Neuroendocrine Neoplasia Tailoring Treatment and Prognosis

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(met een samenvatting in het Nederlands)

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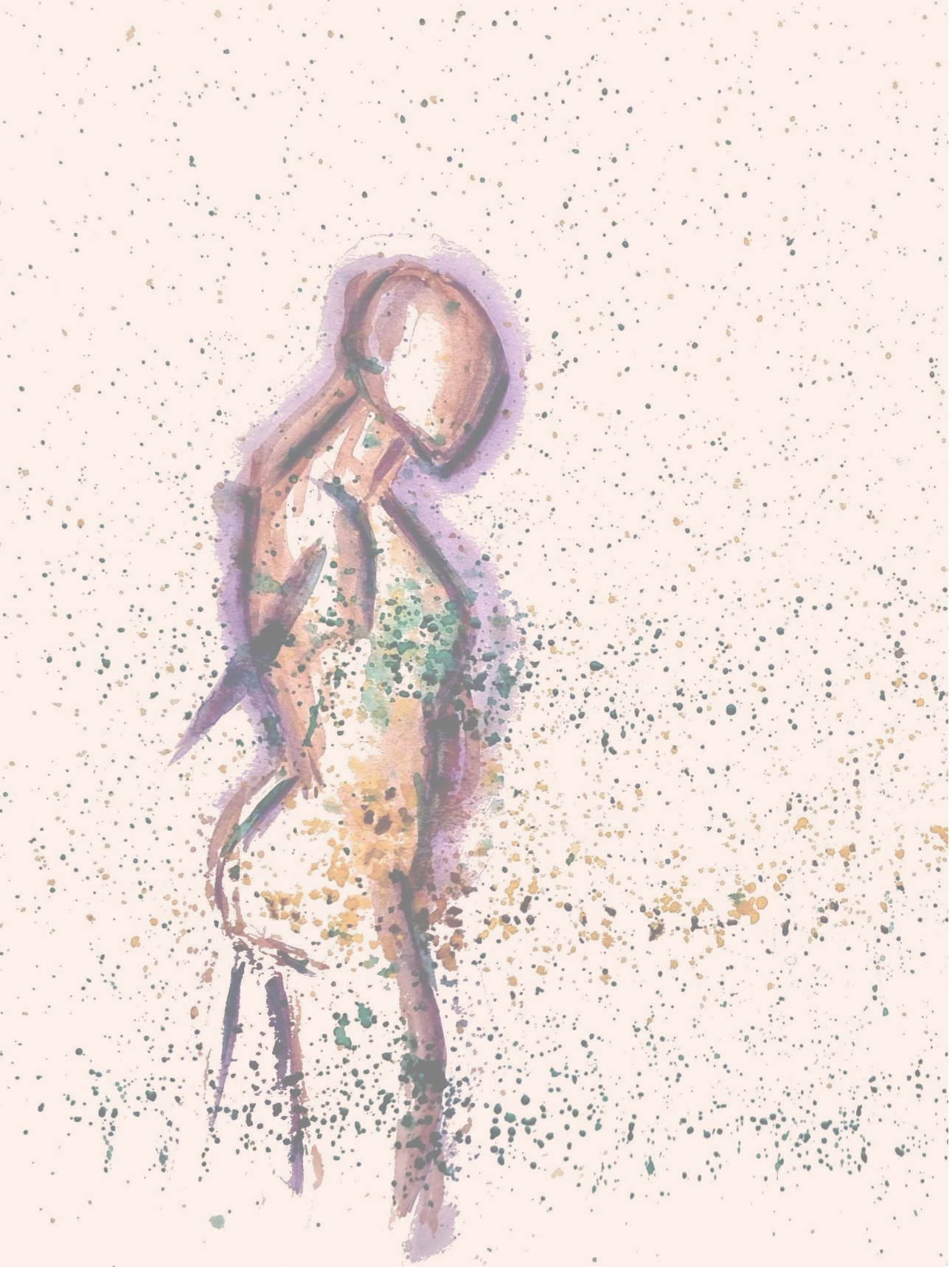

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General Introduction Thesis

Neuroendocrine Neoplasms

Ever since the first description of a neuroendocrine neoplasm, the 'Karzinoid' by dr. Oberndorfer in 1902,[1] clinicians have attempted to adequately characterise, grade and treat these heterogeneous malignancies. Still, despite elaborate and scrupulous efforts, an unmet need remains for further delineating these rare, often unpredictable malignancies and providing adequate prognostication and treatment strategies for patients and clinicians. The most important historical change in classification that took place was the distinction of patients who have quite the favourable disease course, even when metastatic disease may be present, from patients with aggressive cancers with very poor outcomes. This was the classification instated in 2000 by the World Health Organization (WHO) that identified the low grade, well-differentiated neuroendocrine tumours (NET) as a different entity than the high grade, poorly differentiated neuroendocrine carcinoma (NEC),[2] and was recently updated in 2019.[3] By making this distinction, patients could be accurately classified not only by disease course, but also by other characteristics, that would aid in the recognition and treatment of NEN. For instance, the well-differentiated nature of NET first of all means that NET have low proliferation indices, hence no response to cytotoxic therapies can be expected, since these often intervene in the proliferation cycle of cancer cells. Contrastingly, the high grade NEC may have very high proliferation indices, and show good responses to cytotoxic therapies.[4] In a similar situation, hormonal syndromes caused by secretion of peptides by the tumour solely occur in well-differentiated NET, whereas these do not have a place in the array of symptoms caused by NEC.

Although great steps for improving the classification and therefore prognostication of NEN have been made, a number of unmet needs remain. In solid tumours, the classical method for prognostication has long been the tumour, node, metastasis (TNM) staging system.[5] However, both the TNM system and the WHO NEN classification do not incorporate other possibly relevant factors for individual prognosis, including continuous variables such as age, or clinical variables such as performance status, gender or provided therapies. In this view, this thesis has attempted to search and identify determinants that aid in more tailored treatment and more accurate prognostication for patients with neuroendocrine neoplasia.

Small intestinal neuroendocrine tumours

The majority of NET arise in the gastroenteropancreatic tract, of which tumours arising in the small intestine (SI-NET) comprise the largest group, with an overall incidence of 0.5-1.42/100 000 persons.[6-8] A pathognomonic characteristic of SI-NET is mesenteric fibrosis (MF), a desmoplastic reaction surrounding the tumour and mesenteric lymph nodes, which leads to moderate-severe fibrosis of the mesentery.[9-11] MF can present with symptoms of ischemia or bowel obstruction, which is associated with a worse survival.[9, 11, 12] Currently, clinicians struggle to determine the amount of MF and whether it will indeed lead to serious morbidity, or will remain indolent. This is especially important in the setting of metastatic disease, where the question rises whether an intervention targeting the primary tumour and associated MF is justified.

Another important characteristic of SI-NET is the secretion of vasoactive peptides, of which the most common is 5-hydroxytryptamine, also called serotonin.[13] In the setting of localized disease, the excess of serotonin secreted by the tumour is metabolized by the liver to the inactive metabolite 5-hydroxyindoleacetic acid (5-HIAA).[14] Yet when liver or retroperitoneal metastasis is present, the hepatic metabolization is bypassed and peptides secreted by the tumours may access the circulation, giving rise to the carcinoid syndrome (CS), which occurs in 30-40% of patients with SI-NET.[15, 16] In 30% of patients with CS, elevated systemic levels of serotonin cause carcinoid heart disease (CHD). CHD consists of fibrotic changes of the endocardium, which leads to thickening and retraction of the heart valves.[10, 17, 18] Similar to the hepatic route, metabolization of serotonin to 5-HIAA occurs in the lungs, therefore CHD only effects the right-sided heart in most cases.[19, 20] To date, it was not possible to predict which patients develop CHD, and which do not, leading to frequent echocardiographic screening of all patients with elevated serotonin. Similarly, since such rare occurrence of the disease, large epidemiological studies are grossly lacking, and the effect of surgical valve replacement in patients with CHD has been highly understudied. This thesis provides an insight in the aforementioned gaps in the knowledge of small intestinal NET, and with this offers guidance for clinicians treating NET.

Bronchopulmonary neuroendocrine tumours

Another primary tumour location for NET is in the bronchopulmonary tract (bpNET). These tumours have a somewhat different nomenclature, and bpNET are classified in typical carcinoid (TC) and atypical carcinoid (AC). Classification is based on histopathological features, including mitotic count, the presence or absence of necrosis and a variety of cytological and morphologic features.[21] bpNET may arise sporadically or in the context of a hereditary predisposition, *e.g.* Multiple Endocrine Neoplasia type 1 (MEN1).[22] Another context in which bpNET may arise, is Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia (DIPNECH), a proliferation of neuroendocrine cells of the bronchoepithelium.[23] Although some epidemiological studies exist that describe the occurrence and prognosis of sporadic bpNET,[24] and even less of bpNET in MEN1 of DIPNECH,[22, 25] to date none had described and followed up bpNET in all different contexts.

Neuroendocrine carcinoma

Although NET have low proliferation indices and a relatively favourable survival compared to other malignancies, this can not be said of their poorly differentiated counterpart. As mentioned before, the Ki67-index of NEC often exceeds the cut-off value for the diagnosis of 20%, and has been described to range from 40-100%.[4] This is inevitably accompanied by rapid progression of disease and translates to a poor prognosis, with overall survival ranging from 11 to 20 months.[26] The majority of NEC are of pulmonary origin in the form of either small cell lung carcinoma (SCLC) or large cell neuroendocrine carcinoma (LCNEC).[3, 27, 28] Extrapulmonary NEC (EP-NEC) mostly originate from the gastroenteropancreatic tract, accounting for around

35-55% of all NEC.[29] Currently, the mainstay of EP-NEC treatment consist of cytotoxic regimens that are mostly based on regimens known from small cell lung carcinoma (SCLC). Since EP-NEC may have a different aetiology, arising from neuroendocrine cells, combined with the poor prognosis, there is a strong need to evaluate other possible treatment options that may benefit the duration as well as quality of life of patients with EP-NEC.

Another EP-NEC is the Merkel cell carcinoma (MCC), that arises from the skin and is currently regarded as a separate entity.[30, 31] MCC is an aggressive small cell NEC that occurs mostly in elderly patients and has an incidence of 0.5-0.8/100 000.[31-34] It has a unique pathogenesis, since in 80% of the patients in the Northern hemisphere, MCC is caused by the Merkel cell polyomavirus.[35-38] The remaining 20% has a oncogenic pathway that is associated with ultra-violet radiation exposure.[39] Similarly to other EP-NEC, the treatment of MCC has long been based SCLC guidelines, and consisted of surgery with postoperative radiotherapy in locoregional disease, and polychemotherapy in advanced disease.[31] Over the past decades however, changes have occurred in the treatment of MCC, the most practice-changing of which was the introduction of immunotherapy for the treatment of MCC in 2017. Before immunotherapy, the five-year survival rate of patients with advanced MCC was merely 7-12%. Recent updates of the initial trials of immunotherapy for patients with advanced MCC, showed survival rates of 59 and 26% three- and five-years survival, respectively, underscoring the true change in outcome that has been introduced.[40, 41] However, the question remains whether these impressive results of clinical trials are upheld in real-world oncological outcomes.

In this thesis, various deviations from previous, possibly outdated guidelines for NEC are described, including new treatment strategies for EP-NEC and real-world evaluations of current treatment strategies for MCC.

Thesis outline

Part I Tailoring prognosis in neuroendocrine neoplasia

As stated in the General Introduction, great efforts have been made to ensure uniform nomenclature and classify tumours according to aggressiveness of disease. Unfortunately, the rarity of NET, with an overall incidence ranging from 1.8-5.2/100 000,[42] limits the initiation of large studies to provide more in-depth analysis in the prognosis of patients with NET. Luckily, over the past decades, institutional databases of patients with rare diseases have become more common. As such, the Netherlands Cancer Institute and University Medical Centre Utrecht, a joint European Neuroendocrine Tumour Society (ENETS) Centre of Excellence, has set up an institutional NEN database, in which patient data is collected from 2000 on, and continues prospectively. This resource, together with (inter)national collaborations provides the opportunity to fill current gaps of knowledge or validate previously found results from other studies.

In 2010, a nomogram was developed that included patient and tumour characteristics of patients with SI-NET for the prediction of disease specific survival.[43] Such a nomogram could aid in providing a prognosis for patients and clinicians, and preferably could even tailor treatment decisions in clinical practice. In **Chapter 2**, with data from our institutional NEN database, we aimed to validate this nomogram in a large cohort of patients with SI-NET and assess whether this could serve as a practical tool in Dutch clinical practice. Additionally, in **Chapter 3**, we hypothesized that this nomogram and the clinical parameters included therein could be further improved by addition of a blood-based molecular genomic analysis that has been developed for NET, namely the NETest®.[44]

Sequencing of patient tumour material for possible mutations that may be of clinical benefit is increasingly being incorporated in oncological clinical practice.[45] Mutations that are known to drive oncogenic pathways are known to be common in many tumours,[46] but have yet been poorly elucidated in NET. In **Chapter 4**, we have combined results from whole genome sequencing and next generation sequencing to investigate the presence of driver mutations in metastatic SI-NET and the clinicopathological significance thereof.

Since a relatively large proportion of patients with SI-NET present with metastatic disease at diagnosis, curative surgery is often no longer an option.[47] Nevertheless, due to the mesenteric fibrosis with which SI-NET are associated, there is an ongoing debate whether resection of the primary tumour (PTR) should be performed even when other metastatic lesions can not be surgically removed.[48] Previously published retrospective studies that describe the survival benefit of PTR are likely highly confounded (i.e. patients that have lower disease burden or are more fit are more likely to receive surgery).[49, 50] In **Chapter 5**, we made use of an unique situation for the investigation of this research question. We investigated the influence of PTR on survival in two ENETS Centres of Excellence, the Netherlands Cancer Institute in Amsterdam and the

Aintree University Hospital in Liverpool who had adopted contrasting treatment approaches: upfront surgical resection *versus* watch and wait, respectively.

The occurrence and disease course of CHD can be capricious. First, although CHD is known to be caused by serotonin, albeit by a currently not fully elucidated mechanism, not all patients with elevated serotonin levels develop CHD.[51] Therefore current guidelines advice to screen for CHD by performing 1-2 yearly echocardiography.[52] Nevertheless, patients might develop CHD rapidly in between screening moments, whereas other patients may never develop CHD and undergo frequent and unnecessary visits to the outpatient clinic. In **Chapter 6**, we sought to validate known biomarkers as well as potential new biomarkers in both the prediction and detection of CHD, thereby identifying patients at increased risk of CHD, as well as patients that could be released from intensive echocardiographic screening.

This identification of patients at risk of developing CHD may further aid in timely intervention for CHD, such as valve replacement surgery.[53] Over the past decades, screening and treatment of CHD has improved, but CHD remains a poor prognostic factor in patients with SI-NET.[54] In **Chapter 7**, we describe the temporal influences on survival in the largest European cohort of patients with CHD to date.

As mentioned earlier, bpNET may arise sporadically, in the context of the MEN1 syndrome, or in a DIPNECH background. Until now, all these bpNET were considered the same entity, which is also reflected in the recently updated international guidelines.[25,26] However, based on clinical experience and earlier reports on the natural course of sporadic bpNET, MEN1-related bpNET and DIPNECH-related bpNET, the question arose whether these subtypes are in fact different entities; MEN1- and DIPNECH-related bpNET rarely metastasize or lead to bpNET-related death,[22] while the prognosis of sporadic bpNET seems more heterogeneous – and perhaps worse than non-sporadic forms of bpNET.[55] In **Chapter 8**, we provide the first head-to-head comparison between sporadic bpNET, and bpNET that arise in the context of MEN1 or DIPNECH.

