Magnetic resonance imaging (MRI) might be used to characterize metastatic lesions and for pre-surgery planning; it might also be used for long-term follow-up of younger patients, in order to reduce radiation doses. CT and MR enteroclysis have high sensitivity (100% and 86-94%) and specificity (96% and 95-98%) for the detection of small bowel tumours [10].

Other imaging modalities might be employed in specific cases. Contrast-enhanced ultrasonography can detect liver metastases, however the modality is investigator-dependent. Endoscopic procedures including colonoscopy, video-capsule endoscopy and double-balloon enteroscopy may be used, if available.

Somatostatin receptor imaging (SRI)

Radioligands binding to somatostatin receptor (SSTR) -2, which is expressed by most Si-NET, are used to visualize the tumours. The older somatostatin receptor scintigraphy (SRS) which used ¹¹¹Indium as a radionuclide is largely replaced by positron emission tomography (PET), which uses ⁶⁸Gallium as a radiotracer. Addition of ⁶⁸Ga-DOTATATE-PET to ¹¹¹Indium-SRS resulted in detection of additional lesions which would cause changes in planned treatment in one third of patients in one study [11]. Other type of tracers such as ¹⁸fluoro-dihydroxyphenylalanine (¹⁸F-DOPA) have limited availability. Small studies and a meta-analysis point to a slightly higher per-lesion detection sensitivity when using ¹⁸F-DOPA-PET [12,13]. Even if ¹⁸F-DOPA-PET might be useful in special cases, the evidence does not seem to support its more wide adoption [14]. ⁶⁴Cu-DOTATATE is a newer tracer with theoretical advantages in terms of transportability and detection rate [15] over ⁶⁸Ga-DOTATOC/DO-TATATE/DOTANOC but is currently not licenced for use in Europe.

Due to their low proliferation indexes, ¹⁸Fluorodeoxyglucose (¹⁸FDG)-PET was not considered as useful in the diagnosis of Si-NET, but as potentially useful in the case of de-differentiated tumours, with higher Ki-67. However a recent Danish study showed that up to 40% (35/90) of Si-NET patients have positive uptake on ¹⁸FDG-PET, even among G1 and G2 tumours [16]. More importantly, across all tumour types included in this cohort, ¹⁸FDG-PET-positivity was prognostic for both overall survival (OS) and progression free survival (PFS), as well as for OS following treatment with peptide receptor radionuclide therapy (PRRT). Dual imaging with both SSTR- and ¹⁸FDG-PET might provide additional prognostic information [17,18] and is advocated in

both Swedish and European guidelines for G3 and selected G2 tumours with higher Ki-67 [14,19].

In addition to its role in the diagnostic work-up of Si-NET, for the detection of both the primary tumour and metastases, SRI can be used to determine eligibility for PRRT, and for treatment monitoring. As SRI might be negative in cases of small tumours, in tumours with lower SSTR-2 expression and in the rare case of undifferentiated Si-NET, it should be combined with diagnostic high-quality cross-sectional imaging.

Symptoms

Si-NET are sometimes found during investigation for a primary tumour in a patient with known metastases, or as incidental finding in radiology performed for other reasons. When symptomatic, they often present with non-specific symptoms like abdominal pain [4]. Hormone-related mesenteric fibrosis may result in mechanical small bowel obstruction, but also in vascular encasement and ischemia. The typical “carcinoid syndrome”, characterised by diarrhoea and flushing due to the secretion of serotonin and other vasoactive substances, presents in 13-22% of cases at diagnosis [4,20]. The development of right heart valve fibrosis (carcinoid heart disease, CHD) is a serious comorbidity present in 3% of patients at baseline and 4% during later stages [21]. It is associated with persistently high hormone levels. In extreme cases, a life-threatening form of carcinoid syndrome (carcinoid crisis) might be caused by for example surgery or anaesthesia.

Biomarkers

No single biomarker has the sensitivity and specificity to be considered as a diagnostic tool [22]. However, in clinical praxis, two monoanalytes, plasma CgA and urine or serum 5-hydroxyindoleacetic acid (5-HIAA) are used to monitor the course of Si-NET during treatment of non-curable tumours, and may also have a role in the detection of recurrence after potentially curative surgery or other treatments.

CgA is a polypeptide secreted by neuroendocrine cells in general. Although CgA correlates to tumour burden [23], its utility as a diagnostic tool is limited, as it might be falsely elevated in several non-cancerous conditions, including liver and kidney disease, and in the case of simultaneous use of proton pump inhibitors such as omeprazole [24]. Additionally, as there exist multiple assays for the measurement of CgA, with different cut-off values, results obtained in different labs might not be directly comparable [24].

5-HIAA, a by-product of serotonin, the main hormone produced by Si-NET, was traditionally measured as the mean value of two 24-hour urine collections. It is a reliable marker for the diagnosis of carcinoid syndrome, and might correlate with the development of carcinoid heart disease [25]. Patients have to restrict intake of serotonin-rich food during the period of urine collection. In recent years, measurement of 5-HIAA in serum or plasma has shown to be comparable with 24h-urine measurements [26,27] and has gained popularity in clinical practice due to its convenience. However the method might be less optimal in cases of impaired renal function [26].

Additional circulating markers might correlate with specific symptoms, such as flushing (tachykinins) and with higher grade tumours (neuron-specific enolase, NSE, lactate dehydrogenase, LDH or aspartate transaminase, AST) but are not used in clinical routine for monitoring Si-NET. The N-terminal pro-brain-natriuretic peptide (NT-ProBNP) reflects the degree of heart strain in the patients with carcinoid heart disease; ENETS recommends its use for screening and follow-up of patients with suspicious symptoms in a recent guidance paper [28].

Treatment

Operation is the cornerstone of treatment for localized disease. Unlike most other tumour types, where metastatic disease often precludes surgical approaches, debulking procedures are meaningful in selected cases, especially those with high symptom burden.

Most Si-NET express SSTR and somatostatin analogues (SSA) are the most used treatment for both symptom and tumour control. Other medical treatments include PRRT, everolimus, Interferon-alpha (IFN α) and telotristat ethyl [3].

Surgical and invasive procedures

Surgery of the primary tumour and metastases is the initial treatment of choice, if radical resection is feasible. Resection with curative intention results in excellent 5-10 year survival rates of 80-100% in local and regional disease. Surgery should therefore be planned with the goal of achieving R0 resection, including lymph node dissection.

Although centralization of surgery has been advocated, a recent systematic review showed contradicting results regarding 90-day post-operative morbidity and mortality in low- and high-volume centres [29]. The surgical approach should attempt a systematic lymphadenectomy while reducing the risk for a short bowel syndrome. It has been suggested that at least 8 lymph nodes should be extracted for examination, and that >4 positive lymph nodes are associated with shorter recurrence-free survival [30]. Manual palpation should

be performed in order to locate additional concomitant tumours. Peritoneal metastases might be dissected.

If an R0 resection cannot be accomplished, an operation might still be offered if bowel circulation is threatened due to tumour extension. Surgery for mesenterial fibrosis, a serious event during the course of Si-NET, which results in shorter OS and deterioration of quality of life with abdominal pain and obstruction, remains controversial and possibly limited to symptomatic patients [31,32]. Primary operation in cases of asymptomatic tumours is still controversial. Several retrospective and registry analyses had shown longer survival after non-radical operation of the primary tumour [31,33,34]. However patients operated are often younger and fitter. A recent study analysed propensity-score matched patients treated with prophylactic surgery within 6 months from diagnosis vs nonsurgical treatment or delayed surgery in a Swedish cohort. Although the prophylactically operated patients performed significantly better in the unmatched cohorts, the survival benefit was lost in the matched cohorts [35]. Other institutional series show contradicting results [36]. The difference might be due to the higher percentage of patients treated with delayed resection in the Swedish cohort.

Resection of limited liver disease should be considered. Cytoreductive liver surgery may be considered if the bulk of disease can be operated safely, with a goal to reduce tumour burden and hormonal secretion, and therefore achieve better symptom control. Other types of locoregional treatment of liver metastases are radiofrequency or microwave ablation, transarterial liver embolization (TAE) and selective internal radiation therapy (SIRT).

Somatostatin analogues (SSA)

SSA (octreotide and lanreotide) are indicated for tumour growth inhibition in SSTR-positive Si-NET, and for the control of symptoms related to the carcinoid syndrome. Octreotide is available as a short-acting subcutaneous and as a long-acting (Octreotide LAR) intramuscular formulation. Lanreotide is only available as a long-acting formulation.

Treatment is often started with a short test of subcutaneous octreotide (50-100 µg 2-3 times a day over a few days) to test for tolerability, before using the long-acting formulations.

The optimal starting doses of the latter have not been formally investigated; historically, treatment was initiated with below-label doses of SSA, whereas nowadays Octreotide LAR 30 mg or lanreotide 120 mg every 4 weeks are typically used, based on two phase 3, placebo-controlled clinical trials (PROMID and CLARINET). PROMID included exclusively treatment naïve G1 tumours treated with Octreotide LAR; median time to tumour progression favoured the SSA group at 14 vs 6 months [37]. CLARINET investigated the use of lanreotide in non-functioning gastroenteropancreatic (GEP)-NET with

Ki-67 <10% (4% with progression at baseline) and showed a 2-year PFS of 65% vs 33% in favour of SSA [38].

Treatment with SSA usually continues as long as there is evidence of clinical benefit, and often after disease progression, concomitantly with additional medical or interventional treatments. Increase of SSA dose is often used as a first step after progression of Si-NET on first-line SSA, based on retrospective publications and on data from the control arms of two randomized trials, reporting median PFS of 6.8 and 8.4 months with the use of above-label SSA doses [39–43]. This practice is also supported by a prospective phase 2 trial (CLARINET FORTE) with above-label dose of lanreotide that recently reported moderate efficacy, with a median PFS of 8.3 months in the Si-NET subgroup. Only 22 of 51 patients had Ki-67 >2% and only 4 patients >10%; the latter had a median PFS of 5.5 months [44]. Based on this data, treatment intensification with above-label SSA doses has been advocated mainly for G1 and lower G2 patients, with Ki-67 indexes <10%. Recently, however, treatment-naïve patients with Ki-67 >10% who were treated with above-label SSA doses in the control arm of the NETTER-2 study achieved a median PFS of 8.5 months [45]. This might imply a higher than expected efficacy of high-dose SSA even in the high-G2 population.

Peptide Receptor Radionuclide therapy (PRRT)

PRRT is indicated for the treatment of SSTR-positive G1-G2 Si-NET. ¹⁷⁷Lu-DOTATATE (Lutathera) is nowadays registered based on the results of the NETTER-1 study, which showed higher 20-month PFS rate (65% vs 11%) for PRRT compared with an above-label dose of SSA in progressive SSTR-positive Si-NET (30% G2 tumours) [43]. Longer follow-up showed a median OS of 48 and 36 months, respectively [46]. Recently, the NETTER-2 trial compared treatment with high dose SSA with the combination of ¹⁷⁷Lu-DOTATATE and SSA in newly diagnosed well-differentiated GEP-NET patients with Ki-67 between 10-55%. Median PFS favoured the combination arm (8.5 months vs 22.8 months). One-third of the patients had Si-NET. The hazard ratio (HR) for PFS for the Si-NET subgroup was similar to the HR for the overall population (0.30 vs 0.28) [45]. As OS results are immature, it remains to be seen whether the PFS benefit will translate in OS difference.

Treatment with PRRT consists of four doses of 7.4 GBq every approximately two months. Other approaches, including combination with other radio-labelled molecules and chemotherapeutics, and a dosimetry-based treatment are being investigated. The latter has been common practice in Sweden [47] and is now formerly investigated in a prospective, randomized clinical trial (STARTNET). Common side-effects include nausea and vomiting, haematological and renal toxicity. Development of leukaemia and myelodysplastic syndromes are unusual (2% in the NETTER-1 study [46]) but serious complications.

The value of SSA maintenance after treatment with PRRT has not been adequately studied. Maintenance therapy in symptomatic or high volume tumours has been standard praxis. However a prospective, randomized, single-centre study in well-differentiated NET of various origins failed to show any benefit in terms of PFS or OS [48]. Similarly, there is no prospective data on rechallenge with PRRT after progression. However, a recent meta-analysis of retrospective studies on retreatment with ¹⁷⁷Lu-DOTATATE showed a median PFS of 13.4 months and an OS of 26.8 months from the start of retreatment, while the rate of grade 3 and 4 events were comparable to initial therapy [49]. Retreatment with PRRT is thus a reasonable choice in fit patients with adequate SSTR expression who responded after the first PRRT treatment.

Everolimus

Based on the results of the RADIANT-4 trial, everolimus is registered for the treatment of progressive G1/G2 non-functional Si-NET at a dose of 10 mg daily per os. RADIANT-4 showed a longer median PFS for everolimus compared to placebo (11 vs 4 months) in non-functioning NET of lung or gastrointestinal origin including one third G2 tumours [50]. However, a post-hoc subgroup analysis did not show a benefit from everolimus in the more indolent ileal NET [51]. Common side effects include stomatitis, diarrhoea, hyperglycaemia and non-infectious pneumonitis and often lead to dose reductions. Given the good tolerability and quality of life improvements associated with treatment with PRRT [52], everolimus is generally considered a third-line option after failure of treatment with PRRT for SSTR-positive patients. However, it might be considered earlier in cases of patients with low or negative SSTR expression. The COMPETE phase 3 trial will compare everolimus with ¹⁷⁷Lu-edotreotide in patients with well-differentiated, SSTR-positive tumours.

Interferon-alpha (IFNa)

IFNa has been registered for the treatment of Si-NET associated with carcinoid syndrome, but not for its antiproliferative activity. It has limited availability, but might be a reasonable option as an addition when treatment with SSA fails, and in tumours not suitable for SSA, such as in patients who cannot tolerate SSA, or who have SSTR-negative tumours. IFNa 2b (IntronA) is administered at a standard dose of 3-5 MU three times a week. A pegylated formulation (PegIntron) is available for administration once weekly at a dose of 50-150 µg/week. Common side-effects include fatigue, fever and flu-like symptoms, myelosuppression, depression and autoimmune conditions

Three small randomised trials showed some benefit for the combination of SSA and IFNa compared to single SSA, but they could not show a statistically significant OS difference [53]. As there are no head-to-head prospective, randomized trials comparing the different medical treatments, two recent network

meta-analyses examined the efficacy of IFNa in combination with SSA in non-pancreatic NET [54,55]. In both meta-analyses, PRRT+SSA was the most effective intervention (HR for PFS 0.07-0.08), followed by bevacizumab+SSA (HR 0.18-0.22), everolimus+SSA (HR 0.12-0.31), IFNa+SSA (HR 0.23-0.27) and single SSA (HR 0.34-0.40).

Tryptophan hydroxylase inhibitors

Telotristat ethyl is registered for the treatment of secretory diarrhoea related to the carcinoid syndrome, which is refractory to treatment with only SSA. Two randomised trials assessed the efficacy of telotristat ethyl in patients with ≥ 4 bowel movements per day while on SSA, and showed a $>40\%$ response in terms of reduced bowel frequency, and $>80\%$ reduction in 5-HIAA levels, with a favourable safety profile. Most notable adverse events were nausea and dose-dependent increases in hepatic enzymes [56,57].

Non-registered treatments

Angiogenesis has been explored as a rational target in multiple small Si-NET trials. The pivotal trial of sunitinib, the only anti-angiogenic tyrosine kinase inhibitor currently registered for treatment of pancreatic NET did not include Si-NET [58]. Axitinib [59], pazopanib [60], lenvatinib [61], surufatinib [62] and cabozantinib [63] have demonstrated some activity in phase 2 clinical trials, and cabozantinib showed prolonged PFS in a phase 3 setting [64]. As of today, though, no tyrosine kinase inhibitor has been approved for use in Si-NET.

SSTR-negative tumours

Tumours with low expression of SSTR remain a therapeutic challenge, as PRRT is not effective in this population, everolimus is only approved for non-functional Si-NET and use of SSA is less documented. A propensity-score matched analysis of 69 SSTR-negative and 69 SSTR-positive patients showed a median OS of 38 vs 131 months, with SSTR-negativity as an independent negative prognostic factor of OS in adjusted analysis. Median PFS for SSTR-negative and SSTR-positive patients treated with SSA was 15 and 47 months, and median OS did not differ for SSTR-negative patients depending on whether they were treated with SSA or not [65]. Both IFNa, everolimus and other tyrosine kinase inhibitors might be reasonable options, if available.

Chemotherapy

Cytotoxic chemotherapy has, in general, no role in low-grade Si-NET. Various chemotherapeutics have been historically tested with low response rates, reported to be 11.5% in a meta-analysis [66]. However this study included mixed populations of gastrointestinal NET, and the response rate of the more indolent si-NET might be even lower. Chemotherapy regimens most usually used include temozolomide, capecitabine and 5-Fluorouracil-based combinations [67–70]. In the case of a very rare, poorly differentiated NEC, platinum-based chemotherapy might be used [71].

Prognostic markers

Clinical parameters

Age

Multiple studies have associated age with both cancer-specific survival (CSS) and OS. In most cases, a year of increasing age is linked to 2-3% increase in mortality [72,73]. This increase seems to be consistent across multiple age groups. For example, a Swedish study showed that in Si-NET patients aged <50, 50-59, 60-69, 70-74 and ≥ 75 years, the risk for deaths steadily increased (HR for OS: 1; 1.77; 3.68; 6.20; 11.28, respectively) [74].

Sex

Male sex seems to consistently confer a slightly higher risk for death from Si-NET, but the result has often been non-significant in all but the largest database series. In a Surveillance Epidemiology and End Results (SEER) database analysis of a mixed GEP-NET population, being a female conferred a 25% reduction in risk for death [75]. Modlin et al similarly reported a lower HR for death of 0.8 for Si-NET female patients [76]. On the other hand in two purely Si-NET Swedish cohorts, the authors reported 12-20% higher risk for death for male patients, with the results not being significant [72,74].

Interestingly, the latest SEER analysis including only metastatic Si-NET shows no difference, with a HR of 1.02 [77].

Symptom burden

The presence of carcinoid syndrome at baseline has uncertain effect on disease outcome. Baptiste et al reported decreased 3-year relapse-free survival, but not OS in patients presenting with symptomatic disease [78]. Three institutional cohorts similarly reported no effect on OS, with HR between 0.95 and 1.05 [21,74,79]. An older report showed some association between OS and hormonal symptoms in unadjusted analysis, but this was lost in adjusted analysis [23]. As this was a population treated before SSA became standard of

care for symptom control, the results of that study might not represent the modern landscape.

Year of treatment start

Some but not all studies report a trend to improved survival in patients diagnosed in recent years. A SEER database analysis showed longer OS for patients with metastatic Si-NET diagnosed after 2007 (HR 0.79), with the result being borderline significant [77]. A similar HR was reported in the above-mentioned Swedish cohort, with the result though not reaching significance, possibly because of the limited number of patients [72].

Carcinoid heart disease (CHD)

Multiple reports associate CHD to shorter OS in unadjusted analysis [74,79,80]; however the effect is often lost after adjustment for potential confounders [79,80]. For example, Bergestuen et al reported a HR for OS of 1.76 in unadjusted analysis but 0.7 in the adjusted analysis of a Norwegian Si-NET cohort.

Performance status

Eastern cooperative group performance status (ECOG PS) ranks patients in a scale of 0 (indicating no symptoms) to 4 (seriously compromised). At baseline, it is a powerful prognostic factor for OS and treatment effect in multiple cancers. Chou et al reported that survival was 70% worse for patients with ECOG PS 2-3 vs those with a PS of 0-1 in a mixed cohort of GEP-NET (HR 0.30 in favour of the low PS group) [81]. Laskaratos et al similarly reported a trend to worse survival in Si-NET patients with desmoplasia and increasing PS (1 vs 0, HR 1.46; 2 vs 0, HR 2.17) [79], while Levy et al in a larger cohort of 400 Si-NET found a PS of 2 to be a strong predictor of CSS (HR 4.4 in adjusted analysis) [82].

Baseline metastatic status

Liver

Liver is often the first distant metastatic site in Si-NET, with the incidence of liver metastases reported between 31-56% at initial diagnosis [4,21] and >70% in advanced disease [79]. A study of midgut carcinoids showed that the presence of liver metastases at diagnosis conferred a higher risk of death (HR 2.3) [83]. The relationship between liver metastases and survival outcomes has been hard to quantify, and several different cut-offs have been proposed. In a post hoc analysis of the CLARINET mixed GEP-NET study population, the authors used cut-offs of 10%, 25% and 50%. Increasing metastatic volumes resulted in increasing HRs, but the differences became significant only for liver involvement >25% [84]. Multiple series have used cut-offs of 25% and

50%. Only one of them showed a significant correlation in adjusted analysis [79,85]. In the PROMID trial, a cut-off of 10% was used; patients with more than 10% liver involvement had a higher risk of death, with a HR of 2.49 [37].