Part II Tailoring treatment in neuroendocrine neoplasia

Most patients (75%) with MCC present with localized disease, of whom nearly half have lymph node involvement at diagnosis.[56] Since MCC is considered to be highly sensitive to radiation, postoperative radiotherapy (PORT) has been implemented in the standard of care for patients with MCC.[57] Based on a large study of overall survival of patients with stage I-III MCC, PORT is recommended in all primary MCC.[57] Yet since patients with MCC are often of an elderly and more frail population, and the adherence to PORT guidelines has been shown to be relatively poor,[58] it is possible that overall survival benefit of PORT is driven by treatment decision to administer PORT, rather than true treatment effect. To investigate this, we combined the data of three Dutch referral centres for MCC in **Chapter 9**, and studied the effect of PORT on recurrence, disease specific mortality and overall survival.

Unfortunately, although great efforts are made to contain MCC, metastatic disease can not be completely prevented. In other cases, patients already present with metastatic disease.[59] As mentioned previously, until 2017, advanced MCC was treated with polychemotherapy, albeit with poor outcomes. Since the introduction of immunotherapy, of which avelumab, a programmed-cell-death-1 inhibitor, was the first to be granted approval for treatment of advanced MCC.[60] Since then, avelumab was swiftly incorporated in standard clinical practice for MCC in all four referral centres for MCC in the Netherlands. In **Chapter 10**, we describe the first cohort of MCC patients treated outside of a clinical trial or pharmaceutical expanded access programme, hence giving an overview of the true treatment effect in clinical practice.

Since survival in EP-NEC is poor, clinicians are frantically searching for therapies that would improve the survival in these patients. Yet the rarity and aggressiveness of disease hampers the initiation and follow-up of studies including EP-NEC, due to long and difficult accrual and rapid progression. Mutations in the mammalian target of rapamycin (mTOR) are present in various cancers, including well-differentiated NET.[61, 62] Inhibition of the mTOR signalling pathway has shown antiproliferative effects in cell lines and primary cultures of human neuroendocrine tumours.[61] A therapeutic intervention that specifically targets this mTOR pathway is everolimus. The poor efficacy of current treatment options for EP-NEC, as well as the widely accepted anticancer activity of everolimus in patients with NEN, provided the rationale for **Chapter 11**, a phase 2 study of everolimus in combination with cisplatin in patients with advanced EP-NEC.

Finally, in **Chapter 12**, this thesis concludes with a general discussion, in which we summarize the main findings of the studies, discuss the clinical implications thereof and address future perspectives.

Chapter 13 provides an overall summary in English and in Dutch.

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Part I

Tailoring Prognosis in Neuroendocrine Neoplasia

2

Survival in Patients with Neuroendocrine Tumours of the Small Intestine: Nomogram Validation and Predictors of Survival

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Abstract

Neuroendocrine tumours of the small intestine (SI-NET) are rare and heterogeneous. There is an unmet need for prognostication of disease course and to aid treatment strategies. A previously developed nomogram based on clinical and tumour characteristics aims to predict disease-specific survival (DSS) in patients with a SI-NET. We aimed to validate the nomogram and identify predictors of survival. Four hundred patients with a grade 1 or 2 SI-NET were included, between January 2000 and June 2016. Predicted 5- and 10-year survival was compared to actual DSS. Multivariable analysis identified predictors for actual DSS. We found that in low-, medium- and high-risk groups 5-year nomogram DSS vs. actual DSS was 0.86 vs. 0.82 ($p < 0.001$), 0.52 vs. 0.71 ($p < 0.001$) and 0.26 vs. 0.53 ($p < 0.001$), respectively. Ten-year nomogram DSS vs. actual DSS was 0.68 vs. 0.69 ($p < 0.001$), 0.40 vs. 0.50 ($p < 0.001$) and 0.20 vs. 0.35 ($p < 0.001$), respectively. Age, WHO-performance score of 2, Ki-67 index ≥10, unknown primary tumour, CgA > 6x ULN and elevated liver tests were identified as independent predictors for a worse DSS. This shows that the nomogram was able to differentiate, but underestimated DSS for patients with a SI-NET. Improvement of prognostication incorporating new emerging biomarkers is necessary to adequately estimate survival.

Introduction

Neuroendocrine tumours (NET) represent a heterogeneous group of rare tumours, most commonly presented in the gastrointestinal and bronchopulmonary tract.[1, 2] The incidence of NET is increasing, with a reported incidence of 6.61 per 100,000 individuals in 2011.[3, 4] NETs of the small intestine (SI-NET) are, after pulmonary NET, the second most common NET and the most frequent malignancy of the small intestine.[4-7] Up to 43% of patients have metastases at time of diagnosis, predominantly in the liver.[1, 3, 8, 9] Currently, the only potentially curative treatment for patients with SI-NET consists of surgery.[10, 11] Unfortunately, only a minority of patients (20–30%) with metastasised NET are eligible for curative surgery.[10, 12, 13] In the palliative setting salvage surgery, surgery of the primary tumour, liver-directed therapies, somatostatin receptor analogues (SSAs) and peptide receptor radionuclide therapy (PRRT) are available.[11, 14, 15] Over the past decades, survival rates have increased, most likely due to the expanding therapeutic possibilities in the palliative setting and improved diagnostic techniques.[4] As a consequence, even though most patients present with metastatic disease, survival has been shown to be favourable, with a 5-year survival rate of 75%.[16-18]

Predicting prognosis for an individual patient with a SI-NET remains challenging due to their heterogeneous disease course.[19] Several studies have identified prognostic factors mainly based on clinical and tumour characteristics. Nevertheless, their role in daily clinical practice remains limited.[20-22] Additionally, in recent years, the genomic landscape of SI-NET has been under increasing investigation and identified several molecular prognostic factors.[23, 24] However, these factors have not yet been widely implemented into clinical practice. Alternatively, the scientific focus has undergone a shift towards the development of 'liquid biopsies': blood-based biomarkers that can be used in clinical practice to predict disease presence or prognosis. These may constitute of circulating tumour cells (CTC), miRNA or circulating tumour transcripts. In several malignancies, liquid biopsies were able to predict prognosis.[25-28] In neuroendocrine tumours, both CTC and circulating tumour transcripts showed promising results for monitoring disease.[29-31] For instance, the presence of ≥one CTC in blood samples of 178 patients with NET was shown to be independently associated with worse overall- and progression-free survival.[30] In a recent study of 152 patients with GEP-NET, circulating tumour transcripts (NETest®), using a cut-off of 33%, have been shown to be the strongest predictor for disease progression.[29] Yet, often in these studies the value of clinical and tumour characteristics is underappreciated. Despite the identification of several prognostic factors and biomarkers, currently there remains an unmet need for adequate prognostication to predict disease course and survival for individual patients with SI-NET.

In solid tumours, the classical method for prognostication has long been the tumour, node, metastasis (TNM) staging system.[32] Additionally, in SI-NET, several pathological grading systems have been established over the past decades. The final adjustment to this system dates from 2017, wherein a reclassification has taken place of grade III neuroendocrine carcinoma (NEC) to well-differentiated grade III NET and poorly differentiated NEC.[33] However, both the TNM

system and NET grading system fail to incorporate other possibly relevant factors for individual prognosis, including continuous variables such as age, or clinical variables such as performance status or gender. In this view, more elaborate statistical models for prognostication, i.e., medical nomograms, have been developed for several cancer types.[34-36] Nomograms have been shown to outperform the TNM staging system in predicting recurrence free- or disease-specific survival in several studies, demonstrating the clinical benefit of such models.[35-37] In 2010, Modlin et al. developed the first SI-NET nomogram based on clinical and tumour characteristics, to estimate an individual 5- and 10-year disease specific survival (DSS).[20] Two studies have aimed to validate this nomogram for clinical use in daily practice. One study included 121 patients who underwent surgery with curative intent for a SI-NET; another validated the nomogram in 70 patients with a SI-NET.[18, 38] To date, large validation studies have not been performed to assess the usefulness of the previously established nomogram and with that the value of clinical patient and tumour characteristics, in a real-world cohort of patients with a SI-NET with various stages of disease and treatment modalities.

We aim to assess whether prognostication based on this nomogram and the constituting clinical and tumour characteristics is suitable, especially considering the shifting focus to new emerging biomarkers. Therefore, in this study, we evaluate the prognostic ability of the nomogram in a large patient cohort treated in a European Neuroendocrine Tumour Society Centre of Excellence (ENETS CoE). In addition, prognostic predictors for survival were identified, which could contribute to further development of a prognostic model.

Patients and Methods

Patients

All patients with a well-differentiated, grade I or grade II SI-NET referred to the Netherlands Cancer Institute (NCI) and University Medical Centre Utrecht (UMCU), an ENETS CoE, January 2000–June 2016, were included for retrospective analyses. To avoid misclassification of grade III NET/NEC, grade III NET were excluded due to recent reclassifications in grading systems. Primarily, diagnosis was histopathology confirmed. When histopathological examination was not sufficient for a definitive diagnosis or in case of an unknown primary tumour, the consensus of a multidisciplinary expert panel was used to establish definitive diagnosis and assign the primary tumour type. Consensus was reached with the use of various parameters, such as elevated serum biomarkers: Chromogranin A (CgA), serotonin in thrombocytes or urinary 5-hydroxyindoleacetic acid (5-HIAA), typical desmoplastic fibrotic reaction in a mesenterial mass on imaging or functional symptoms referred to as the carcinoid syndrome (CS) or the presence of carcinoid heart disease (CHD). CS was considered flushing, diarrhoea and/or wheezing. CHD was confirmed with echocardiography. All relevant baseline and follow up characteristics were extracted from the longitudinal institutional neuroendocrine neoplasia database, which includes all patients treated in the centre. Since our centre functions as a tertiary referral centre, date of referral and consequently disease and clinical characteristics at time of referral were considered baseline for referred patients > 3 months after diagnosis.

Urinary 5-HIAA and serotonin in thrombocytes levels > upper limit of normal (ULN) were combined into one variable: 'elevated serotonin', since urinary 5-HIAA was replaced by the latter in clinical follow up. Follow-up, vital status and cause of death were recorded. The study was conducted in agreement with the NCI/UMCU ethical guidelines and all patients gave consent for the use of their medical data as per institutional protocol.

Handling of Missing Variables

Missing values were predicted using multiple imputation. Variables that were assumed to be missing not at random were excluded from multiple imputation (tumour size, Ki-67, tumour grade, World Health Organisation (WHO) performance score and ethnicity). To establish patterns of missing values in the remaining variables Little's missing completely at random (MCAR) was performed. CgA, serotonin and liver tests (including both liver function tests as liver enzymes) were found to be MCAR and were imputed using the fully conditional specification method. For imputation of continuous variables, a linear regression was used and for dichotomous variables logistic regression was used. The minimum amount of imputations was determined by the maximum percentage of missing data in the variables.

Nomogram and Prognostic Indicators

For all patients, individual predicted survival according to the nomogram was calculated. Nomogram survival reflects the predicted 5-year or 10-year DSS. For variables in which missing values were not imputed, best possible and worst possible scenario was created: missing values were assigned no points (scenario 1) or highest possible points (scenario 2), respectively. Hereafter, patients were divided in three equal strata: low-, medium- and high-risk stratum, according to their predicted survival probability. Actual DSS for these strata was calculated using Kaplan–Meier curves and was compared to the nomogram survival (for both scenarios 1 and 2) using paired signed rank test. DSS for the three risk strata were compared using the logrank test.

Additionally, the predictive value of the nomogram was evaluated in three patient categories. These categories were assumed to differ in a-priori survival probability: group 1 who underwent surgery with curative intent, group 2 who underwent surgery in a palliative setting, such as resection of primary tumour in metastatic setting or debulking surgery, and the final group 3 consisted of patients who were not eligible for surgical treatment. For these subgroups, nomogram survival was compared to actual DSS as well.

In our institutional patient cohort (both the original dataset as well as in the imputed dataset) a separate analysis to identify independent prognostic indicators for actual DSS was performed.

Statistics

Variables were analysed using descriptive statistics: median with interquartile range (IQR) for continuous variables, frequency and percentage for categorical variables. DSS was calculated from date of diagnosis or date of referral for patients > 3 months after diagnosis. Patients alive

before reaching one of the endpoints and patients who died of other causes were censored at their last time of follow-up or death, respectively. DSS and possible prognostic indicators were analysed using Kaplan–Meier curves, the logrank test and Cox's proportional hazards regression. Variables with a *p*-value < 0.2 were included in multivariable analysis. Variable selection for multivariable analysis was performed using backward stepwise selection retaining variables with a *p*-value < 0.05. To avoid collinearity, the absence/presence of CS and CHD were combined in one variable. The same was done for tumour grade and Ki-67, combining these variables in grade I, grade II and <5% Ki-67, grade II and Ki-67 ≥ 5% but < 10% and grade II ≥ 10%. Statistical analyses were performed using IBM SPSS Statistics software, version 25.0.

Results

Patients

A total of 400 patients were included. Patient characteristics at baseline and nomogram variables can be found in Table 1. In the cohort, 192 patients (48%) were male and patients had a WHO performance status of 0, 1, and 2 in 161 (40%), 129 (32%) and 34 (9%) patients, respectively. Median age was 63 years (IQR 55–71). A total of 244 patients (61%) was referred within 3 months of diagnosis. Median time to referral for the remaining patients was 18 months (IQR 7–57). The ethnicity records were missing in 130 patients (33%). However, within the remaining 270 patients the majority (*n* = 253, 93%) was Caucasian, 7 patients (3%) were Black and 10 patients (4%) had another ethnicity. In 96 patients (24%) no primary tumour could be identified but consensus was reached by the multidisciplinary expert panel on the origin of the tumour. Most patients (*n* = 267, 67%) presented with functional symptoms. In 24 patients (6%) CHD was present at time of referral. Over three-quarters of patients (*n* = 305, 76%) had distant metastases at referral, of whom 236 patients (77%) had liver metastases.

WHO grade I tumours accounted for the majority of patients (*n* = 265, 66%); grade II tumours were seen in 94 patients (24%). In 41 patients (10%), no distinction could be made between grade I and grade II. Nevertheless, these tumours were recognized as well-differentiated, low-grade tumours. Tumour size was determined by pathology reports and was available for patients that underwent surgery for their primary tumour (*n* = 138, 35%). Tumour size (cm) was <2, 2–2.5, 2.5–3 and >3 in 44 patients (11%), 22 patients (6%), 26 patients (4%) and 46 patients (12%), respectively. In approximately half of the patients (*n* = 202, 51%) Ki-67 index was <5%; 52 patients (13%) had a Ki-67 index between 5 and 10%, and 22 patients (6%) had a Ki-67 index ≥ 10%. After imputation, 107 patients (27%) had CgA levels > 6x ULN, 163 patients (41%) had elevated serotonin levels, and 35 patients (9%) had elevated liver tests.

Primary Treatment

In our cohort, 175 patients (44%) underwent surgery: 26 patients (7%) with curative intent and 149 patients (37%) with palliative intent. Nine patients (2%) underwent liver surgery for metastases. Somatostatin analogues were used by 152 patients (38%) whereas 21 patients (5%) were treated with nuclear- or radiotherapy, 4 patients (1%) were treated with liver embolization.