Other series have studied the number of metastasis: In a Swedish cohort, fewer than 5, 5-10 and >10 liver metastases were associated with HR for death of 1.58, 2.55 and 2.9, respectively, compared to the absence of liver metastases [74]. In an older cohort, the presence of more than 5 metastases conferred a higher risk for death in unadjusted analysis (HR 3).

Bone metastases

Bone metastases are often a late event and are reported at around 2% of patients at baseline [4,21], whereas one institutional series focusing on metastatic Si-NET with desmoplasia reported significantly higher rates [79]. In this series, bone metastases were associated with OS in unadjusted, with the result being borderline significant in adjusted analysis (HR 1.70). In a German mixed GEP-NET cohort, synchronous bone metastases were coupled to almost 3 times higher risk of death (HR: 2.5).

The frequency of bone metastases increases in more advanced disease. A large Swedish cohort of 753 Si-NET patients found that 23% had bone metastases in SSTR-PET [86]. In 138 patients with survival data, having more than five bone metastases was associated with shorter 5-year OS (21 vs 58%) [87].

Lung metastases

Lung metastases have been reported at approximately 1% of patients at baseline [21] and as high as 8% [79] at later stages. Impact on OS remains uncertain, with the result being borderline significant in an institutional cohort (HR 1.93) [79].

Other metastases

Distant abdominal lymph node metastases were associated with shorter OS in a Swedish cohort (HR 1.47) [88]. Ovarian metastases seemed to confer a higher risk for death in a small study including 35 patients with metastatic Si-NET (HR 2.8); the result was borderline significant in adjusted analysis [89]. However, another study failed to show any difference in median OS between patients with and without ovarian metastases ($p=0.76$) [90]. Peritoneal metastases have recently gained interest, as they seem to be associated with considerably higher risk for death or progression in some reports. They are found at 9-37% at diagnosis [4,21,91]. In a national registry with mixed GEP-NET tumours, in which metastases from Si-NET accounted for half of the peritoneal metastases, having a tumour extending to the peritoneum conferred a higher risk for death (HR 2.3). However, among all patients with metastatic disease, the additional presence of peritoneal metastases was not associated with worse survival [91]. In a large cohort of Si-NET, the presence of peritoneal carcino-

matosis, as detected perioperatively, was associated with lower long-term survival rates, with 10-year OS of 32 vs 54%. The result was statistically significant [74].

The presence of synchronous metastasis was associated in a study with a higher risk of death from any cause, with a HR of 2.4. Both extrahepatic and extra-abdominal disease at diagnosis appear to be negative prognostic factors. In a study of metastatic Si-NET, extrahepatic involvement was related to worse 5-year CSS, 68 vs 88%. The difference was significant in adjusted analysis [85]. The presence of extra-abdominal metastases was also associated with a HR for OS of 2.1 [74].

Biomarkers

CgA

Higher CgA levels at baseline are associated with shorter survival [31] while significance of CgA changes for predicting worse response to treatment is still unclear, with two recent reviews reaching contradicting conclusions [24,92]. Post hoc analysis from phase 3 trials showed that an early decrease in CgA related to a decreased risk of progressive disease for SSA [93] and everolimus [94] but not for PRRT [95]. In any case, a recently published prospective study showed only a weak association between changes of CgA and changes in tumour burden, and CgA can thus not be used as a sole marker of disease progression [96].

5-HIAA

Less is known about the prognostic and predictive value of 5-HIAA in Si-NET. Two studies examined the prognostic value of baseline 5-HIAA at a cut-off of 10 times the upper limit of normal (ULN), with only one reporting a significant correlation [79,97].

Inflammation markers

Cancer-associated inflammation has been associated with prognosis and treatment response in various cancer types. Several inflammation parameters are easy to measure in routine blood tests. Maurer et al reported that normal levels of C-reactive protein (CRP) were associated with lower risk of death (HR 0.38) in 138 patients with unresectable, stage IV Si-NET [98]. In another study of 281 patients with intestinal NET operated with curative intent (56% of which were Si-NET), a neutrophil to lymphocyte ratio (NLR) of ≥ 2.47 was associated with higher risk of lymph node metastases and with shorter OS (116 vs 160 months) [99]. Recently a systematic review and meta-analysis of the prognostic significance of inflammation biomarkers in GEP-NET of various origins confirmed the association between higher CRP, NLR and platelet to

lymphocyte ratio (PLR) levels and shorter OS. Additionally, higher NLR levels were associated with shorter disease-free survival (DFS) and higher CRP and PLR levels were associated with shorter PFS in this mixed population [100].

Genomic assays

Genomic assays such as the NETest® have been argued to provide a more precise alternative [101]. However, trials to date have examined the prognostic ability of the NETest® in relation to disease status and progression instead of OS, and their role is yet to be determined [102].

Other

In an analysis of data from a prospective trial of patients treated with SSA with the addition of interferon or bevacizumab, elevated NSE levels were found to be prognostic for both OS and PFS [103]. Increased circulating neurokinin A (NKA) levels has been reported to be a marker of worse survival at a cut-off of 50 ng/ml. More importantly, patients achieving a reduction of NKA below these levels through treatment had a longer median OS [104]. Plasma kallikrein-14 levels at the time of diagnosis have been shown to predict early major CgA responses after treatment with SSA in the prospective Nordic EXPLAIN biomarker study, which examined the use of a 93 plasma protein biomarker panel [105]. This multiple biomarker/machine learning approach could be used to detect Si-NET and predict the risk of progressive disease [106,107].

Grade, Ki-67 and mitotic count

Cell cycle

A proliferating cell progresses through a series of well-characterized, tightly regulated events which are divided into four phases: In G1 (Gap phase 1), the cell grows in size and prepares the necessary enzymes and organelles. In S phase the actual DNA replication occurs, whereas in the G2 (Gap 2) phase, growth continues and the cell prepares for mitosis (M phase). The proliferation rate of tumour cells is considerably higher than that of most normal cells.

Mitosis represents only 5% of the total time a cell needs to divide. In a human cell culture, interphase (G1 to G2 phase) would take 23 hours of a 24 hour cycle, while M phase would occupy 1 hour [108]. Not all cells entering G1 or G2 phases will successfully complete mitosis; cells that have not successfully completed all necessary steps or that have accumulated critical errors during DNA replication are stopped in a number of checkpoints, the most well-known of which are the G1/S and G2/M checkpoints. Counting mitoses rather than dividing cells might thus more accurately represent a tumour's rhythm of proliferation.

Grade and Ki-67

Ki-67 is probably the most important prognostic factor for NET, and forms the basis of the WHO classification system. It is an antibody against a protein which is abundant in the G1, S, G2 and M phases of the cell cycle, but is not expressed during the resting (G0) phase. Ki-67 functions as a biological surfactant, allowing the mitotic chromosomes to separate [109]. It can be used to distinguish cells which are actively dividing, and thus estimate the Ki-67 index, that is to say the ratio of tumour cells in the proliferation phase.

The Ki-67 index is assessed in Ki-67-stained tissue slides. Assessment might be done through quickly scanning the slide and giving a rough estimation (“eye-balling”), or by manually counting stained cells. Initially, a pathologist identifies areas with high proliferation “hot-spots” by examining the entire slide at low magnification. Then a high-power field (HPF, typically 40x objective) is used to count the proportion of Ki-67-positive cells in relation to the total number of tumour cells in this area. Typically a pathologist needs to count between 500 to >2000 tumour cells in immunohistochemically stained slides, and estimate the percentage of cells that stain positive for the Ki-67 antibody [110].

An increasing Ki-67 correlates with increased risk for death, with a HR of 1.12-1.19 [73,85,111] and for progression with one study reporting a HR of 1.14 [73]. Increasing grade correlates with shorter survival. For example, an institutional Si-NET cohort validated the ENETS grading system, showing that G2 tumours had almost twice, and G3 tumours 38 times as high risk of death as G1 tumours [21].

The current grading system suggests cut-offs of 3% and 20% for the classification of tumours as G1, G2 and G3. In one study, a lower cut-off of 1% did not provide additional information to distinguish between G1/G2 tumours [72]. On the other hand, a higher cut-off has been suggested as showing more discriminative power. In one study, the adjusted risk of death was 2.4 times higher for G2 vs G1 tumours at a cut-off of 2%, and 4 times higher at a cut-off of 5% [73]. A similar increase in the risk of death with a HR of 4 for patients with Ki-67 >5% was reported in another study; all cut-offs tested yielded significant results, but the authors concluded that a cut-off of 5% might better identify a small group of high-risk, well-differentiated tumours who might benefit of closer follow-up [111].

Heterogeneity

Tumours are heterogeneous. As a rule, Ki-67 is calculated in hot spots. Some, but not all reports support the idea that Ki-67 might differ between the primary tumour and metastases. In a mixed GEP-NET cohort with 80% Si-NET, one third of the patients had different grade in the metastases compared to the primary. Notably though, in 10/35 cases where grade differed, biopsies from the metastatic sites showed a lower rather than higher grade. Most interestingly,

5-year PFS and OS were lower in patients with increased versus those with stable grade (55 vs 8%, and 92 vs 54%) [112]. In another mixed cohort, patients were re-assessed with new biopsies at the time of disease progression. A grade change was seen only in pancreatic, but not in Si-NET, with the median Ki-67 also remaining unchanged [113].

Mitotic count

Another way of quantifying tumour proliferation is through counting mitoses. This can be done in routine haematoxylin-eosin (H&E) slides. Similarly to Ki-67 index estimation, a pathologist starts by identifying “hot-spots” and then uses a HPF to count cells actively undergoing mitoses. Traditionally, the number of mitoses per 10 HPF is reported. However, as the area covered by a HPF is different for different microscopes [114], results are often reported per 2 mm².

There is some correlation between MC and survival, and a cut-off of 2 mitoses per 10 HPF is used as a limit between G1/G2 tumours. As is the case with Ki-67, the use in various studies of arbitrary, post hoc selected cut-offs precludes firm conclusions. In one cohort, increased number of mitoses was related to higher risk for death with a HR of 1.19 in non-metastatic and 1.12 in metastatic Si-NET [111]. The authors tested 11 different cut-offs, concluding that all but 4, 5 and 6 were significant. On the contrary, Strosberg et al reported that a MC >5 rather than >2/10 HPF provided best discriminative power [21].

Caveats of Ki-67 and mitotic count

Even if Ki-67 index and MC are well-established methods used in several tumour types, there are several considerations regarding accuracy and time constraints. The quickest method to estimate Ki-67 is eye-balling. However, eye-balling comes at the expense of significant interobserver variability and is not recommended [115]. On the other hand, the method WHO endorses, manual counting in hotspots, is quite resource-intensive, as it requires counting of up to 2000 cells. Location and size of the selected hotspot as well as heterogeneity might also influence the result. Huang et al recently showed, for example that choosing a smaller hotspot area resulted in a higher Ki-67 index. The whole process could take up to 10 minutes per case [110]. Automated systems and AI-driven approaches might solve these issues in the future. However as of now, these attempts have only been partially successful. Lea et al recently showed excellent agreement with a correlation coefficient of 0.96 when comparing pathology review of Ki-67 with a digital analysis system, and kappa value of 0.86 for grade estimation [116]. On the other hand, Hacking et al found that performance of their digital image analysis system was suboptimal [117]. It is also worth noting that, although Ki-67 is associated with outcomes after treatment with SSA, this association is less certain when it comes to treatment with PRRT.

Similar to estimating the Ki-67 index, counting mitoses can be influenced by tumour heterogeneity and interobserver variability. The most usual problem is correctly distinguishing cells undergoing apoptosis or necrotic from mitotic cells [118]. The correct identification of hotspots, poor tissue preparation, artefacts such as crashed cells and the variation of the size of HPF for different microscopes might pose additional challenges.

Phosphohistone-H3 (PHH3)

Histones are proteins related to chromatin structure. PHH3 is a histone H3 isoform phosphorylated during cell proliferation. As the phosphorylation of histone H3 only occurs when cells are dividing, PHH3 could be used for identifying mitotic cells, mitigating some of the disadvantages of Ki-67 and MC. PHH3 is not found during apoptotic procedures; thus the risk for incorrect characterization of apoptotic cells as dividing cells is reduced. PHH3-based MC have demonstrated near-perfect interobserver agreement [119]. Additionally, in comparison to Ki-67, PHH3 stains cells during the late G2 and M phases and might be a more accurate marker of mitosis. Of note, tumours graded based on PHH3 MC have higher grade [120].

PHH3 has been studied as a marker of proliferation activity in several tumours including breast cancer, melanoma and meningiomas. It has also been assessed in conjunction with pancreatic and lung neuroendocrine tumours, but not in Si-NET [110,116,119–127].

Other predictive and prognostic markers

Survival has been reported to be better for patients attending a specialist clinic [104]. Smoking did not show a significant correlation to survival [79]. Tumour growth rate assessed using consecutive CT scans has been suggested as a novel biomarker. In a post hoc analysis of a randomized prospective trial of patients treated with SSA vs placebo, a growth rate of >4% per month was found to be associated with worse survival [84]. Unfortunately, this biomarker is hard to assess outside a research facility and has currently limited value in clinical praxis.

IHC staining for SSTR expression has been explored as a potential marker and seems to have some value in univariable analysis. In one study, SSTR2-positive Si-NET had longer OS (HR 0.37), as well as longer PFS after treatment with SSA (HR 0.40) compared with SSTR2-negative tumours, after adjustment for age, sex, tumour stage and Ki-67. There was no survival advantage for SSTR1, 3, 4 or 5-positive tumours [128]. However, indirect information on SSTR expression is routinely derived in clinical practice through SRI, and it would be more relevant to examine whether IHC staining provides additional information. Indeed, in a study of mixed GEP-NET patients selected for treatment with PRRT based on positive SRS, 93% of patients were

also positive for SSTR2a based on immunohistochemistry. However there was no correlation between IHC positivity and best response to PRRT [129].

Follow-up

Si-NET can metastasize late and therefore follow-up might be lifelong and depends on whether resection with curative intent has been performed. Controls include tumour markers (CgA, 5-HIAA) and cross-sectional imaging (3-phase CT or MRI). ENETS recommends, follow-up with 6-12 month intervals in radically resected tumours, except in the case of higher grade tumours, where imaging might be performed at 3 months. Patients who have not been treated with curative intent are followed initially every 3-6 months, and intervals can be later extended for slowly growing tumours [20].

SRI may be performed preoperatively to determine tumor spread and is usually performed post-operatively, in order to determine the presence of residual disease. It is often performed in cases of tumour progression, when treatment with PRRT is considered in order to determine eligibility and when there is discrepancy between radiological, clinical and biochemical findings.

Aims

The aims of the present studies were:

I. To investigate the prognostic value of baseline CgA and 5-HIAA and of the early biochemical response to treatment in G2 Si-NET, and to compare different cut-off values used in the literature.

II. To evaluate whether standard medical treatments are equally effective in G2 Si-NET and in distinct subgroups based on the proliferation marker Ki-67.

III. To investigate whether mitotic count, as estimated by PHH3, might better predict outcomes of patients with Si-NET, compared to Ki-67.

IV. To estimate whether the addition of inflammation markers in models based on standard clinical prognostic factors further improves the models, and to compare the prognostic value of various inflammation markers.

Materials and Methods

All patients with metastatic G2 Si-NET diagnosed between 2000 and 31 May 2017 (paper I, n=184) or 2019 (paper II, n=212) and treated at the Department of Endocrine Oncology, Uppsala University Hospital, a tertiary referral centre, and at the Department of Oncology, Ryhov County Hospital, a regional hospital, were eligible for inclusion. Patients operated radically and not relapsing during the study period were not included. Subsequently PHH3 and Ki-67 stains for all patients with available formalin-fixed, paraffin-embedded (FFPE) tumour blocks were evaluated (paper III, n=73). Following approval from the Uppsala ethical review board (Dnr 2017/403), data on patients' clinical status including ECOG PS, treatments given, laboratory tests and cause of death were extracted from the hospitals' medical records. Survival status was censored at respective study end, or at last known contact. Causes of death due to tumour progression, adverse events, surgical morbidity and cases where cause of death was indeterminate but cancer-related death likely, were classified as cancer-specific mortality. Patients dying from causes not related to their NET tumour were censored at time of death.

For paper IV, all patients with progressive metastatic or unresectable NET, adequate somatostatin receptor expression and organ function who were treated with PRRT between 2005-2015 based on mostly a dosimetry-guided protocol, were retrieved from a prospective internal database. For this study, overall survival was the primary endpoint, defined as time from PRRT start to death from any cause. Baseline blood tests and clinical data were extracted from hospital records.

Patient characteristics

For paper I, we included 184 patients with metastatic G2 Si-NET. Of those, 182 had been treated with SSA, 93 with IFNa and 92 with PRRT. Four patients were re-treated with IFNa and 13 with PRRT. Ki-67 at treatment start was 7, 6 and 8% for the different treatment groups. Baseline CgA was 6.8, 8.0 and 16.5xULN, and baseline 5-HIAA 4.6, 4.2 and 6.1xULN.

For paper II, we included 28 additional patients (n=212). Among the 212 patients, 85 (40%) were female. Median age at start of treatment was 65 years.

The primary tumour was operated in 151 patients (71%). The cohort included 210 treatment cases with SSA, 141 with PRRT (116 as first treatment and 25 rechallenges), 104 with IFNa (95 as first treatment and 9 rechallenges), 29 with everolimus, and 17 with chemotherapy.

In paper III, we included all 73 patients with available FFPE blocks. Baseline characteristics were comparable with the original study population (age 62 years, female 44%). Median Ki-67 was 4% and median PHH3-estimated MC was 2. Some of the patients in papers I-II initially had grade 1 tumours, and their grade was changed to G2 in subsequent biopsies performed upon tumour progression. As for the purpose of the study only baseline biopsies were considered, 32% of the included patients had G1 tumours at baseline.

For paper IV, 557 patients were eligible for inclusion, and 347 patients had all biomarkers available. Median age at PRRT start was 63 years and the majority (281 patients, 51%) had Si-NET, and grade 2 tumours (66%). Approximately one in three patients had high derived neutrophil to lymphocyte ratio (dNLR) and low albumin, and one in five had CRP above the upper limit of normal.

Table 2. Baseline demographics

	Paper I		Paper II				Paper III	Paper IV
	SSA (n=182)	IFNa (n=97)	PRRT (n=105)	SSA (n=210)	IFNa (n=104*)	Everolimus (n=17)	PRRT (n=141*)	All
Sex, n (%): Female	73 (40%)	40 (41%)	40 (38%)	84 (40%)	42 (40%)	13 (45%)	52 (37%)	32 (44%)
Age, median (IQR)	65 (58-71)	61 (54-68)	66(58-71)	65 (58-72)	61 (54-69)	70 (63-72)	67 (60-73)	62 (57-69)
Performance status, n (%)								
0	65 (58%)	34 (59%)	52 (55%)	79 (56%)	36 (54%)	4 (31%)	2 (29%)	68 (50%)
1	31 (27%)	15 (26%)	33 (35%)	43 (30%)	22 (33%)	5 (38%)	2 (29%)	48 (36%)
≥2	16 (14%)	9 (16%)	10 (11%)	20 (14%)	9 (13%)	4 (31%)	3 (43%)	19 (14%)
Line of treatment:								
1	168 (93%)	60 (63%)	9 (9%)	196 (93%)	60 (58%)	3 (10%)	9 (53%)	10 (7%)
2	12 (7%)	29 (30%)	60 (58%)	14 (7%)	34 (33%)	4 (14%)	2 (12%)	76 (54%)
≥3	0 (0%)	7 (7%)	35 (34%)	0 (0%)	9 (9%)	22 (76%)	6 (35%)	55 (39%)
Ki-67 (%), median (IQR)	7 (4-10)	6 (4-10)	8 (5-12)	7 (4-10)	6 (4-9)	8 (6-11)	9 (8-15)	8 (5-12)
Ki-67 (%), n (%)								
3-5				71 (34%)	39 (39%)	6 (24%)	2 (12%)	38 (27%)
5-10				87 (42%)	46 (46%)	12 (48%)	7 (44%)	61 (43%)
10-20				49 (24%)	14 (14%)	7 (28%)	7 (44%)	42 (30%)