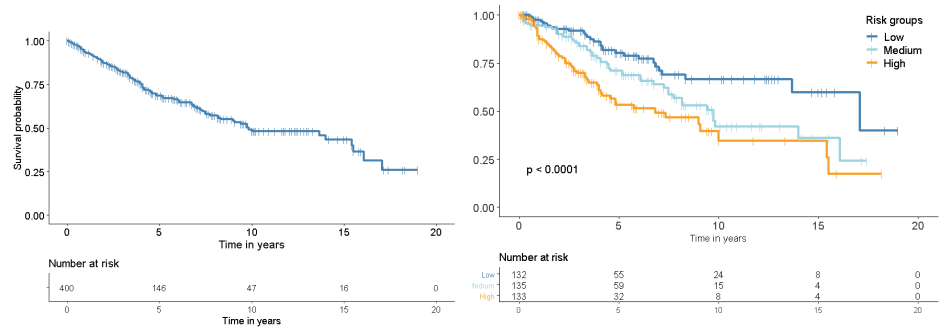
Patient Characteristics	*n* (%) or Median (IQR)	Imputed *n* (%)
Sex		
Male	192 (48)	
Female	208 (52)	
Age at baseline *	63 (55–71)	
WHO PS		
0	161 (40.2)	
1	129 (32.3)	
2	34 (8.5)	
Missing	76 (19)	
Ethnicity *		
Caucasian	253 (63.2)	
Black	7 (1.8)	
Other	10 (2.5)	
Missing	130 (32.5)	
Primary tumour		
SI	304 (76.0)	
Unknown primary	96 (24.0)	
Carcinoid syndrome *	267 (66.8)	
CHD *	24 (6.0)	
Distant metastases at baseline	305 (76.3)	
Liver metastases at baseline *	236 (59.0)	
Tumour grade *		
1	265 (66.2)	
2	94 (23.5)	
Unknown	41 (10.3)	
Tumour size *		
<2	44 (11.0)	
2–2.5	22 (5.5)	
2.5–3	26 (3.8)	
>3	46 (11.5)	
Missing	127 (31.8)	
Max Ki-67 index *		
<5	202 (50.5)	
<10	52 (13.0)	
≥10	22 (5.5)	
Missing	124 (31.0)	
Elevated CgA >6x ULN *	98 (24.5)	107 (26.8)
Missing	88 (22.0)	
Elevated serotonin *	143 (35.8)	163 (40.8)
Missing	212 (53.0)	

Patient Characteristics	*n* (%) or Median (IQR)	Imputed *n* (%)
Elevated liver tests *	33 (8.3)	35 (8.8)
Missing	53 (13.3)	
Treatment		
Groups		
1. Surgery with curative intent	26 (6.5)	
2. Surgery with palliative intent	149 (37.3)	
3. No surgery	225 (56.2)	
SSAs *	152 (38.0)	
Surgery	175 (43.8)	
Liver surgery *	9 (2.3)	
PRRT/RT	21 (5.3)	
Embolization	4 (1.0)	

Table 1. Baseline characteristics for the institutional cohort and imputed variables.* Nomogram variable; WHO PS: World Health Organisation Performance Score; G1: grade 1; G2: grade 2; SI: small intestine; UP: unknown primary; CS: carcinoid syndrome; CHD: carcinoid heart disease; CgA: chromogranin A; liver tests: any elevation of alkaline phosphatase, gammaglutyltransferase or bilirubin; ULN: upper limit of normal; SSAs: somatostatin analogues; PRRT: peptide receptor radionuclide therapy; RT: radiotherapy.

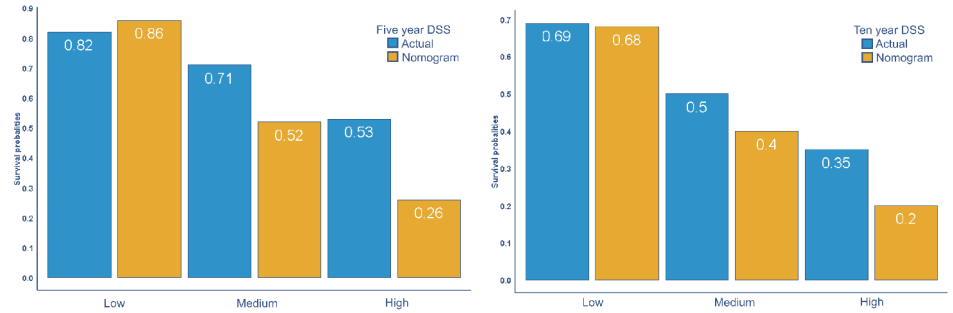
Nomogram Survival and Actual DSS

Median follow up time was 5.0 years with a median actual DSS of 9.8 years for patients from the institutional cohort (Figure 1a). At the end of follow up, 80 patients (20%) died of their SI-NET, 50 patients (13%) died of an unknown cause of death, 21 patients (5%) died of other causes and the remaining 249 patients (62%) were alive at end of follow up. Considering the separate strata, median actual DSS was 17.1 in the low-risk group, 9.8 years in the medium-risk group, and 6.8 years in the high-risk group. The nomogram was able to differentiate between low-, medium- and high-risk groups ($p < 0.001$, Figure 1b). The predicted nomogram survival compared to the actual DSS for scenario 1 can be found in Figure 2a,b. In this scenario, patients in the low-, medium- and high- risk group had a 5-year predicted nomogram survival of 86%, 52% and 26%, compared to an actual 5-year DSS of 82%, 71% and 53%, respectively (Figure 2a, $p < 0.001$). The 10-year predicted nomogram survival was 68%, 40% and 20% compared to the actual 10-year DSS of 69%, 50% and 35% in the low-, medium- and high-risk group, respectively (Figure 2b, $p < 0.001$). Similar significant differences in DSS were seen for scenario 2 and in the different treatment subgroups. The nomogram overestimated 5-year DSS in the low risk group, but underestimated DSS in all other groups. The predicted nomogram survival for scenario 1 and 2 and actual DSS divided by subgroups can be found in the Supplementary Material (Table S1,2).



A B

Figure 1. Kaplan–Meier curves for disease specific survival: (**a**) Disease specific survival curve of the institutional cohort; (**b**) Disease specific survival curves for low-, medium- and high-risk strata. Logrank test was performed for comparisons between survival.



A B

Figure 2. Actual disease specific survival vs. nomogram disease specific survival: (**a**) Five-year DSS categorised in low-, medium- and high-risk group, scenario 1; (**b**) Ten-year DSS categorised in low-, medium- and high-risk group, scenario 1.

The difference between nomogram survival and actual DSS ranged from 1% to 46%. The median difference was 24% (IQR 16–39) for 5-year survival and 20% (IQR 10–28) for 10-year survival. For all scenarios and treatment groups, the difference in predicted nomogram survival and actual DSS was the smallest for patients in the low-risk group, (median difference in survival 20%, IQR 1–29) compared to medium-risk (median difference in survival 22%, IQR 17–44) and high-risk group (median difference in survival 24%, IQR 9–36).

Prognostic Indicators

Univariable analysis identified age (HR 1.06), WHO performance score of 2 (HR 4.0), Ki-67 ≥ 10% (HR 1.3), grade 2 tumours (HR 1.8), an unknown primary tumour (HR 1.3), distant (HR 2.2) and liver metastases (HR 2.5), the presence of CS (HR 1.7) and CHD (HR 2.3), elevated CgA > 6

ULN (HR 4.3) and any elevated liver test (HR 4.9) to be associated with actual DSS (Table 2). In multivariable analysis age (HR 1.07), WHO performance status of 2 (HR 4.4), an unknown primary tumour (HR 3.2), Ki-67 index ≥ 10% (HR 12.6), CgA > 6 times ULN (HR 3.2) and elevated liver tests (HR 3.1) remained independent predictors for DSS in both the imputed as well as the non-imputed dataset. Results for multivariable analyses for both datasets can be found in Table 3.

Variable	HR	*p*	CI
Age	1.06	<0.001	1.04–1.08
Gender	0.72	0.067	0.51–1.02
Ethnicity			
Caucasian	1		
Black	1.11	0.882	0.27–4.55
Other	1.01	0.992	0.25–4.12
WHO PS			
0	1		
1	1.72	0.014	1.11–2.67
2	4.03	<0.001	2.27–7.14
Grade			
1	1		
2	1.83	0.003	1.23–2.74
Ki-67 index			
G1	1		
G2			
<5	1.00	0.994	0.46–2.16
<10	1.28	0.429	0.69–2.36
≥10	4.0	<0.001	2.16–7.40
Primary			
SI	1		
Unknown primary	1.34	<0.001	1.23–1.46
Distant metastases	2.23	0.001	1.39–3.68
Liver metastases	2.51	<0.001	1.70–3.70
CS	1.72	0.011	1.13–2.60
CHD	2.27	0.002	1.34–3.83
CgA > 6x ULN	4.28	<0.001	2.83–6.49
Serotonin > ULN	1.07	0.814	0.59–1.94
Liver tests > ULN	4.94	<0.001	2.92–8.36

Table 2. Univariable analysis for disease specific survival. WHO PS: World Health Organisation Performance Score; G1: grade 1; G2: grade 2; SI: small intestine; UP: unknown primary; CS: carcinoid syndrome; CHD: carcinoid heart disease; CgA: elevated chromogranin A; liver tests: any elevation of alkaline phosphatase; gammaglutyltransferase or bilirubin. ULN: upper limit of normal.

	Original Dataset			Imputed Dataset		
Variable	**HR**	***p***	**CI**	**HR**	***p***	**CI**
Age	1.07	<0.001	1.04–1.10	1.07	<0.001	1.04–1.09
WHO PS						
0	1			1		
1	1.11	0.756	0.58–2.14	1.13	0.676	0.62–2.07
2	4.40	<0.001	2.09–9.20	4.14	<0.001	2.01–8.51
Ki-67 index						
G1	1			1		
G2						
<5	2.38	0.121	0.79–7.11	2.51	0.067	0.94–6.73
<10	1.04	0.918	0.46–2.36	0.99	0.981	0.44–2.22
≥10	12.61	<0.001	5.51–28.84	11.56	<0.001	5.15–25.93
Primary						
SI	1			1		
UP	3.24	<0.001	1.42–4.74	2.32	0.003	1.32–4.01
CgA > 6x ULN	3.24	<0.001	1.91–5.46	3.43	<0.001	2.06–5.70
Liver tests > ULN	3.10	0.004	1.45–6.63	3.12	0.003	1.46–6.60

Table 3. Multivariable analysis for disease specific survival in original dataset and imputed dataset. WHO PS: World Health Organisation Performance Score; G1: grade 1; G2: grade 2; SI: small intestine; UP: unknown primary; CgA: elevated chromogranin A; liver tests: any elevation of alkaline phosphatase; gammaglutyltransferase or bilirubin. ULN: upper limit of normal.

Discussion

In this study, a previously designed nomogram, based on clinical and tumour characteristics, identified low-, medium- and high-risk groups in patients with SI-NET. However, 5- and 10-year survival was underestimated for all scenarios and treatment groups, except for 5-year DSS in the low risk group. In our population, in multivariable analysis age, a WHO performance status of 2, an unknown primary tumour, Ki-67 index ≥ 10%, elevated CgA > 6x ULN, and elevated liver tests were the strongest independent predictors for a worse DSS.

On the whole, predicted DSS by the nomogram was lower than the actual observed DSS. The low-risk subgroup for 5-year DSS in scenario 1 was the only subgroup where the opposite had occurred. This could be explained by the fact that in this scenario lowest possible nomogram scores were assigned to missing values in non-imputed variables, hence leading to an overestimation of DSS by the nomogram. The low-risk subgroup is the most susceptible to having assigned low nomogram points, because by definition, this group would have the highest DSS, and thus, the lowest nomogram score. This is illustrated by the fact that the difference between actual and nomogram DSS increases dramatically in scenario 2,

resulting in poorer nomogram predicted DSS. Therefore, it is established that the nomogram underestimates actual DSS across all risk groups.

The evaluated nomogram is based on a large dataset from 7455 patients from the SEER database and variables were selected and weighed after extensive analyses of literature-curated data. However, the nomogram itself was initially validated in only 33 patients. Two earlier studies have attempted to validate the predictive properties of the SI-NET nomogram. Clift et al. (2017) showed that the predicted nomogram DSS matched the observed 5-year and 10-year DSS in a cohort of 70 patients.[38] This difference between our cohorts is not easily explained; our populations were quite comparable with regard to the baseline characteristics underlining the caution that should be taken by extrapolating findings from one population to another. However, patients were more often treated with PRRT (21% compared to 5% in our cohort), which suggests that their patients might have had more extensive disease burden.

In the study performed by Kelly et al. (2019), the nomogram was able to predict survival of patients in a cohort of 121 patients who underwent surgery with curative intent.[18] However, the nomogram score was not identified as an independent predictor for survival in multivariable analysis. The authors argued that this might be explained by their high survival rate of patients at the end of their follow up period (90.9%). Similarly, our patients had a significantly higher DSS than was estimated by the nomogram. Nevertheless, both Clift et al. and Kelly et al. recognized the prognostic potential of a nomogram based on clinical and tumour characteristics, with the need for extensive validation and possibly improvement. Subsequently, Kelly et al. recently developed a new nomogram for patients specifically from the United States.[39]

Several studies have investigated survival of patients with NET over different time periods and also found that survival increases in patients diagnosed in more recent time periods.[3, 4] This is primarily due to new systemic treatment modalities that have emerged which have a beneficial effect on NET-related survival. SSAs made an entrance in 1987 and was the first systemic treatment option specifically for NET. Initially, SSAs were found to reduce symptoms of carcinoid syndrome, but an antiproliferative effect was shown in the PROMID and CLARINET study.[40-42]

PRRT was introduced in 2008. This treatment uses a radiolabelled somatostatin analogue to achieve local intratumoural nuclear therapy and showed a survival benefit for patients with a SI-NET treated with PRRT in the NETTER-1 study.[43] Additionally, diagnostic techniques for NET have improved, earlier detection could contribute to an improved survival.[4] Likewise, the underestimation of survival by the nomogram could be explained by the fact that the nomogram was based on studies from 1997 and 2010 and on SEER data from patients diagnosed between 1977–2007. As a consequence, the nomogram was probably based on a poorer survival outcome compared to our cohort.

We found that a Ki-67 index ≥ 10% was associated with a worse DSS. This cut-off value is currently not used in clinical practice. The cut-off point most suited for distinguishing between grade I and II NET has been the subject of debate since the introduction of the Ki-67 index in the ENETS grading system in 2007. In a systematic review by Richards-Taylor et al. it was postulated that grade II and Ki-67 index ≤ 5% NET was more similar to grade I NET, compared to a grade II NET with Ki-67 index ≥ 5%.[44] Although in our cohort, a Ki-67 index < 5%, or Ki-67 index < 10% could not be identified as a separate prognostic factor for DSS, it does support the notion that the current Ki-67 index subdivision used for grading SI-NET might be insufficient for adequate prognostication. Future studies should aim to determine which cut-off values, if any, albeit in combination with other histopathological characteristics, would be more suitable.

An elevated CgA at referral of > 6x ULN was associated with a shorter DSS. Several studies have shown the prognostic value of baseline CgA as well.[45-47] This is in line with previous studies indicating that CgA is a marker of bulky disease, which is associated with poor survival.[48] Others have discussed that a change of CgA (of 25–20%) might be a better prognostic predictor than a single measurement, however this needs further validation.[47, 48] Currently, there is a debate on the prognostic potential of CgA, since even in patients with metastatic disease, CgA is often within the normal range.[29] This suggests there might be room for improvement to decide the optimal threshold.

Remarkably, liver metastases did not prove to be an independent prognostic predictor for DSS. Many other studies did show this association.[7, 49-51] However, we included an unknown primary tumour as a separate variable in our multivariable analysis, which likely influenced this outcome since it overlaps with the presence of (liver) metastases. Yet, any elevation of liver test was associated with a worse DSS. It supports the notion that the extent of liver metastases (resulting in elevated liver test), might be more important than the presence of liver metastases alone.[51, 52]

A major strength of this study is the large patient population with detailed data on treatment and DSS. Moreover, our patient population treated in an ENETS CoE entails a representative patient population with a considerable follow up period incorporating all available therapies for patients with SI-NETs. Furthermore, we calculated the predicted nomogram survival and actual DSS for both best- and worst-case scenarios and included patients from all possible treatment categories. Additionally, to avoid confounding by indication, surgical treatment was excluded from analysis, since the policy in our centre is to perform a resection if technically achievable with either curative or palliative intention. Instead, nomogram survival and actual DSS was compared in different treatment groups. This ensures that the nomogram was evaluated in all possible real-world and clinically relevant patient groups, and assessed for its clinical validity in daily clinical practice.

Nevertheless, several limitations should be taken into consideration. Unfortunately, the included biomarkers were not always available because of changes in clinical practice over time. For example, CgA was measured from 2004, simultaneously abandoning urinary 5-HIAA

as a biomarker, while serotonin in thrombocytes was introduced in 2010. However, the variable use of biomarkers is consistent with other studies involving NETs.[11, 53]

Another downside of a retrospective cohort study is missing data. This is inherent to collecting data in a longitudinal clinical database compared to data collection within the framework of a clinical trial. We handled missing data with the use of multiple imputation. By only imputing variables which we found to be completely at random, imputed values are not expected to be biased and statistical power could be maintained. Survival analyses were performed with an imputed and non-imputed dataset. For missing and non-imputed data, a best- and worst-case scenario was calculated assigning no points or the highest points possible. In both the imputed as non-imputed dataset and in various scenarios, our results were highly comparable. This suggests that, despite the retrospective character, our results are robust.

On the whole, while nomograms can fulfil an important role in personalized cancer care, when it accurately models clinical outcome,[36] this nomogram in its current form and the clinical characteristics constituting the nomogram unfortunately appear insufficient to accurately predict individual prognosis. Recent advances in identifying (molecular) prognostic factors and the development of liquid biopsies such as CTC, or circulating tumour transcripts (the NETest®), appear to be a valuable addition for individualized prognostication.[31, 54] Prognostic studies often focussed either solely on clinical and tumour characteristics[3, 4, 7, 10] or the sole predictive value of a biomarker alone (subsequently abandoning the use of clinical and tumour characteristics).[29, 53, 55] Our study confirms the prognostic potential of a nomogram based on clinical and tumour characteristics while underlining the need for improvement. Future studies should aim to combine clinical and tumour prognostic factors with potential new (molecular) biomarkers. By doing so, an advanced method of prognostic modelling for individual patients could be achieved.

Conclusions

A nomogram based on clinical data and tumour characteristics was the first extensive attempt for individual prognostication. The nomogram was able to differentiate between survival for patients in the low-, medium- and high-risk groups. However, the nomogram underestimated survival for 5- and 10-year survival for all but one scenario and all treatment groups. Age, WHO performance status of 2, an unknown primary tumour, Ki-67 index ≥ 10%, elevated CgA > 6x ULN, and elevated liver tests were identified as independent predictors for a worse DSS. Our findings imply that improvement of individualized estimation of prognosis is desirable. Future studies should aim to combine clinical and tumour prognostic factors with potential new (molecular) biomarkers.

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Supplementary material

Stratum	Actual 5Y DSS	Nomogram 5YDSS	P	Actual 10Y DSS	Nomogram 10YDSS	P
Scenario 1						
Low	0.82	0.86	<0.001	0.69	0.68	<0.001
Medium	0.71	0.52	<0.001	0.50	0.40	<0.001
High	0.53	0.26	<0.001	0.35	0.20	<0.001
Scenario 2						
Low	0.89	0.64	<0.001	0.79	0.50	<0.001
Medium	0.70	0.25	<0.001	0.46	0.20	<0.001
High	0.47	0.08	<0.001	0.29	0.08	<0.001

Table S1. Nomogram survival and actual DSS. Stratum: column indicating low-, medium- or high risk groups based on nomogram scores; 5Y DSS: five year disease specific survival, 10Y DSS: 10-year disease specific survival

	Actual 5Y DSS	Nomogram 5YDSS	P	Actual 10Y DSS	Nomogram 10YDSS	P
Scenario 1						
Curative surgery						
Low	0.96	0.88	<0.001	NE	0.70	<0.001
Medium	NE	0.56	<0.001	NE	0.43	<0.001
High	NE	0.33	<0.001	0.67	0.23	<0.001
Palliative surgery						
Low	0.82	0.86	<0.001	0.78	0.68	<0.001
Medium	0.67	0.49	<0.001	0.46	0.38	<0.001
High	0.50	0.26	<0.001	0.33	0.20	<0.001
No surgery						
Low	0.81	0.85	<0.001	0.69	0.68	<0.001
Medium	0.68	0.52	<0.001	0.49	0.40	<0.001
High	0.50	0.26	<0.001	0.36	0.19	<0.001
Scenario 2						
Curative surgery						
Low	0.93	0.69	<0.001	NE	0.54	<0.001
Medium	NE	0.23	<0.001	NE	0.16	<0.001
High	NE	0.08	<0.001	0.67	0.08	<0.001
Palliative surgery						
Low	0.83	0.67	<0.001	0.78	0.53	<0.001
Medium	0.67	0.24	<0.001	0.42	0.18	<0.001
High	0.50	0.08	<0.001	0.36	0.08	<0.001
No surgery						
Low	0.91	0.59	<0.001	0.74	0.45	<0.001
Medium	0.69	0.23	<0.001	0.36	0.18	<0.001
High	0.45	0.08	<0.001	0.30	0.08	<0.001

Table S2. Nomogram survival and actual DSS. Stratum: column indicating low-, medium- or high risk groups based on nomogram scores. 5Y DSS: 5-year disease specific survival, 10Y DSS: 5-year disease specific survival. NE: no events in group.

3

Is addition of NETest to clinical parameters valuable in the prognostication of patients with small intestinal neuroendocrine tumours?

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Small intestinal neuroendocrine tumours (SI-NET) are rare epithelial malignancies, with an age-adjusted incidence of 0.81/100,000 person-years in the Netherlands.[1] The disease course of SI-NET is overall favourable compared to other malignancies, with a five-year overall survival ranging between 57-75% for stage I-II to IV, respectively.[1] Nevertheless, the heterogeneity in disease course of SI-NET is large, since some patients may live over two decades with metastatic disease, whereas others die swiftly after diagnosis, with seemingly similar disease characteristics.[2] To aid in the prognostication of patients with SI-NET, several methods have been developed over the past years. First, prediction models such as nomograms have been developed for disease specific survival (DSS) based on large amounts of patient data, to assess the joint predictive value of known patient and tumour characteristics.[3] This model has been validated by our group in a large cohort of 400 patients with SI-NET, where it was found that the nomogram was able to discriminate between risk groups, but unfortunately, underestimated DSS in this cohort.[4] Besides patient and tumour characteristics, more novel methods have been developed recently, such as the blood molecular genomic analysis: NETest®. This is a multianalyte algorithmic analysis which provides an activity score, derived from circulating transcripts of 51 target genes involved in tumour biology. The NETest® has been shown to have a strong predictive value for progression free survival (PFS) in patients with gastroenteropancreatic NET (GEP-NET).[5] We hypothesized that the addition of the NETest® to the previously validated nomogram would aid in the prognostication of DSS and possibly PFS in patients with SI-NET. To investigate this, we included consecutive patients with a SI-NET that were referred to the Netherlands Cancer Institute/University Medical Centre Utrecht European Neuroendocrine Tumour Society (ENETS) Centre of Excellence between 2000-2018. From a random subgroup of these patients a NETest® sample was collected at cross-sectional time points between 2014-2017. Multivariable backwards cox regression including all variables that were part of the nomogram was performed to identify clinical parameters with the strongest association with DSS. These parameters were included in the multivariable models for DSS and PFS. Subsequently, NETest® outcomes with a cut-off of 40% were included in the models. A cross-sectional design was used for models including the NETest®, where DSS and PFS were defined as time of NETest measurement until death from SI-NET or Response Evaluation Criteria in Solid Tumours (RECIST) 1.1 documented progression, respectively. Since the cohort with NETest sample was smaller, with inherently less DSS events, additional backwards regression was performed to identify whether NETest® was a significant predictor for DSS. Patients that died of other causes or were alive at end of follow up were censored, patients that died of unknown causes were considered to have died of SI-NET. A total of 523 patients with a SI-NET were included. From these patients, a NETest® sample was collected from 104 (19.9%) patients. Median DSS for all patients was 14.0 years (confidence interval [CI] 1.01-17.9). Median DSS for the cross-sectional approach was not reached, and median PFS was 3.2 years (CI 2.1-4.3). Backwards regression of the complete cohort yielded a model with seven significant clinical parameters. These were age (hazard ratio [HR] 1.05, p<0.001), regional (HR 2.73, p=0.029) and distant (HR 2.23, p=0.011) disease stage, Ki67-index ≥10 (HR 2.52, p=0.003), carcinoid heart disease (HR 2.07, p=0.007), elevated Chromogranin A (HR 2.72, p<0.001), serotonin (HR 3.23, p=0.007) and liver tests (HR 1.89, p=0.014). Results from the backwards regression model can be found in Table

1. When the cross-sectional approach was performed for DSS with the addition of NETest®, the cut-off of 40% was not a significant predictor in the model (HR 1.5, p=0.629). Also, backwards regression of the cross-sectional model identified only a Ki67-index ≥10 (HR 8.84, p=0.015) as a significant contributor to DSS. Contrastingly, when the same approach was performed for PFS, only NETest® (HR 3.53, p<0.001) and age (HR 1.03, p=0.041) were significant predictors for PFS. Results of the PFS model can be found in Table 2.

Characteristic	HR	CI	p-value
Age at referral	1.05	1.03-1.07	<0.001
Stage			
Local	1		
Regional	2.73	1.11-6.70	0.029
Distant	2.23	1.21-4.13	0.011
Ki67-index			
G1	1		
G2			
<5	0.91	0.45-1.81	0.782
<10	0.76	0.42-1.36	0.357
≥10	2.52	1.38-4.61	0.003
Unknown	2.13	1.24-3.66	0.006
Carcinoid syndrome			
No functional symptoms	1		
Functional symptoms	1.29	0.83-2.01	0.265
CHD	2.07	1.22-3.53	0.007
Chromogranin A			
Elevated	2.72	1.79-4.14	<0.001
Unknown	2.17	1.32-3.58	0.002
Serotonin			
Elevated	3.23	1.37-7.61	0.007
Unknown	3.96	1.66-9.43	0.002
Liver tests			
Elevated	1.89	1.14-3.13	0.014
Unknown	1.28	0.67-2.44	0.449

Table 1. Variables identified through backwards cox regression. HR: hazard ratio, CHD: carcinoid heart disease.

These results show that the NETest® is superior in predicting PFS over known relevant patient and tumor characteristics, but unfortunately fails to be of added value in the prediction of DSS. This is in in line with a previous independent study, where the NETest® proved to be the strongest predictor for disease progression while CgA was a stronger predictor for overall survival in a head-to-head comparison.

Our results also illustrate the poor correlation between PFS and OS in GEP-NET. PFS is an inadequate surrogate marker in neoplasms that have relative long periods of survival, after progression is being concluded (postprogression survival; PPS). In GEP-NET, it is unclear if systemic therapy alters tumor biology and influences survival in the long term. Large studies like RADIANT-2 and NETTER-1 did not show an overall survival benefit for everolimus and PRRT respectively, while PFS was significantly longer in treatment subgroups.

The NETest® score is based on the summed expression of biologically relevant gene clusters, involved in neoplastic processes such as tumor proliferation. A parameter that seeks to reflect the molecular biology of a tumour is more susceptible to systemic therapy and other factors influencing the tumor micro-environment, therefore making it more volatile and less reliable for long-term prediction. In contrast, static patient- and tumor characteristics present at diagnosis, like disease stage, liver function and carcinoid heart disease, hardly change after treatment initiation and have important implications for DSS.

These outcomes, with recent survival data from large studies, questions whether current study endpoints actually reflect patient benefit but also underscores the importance of accurate patient selection to optimize treatment outcome. Accurate delineation of the disease status is one of the many parameters involved in treatment decision making. Besides, from patient point of view, the prevention and treatment of symptoms associated with progression can be as important as DSS. Therefore, PFS and DSS must be seen as two separate clinical outcomes, both being of clinical relevance, instead of one being surrogate for the other. Prognostication in SI-NET should be based on static clinical parameters like age, disease stage at diagnosis, Ki-67 index, functional status (reflected by serotonin), presence of carcinoid heart disease and tumor load (reflected by CgA). These parameters have a predictive value over longer term for patients. Disease progression is best predicted by age and target gene expression, as reflected by the NETest. This gene signature is the best predictor of tumour behavior in short term, but has no added value in DSS. Future research is needed to determine if accurate delineation of disease status, with help of the NETest®, can improve patient selection before treatment initiation and subsequently increase DSS.

Characteristic	HR	CI	p-value
Age at NETest®	1.03	1.00-1.07	0.040
Stage*			
Regional	1		
Distant	1.32	0.43-4.08	0.631
Ki67-index			
G1	1		
G2			
<5	1.61	0.62-4.22	0.332
<10	1.49	0.62-3.60	0.371
≥10	1.86	0.51-6.78	0.344
Unknown¥	n/a	n/a	n/a
Carcinoid syndrome			
No functional symptoms	1		
Functional symptoms	1.45	0.63-3.33	0.381
CHD	1.06	0.31-3.61	0.925
Chromogranin A			
Elevated	1.14	0.46-2.84	0.779
Unknown¥	n/a	n/a	n/a
Serotonin			
Elevated	1.71	0.70-4.20	0.243
Unknown	1.82	0.49-6.71	0.370
Liver tests			
Elevated	1.06	0.55-2.03	0.873
Unknown	1.99	0.23-17.87	0.531
NETest®			
≤40%	1		
>40%	3.53	1.87-6.66	<0.001

Table 2. Multivariable cox regression for progression free survival, including NETest®. HR: hazard ratio, CHD: carcinoid heart disease. * At the cross-sectional time point no patients with local disease stage were included. ¥ At the cross-sectional time point no patients had an unknown Ki67-index or Chromogranin A.

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4

Driver mutations occur frequently in metastases of well-differentiated small intestinal neuroendocrine tumours

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Abstract

Aims

This study aims to investigate the clinicopathological significance of driver mutations in metastatic well-differentiated SI-NETs

Methods and results

Whole genome sequencing (WGS) of 35 and next generation sequencing (NGS) of 8 metastatic SI-NETs was performed. Biopsies were obtained between 2015 and 2019. Tumours were classified using the 2019 WHO classification. WGS included assessment of somatic mutations in all cancer related driver genes, tumour mutational burden (TMB) and microsatellite status. NGS entailed a cancer hotspot panel of 58 genes. Our cohort consisted of 21% G1, 60% G2 and 19% G3 SI-NETs. Driver mutations were identified in approximately 50% of SI-NETs. In total 27 driver mutations were identified, of which 74% in tumour suppressor genes (e.g. *TP53, RB1, CDKN1B*) and 22% in proto-oncogenes (e.g. *KRAS, NRAS, MET*). Allelic loss of chromosome 18 (63%), complete loss of *CDKN2A* and *CDKN1B* (both 6%) and CDKN1B mutations (9%) were most common. Potential targetable genetic alterations were detected in 21% of metastasized SI-NETs. All tumours were microsatellite stable and showed low TMB (median 1.10, IQR 0.87-1.35). Ki67 proliferation index was significantly associated with the presence of driver mutations (p=0.015).