<i>PHH3 (median (IQR))</i>				2 (0-5)
<i>CgA xULN, median (IQR)</i>	6.8 (2-34)	8 (2-45)	17 (5-32)	
<i>CgA, dichotomized</i>				
<5	49 (41%)	31 (44%)	27 (27%)	
5-10	17 (14%)	6 (9%)	12 (12%)	
>10	55 (46%)	34 (48%)	62 (61%)	
<i>5-HIAA xULN, median (IQR)</i>	5 (1-16)	4 (1-15)	6 (3-15)	
<i>5-HIAA, dichotomized</i>				
<5	68 (55%)	40 (58%)	42 (42%)	
5-10	16 (13%)	7 (10%)	24 (24%)	
>10	39 (32%)	22 (32%)	35 (35%)	
<i>Tumour location</i>				
<i>small intestine</i>				281 (51%)
<i>pancreas</i>				139 (25%)
<i>other</i>				131 (24%)

SSA: Somatostatin analogues, IFN α : interferon-alpha, PRRT: peptide receptor radionuclide therapy, IQR: interquartile range, CgA: chromogranin A, 5-HIAA: 5-hydroxyindoleacetic acid

Biochemical markers (Paper I)

Biomarkers were collected at baseline, and at the 6-month visit. In a minority of patients undergoing interventional procedures within this 6-month period, last biomarker control before intervention was accepted, as long as it was at least 3 months after treatment start; otherwise these patients were excluded from analysis. For the measurement of 5-HIAA, patients were provided with one or two receptacles and were asked to collect urine for one or two 24-hour periods prior to the planned visit and to maintain a serotonin-poor diet for 72 hours beforehand. 5-HIAA was measured as a single sample or as two samples on consecutive days; whenever two samples were examined, the mean value was used. Samples were analysed using high-performance liquid chromatography. Plasma samples for CgA were collected in chilled heparinised vacutainer tubes after fasting overnight. All samples before and during PRRT were measured at Uppsala University Hospital using the EuroDiagnostica kit (Malmö, Sweden) for CgA. Samples before and during other treatments were measured at Uppsala University Hospital (85% of evaluable samples for patients treated with SSA or IFN) or at the patient's local laboratory. In each case, baseline and 6-month tests were conducted at the same laboratory. Levels of CgA and 5-HIAA were described as times the upper limit of normal (xULN) of the reporting laboratory.

Biochemical partial response (PR) was defined as a reduction of baseline CgA or 5-HIAA by at least 50% and biochemically progressive disease (PD) as an increase by at least 25%. Patients with values at 6 months between -50% and +25% of baseline were deemed as having biochemically stable disease (SD).

Ki-67 (Paper II)

Ki-67 was analysed both as a continuous non-linear variable, using restricted cubic splines with three degrees of freedom in cox models, and as a categorical variable in $\leq 5\%$, $>5\%$ to 10% , $>10\%$ groups.

PHH3 mitotic index and Ki-67 proliferation index (Paper III)

FFPE sections were immunohistochemically stained with fully automated protocols for PHH3, CgA and Ki-67. In CgA-positive areas, the region with highest labelling for Ki-67 ("hotspot") was selected under low magnification. Subsequently the Ki-67 proliferation index (defined as the ratio of Ki-67 positive nuclei to the total number of nuclei in at least 500 cells) and the PHH3

mitotic index (defined as the number of tumour cells with prominent PHH3 staining in 10 consecutive high-power fields) were estimated.

Inflammation scores (Paper IV)

All patients hospitalized for the initiation of treatment with PRRT had routine blood tests. For the purpose of this study, inflammation markers and scores previously examined in NET studies were evaluated: White blood cell (WBC), neutrophil and thrombocyte counts, CRP, albumin, dNLR, defined as neutrophil/(WBC-neutrophil), Glasgow Prognostic Score (GPS, calculated from CRP and albumin assigning one point each to CRP >10 mg/L and albumin < 35 g/L), CRP to albumin ratio, platelet count x CRP and erythrocyte sedimentation rate (ESR).

Statistical methods

Statistical analysis was performed with R (R Foundation for Statistical Computing, Vienna, Austria), using the chi-square test for dichotomous variables, t-test or Kruskal-Wallis test for continuous variables and semi-parametric cox models for censored variables. CSS, calculated from treatment start to cancer-related death, OS, calculated from treatment start to death of any cause, and PFS calculated from start of each treatment to radiological progression (defined as any unequivocal increase in the size of known tumours or detection of new lesions, in scans performed every 3-6 months, unequivocal clinical progression or death), were analysed using the Kaplan-Meier method and between-group differences were analysed using a log-rank test. HR and confidence intervals (CI) were estimated from the Cox proportional hazards model.

Optimal cut-off points were calculated with R packages *Survminer* and *maxstat* using the maximally selected rank statistics, a method that allows the evaluation of cut-off points, which provide the classification of different risk groups in a quantitative or ordered predictor variable. Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) were estimated with R package *timeROC* (paper I).

Adjusted survival curves, which represent expected survival curves corrected for co-variables on the basis of a Cox model, were created with the *surminer* package 0.4.9 [130,131] (paper II).

In order to validate the estimated optimal cut-off for PHH3, an internal cross-validation was performed. We splitted the sample into training and testing datasets and repeated that 1000 times. We then produced a frequency distribution of the optimal cut-offs (paper III).

Akaike's information criterion (AIC) was used to compare the prognostic value of various inflammation markers. AIC estimates the goodness-of-fit and

can be used to estimate the prediction error and to compare statistical models derived from the same dataset. Generally, a lower AIC shows a more accurate model. If two models differ by <4 , they are considered roughly equivalent, whereas if the difference is 4-10, there is some evidence that the model with the lower AIC is better, whereas if AIC difference is >10 , there is strong evidence that the model with the lower AIC is more accurate. AIC was estimated with AICcmodavg package 2.3 (paper III, IV).

Results

Paper I

We examined the association between baseline CgA and 5-HIAA levels as continuous and dichotomous variables, and treatment outcomes. Baseline CgA was consistently prognostic for both CSS and PFS for all treatments, whereas 5-HIAA was prognostic for CSS after treatment with IFNa and PRRT, but not single SSA, and for PFS after treatment with PRRT.

For patients treated with SSA, dichotomizing CgA at 2xULN provided good discrimination for both CSS (HR 5.30, $p < 0.01$) and PFS (HR 2.22, $p = 0.01$). The estimated optimal cut-offs for 5-HIAA were 4xULN for CSS and 1xULN for PFS. An approximation with dichotomization at 5xULN provided modest discrimination for CSS (HR 2.18, $p = 0.02$).

For patients treated with PRRT, a CgA cut-off at 5xULN provided good discrimination (HR 8.44, $p < 0.01$ for CSS and 5.94, $p < 0.01$ for PFS). For 5-HIAA, higher cut-off values were more discriminative, especially for CSS (HR 6.30, $p < 0.01$) and moderately for PFS (HR 2.34, $p = 0.01$).

Six-month reductions of CgA and 5-HIAA correlated well with PFS and CSS after treatment with single SSA, both as continuous and dichotomous variables (Fig. 1A). However, after SSA dose intensification, there was only a non-statistically significant correlation of early CgA changes with CSS (HR 1.11, $p = 0.08$) and no correlation between early 5-HIAA changes and either CSS or PFS.

Similarly, there was no correlation between 6-month changes of CgA and 5-HIAA with CSS or PFS after treatment with PRRT (Fig. 1B). We further examined whether different baseline SSA doses could potentially mask the early effect of PRRT on CgA and 5-HIAA. After adjusting in a cox model, early reduction of CgA, but not 5-HIAA, had a borderline significant correlation with CSS (HR 1.45, $p = 0.04$).

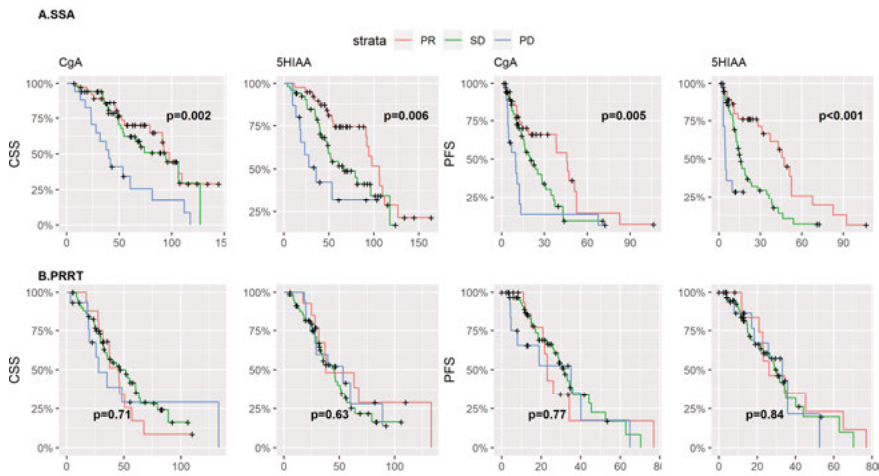


Fig. 1. Cancer-specific survival (CSS) and progression-free survival (PFS) after treatment with somatostatin analogues (SSA) are longer for patients with biochemical response at 6 months, while there is no difference in patients treated with peptide receptor radionuclide therapy (PRRT). CgA: Chromogranin A, 5-HIAA: 5-hydroxyindoleacetic acid, PR: partial response, SD: stable disease, PD: progressive disease. Adapted from Papantoniou et al, 2021; 10.1007/s12020-020-02534-8.

In the case of IFNa, we examined patients having progressive or stable disease together and compared them with responders. HR for CSS for responders vs non-responders was statistically significant for 5-HIAA (HR 2.59, $p=0.04$) but not for CgA (HR 1.91, $p=0.14$). The small number of patients precludes firm conclusions.

We finally examined whether early treatment intensification in non-responders could result in longer CSS. Among 83 cases with early biomarker progression, 39 changed treatment within a year. In an adjusted analysis including factors associated with a more aggressive outcome (CgA, 5-HIAA, age, Ki-67) there was a non-significant trend in favour of early treatment intensification (HR 0.70, $p=0.28$).