Conclusion Driver mutations occur in 50% of metastasized SI-NETs and their presence is associated with high Ki67 proliferation index. The identification of targetable mutations render these patients potentially eligible for targeted therapy.

Introduction

Well-differentiated neuroendocrine tumours (NETs) represent a group of rare tumours characterized by a relatively indolent disease course. Well-differentiated NETs harbour relatively few genomic mutations and are often characterized by changes in the methylation machinery.[1] Neuroendocrine carcinomas (NECs) in contrast, have an aggressive clinical course and a dismal prognosis. Gastro-entero-pancreatic (GEP) NECs share oncogenic pathways with adenocarcinomas and have a relatively high mutational burden. To illustrate, the genetic make-up of GEP-NECs includes loss of heterozygosity of *APC, TP53* and *DCC* tumour suppressor genes as well as mutations in *TP53, KRAS* and *BRAF* genes, which are typical for gastrointestinal adenocarcinomas.[2]

Neuroendocrine neoplasia (NEN) are graded according to the World Health Organisation (WHO) grading system as grade 1, 2 or 3, based on proliferation rate, as quantified by mitotic and Ki67 proliferation index. Until 2017, all NEN of the digestive tract with a Ki67 proliferation index >20% were classified as NEC, regardless of clinical disease course or tumour morphology. In 2016, it was observed that a group of well-differentiated neuroendocrine tumours of the pancreas displayed a Ki67 proliferation index >20%.[3] These tumours were classified as grade 3 well-differentiated neuroendocrine tumours. This term was adopted by both the WHO classification of neuroendocrine tumours as published in 2017 and subsequently by the WHO classification of tumours of the digestive system,[4] concerning all NEN arising throughout the gastrointestinal tract and the hepatopancreaticobiliary organs. Mutational status of neuroendocrine neoplasms is currently not integrated in the clinicopathological classification. At initial presentation, histological grades of well-differentiated NETs can vary from grade 1 to 3. It is now assumed that NETs can progress from grade 1 to grade 2 to grade 3. The factors underlying such progression are currently unknown. In contrast, to our knowledge progression of well-differentiated NETs to NECs has not been reported. At time of diagnosis, 27-43% of patients with small intestinal NETs (SI-NETs) have metastatic disease.[5-7] For patients with metastatic disease, treatment is based on the availability of several treatment modalities, e.g. somatostatin analogues, peptide radionuclide receptor therapy (PRRT) and liver directed therapies. These treatment modalities generally slow down clinical progression but do not provide curation for the disease. However, no therapies are currently available which specifically target genetic alterations in NETs. The present study aims to investigate the presence of driver mutations in metastatic SI-NETs and to explore the clinicopathological significance of these mutations, by investigating whether they are related to tumour characteristics such as tumour grade and whether they provide a rationale for targeted therapy.

Materials and methods

Patient cohort and study procedures

For the analyses, patients with metastatic SI-NETs were selected, whom were included under the study protocol (NCT01855447) of the Center for Personalized Cancer Treatment (CPCT). The

CPCT-02 protocol was approved on the first of August 2011 by the medical ethical committee of the University Medical Center of Utrecht (NL35781.041.11) and was conducted in accordance with the Declaration of Helsinki. Patients were eligible for inclusion if the following criteria were met: (1) age ≥ 18 years; (2) locally advanced or metastatic solid tumour; (3) indication for new line of systemic treatment with registered anti-cancer agents; (4) safe biopsy according to the intervening physician. The biopsies analysed for this study were taken between April 2016 and February 2019. All patients (n=35) provided informed consent. The study procedures consisted of the collection of matched peripheral blood samples for reference DNA and image-guided biopsy of a single metastatic lesion.

Whole genome sequencing data

WGS was performed on fresh frozen samples. One or two biopsies were selected with no visible necrotic tissue and freeze sections were cut to ensure a sufficient tumour cell percentage (>20%). Collection and whole genome sequencing of samples at Hartwig Medical Foundation (HMF) was performed according to the standard procedures as described in detail previously by Priestley et al.[8] All procedures at HMF are automated as much as possible and the Illumina® HiSeqX and NovaSeq6000 platforms are used for sequencing. During the process, shallow whole-genome sequencing is first used to determine an accurate tumour purity of the received and processed tumour samples before continuing full sequencing of the samples with sufficient tumour content (molecular tumour cell percentage >20%). Sequencing data is analysed with an optimized in-house bio-informatic pipeline designed to detect all types of somatic alterations, including single and multiple nucleotide substitutions (SNV and MNV), insertions and deletions (indels), copy number alterations (amplifications and gene copy losses) and genomic rearrangements and structural variants (e.g. gene fusions) in 508 cancer related driver genes (Appendix 1).[9] Furthermore, tumour mutational burden (TMB) and microsatellite stability score are provided. The tumour mutational burden score represents the number of all somatic variants across the whole genome of the tumour per Mega base (Mb). Tumour mutational load is the total number of somatic missense variants across the whole genome of the tumour. Patients with a mutational load over 140 could be eligible for immunotherapy. The microsatellite stability score represents the number of somatic inserts and deletes in (short) repeat sections across the whole genome of the tumour per Mb. The score is considered as a marker for instability in microsatellite repeat regions. Tumours with a score greater than 4.0 are considered microsatellite unstable (MSI). A comparison between the tumour biopsy and blood sample is performed to filter out germline polymorphisms and in order to be able to report somatic variants only. All code and scripts used for analysis of the WGS data are available via Github.[10] HMF has established procedures for WGS under ISO17025 accreditation. The genomic data is presented in a detailed molecular patient report which describes all variants which are relevant for cancer treatment decision making and gives a visual overview of the genomic data using CIRCOS plots. Appendix 2 provides more information on the interpretation of CIRCOS plots.

Clinical and WGS data

WGS data and corresponding clinical data were obtained from HMF under data request number DR-070 on the 5th of June 2019. Both WGS and clinical data are freely available for academic use from HMF (https://www.hartwigmedicalfoundation.nl/) through standardized procedures and after approval by the Data Access Board. Germline data was not included in the request.

Clinical and NGS data

In routine diagnostic practice, there was an opportunity to perform NGS on 8 liver biopsies of metastasized SI-NETs. Biopsies were received between August 2015 and November 2019. In all patients, NGS with a cancer hotspot mutation panel of 58 genes was performed. All patients consented for the use of their clinical information according to the opt-out consent procedure at the Netherlands Cancer Institute. By default specific clinical information may be used for research, unless a patient explicitly states he or she objects.

Histopathology

Of all patients (n=43), diagnosis was confirmed by histopathological revision of representative slides, consisting at least of hematoxylin and eosin slides and the following immunohistochemical stainings: Ki67, chromogranin and synaptophysin. The slides were revised by an experienced NET pathologist (JB) using the criteria of the WHO classification of tumours of the digestive system 2019 to ensure only well-differentiated SI-NETs were included in this study and neuroendocrine carcinomas were excluded. For 16 patients (37%), slides stained for Somatosatin Receptor 2A (SSTR2A) were available for assessment.

Immunohistochemical stainings

Formalin-fixed paraffin embedded (FFPE) sections were obtained from biopsies and from resection specimens. Four-micrometer FFPE slides were immunohistochemically stained using the following antibodies: anti-Chromogranin A (LK2H10) primary antibody (Roche, ready to use), Synaptophysin (27G12) (Leica/Novocastra, 1:50 to 1:100), Ki67 Antigen, MIB 1 Concentrate (Agilent/Dako, 1:100) and Recombinant Anti-Somatostatin Receptor 2 antibody (UMB1)-C-terminal (ab134152) (Abcam, 1:400 to 1:800). Immunochemistry was performed on BenchMark Ultra equipment (Ventana Medical System Inc., Tucson, AZ). Positive SSTR2A staining was defined as moderate to strong staining, including circumferential staining, essentially as described by Körner et al.[11] and Mehta et al.[12] The proportion of stained tumour cells was expressed in percentages with increments of 10.

Statistical analysis

Patient and tumour characteristics and DNA sequencing results were described using descriptive statistics. Association between Ki67 proliferation index and presence of driver mutations was assessed using the Mann-Whitney U test. Disease specific survival (DSS) was defined as time from biopsy to disease specific death or date of follow-up. Patients alive or lost to follow-up were censored. DSS was analyzed using the Kaplan-Meier method. IBM SPSS v25 (SPSS Inc., Chicago, IL) was used to perform all statistical analysis.

Results

Patients and tumour characteristics

Baseline patient characteristics are shown in Table 1. Of this cohort (n=43), the median age at diagnosis was 61 years (IQR 56-67). Fifty-three percent of patients were male. Of the total of 43 tumours, 9 were grade 1 (21%), 26 grade 2 (60%) and 8 were grade 3 (19%). All tumours, irrespective of grade were 100% Synapthophysin and Chromogranin positive. SSTR2A expression was positive in all grade 1 tumours and ranged from 50% to 100% in the grade 2 and 3 tumours. There was no significant correlation between SSTR2A expression and mutational status (p=0.840).

Patient characteristics	**N=43**
Median age at diagnosis (IQR)	61 (56-67)
Sex n(%)	
Total	43
Male	53
Female	47
Grade n(%)	
Total	43
G1	9 (21)
G2	26 (60)
G3	8 (19)

Table 1 Baseline characteristics for all 43 included patients with metastasized SI-NETs.IQR: interquartile range, G1: grade 1, G2: grade 2, G3: grade 3.* According WHO classification of tumors of the digestive system 2019.

Whole genome sequencing

WGS data on 35 metastatic NET samples obtained from HMF revealed a total of 23 driver mutations in 17 patients (49%). Of all driver mutations (n=23), 17 (74%) were present in tumour suppressor genes (*e.g. TP53, RB1, ATM, CDKN1B, SMAD2*) and 5 (22%) in proto-oncogenes (*KRAS, NRAS, CTNNB1*). All tumours were microsatellite stable (microsatellite stability score). The tumour mutational burden and load of all tumours was low with a median of 1.098 variants per Mb (IQR 0.870-1.350) and a median of 21 (IQR 10.5-28.0), respectively. In Figure 1, TMB of all samples is shown. The above mentioned WGS findings are shown in Table 2 and 3. Allelic loss of chromosome 18 was present in 63% of tumours. Other recurrent events were complete loss of *CDKN2A* and *CDKN1B* (both 6%) and *CDKN1B* mutations (9%).

WGS results	**N (%)/median (IQR)**
Mutational status	
Total	35
Patients with driver mutations	17 (49)
Driver mutations	23
Tumor suppressor genes	17 (74)
Proto-oncogenes	5 (22)
Unknown	1 (4)
No genomic aberrations	18(51)
Tumor mutational load (n=25), median (IQR)	21 (10.5-28.0)
Tumor mutational burden (variants per Mb) (n=25), median (IQR)	1.098 (0.870-1.350)
Microsatellite status (n=25), median (IQR)	0.0311 (0.0233-0.0495)

Table 2 Whole genome sequencing findings for 35 patients with metastasized SI-NETs. IQR: interquartile range, WGS: whole genome sequencing

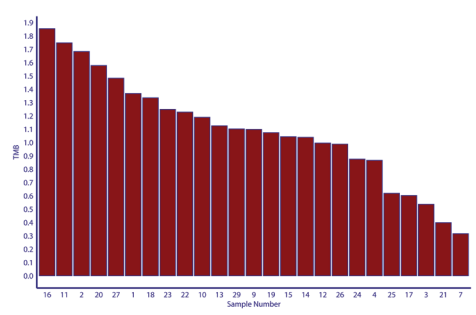

Figure 1 Tumor mutational burden per metastasized SI-NET sample**.** The TMB score represents the number of all somatic variants across the whole genome of the tumor per Mega base (Mb).

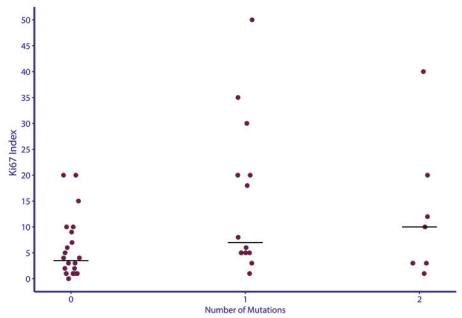

Figure 2. Number of mutations per patient compared with the Ki67 proliferation index. The horizontal line represents the median Ki67 proliferation index per number of mutations. For zero mutations, the median Ki67 proliferation index was 3.5 [interquartile range (IQR) 1.0–9.25]; for one mutation, the median Ki67 proliferation index was 7.0 (IQR 5.0–22.5); and for two mutations, the median Ki67 proliferationindex was 10.0 (IQR 3.0–20.0).

Next generation panel sequencing

In 8 patients NGS was conducted as part of routine diagnostic practice. NGS identified 4 tumours with driver mutations. The specific mutations of these tumours are shown in Table 4.

Samples	Driver mutations
Sample 4	SMAD2 c.1090C>T, p.Gln364 (TS) CDKN1B, c.92_03insCC, p.Leu32fs (TS)
Sample 9	TP53, c.19G>C, p.Asp7His (TS)
Sample 10	CDKN1B, c375_378delTGAG, p.GLu126fs (TS)
Sample 12	URB5, c.3622_3624delTGT, p.Cys1208del (TS)
Sample 14	KRAS, c.64C>A, p.Gln22Lys (PO)
Sample 15	SPEN, c.785C>A , p.Ala262Glu (TS)
Sample 16	DICER1, c.5113G>A, p.Glu1705Lys (TS)
Sample 17	PBRM1, c.4610A>G, p.Gln1537Arg (TS)
Sample 19	KMT2D, c.12667C>T, p.Gln4223 (TS) TCF7L2, c.1268A>G, p.Tyr423Cys (TS)
Sample 20	NRAS, c.37G>C, p.Gly13Arg (PO)
Sample 24	CTNNB1, c.110C>G, p.Ser37Cys (PO) PSIP1 (gene), c.283C>T (variant), p.Gln95 (impact) (?)
Sample 25	CDKN1B, c.280delC, p.Gly97fs (TS) ATM, c.5495_6496+2delAAGT, p.Glu1832fs (TS)
Sample 26	BCL9L, c.4283_4284dupTG, p.Thr1429fs (TS)
Sample 27	RB1, c.2357C>T, p.Arg787 (TS) PBRM1, c.2715_2718delGAGA, p.Glu908fs (TS)
Sample 28	GRIN2A, c.3321_3322insTTTTTTAATGATACGGC, p.Lys1107_ Thr1108insPhePheAsnASpThrAla (TS)
Sample 33	KRAS, A146V (PO) GNAS, R210H (PO)
Sample 35	CDKN1B, G97Vfs*22 (TS)

Table 3 Mutations with high driver likelihood identified by whole genome sequencing.TS: tumor suppressor gene, PO: proto-oncogene.

Association between driver mutations and Ki67 index

When comparing Ki67 proliferation index with the presence and absence of driver mutations, it was observed that patients with driver mutations had a significantly higher Ki67 index than those without driver mutations (p=0.015).

Samples	Driver mutations
Sample 40	CTNNB1 c.134C>T p.Ser45Phe (p.S45F) NM_001904.3 (PO)
Sample 41	TP53 [ENST00000269305.4]: codon 1-19, 21-257, 259-261, 263-394: c.1009C>T (p.Arg337Cys) (TS)
Sample 42	TP53 [ENST00000269305.4]: codon 1-19, 21-257, 259-261, 263-394: exon 5: c.404G>C (p.Cys135Ser) (TS) MET amplification of 6 amplicons
Sample 43	TP53 NM_000546.5 intron 4 c.376-1G>T p.? (p.?) (TS)

Table 4 Driver mutations identified by next generation panel sequencing.