Paper II

Median CSS for the 210 patients treated with SSA, either as single treatment or in combinations, was 77 months. Median PFS was 12.4 months for those starting treatment with SSA monotherapy, and 19 months for all patients. Survival was largely similar for patients with PS 0 and 1 (CSS 92 and 91 months, PFS 28 and 25 months), whereas it was significantly worse for those with PS \geq 2 (CSS 24 months, PFS 6 months).

Our cohort included patients starting treatment at both standard and below-label doses of SSA. In an analysis adjusted for age, PS, Ki-67, liver metastases, subsequent PRRT use and CgA, patients starting treatment on below-label doses had significantly worse prognosis (HR=2.33, 95% CI 1.22-4.48, p=0.01). However the difference was obvious only in the group of patients with Ki-67 5-10% (n=74, 53 vs 87 months, p=0.002) but not in those with Ki-67 \leq 5% (n=67, 109 vs 120 months, p=0.54).

Treatment intensification with above-label SSA doses were more efficient when used for better symptom control or biochemical progression than for radiological progression (respective PFS of 22, 9 and 6 months) and at the first intensification step (9 vs 6 months).

Interferon (n=95) was often started either in parallel with SSA as first-line treatment (CSS 105 months, PFS 32 months) or sequentially (PFS 6 months). Everolimus (n=29) was used mostly at later lines, with a median PFS of 5 months. PFS after chemotherapy (n=17, mostly temozolomide-based) was 9 months, after initial PRRT treatment (n=116) 30 months, and after PRRT re-challenge (n=25) 13 months.

G2 Si-NET include tumours with a wide range of Ki-67 between 3-20%. We analysed CSS and PFS according to Ki-67 group for treatment with SSA and PRRT. Median CSS for 1st line treatment with SSA in the 3-5%, 5-10% and 10-20% subgroup was 111, 70 and 49 months. Median PFS was 31, 18 and 10 months. In the case of PRRT, differences were minimal, with respective median CSS of 56, 39 and 34 months and PFS of 29, 25 and 25 months (Fig. 2).

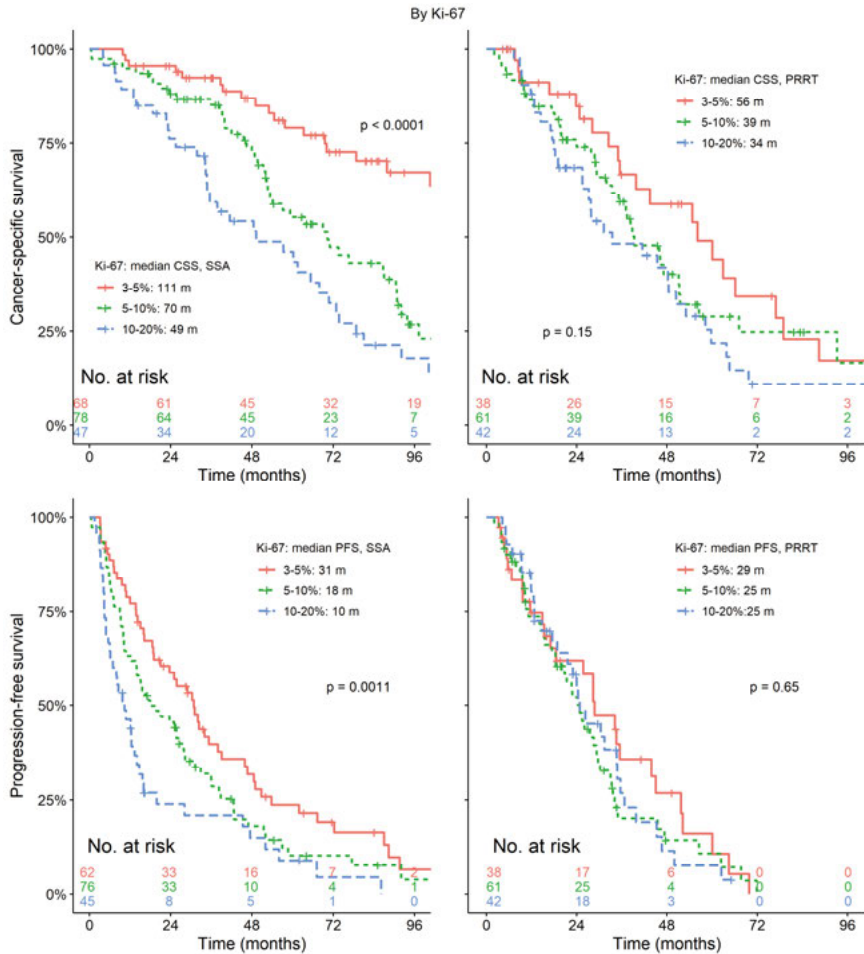


Fig. 2. Cancer-specific (CSS) and progression-free survival (PFS) for somatostatin analogues (SSA) and peptide-receptor radionuclide treatment (PRRT) by Ki-67 at 5 and 10% cut-offs. Reprinted from Papanthiou et al, 2023; 10.1530/ERC-22-0316.

We finally assessed treatment efficacy in relation to SRI. Median PFS was lower in SSTR- patients treated with SSA single (7 vs 14 months, $p=0.008$) but not in those treated with the combination of SSA and IFNa (16 vs 36 months, $p=0.43$) in first line, nor in those treated with single IFNa (13 vs 6 months, $p=0.56$). After adjusting for the higher proliferation index Ki-67 of the SSTR- group, the risk of the SSTR- patients for cancer-related death and for disease progression remained significantly higher in the SSA group, while there was no significant difference or trend in the IFNa group.

Paper III

Both Ki-67 and PHH3 showed a significant association with CSS in patients with Si-NET. Specifically, tumours labelled as G1 based on their Ki-67 expression had a median CSS of 128 months, compared to 95 months for G2 tumours ($p = 0.007$). Similarly, when PHH3 was used to classify tumours, G1 tumours exhibited a median CSS of 149 months compared to 88 months for G2 tumours ($p = 0.001$). In unadjusted Cox regression models, both variables were significantly associated with CSS (HR 1.18 and 1.16, respectively). When comparing the Ki-67 and PHH3-based models, the latter showed slightly higher c-index (0.71 vs 0.68, higher is better) and lower AIC (219 vs 223, lower is better) (Fig.3).

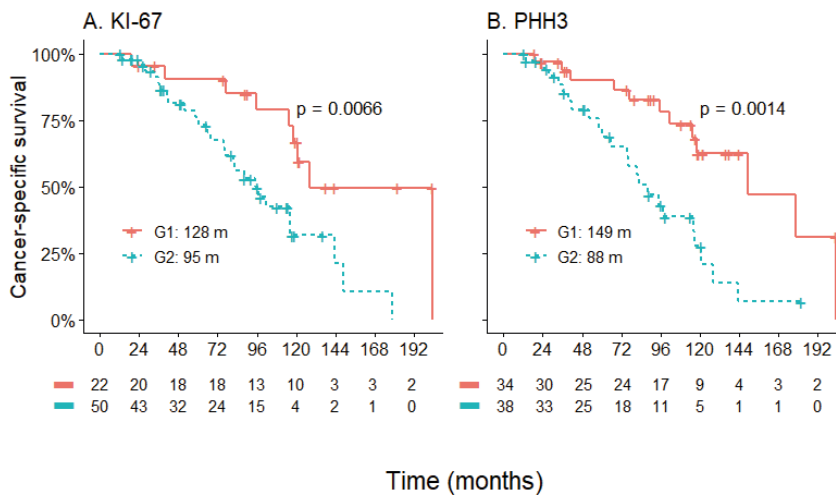


Fig. 3. Cancer-specific survival by grade, as assessed by A. Ki-67, B. PHH3.

In 52 patients with available data for treatment with first-line SSA, PHH3 was the only proliferation parameter exhibiting significant association with PFS (HR: 1.12, 95% CI: 1.03-1.21). A similar trend between PHH3-estimated proliferation index and PFS was seen in the 26 patients later treated with PRRT (HR: 1.11, 95% CI 1.00-1.24), but not for Ki-67 (HR: 0.93, 95% CI 0.82-1.07).

We finally estimated a cut-off for mitotic count by PHH3 of >2 mitoses per 10 HPF to better differentiate between G1 and G2 tumours in our data (179 vs 82 m, $p < 0.0001$). After splitting the dataset into training and testing samples, and repeating the process 1000 times, having >2 mitoses per 10 HPF was the most commonly suggested cut-off. At this cut-off, the median HR was 4.92 for G2 vs G1 tumours, and was significant in 95% of the test samples.

Paper IV

Inflammatory parameters and scores based on CRP and albumin, were associated with OS in patients treated with PRRT. In an unadjusted analysis, shorter OS was seen in patients with higher CRP, GPS, dNLR and CRP/albumin ratio as well as lower albumin at baseline. In the subgroup of patients with Si-NET, albumin (HR: 0.89, 95% CI 0.86-0.93), CRP (HR: 1.03, 95% CI: 1.02-1.04) and GPS (1 vs 0, HR 1.68, 95% CI 1.07-2.63, 2 vs 0, HR: 3.72, 95% CI 2.19-6.34) influenced OS. Similar results were attained for NET of other origins. The direction of effect was similar in tumours irrespective of grade, but reached statistical significance only in higher grade tumours. In adjusted analysis, all variables except for dNLR remained associated with OS (Table 3).

Table 3. Association between inflammation markers and OS after treatment with PRRT

		n	HR (unadjusted)	p	HR (adjusted)*	p
CRP		351	1.02 (1.02-1.03)	<0.001	1.02 (1.01-1.02)	<0.001
Albumin		556	0.89 (0.87-0.91)	<0.001	0.91 (0.87-0.95)	<0.001
White blood cells		556	0.99 (0.94-1.03)	0.555	0.96 (0.89-1.03)	0.279
Neutrophils		555	1.05 (0.98-1.12)	0.136	1.00 (0.91-1.10)	0.989
Thrombocytes		556	1.00 (1.00-1.00)	0.496	1.00 (1.00-1.00)	0.906
Albumin, Dichotomized	Low	178	-	-	-	-
	Normal	378	0.43 (0.35-0.54)	<0.001	0.39 (0.27-0.55)	<0.001
CRP, dichotomized	Normal	279	-	-	-	-
	Elevated	72	2.02 (1.47-2.79)	<0.001	1.75 (1.18-2.59)	0.005
dNLR		555	1.19 (1.08-1.31)	<0.001	1.11 (0.98-1.25)	0.111
dNLR, dichotomized	Low	364	-	-	-	-
	High	191	1.44 (1.16-1.78)	0.001	1.37 (0.99-1.90)	0.060
GPS	0	210	-	-	-	-
	1	99	2.05 (1.51-2.77)	<0.001	1.67 (1.14-2.44)	0.008
	2	42	3.59 (2.40-5.36)	<0.001	3.60 (2.24-5.79)	<0.001
CRP/albumin ratio		351	2.27 (1.86-2.78)	<0.001	1.84 (1.43-2.37)	<0.001
(Plt*CRP)/1000		351	1.08 (1.05-1.10)	<0.001	1.06 (1.03-1.08)	<0.001

*Adjusted for age, Ki-67, logarithmically transformed normalized Chromogranin A (CgA), prior treatment lines (0, 1, ≥ 2), performance status (PS, 0, 1, ≥ 2) and stratified by tumour type (small intestinal vs pancreas vs other). All patients were entered in unadjusted analyses. For adjusted analyses, only patients with complete lab tests and Ki-67 (n=273) were used. To adjust for multiple comparisons, p < 0.004 was considered significant. CRP: C-reactive protein, dNLR: derived neutrophil to lymphocyte rate, GPS: Glasgow prognostic score, PLT: platelets, HR: Hazard ratio

We subsequently examined which inflammatory parameter had most prognostic value. For this purpose, we compared the unadjusted and adjusted models presented in Table 3, with their AIC. AIC can be used to compare two models derived from the same dataset; a lower AIC denotes a model which has a lower prediction error, and is thus more accurate. Models based on albumin, alone or in combination with CRP, were associated with lower AIC, less prediction error, and thus could more accurately predict OS (Fig.4).

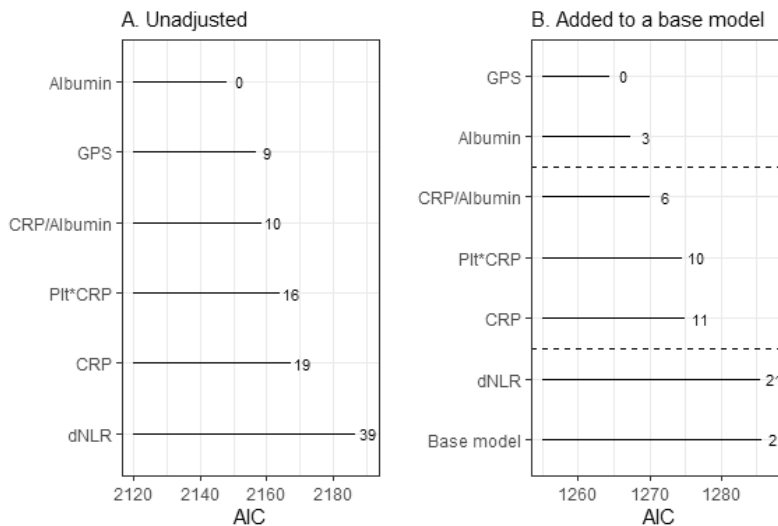


Fig. 4. Predictive accuracies of unadjusted and adjusted models of inflammation markers. Lower values of Akaike's information criterion (AIC) suggest a lower prediction error (a more informative model which better fits the data). Base model includes age, tumour type, Ki-67, log(CgA), previous treatment lines and performance status (PS). All parameters except for derived neutrophil to lymphocyte ratio (dNLR) improved the base model. Albumin was the single variable that mostly improved the base model. Glasgow prognostic score (GPS), a composite score combining C-reactive protein (CRP) with albumin, marginally further improved the adjusted model. CgA: Chromogranin A, Plt: Platelets. Adapted from Papantoniou et al, 2025; 10.1111/jne.13379.

Discussion

Paper I and II

Traditionally, Si-NET tumours have been studied as a uniform group, regardless of their grade, and often together with tumours from other gastroenteropancreatic sites. With G2 tumours constituting approximately one-fourth of all Si-NET, study results are more representative of the G1 group. As G2 tumours have variable clinical course and possibly a different molecular background, it remains uncertain whether those results can be extrapolated to the G2 subgroup.

Moreover, those tumours with higher proliferation indexes often have an unpredicted clinical course, with some of them progressing slowly like their G1 counterparts, whereas in other cases the patients deteriorate quickly. None of the factors studied to date has prognostic power to sufficiently discriminate between those groups, and, with the exception of positivity in SRI, no single variable is predictive of treatment response.

In our work, we have focused on the subgroup of G2 Si-NET patients. In this retrospective bi-institutional cohort study, we have revisited the three prognostic markers mostly used in clinical praxis, CgA, 5-HIAA, and Ki-67, and we evaluated their prognostic value for treatment with SSA and PRRT. We found that baseline CgA, and to a lesser extent 5-HIAA, are predictive of survival for both treatments, whereas their early reductions are prognostic only for treatment with SSA. We also found that benefit from PRRT was largely independent of Ki-67 levels in the 3-20% range; the opposite was true for SSA.

Paper I

In this paper, we hypothesized that baseline tumour markers and their dynamics early under treatment can be prognostic of treatment effect. Several studies have correlated elevated baseline CgA levels to poor outcomes [23,76,79,95,97,97,103,132–137] in various types of NET. CgA is usually assessed as a dichotomous variable, with a wide range of cut-offs, between 1x and 10xULN, making results difficult to compare. We assessed the most usual cut-offs used in literature, and found cut-offs in a more narrow range to be

most relevant: a cut-off of 2xULN provided best discriminative value during treatment with SSA and 5xULN for treatment with PRRT.

The relation between 5-HIAA levels and treatment outcomes has been less documented, with two studies showing contradicting results [79,97]. We confirmed the prognostic significance of 5-HIAA for treatment with PRRT, but we could not find a significant association for treatment with SSA. Finding a consistent optimal cut-off proved elusive; optimal discrimination was seen in most treatment cases at cut-offs of 10xULN or higher, but this varied widely between different treatment scenarios.

Limited data from mixed GEP-NET populations suggests that early CgA changes under treatment might relate to a lower risk of progression for SSA [93] and everolimus [94], but not for PRRT [95]. There is no data associating early 5-HIAA responses with survival outcomes. Only a weak association between changes in CgA and radiologically assessed tumour burden has been reported [96], especially in the case of PRRT [138]. As a result, two recent reviews reached different conclusions regarding the prognostic significance of early CgA changes [24,92]. We add to this ongoing discussion and confirm that 6-month changes of CgA correlate with CSS and PFS in patients treated with SSA, but not in those treated with PRRT. We also present the first data showing a strong correlation between early 5-HIAA changes and survival outcomes in SSA-treated patients.

Paper II

We hypothesized that due to their more aggressive behaviour, G2 tumours might respond worse to standard treatments, and especially to cytostatic treatments, such as SSA.

The landmark PROMID trial, which established long-acting SSA as the cornerstone of Si-NET treatment only included slowly-proliferating G1 patients with Ki-67 <2% and reported a median PFS of 14.3 months. A second trial (CLARINET) included GEP-NET of various origins with Ki-67 up to 10%. Despite the higher proliferation index, the PFS of G2 Si-NET patients in our study was 12.4 months, very similar to that reported in G1 patients in PROMID, thus suggesting that SSA is active in this patient group. We noted, however, that both PFS and CSS after treatment with SSA were significantly shortened at higher Ki-67 levels, implying that SSA efficacy is dependent on proliferation index, and that tumours with higher proliferation rates might be considered for follow-up at shorter intervals, and possibly for alternative treatment strategies. On the other hand, we did not notice any significant deterioration of PRRT efficacy throughout the 3-20% range. It is probable that a primarily cytotoxic treatment like PRRT retains its efficacy against rapidly proliferative tumours. This finding is consistent with the NETTER-1 trial, where

PRRT had similar HR for both G1 and G2 tumours [43]. Indeed, in small retrospective series, PRRT but not SSA seem to be effective in tumours with even higher proliferation indexes [139,140].

Increases of SSA doses to above-label levels has traditionally been the first step of treatment intensification. We observed a modest median PFS of 6 months after increasing SSA dose in patients progressing radiologically on the initial dose. Three prospective series have reported similar PFS gains of 6.8-8.4 months [42–44]. The small difference might reflect the use of RECIST criteria (which has been shown to give longer estimates of PFS in slowly growing tumours [141]), the lower final SSA dose in our study, or the more aggressive nature of G2 tumours. Interestingly, some of the patients included in our study were treated with below-label doses of SSA. These patients had worse OS if they had Ki-67 5-10%, but not if they had lower Ki-67, implying that below-label doses might be adequate for the slower-proliferating tumours.

The last group of patients we examined was those with low SSTR expression. This is a particularly challenging group, as they have limited treatment options and worse outcomes [65]. Unlike SSA, IFNa seemed to be equally effective in these patients.

Paper III

In this study, we hypothesized that PHH3 might outperform Ki-67 as a prognostic marker for CSS and for response to treatment in patients with Si-NET. We showed that not only are PHH3-estimated MC associated with CSS, but also with PFS after treatment with SSA and possibly with PRRT. The associations appeared to be slightly stronger with PHH3 than with Ki-67. Moreover, we hypothesized that a “standard” WHO mitotic cut-off of <2 per 10 HPF for H&E slides might not apply to mitoses, as counted by PHH3. Indeed, a slightly higher cut-off of >2 per 10 HPF seemed to better differentiate between lower and higher grade.

The current WHO classification depends on the estimation of Ki-67 and MC on H&E slides. Ki-67 is an antibody against a chromatin protein expressed throughout the G1, S, G2 and M phases of cell proliferation. “Eyeballing”, the fastest way of estimating Ki-67, is coupled to considerable interobserver variability [115]. WHO recommends manual counting in hotspots, a resource-intensive method that can take up to 10 minutes per case [110]. Tumour heterogeneity, hotspot selection and field size might also impact the estimation of Ki-67. Automated systems have been tried, with mixed results [116,117]

Similarly, counting mitoses in H&E stains comes with the risk of misidentifying apoptotic, necrotic or crashed cells as proliferating cells [118]. PHH3 is expressed almost exclusively in the M phase of cell division and staining for PHH3 makes recognising mitotic cells both easier and faster. For example,

a study in pancreatic NET showed near perfect interobserver agreement (intraclass correlation coefficient $k \geq 0.98$) and shorter time (1.68 min vs 3.67 min per 50 HPF) when PHH3 was used [119].