CIRCOS plots

In general, metastasized SI-NETs show little genomic aberrations resulting in a relatively empty CIRCOS plots. Figure 3 shows the histological features and CIRCOS plot of a grade 3 metastasized SI-NETs with driver mutations in KMT2D and TCG7KL2 and full loss of CDKN2A. In Figure 4, the histological features and a CIRCOS plot of a grade 2 metastasized SI-NET without driver mutations are shown. The CIRCOS plot of Figure 3 shows more genetic aberrations (e.g. somatic mutations, translocations and an amplification on chromosome 1) compared to the CIRCOS plot of Figure 4.

Targeted therapy

Potential actionable genetic alterations were detected in 9 (21%) patients in the BRCA pathway, the cyclin D/cyclin-dependent kinases 4-6 –retinoblastoma protein pathway, RAS/REF/MEK/ERK pathway and the HGF/MET pathway. These patients could be eligible for targeted therapy (off label). Table 5 shows potential actionable driver mutations and their potential targeted therapy.

Actionable driver mutation	Potential precision drugs
CDKN1B	CDK4/6 inhibitors
KRAS	RAS/REF/MEK/ERK inhibitors
NRAS	RAS/REF/MEK/ERK inhibitors
GNAS	RAS/REF/MEK/ERK inhibitors
ATM	PARP inhibitors
MET amplification	MET inhibitors

Table 5 Actionability of identified driver mutations.

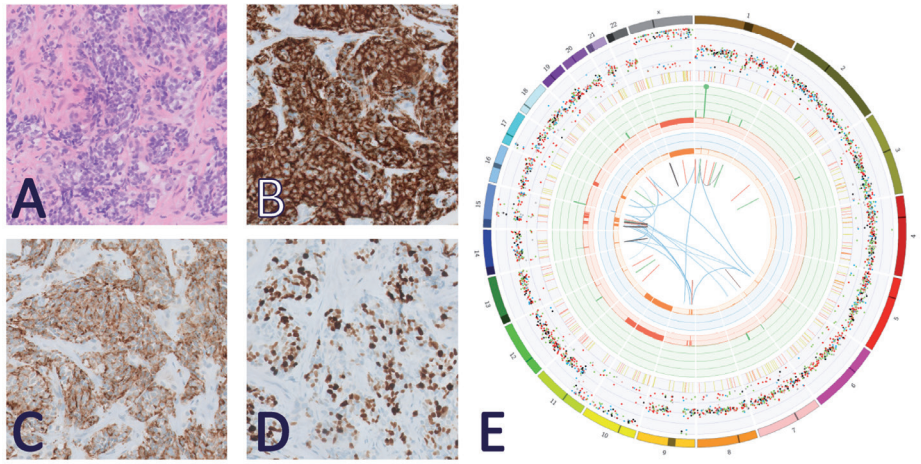

Figure 3. SI-NET grade 3 with driver mutations in KMT2D and TCF7L2 and CDKN2A loss. A: H&E staining, B: Synaptophysin staining: 100% positivity, C: Chromogranin staining: 100% positivity, D: Ki67 staining: 40%, E: CIRCOS plot.

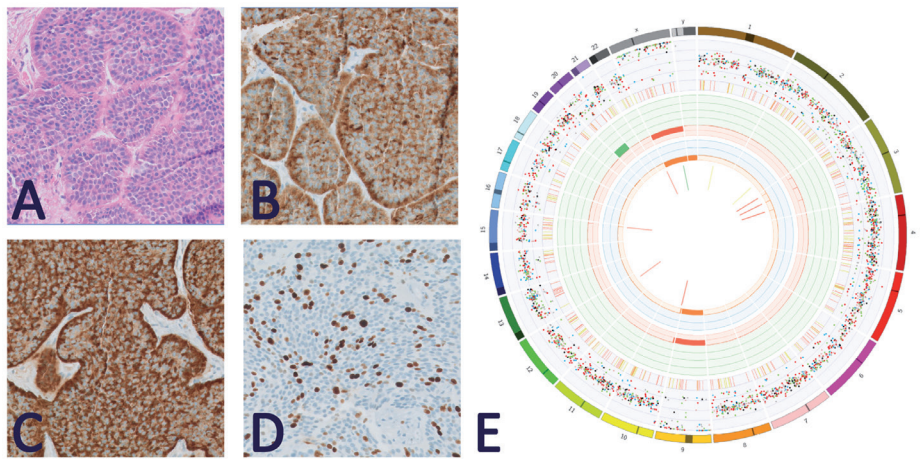

Figure 4. A grade 2 small-intestine neuroendocrine tumour without driver mutations.A, Haematoxylin and eosin staining.B, Synapto-physin staining: 100% positivity.C, Chromogranin staining: 100% positivity.D, Ki67 staining: 10%.E, CIRCOS plot.

Disease specific survival

After a median follow up of 25 (IQR 15-33) months, median disease specific survival was not reached as shown in Figure 5. Survival times did not differ significantly between patients with or without driver mutations (p=0.618) as is shown in Figure 6, nor a difference in DSS between tumour grades was seen (p=0.636).

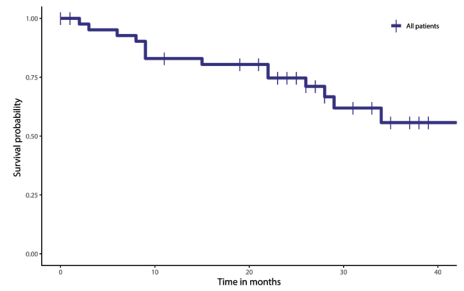

Figure 5. Disease-specific survival (DSS) in months for all patients; the median DSS is not reached.

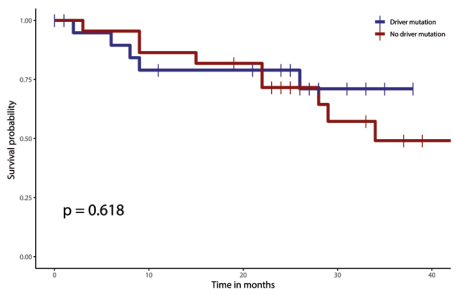

Figure 6. Disease-specific survival in months for patients with driver mutations and without driver mutations.

Discussion

The recent WHO classification of NEN (2017) sharply distinguishes well-differentiated NET from poorly differentiated NEC. This distinction is based on pathologic (e.g. morphology and proliferation rate) and clinical features. At present, alterations in the genome of NEN do not contribute to the current classification, despite the paradigm shift in the classification of many other tumour types which has been caused by molecular subtyping in the past decade.

The distinction between NET and NEC has serious clinical implications in terms of treatment and prognosis. To illustrate, advanced GEP-NECs are treated with platinum based chemotherapy and have an overall survival of less than 12 months[13] whereas advanced GEP-NETs are treated with multiple modalities and have an overall survival (largely dependent on primary tumour location) of approximately 33 months.[14]

In this study, we aimed to investigate the presence of driver mutations in metastatic SI-NETs and to explore their clinicopathological significance. We show that well-differentiated SI-NETs are mutationally quiet tumours with few genomic disruptions, which is in concordance with earlier studies (as reviewed in SI-NETs[15]). Surprisingly, despite this low number of genomic disruptions, 50% of SI-NETs harbour driver mutations in cancer genes, including mutations in genes which are frequently affected in NEC, such as *TP53, RB1, KRAS* and *NRAS*. Our results are corroborated by WGS data of 25 well-differentiated SI-NETs of the MSK IMPACT study, which show complete loss of *CDKN2A* in 12% and driver mutations in 4.0% in *SMAD2, KRAS, RB1* and *TP53*.[16] This data was accessed through an open-access resource named cBioportal for Cancer Genomics (http://cbioportal.org).[17] Of note, the biopsies which are included in this open-access resource are not reviewed by a pathologist whereas expert revision of all biopsies included in this study took place.

In our cohort, with a median follow up of 25 months, the presence of driver mutations did not affect disease specific survival which suggests that one or two driver mutations alone do not necessarily alter the clinical behaviour of metastasized SI-NETs. However, the identification

of potential actionable genetic alterations in 21% of patients in our cohort is promising since it provides a rationale for the introduction of targeted therapy in the treatment of NET. For instance, in our study we found potential targets in the BRCA pathway, which would suggest that targeting DNA repair mechanisms may be effective in NET, e.g. through the use of Poly(ADP-ribose) Polymerase (PARP) inhibitors. Other targetable pathways included the cyclin D/cyclin-dependent kinases 4-6–retinoblastoma protein, RAS/REF/MEK/ERK and the HGF/MET pathway. Furthermore, this study shows that SI-NETs have an invariably low tumour mutational load (median 21, IQR 10.5-28.0) and maintain chromosomal stability. In contrast to NECs, which have a high number of copy number alterations and a high mutational load.[18,19] Chromosomal stability and tumour mutational load therefore can be of practical aid in the distinction between NEC and NET. Loss of heterozygosity chromosome 18 was common in this cohort (63%), which is in accordance with earlier studies on primary SI-NETs (44-100%).[13, 18-28] Similarly, *CDKN1B* mutations occurred in 9%, which is also in accordance with earlier findings (4.5-11%).[20,23,30-35] A complete loss of *CDKN2A* was found in 6% of SI-NETs. Loss of *CDKN2A* is an unspecific finding which is frequently encountered in metastasized solid tumours. In fact, *CDKN2A* has been identified in a pan cancer whole genome analysis of 2399 metastatic tumours as the most significantly deleted gene (n=415 (17%)).[8]

In conclusion, this study shows that well-differentiated metastasized SI-NETs do harbour driver mutations, which means that their presence is not exclusive to NECs. Consequently, the distinction between well-differentiated grade 3 NETs and poorly differentiated NECs should therefore not solely rely on the presence of driver mutations, and rather be made on clinical and pathologic characteristics, such as a previous history of well-differentiated NET, a prolonged clinical course and well-differentiated morphology. The relationship between Ki67 proliferation index and the presence of driver mutations may suggest that these mutations may have contributed to tumour progression, i.e. progression from low to higher grade NET. However, this progression is not reflected in a decrease in disease specific survival and only in some patients by incomplete loss of SSTR2A expression. Our data support the notion that NET and NEC are two different disease entities and that progression of well-differentiated NET into poorly differentiated NEC is unlikely to occur.

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5

Primary tumour resection is associated with improved disease specific mortality in patients with stage IV small intestinal neuroendocrine tumours (NET): a comparison of upfront surgical resection *vs.* a watch and wait strategy in two specialist NET centres.

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Abstract

Introduction

Small intestinal neuroendocrine tumours (SI-NET) often present with metastatic disease. An ongoing debate exists whether to perform primary tumour resection (PTR) in patients with stage IV SI-NET, without symptoms of the primary tumour and inoperable metastatic disease. The aim of this study was to compare a treatment strategy of upfront surgical resection versus a surveillance strategy of watch and wait.

Methods

This was a retrospective cohort study of patients with stage IV SI-NET at diagnosis, between 2000-2018, from two tertiary referral centres (Netherlands Cancer Institute [NKI] and Aintree University Hospital [AUH]) who had adopted contrasting treatment approaches: upfront surgical resection *versus* watch and wait respectively. Patients without symptoms related to the primary tumour were included. Multivariable intention-to-treat (ITT), per-protocol (PP), and instrumental variable (IV) analysis using 'institute' as an IV were performed to assess the influence of PTR on disease specific mortality (DSM).

Results

A total of 557 patients were identified, 145 patients remained after exclusion of stage I-III disease or symptoms of the primary tumour: 93 NKI, and 52 AUH. The cohorts differed in performance status (PS, p=0.006) and tumour grade (p<0.001). PTR was independently associated with reduced DSM irrespective of statistical methods employed: ITT HR 0.60, p=0.005; PP HR 0.58, p<0.001; IV HR 0.07, p=0.019. Other factors associated with DSM were age, PS, high CgA and somatostatin analogue treatment.

Conclusion

This study, taking advantage of contrasting institutional treatment strategies, has identified PTR as an independent predictor of DSM. Future prospective studies should aim to validate these results.

Introduction

Neuroendocrine tumours (NET) are rare tumours with an overall incidence of 1-5/100,000.[1] The most common site for NET is the gastro-entero-pancreatic (GEP) tract, of which NET of the small intestine (SI-NET) comprise the largest group, with incidence rates of 0.7-1.63 per 100,000.[1-4] An important characteristic of SI-NET is mesenteric fibrosis, a desmoplastic reaction surrounding the tumour and mesenteric lymph nodes, which leads to moderate-severe fibrosis of the mesentery.[5-7] Mesenteric fibrosis with resultant bowel involvement can present with symptoms of colicky abdominal pain and physiological derangements as a result of complications such as ischemia, bowel obstruction, invagination, and even perforation, arguably associated with a poorer outcome.[5,7,8]

Nevertheless, PTR in patients with stage IV SI-NET who do not have symptoms of the primary tumour or mesenteric fibrosis and have inoperable metastatic disease, has long been the subject of debate.[9] A number of retrospective studies have identified a favourable association of PTR with survival, regardless of the extent of mesenteric fibrosis.[10-14] Based on such studies, the European Neuroendocrine Tumour Society (ENETS) has recommended that palliative PTR "*should be attempted because the overall outcome is better in patients after primary tumour resection, although a causal relationship [between PTR and survival] has not been proven to date.*"[15]

As implied by the ENETS guidelines, the lack of prospective studies to resolve this contentious issue prevents directive recommendations to be formulated. To date, one study in an attempt to control for confounding by indication, performed a propensity score matched analysis of 91 pairs of patients with stage IV SI-NET. No association of PTR with overall survival (OS) was found which further fuelled the debate.[16]

To establish whether, in the presence of distant metastases, there is an advantage of PTR over a 'watch and wait' approach, more in-depth studies are warranted. Due to the ambivalent results in the literature and guidelines, referral centres for NET have adopted contrasting approaches to this issue. We compared the approaches of two ENETS Centres of Excellence: The Netherlands Cancer Institute (NKI) in Amsterdam, the Netherlands, has chosen to resect primary tumours (and, if present, accompanying pathological lymph nodes or mesenteric fibrosis) at the time of initial diagnosis whenever technically possible. In contrast, surgeons at Aintree University Hospital (AUH) in Liverpool, United Kingdom, only resect the primary tumour if symptoms of mesenteric fibrosis or the primary tumour itself, such as ischemia or bowel obstruction occur. In this study, we aim to make use of this naturally occurring treatment allocation to investigate the association of PTR with survival in patients with SI-NET.

Methods

Patients

All consecutive patients with a histopathologically and radiologically confirmed stage IV SI-NET, referred to the NKI or AUH between 2000-2018, were eligible for inclusion. Patient and tumour characteristics were retrieved from electronic patient records retrospectively. Tumour staging at time of diagnosis was based on pathological and radiological reports. Patients were adults with stage IV SI-NET, without symptoms attributable to mesenteric fibrosis or the primary tumour such as obstruction or ischemia, were fit for surgery, and conventional imaging did not demonstrate an unresectable mesenteric mass. To avoid possibly including neuroendocrine carcinoma, only patients with well-differentiated tumour morphology were included, and patients with a grade 3 tumour according to the latest WHO classification for neuroendocrine tumours were excluded since the classification and nomenclature of these tumours has changed substantially over the past decades.[17] Chromogranin A (CgA) was included as a measure of disease burden. Since both institutes used different methods and units for CgA determination, the upper limit of normal (ULN) for both institutes and the relative increase thereof was used. CgA was categorised in <ULN, <2x ULN (100% increase), <6x ULN (500% increase), <11x ULN (1000% increase) and >11x ULN. This study was performed in accordance with the Declaration of Helsinki and all patients gave consent for their data as per institutional protocol.