In the current study, grading by both Ki-67 and by PHH3 was strongly associated with CSS. When examining though the two proliferation markers as continuous variables in a Cox model, PHH3 could better discriminate survival outcomes, as evidenced by a slightly higher Harrell's c-index and lower AIC. More importantly, PHH3 seemed to better correlate with PFS after SSA, and possibly after PRRT. The higher specificity of PHH3 for the M phase might mean that PHH3 staining better expresses tumour biology, and might be better associated with treatment outcomes.

WHO suggests a cut-off of 2 mitoses per 10 HPF on H&E stains to distinguish between G1 and G2 tumours. PHH3 has previously been studied in pancreatic NET, and various cut-offs have been suggesting, ranging from 2-10 [119,123,124,126]. In our study, a lower cut-off of >2 mitoses per 10 HPF offered better discrimination; this might be in line with the lower proliferation rates usually seen in Si-NET.

Paper IV

A new biomarker should offer prognostic insight regarding disease outcomes; it should also be easy to measure, cost-effective and reproducible [142]. A crucial question is whether it can provide additional information to already established prognostic markers [143]. As inflammation scores are derived from routine blood tests, they are easy to calculate and provide excellent reproducibility. In this paper, we showed that they could also increase the prognostic value of a base model consisting of tumour type, age, PS, CgA and previous treatments. Hypoalbuminemia was the single marker providing the most added predictive information.

Interestingly, neutrophil/lymphocyte related counts, which are commonly associated with outcomes in multiple cancer types, as well as in some smaller retrospective series of NET patients treated with PRRT[144–146] were not associated with OS in our study. More specifically, dNLR was prognostic of outcome in unadjusted, but not in adjusted analyses. This unexpected result might mean that neutrophil-mediated cancer growth might not be as relevant after treatment with PRRT as with classical chemotherapy, or that the more indolent NET do not activate cellular inflammation system in the same way as more aggressive cancers.

On the contrary, we found a strong association between hypoalbuminemia and shorter median OS. Two previous studies had reported contradictory results, with only one showing that albumin levels related to OS and PFS [147,148]. Hypoalbuminemia is a multifactorial condition which commonly occurs in later cancer stages. It has been linked to worse outcomes in several

types of cancer, and with complications and toxicity after surgery, chemotherapy, targeted treatments and radiotherapy [149–153]. Although it is often considered to be a result of low nutritional status, a stronger association with inflammation has been suggested[154]. This is in line with our findings, where albumin was more closely correlated with CRP (Pearson's $r = -0.45$), than with hepatic involvement (point biserial correlation coefficient $r_{pb} = 0.26$) or with body-mass index ($r = 0.16$).

Major findings

- CgA baseline levels are associated with CSS in patients with G2 Si-NET, irrespective of treatment used, and with PFS in patients treated with PRRT.
- A cut-off of 5xULN provides best discrimination in most cases
- Reductions of CgA and 5-HIAA at 6 months from treatment start with SSA, but not with PRRT, have prognostic value.
- Treatment with SSA appears to be effective in G2 Si-NET, a group with more aggressive, rapidly progressing tumours, which had not previously been studied separately. Median PFS in our study was similar to historical data for all-grade Si-NET.
- Patients with lower Ki-67 levels had significantly longer median PFS compared with those with higher Ki-67 levels when treated with SSA, whereas Ki-67 impact on PRRT efficacy seemed to be limited, at least in the 3-20% range.
- Dose intensification provided only short-term disease stabilization.
- PHH3 could serve as a viable alternative to Ki-67 for grading of metastatic Si-NET. It correlates with both CSS and PFS following first-line SSA, and potentially PRRT.
- A threshold of more than 2 PHH3-counted mitoses per 10 HPF differentiates effectively between G1 and G2 Si-NET.
- Inflammation scores are associated with OS in NET patients treated with PRRT, regardless of origin. The effect is clear in patients with G2/3 tumours. These scores could be used as stratification factors in future PRRT trials.
- Hypoalbuminemia emerged as the single marker adding most predictive value to a base model of established prognostic factors.

Future directions

The previous decades have been dominated by single-institution retrospective studies, which often examined all types of NET together. We took a step from this paradigm by focusing on a more homogeneous population of small intestinal tumours of intermediate grade. Future research should examine separately NET of various origins and grade. The G2 Si-NET cohort used as a basis for the first three papers is the largest published series of patients with this relatively rare tumour. However, our work is limited by the fact that most of the patients were treated in a single tertiary referral centre, over a period of 20 years. Our results should therefore be validated in a general, more modern population. The rarity of NET and their indolent course has been an obstacle for most attempts at randomized clinical trials. Until such prospective data becomes widely available, large retrospective multi-institutional series as a result of international collaboration and well-organized registries will provide data with less risk of bias and more representative of the general population.

Monoanalytes like CgA and other simple tests that were examined in this thesis are reaching their limitations. They are often prognostic of disease outcomes, but not predictive of treatment effects. We expect multianalytes, either genomic or biochemical, to start being implemented in everyday clinical praxis.

NET have considerable variability in their clinical manifestations, response to treatment and prognosis, with survival often ranging from a few years to decades. An old prognostic model was developed in the time before PRRT. It is based on 15 clinical parameters, some of which are not always available even in specialized centres, and might be difficult to implement in clinical praxis [155]. Additionally, it was recently shown to sufficiently predict 5-year OS, but underestimate 10-year OS [156]; this might be due to the model's limitations, or to the emergence of newer life-prolonging treatments and the more extensive use of SSA during the last two decades.

We have developed a simple treatment-specific prognostic model for Si-NET patients treated with PRRT. Our model is limited to 5 variables, and includes only easily accessible laboratory and clinical parameters that can be assessed objectively and calculated “bedside” for it to be clinically useful. It does not contain investigatory parameters assessable only in the context of a study, such as percentage of tumour volume, rate of tumour progression or dual PET imaging. It is currently pending external validation.

Sammanfattning på svenska

Neuroendokrina tumörer i tunntarmen (Si-NET) är ovanliga. De växer långsamt och utsöndrar ämnen som kan påverka kroppens funktioner. I Sverige drabbas cirka 1,2 personer per 100 000 invånare varje år. Tumörerna delas in i tre grupper beroende på hur snabbt de växer: Grad 1 (G1) tumörer växer långsamt och ger ofta symtom som diarré och värmevallningar; de kan behandlas med läkemedel som heter somatostatinanaloger (SSA). De sällsynta G3 tumörer växer däremot snabbt och effekten av sedvanliga behandlingar är begränsad.

Min forskning fokuserar på tumörer med intermediär växthastighet (G2), som har hunnit sprida sig (metastaserats). Dessa kan bete sig olika: vissa liknar de indolenta G1 tumörer, medan andra är mer aggressiva.

Det är oklart hur G2-tumörer svarar på SSA, och det finns få andra behandlingsalternativ. En ny studie (NETTER-1) visade att en behandling som kallas PRRT kan vara effektiv. En annan behandling, everolimus, har visats ge begränsat värde för patienter med Si-NET. De flesta studier har undersökt blandade grupper av patienter, där patienter med G2 tumörer är få. Det är därför viktigt att bekräfta effektiviteten av behandlingar i denna unika subgrupp, och att utveckla metoder som kan förutse hur tumörerna kommer att bete sig.

I vår första studie undersökte vi om halten av vissa markörer i blodet – Chromogranin A (CgA) och 5-HIAA – kan förutsäga hur länge patienter med spridda G2-tumörer lever och hur länge de svarar på behandling. Vi såg att höga nivåer av CgA var kopplade till kortare överlevnad, särskilt hos dem som fick PRRT. Intressant nog visade vi att om nivåerna av dessa ämnen inte minskar efter sex månader med SSA, har patienter betydligt sämre effekt av behandlingen vilket kan betyda att man kan behöva byta till en annan behandling snabbare. Däremot hittade vi inget samband mellan förändringar av CgA och 5-HIAA nivåer och effekten av behandling med PRRT.

I vår andra studie fokuserade vi på hur bra SSA och PRRT fungerar för spridda G2-tumörer. Vi påvisade att SSA fungerar även för denna mer aggressiva grupp. Däremot, såg vi att ökning av SSA dosen när sjukdomen förvärras inte såg ut att leda till bättre resultat. Vi såg också att tumörer med högre Ki-67 (en markör för hur snabbt de växer) svarade sämre på SSA, men inte på PRRT, jämfört med patienter med långsammare tillväxt (G1 tumörer). Det är möjligt att patienter med högt Ki-67 kan få bättre effekt om de behandlas med PRRT tidigare under sjukdomens förlopp.

I vår tredje studie testade vi om en ny markör, PHH3, kan förutsäga behandlingsresultat bättre än Ki-67. PHH3 färgar bara celler som befinner sig i delningsfasen av cellcykeln, vilket kan ge en mer exakt bild av hur snabbt tumören växer. Vi såg att PHH3 var lika bra som Ki-67 på att förutsäga överlevnad och möjligen något bättre på att förutsäga hur länge patienten svarar på behandling. Resultaten behöver dock bekräftas i en större studie.

I vår sista studie undersökte vi om inflammationsmarkörer kan förutsäga resultat efter PRRT. Inflammation kan påverka cancer negativt, men detta är mindre tydligt för långsamt växande tumörer som Si-NET. Vi fann att inflammationsmarkörer förbättrade en modell som förutspår överlevnad, och att låg nivå av albumin i blodet var den starkaste markören för sämre överlevnad.

Framtidsperspektiv

Hittills har många studier undersökt alla typer av NET tillsammans. Vi fokuserade istället på en mer enhetlig grupp (G2 Si-NET). Vår studie är den största på denna grupp, men eftersom den gjordes på ett enda sjukhus behöver resultaten bekräftas i större studier.

Eftersom NET är ovanliga och växer långsamt är det svårt att göra stora kliniska studier. Tills dess mer data finns, behövs internationellt samarbete för att samla in information från flera sjukhus.

Vanliga markörer som CgA, 5-HIAA och Ki-67 kan förutsäga sjukdomens utveckling, men inte alltid hur väl behandlingen kommer att fungera. I framtiden kan mer avancerade tester, t.ex. genetiska analyser eller kliniska modeller, ge bättre svar. Som en fortsättning av vårt arbete har vi utvecklat en enkel modell som kan förutsäga överlevnad och behandlingsresultat för patienter som får PRRT. Den baseras på fem lättillgängliga blod- och kliniska tester och håller på att testas.

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