Objectives

The primary objective of this study was to investigate the influence of the exposure on the outcome, namely disease specific mortality. The exposure is defined as upfront PTR, in the presence of stage IV disease, without the presence of symptoms related to mesenteric fibrosis or primary tumour.

Secondary objectives were to identify predictors of disease specific mortality within this bi-institutional cohort.

Statistics - rationale

Most previous retrospective studies have attempted to control for confounding by performing multivariable cox regression, including PTR as a variable of interest (a per-protocol analysis). It is likely however that these methods are not sufficient to completely control for confounding by indication (i.e. more medically fit patients with less burden of disease are more likely to receive PTR). Several methods exist to attempt to control for confounding by indication and unmeasured confounding. One method would be to consider the current scenario as a truly randomised setting, and perform an intention-to-treat analysis.[18] However, when deviations from protocol occur in a relatively large proportion of patients, the treatment effect might become cloaked by a regional effect. In situations with regional differences, instrumental variable (IV) analysis may be performed. IV analysis tries to mimic a randomised study in which treatment assignment is related to the actual treatment received.[19-21] For means of clarity and

comparison with previous literature, we have reproduced the methods from earlier studies, and performed a per-protocol analysis and compared this to an intention-to-treat analysis. To investigate whether further adjustment for confounding by indication or unmeasured confounders is possible in this setting, we have also performed an IV analysis accompanied by a sensitivity analysis in which we evaluate the most extreme scenarios to assess the probability of our results.

Disease specific mortality (DSM) was selected as primary endpoint to assess the effect of PTR on the disease under study. DSM was prioritised over all-cause mortality to avoid possible bias. The use of composite endpoints (combinations of multiple endpoints into one primary endpoint) is common in medical research, especially in clinical trials.[22-24] Yet the use of such endpoints should be judged critically, and when an outcome is (partly) associated with the exposure serious selection bias may occur.[25] This may specifically occur with PTR and all-cause mortality in patients with SI-NET, since patients who are expected to die from other causes will be less likely to receive PTR. To assess the presence of such bias, analysis will be repeated for all-cause mortality and presented in the supplementary data.

Statistics - methods

Three statistical methods for multivariable regression were performed and compared in this cohort. First, a per-protocol analysis was performed in which all patients were categorized according to exposure (PTR yes/no). Second, an intention-to-treat analysis was performed, where patients were categorized according to institute. For both methods multivariable cox regression was performed including all known and measured confounders. Third, IV analysis was performed. The IV analysis allows inference of causality in the presence of unmeasured confounding, yet several assumptions need to be fulfilled: (1) the IV is associated with the treatment under study – this assumption will be tested by calculating the odds ratio (OR) of the intervention across the institutes. An OR of >2 is considered a strong association; (2) the IV is independent of confounders – this will be tested by calculating the standardized difference (Sdif) between confounders across the institutes.[26] A Sdif ≤0.2 is used to indicate a similar distribution of confounders across institutes. Variables that have a Sdif >0.2 will be controlled for in multivariable IV analysis using cox regression; and (3) the IV affects the outcome only through the exposure – this assumption cannot be formally tested, but at this stage there is no reason to assume that the outcome is affected by other factors than the exposure.[21] The IV analysis consists of two stages: in the first stage the exposure is regressed on the IV, and a 'predicted' exposure level is obtained for each subject. In the second stage, the outcome is regressed on these predictions.[27] In this study, 'institute' is considered as the IV, 'PTR' as the exposure, and DSM as the outcome.

Multiple imputation was performed to account for missing values in relevant variables. The number of imputations was determined by the largest percentage of missing values within a variable. Results for multivariable regressions from imputed datasets were pooled according to Rubin's rules.[28-30]

Finally, although multiple imputation is an excellent (least biased) method to account for missing values, it is still only based on the values that are known. To evaluate whether the results retrieved from our dataset could possibly be skewed towards one direction, we constructed an extreme scenario where all patients with missing values for tumour grade (largest proportion missing values) were assigned a grade 1 in the institute that had patients with the least DSM events, and a grade 2 in the institute that had most DSM events, to see whether the effect of PTR upholds in such a scenario.

Additionally, to assess the sole effect of regional influences between the two cohorts, all consecutive patients with a SI-NET that were referred to the AUH or NKI during the study period were included in a cox regression analysis, with surgery of any kind and disease stage (local, regional or distant) as additional variables.

Median with interquartile range (IQR) was used to describe continuous variables, frequency and percentages were calculated for categorical variables. For comparison between groups Fisher's exact test was performed for categorical variables, and the Wilcoxon rank sum test for continuous variables. DSM was defined as time from initial diagnosis until documented SI-NET-related death. Since all patients had stage IV disease, patients who died of unknown causes were considered to have died of SI-NET. Patients who died of other causes or were alive at end of follow-up were censored. For visualization of survival, Kaplan-Meier curves were used. Analyses were performed using R version 4.1.1. R packages 'mice', 'survival' and 'ivtools' were used. The two-stage estimation method for time-to-event outcomes from the packages 'ivtools' was used for IV analysis.

Results

Patients

A total of 557 patients with SI-NET of all stages were referred between 2000 and 2018, 161 (28.9%) to the AUH, and 396 (71.1%) to the NKI. Patients were excluded in the absence of stage IV disease (27.8%); if patients underwent surgery with curative intent (17.2%); the primary tumour or mesenteric fibrosis was unresectable (3.9%); or when the primary was not visible on imaging (0.9%); when patients were deemed inoperable due to their condition (0.7%); or had surgery because of obstruction or ischemia (23.3%). Baseline characteristics for all patients can be found in supplementary Table S1. After exclusion, 145 (26.0%) patients remained, of whom 52 (35.9%) patients were from the AUH cohort, and 93 (64.1%) were from the NKI cohort. A flow-diagram of all patients can be found in Figure 1. Further analyses have been performed in the selected 145 patients.

Differences between cohorts were seen in World Health Organization performance status (PS): in the AUH cohort more patients had PS of 0 (65.4 vs. 53.8%) and a PS of 3 (5.8 vs. 0%, p=0.006), more patients had an unknown tumour grade (36.5 vs. 3.2%, p<0.001) and more patients had died of SI-NET (33.3 vs. 9.7%).

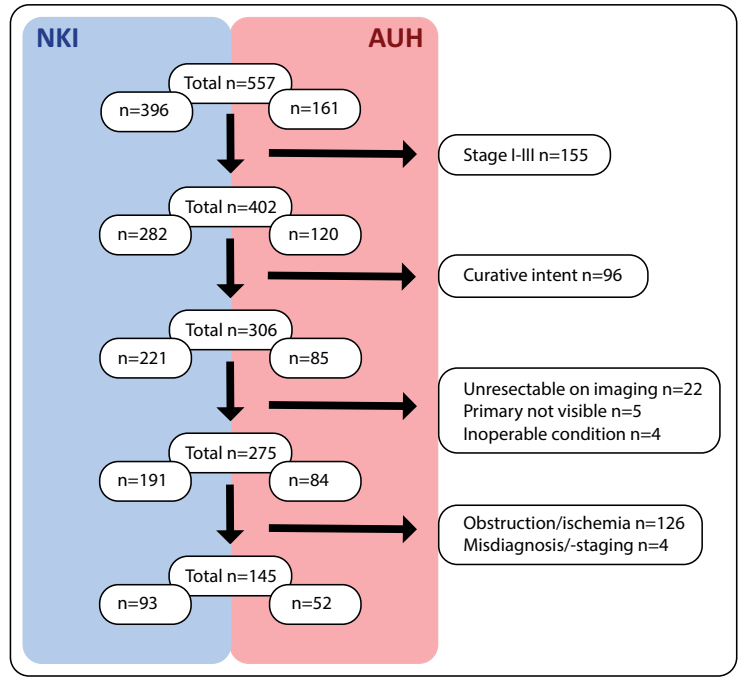

Figure 1. Flowchart of all patients referred to the Netherlands Cancer Institute (NKI) and Aintree University Hospital (AUH).

Less patients died of comorbidities (0 vs. 6.5%, p=0.004). Missing data was present in tumour grade (15.2%), chromogranin A (CgA, 11.0%) and in PS (2.7%). The missing values for PS or grade were mostly due to unavailable or incomplete patient records or pathology reports. Missing values for chromogranin A can also largely be explained by chromogranin A being introduced for use in clinical practice halfway through the first decade of this millennium. Regarding exposure: 73 (78.5%) of patients underwent PTR in the NKI cohort, and four (7.7%) underwent PTR in the AUH cohort. Alongside PTR, six (6.5%) of patients in the NKI cohort underwent liver debulking, whereas none underwent simultaneous debulking surgery in the AUH cohort (p=0.008). All baseline characteristics and comparison between cohorts are summarized in Table 1.

Follow up

In the AUH cohort, 10 (19.2%) patients underwent surgery related to complications of the primary tumour and/or mesenteric fibrosis during follow up, all of these were due to obstruction. Six (11.5%) underwent resection of primary tumour, three (5.8%) had bypass surgery, and one (1.9%) patient underwent laparotomy for adhesiolysis. Median time to surgery was 25.5 months, ranging from 6 to 82 months. Of the 20 patients that did not undergo PTR in the NKI cohort, 1 (5%) patient had surgery a year after diagnosis due to obstruction, yet 4 (20%) patients were lost to follow up.

Patients	NKI n (%)/median (IQR)	AUH n (%)/median (IQR)	*p*-value	SDif
Total	**93**	**52**		
Age at diagnosis	60.1 (54.0-69.8)	66.0 (55.9-73.3)	0.161	0.269*
Sex				
Male	48 (51.6)	27 (51.9)	>0.999	0.009
Female	45 (48.4)	25 (48.1)		0.009
WHO PS			0.006	
0	50 (53.8)	34 (65.4)		0.337
1	37 (39.8)	10 (19.2)		0.654
2	5 (5.4)	2 (3.8)		0.048
3	0	3 (5.8)		0.495
Missing	1 (1.1)	3 (5.8)		0.368
Tumour grade			<0.001	
1	55 (59.1)	17 (32.7)		0.778
2	29 (31.2)	16 (30.8)		0.013
Unknown	3 (3.2)	19 (36.5)		1.299
CgA			0.402	
<ULN	14 (15.1)	4 (7.7)		0.330
<2x ULN	14 (15.1)	8 (15.4)		0.013
<6x ULN	29 (31.2)	13 (25.0)		0.195
<11x ULN	11 (11.8)	7 (13.5)		1.219
>11x ULN	14 (15.1)	15 (28.8)		0.064
Unknown	11 (11.8)	5 (9.6)		0.612
Baseline SSA	51 (54.8)	36 (69.2)	0.112	0.424
Exposure			n/a	n/a
Resection	73 (78.5)	4 (7.7)		
No resection	20 (21.5)	48 (92.3)		
Debulking				n/a
Liver	6 (6.5)	0	0.088	

Table 1. Baseline characteristics. WHO PS: World Health Organization Performance Status, CgA: chromogranin A, ULN: upper limit of normal, SSA: somatostatin analogues, NKI: Netherlands Cancer Institute, Amsterdam, AUH: Aintree University Hospital, Liverpool. *For calculation of the standardized difference the mean values were used.

Regarding additional therapies during follow up, a total of 21 (40.4%) patients received therapeutic intervention other than surgery or SSA in the AUH cohort. This was PRRT in 18 (34.6%) patients, liver-directed therapy in one (1.9%) patient and Meta-Iodo-Benzyl-Guanidine (MIBG) treatment in two (3.8%) patients. In the NKI cohort 41 (44.1%) patients received additional therapies. This was PRRT for 24 (25.8%) patients, liver-directed therapies in 14 (15.1%) patients, MIBG in one (1.1%) patient and radiotherapy for painful metastases in two (2.2%) patients. The proportion of patients receiving additional therapy during follow up were not statistically different (p=0.121).

Patients	NKI n (%)	AUH n (%)
Total	**93**	**52**
Vital status		
Died of disease	9 (9.7)	17 (32.7)
Died of comorbidity	5 (5.4)	0
Alive with disease	74 (79.6)	31 (59.6)
Cause of death unknown	5 (5.4)	4 (7.7)
Died of disease	9 (100)	17 (100)
Disease progression	8 (88.8)	13 (76.5)
Carcinoid heart disease	1 (11.1)	4 (23.5)

Table 2. Causes of death. NKI: Netherlands Cancer Institute Amsterdam, AUH: Aintree University Hospital Liverpool.

Survival

After a median follow up time of 4.9 years (range 0-20.3) for the AUH cohort, and 4.4 years (range 0-20.3) for the NKI cohort: estimated median disease specific survival time for the AUH cohort was 8.9 years (confidence interval [CI] 5.1-12.7) and median survival time for the NKI cohort is 16.5 years (CI not estimable). Survival curves for univariable visualisation of survival across institute and exposure are shown in Figure 2. In the AUH cohort, more patients had died of SI-NET compared to the NKI cohort (33.3% vs. 9.7%, p=0.001). Regarding these patients, 4/17 (23.5%) patients died of carcinoid heart disease (CHD) in the AUH cohort and 1/9 (11.1%) in the NKI cohort. The remaining patients died from disease progression. Regarding death from other causes, no patients were registered to have died from comorbidities in the AUH cohort, whereas five (5.4%) died of other causes in the NKI cohort.

Regarding the sole regional effect between the two institutes, cox regression for DSM of all consecutive patients referred to the AUH or NKI between 2000-2018 (n=557) showed a trend towards improved DSM in the NKI, but this was not significant (HR 0.67, p=0.054). These results are summarized in supplementary Table S1.

Outcomes

In the per-protocol analysis, exposure (PTR) was associated with improved DSM (HR 0.58, CI 0.43-0.78). Other significant predictors for DSM were age at diagnosis (HR 1.07, CI 1.05-1.09), a PS of 2 (2.27, CI 1.24-4.15) and 3 (HR 3.07, CI 1.04-9.05) and treatment with SSA (HR 1.63, CI 1.79-3.91). Results of multivariable per-protocol analysis are shown in Table 3.

In the intention-to-treat analysis, institute was considered the variable of interest, and was significantly associated with DSM. AUH was used as the reference variable, and this resulted in an HR of 0.60 (CI 0.42-0.85) for the NKI cohort. Besides this, age at diagnosis (HR 1.06, CI 1.04-1.08), a PS of 2 (HR 2.57, CI 1.38-4.81) and treatment with a somatostatin analogue (SSA) (HR 2.14, CI 1.45-3.15) were identified as independent predictors for DSM. Results of the intention-to-treat analysis can be found in Table 3.

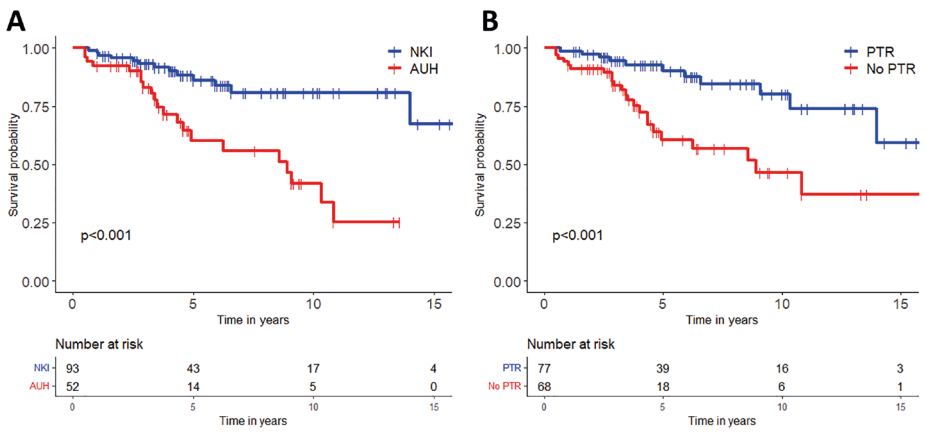

Figure 2. Survival curves for disease specific survival for institutes (A) and exposure (B). NKI: Netherlands Cancer Institute, AUH: Aintree University Hospital. PTR: primary tumour resection.

	Per-protocol analysis			Intention-to-treat analysis		
	HR	**CI**	***p*-value**	**HR**	**CI**	***p*-value**
Female sex	1.11	0.80-1.54	0.551	1.13	0.81-1.57	0.474
Age at diagnosis	1.07	1.05-1.09	<0.001	1.06	1.04-1.08	<0.001
WHO PS						
0	1			1		
1	1.29	0.88-1.91	0.196	1.47	0.99-2.18	0.062
2	2.27	1.24-4.15	0.009	2.57	1.38-4.81	0.004
3	3.07	1.04-9.05	0.044	2.67	0.92-7.73	0.073
Grade						
1	1			1		
2	1.33	0.88-2.00	0.181	1.38	0.91-2.08	0.132
CgA						
<ULN	1			1		
<2x ULN	1.54	0.79-2.99	0.177	1.37	0.71-2.66	0.354
<6x ULN	1.70	0.93-3.10	0.089	1.38	0.75-2.53	0.300
<11x ULN	1.70	0.77-3.75	1.192	1.38	0.59-3.22	0.455
>11x ULN	3.11	1.57-6.17	0.002	2.84	1.44-5.61	0.003
SSA treatment	2.63	1.79-3.91	<0.001	2.14	1.45-3.15	<0.001
Exposure						
No PTR	1			n/a	n/a	n/a
PTR	0.58	0.43-0.78	<0.001			
Institute						
AUH	n/a	n/a	n/a	1		
NKI				0.60	0.42-0.85	0.005

Table 3. Per protocol analysis and intention to treat analysis. WHO PS: World Health Organization Performance Status, CgA: chromogranin A, ULN: upper limit of normal, SSA: somatostatin analogues, PTR: primary tumour resection, NKI: Netherlands Cancer Institute Amsterdam, AUH: Aintree University Hospital Liverpool.

Further, in the IV analysis, the OR of resection of primary tumour in the respective institutes was 43.8, meaning the association with the exposure and the IV (institute) is strong, and the first assumption is fulfilled. For the second assumption, the distribution of confounders across institutes and calculated Sdif can be found in Table 1. Variables for which the Sdif is >0.2 were age at diagnosis, PS, tumour grade and treatment with SSA. These variables were hence included in the multivariable IV analysis. The two-step IV analysis identified PTR (HR 0.07, CI 0.01-0.43), and age at diagnosis (HR 1.04, CI 1.00-1.08) as independent predictors for DSM. Table 4 shows the results of the IV analysis.

Finally, we performed a per-protocol and intention-to-treat analysis for the extreme scenario of all unknown tumour grades being grade 1 in the NKI cohort, and grade 2 in the AUH cohort. In the per-protocol analysis significant associations with DSM were found for PTR (HR 0.30, CI 0.12-0.76) and age at diagnosis (HR 1.05, CI 1.01-1.08). In the intention-to-treat analysis, institute (HR 0.26, CI 0.10-0.67 for the NKI cohort), age at diagnosis (HR 1.04, CI 1.00-1.08) and a PS of 2 (HR 5.41, CI 1.09-26.94) and 3 (HR 5.41, CI 1.04-28.14) were significantly associated with DSM. Results for this sensitivity analysis can be found in Table 4.

To evaluate whether the rates of simultaneous debulking surgery and more liver directed therapies in the NKI cohort might have biased the results, the analysis for the per-protocol, intention-to-treat and instrumental variables analysis were repeated in the cohort in which all patients with debulking surgery and liver directed therapies in the follow up were removed. This resulted in a cohort of 73 patients from the NKI and 52 patients from the AUH. Results of these analyses were nearly identical to the results in the total cohort under study, with no relevant differences in HRs, statistical significance or confidence intervals (data not shown).

Analysis for the per-protocol, intention-to-treat and instrumental variable analysis were repeated with all-cause mortality as a primary outcome. Results were highly similar to those found for DSM and can be found in Table S2 of the supplementary material.

Discussion

In this study, we demonstrate that resection of the primary tumour in patients with stage IV small intestinal neuroendocrine tumours is associated with a lower disease specific mortality. By taking advantage of regional, institutional differences in treatment approaches to people with stage IV SI-NET and performing various statistical methods, we robustly controlled for confounding, given the limitations of a retrospective design.

Our results are concordant with those of previous studies that have investigated resection of primary tumour in the presence of distant metastases.[10,31,32] Zheng, et al. performed a multivariable Cox regression, comparable to our per-protocol analysis, which identified resection of the primary tumour being associated with improved survival (HR 0.48) in a cohort of 1547 patients with GEP-NET, of whom 557 had a SI-NET.[10] The results of another study involving 4252 patients with stage IV SI-NET from the National Cancer Database, also highlighted that primary tumour resection was associated with prolonged survival in multivariable analysis (HR 0.55).[32]

	Instrumental variable analysis			Sensitivity Per-protocol analysis			Sensitivity Intention-to-treat analysis		
	HR	**CI**	***p*-value**	**HR**	**CI**	***p*-value**	**HR**	**CI**	***p*-value**
Female sex	1.37	0.63-3.01	0.438	1.15	0.52-2.53	0.726	1.21	0.55-2.67	0.643
Age at diagnosis	1.04	1.00-1.08	0.036	1.05	1.01-1.08	0.018	1.04	1.01-1.098	0.024
WHO PS									
0	1			1			1		
1	2.18	0.74-6.41	0.157	2.00	0.78-5.13	0.150	2.28	0.89-5.56	0.086
2	3.82	0.57-25.82	0.170	4.55	0.95-21.84	0.058	5.41	1.09-26.94	0.039
3	3.00	0.53-16.91	0.215	5.57	0.99-31.35	0.051	5.41	1.04-28.14	0.045
Grade									
1	1			1			1		
2	1.14	0.41-3.17	0.811	2.65	1.13-6.22	0.026	2.05	0.83-5.03	0.118
CgA									
<ULN	1	0.23-4.65	0.961	1			1		
<2x ULN	1.04	0.06-1.48	0.138	1.00	0.23-4.38	0.996	0.94	0.22-4.01	0.935
<6x ULN	0.27	0.02-2.48	0.134	0.38	0.09-1.64	0.194	0.20	0.09-1.64	0.197
<11x ULN	0.23	0.12-3.16	0.226	0.19	0.03-1.28	0.088	0.12	0.03-1.45	0.115
>11x ULN	0.61			0.76	0.15-3.89	0.740	0.85	0.25-5.54	0.848
SSA treatment	0.88	0.67-2.12	0.787	1.08	0.47-2.53	0.852	1.33	0.57-3.13	0.509
Exposure									
No PTR	1			1			n/a	n/a	n/a
PTR	0.07	0.01-0.43	0.019	0.30	0.12-0.76	0.011			
Institute									
AUH	n/a	n/a	n/a	n/a	n/a	n/a	1		
NKI							0.26	0.10-0.67	0.005

Table 4. Multivariable regression analysis for IV analysis, sensitivity analyses for intention-to-treat analysis and per-protocol analysis. WHO PS: World Health Organization Performance Status, CgA: chromogranin A, ULN: upper limit of normal, SSA: somatostatin analogues, PTR: primary tumour resection, NKI: Netherlands Cancer Institute Amsterdam, AUH: Aintree University Hospital Liverpool.

Only one study has attempted to further control for confounding by indication by performing propensity score matching on a cohort of patients with stage IV SI-NET.[16] Interestingly, their study did not find an association between PTR and OS. This might be because over half of the patients that did not receive upfront PTR in their study, eventually underwent PTR during follow up (53 out of 91 [58.2%]), after a median time of 18 months, whereas in the AUH cohort in our study, only 19.2% underwent PTR during follow up.

Interestingly, we found that treatment with SSA was associated with increased DSM. As known from the large PROMID study, treatment with SSA does not provide long term survival benefit. In the PROMID study it was also found that patients with higher tumour burden had significantly worse survival.[33] In the current paper, it is likely that in patients who received SSA treatment at baseline, more fulminant symptoms of carcinoid syndrome were present, and hence were more likely to have higher tumour burden, resulting in increased DSM.

The reason why PTR was associated with a better outcome remains unclear. Since SI-NET are associated with mesenteric fibrosis, one possible explanation would be that more patients suffered from bowel obstruction or ischemic symptoms and died thereof. In our study, we did not identify patients that died from these causes. All patients in the combined cohort that died of NET-relate causes, died of CHD or disease progression.

In other cancers it has been hypothesised that an interaction between the primary tumour and target organs of metastasis may dictate progression of metastasis.[34-36] Yet contrastingly, studies have also identified feedback mechanisms that work in opposite direction, where the presence of a primary tumour slows progression of metastasis.[37,38] It is possible that interactions between the primary tumour and metastasis exist in SI-NET, but research on this subject is currently lacking. Interestingly, a larger proportion of patients died of CHD in the AUH compared to the NKI cohort; possibly, primary tumour signalling might play a role in the occurrence and progression of CHD. Future studies aimed at prospectively investigating the true effect of PTR in stage IV SI-NET, should also focus on molecular signalling mechanisms.

This study has several limitations worth highlighting. First, although multiple, contemporary methods were performed to control for confounding, the retrospective nature of the study inherently means that selection bias might have occurred. For instance, since we were unable to radiologically assess burden of disease (i.e. no sum of lesions measurements were performed), it is possible that patients in the AUH cohort had more advanced disease at presentation. By including CgA as a marker for disease burden, we attempted to overcome this issue yet the possibility remains that including CgA was insufficient to account for difference of disease between the two cohorts, which is also reflected in the IV analysis. There, the inflated HRs suggest that assumptions 2 and 3 in the IV analysis are - to some extent - violated, preventing us from drawing firm conclusions from this analysis. Second, the possibility exists that the difference in survival between the cohorts might be due to national differences, rather than biological ones. For instance, in a large, international global surveillance study of cancer survival,

the United Kingdom (UK) was found to have a worse 5 year survival of patients with (among others) colorectal cancer, compared to the Netherlands (NL) (i.e. for colorectal, UK vs. NL: 53.8% vs. 60.1%) and other European countries. The authors concluded that differences in survival trends are likely to be attributable to differences in access to early diagnosis and optimum treatment.[39] Although it is not expected that large differences in diagnosis and treatment of patients with SI-NET are present between the two cohorts included in this study, which is also underscored by the analysis of all consecutive patients with SI-NET in both cohorts, a regional effect cannot be ruled out completely. Moreover, the HRs for 'institute' in the intention-to-treat analysis, and 'exposure' in the per-protocol analysis are very similar, even though deviations from protocol occurred in both institutes. This might indicate that the effect of treatment – at least to some extent – is caused by other, unmeasured or unknown factors associated with regional differences. Unfortunately, these are issues that cannot be controlled for outside of an RCT setting, and the current methods remain the best option. Third, inevitably, missing data was present in both cohorts. By performing multiple imputation, we were able to retrieve the least biased results in such a setting, whilst preserving statistical power. Since multiple imputation was performed in the complete cohort of SI-NET in both institutes, a maximal amount of input was used for imputation. Last, although the cohort selected for this study came from a large population of patients with SI-NET treated in two high-volume expert centres, the study cohort remains of limited size, which is also reflected by the large confidence intervals in our results. Nonetheless, this remains the largest cohort to date that has made use of such a naturally selected setting and provides a strong indication of treatment effect. Also, since all patients were treated in expert centres, adherence to treatment protocols as per current, established international guidelines was guaranteed and ensures that patients received similar and adequate treatment during follow up.

Of course, with this study, the debate regarding PTR in patients with stage IV SI-NET has still not been fully resolved, but most likely encourages further research on this subject. Due to the rarity of the disease and the relatively long survival of patients with SI-NET, a prospective study would be challenging albeit not impossible. Future studies should be based in an (inter)national, multicentre setting, where patients are randomised to receive PTR and are followed up until disease progression and disease specific mortality. Such a trial should also include secondary endpoints including postoperative morbidity and mortality, quality of life parameters, influence of PTR on biomarkers and development of carcinoid syndrome and CHD, as well as aim to identify molecular mechanisms for disease progression.

To conclude, this study evaluated the effect of PTR in patients with stage IV SI-NET in two settings where treatment allocation was determined regionally, according to the respective institutional protocols and we applied contemporary statistical methods to assess the effect of primary tumour resection on disease specific mortality. We convincingly demonstrate that primary tumour resection was an independent predictor for disease specific mortality with all multivariable models paving the way for prospective validation to potentially provide evidence for a treatment paradigm shift.

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Supplementary Material

Patients	NKI n (%)/median (IQR)	AUH n (%)/median (IQR)
Total	**396**	**191**
Age at diagnosis	62.1 (53.8-69.7)	64.0 (55.5-71.2)
Sex		
Male	193 (48.7)	88 (54.7)
Female	203 (51.3)	73 (45.3)
PS		
0	191 (48.2)	85 (52.6)
1	124 (31.3)	31 (19.3)
2	29 (7.3)	5 (3.1)
3	1 (0.3)	5 (3.1)
Missing	51 (12.9)	35 (21.7)
Disease stage at diagnosis		
Local	77 (19.4)	17 (10.6)
Regional	37 (9.3)	24 (14.9)
Distant	282 (71.2)	120 (74.5)
Tumour grade		
1	229 (57.8)	52 (32.3)
2	154 (38.9)	46 (28.6)
Unknown	13 (3.3)	63 (39.1)
Carcinoid syndrome	251 (63.4)	87 (66.9)
SSA treatment at baseline	124 (31.3)	40 (24.8)
Surgical treatment at baseline	289 (73.0)	104 (64.6)

Supplementary Table S1. Baseline characteristics for all patients referred to the Netherland Cancer Institute Amsterdam and Aintree University Hospital Liverpool (AUH) between 2000-2018. PS: World Health Organisation Performance Status, SSA: somatostatin analogue.

	HR	CI	*p*-value
Female sex	1.14	0.82-1.59	0.439
Age at diagnosis	1.06	1.04-1.08	<0.001
WHO PS			
0	1		
1	1.47	0.93-2.34	0.104
2	2.67	1.75-7.71	0.040
3	2.91	0.46-18.34	0.100
Grade			
1	1		
2	1.35	0.93-1.98	0.117
CgA			
<ULN	1		
<2x ULN	1.24	0.63-2.41	0.356
<6x ULN	1.14	0.60-2.17	0.682
<11x ULN	1.23	0.52-2.90	0.633
>11x ULN	2.37	1.09-5.14	0.334
SSA treatment	1.65	1.03-2.66	0.039
Surgery*	0.66	0.41-1.05	0.080
Disease stage			
Local	1		
Regional	2.14	1.04-4.40	0.041
Distant	1.55	0.89-2.69	0.122
Institute			
AUH	1		
NKI	0.67	0.44-1.01	0.054

Supplementary Table S2. Cox regression for disease specific mortality for all consecutive patients with a SI-NET (n=557) referred to the Aintree University Hospital (AUH) and Netherlands Cancer Institute (NKI). WHO PS: World Health Organization Performance Status, CgA: chromogranin A, ULN: upper limit of normal, SSA: somatostatin analogues.
* Surgery: any surgical intervention of any kind, regardless of curative or palliative intention.

	Per-protocol analysis			Intention-to-treat analysis			Instrumental variable analysis		
	HR	**CI**	***p*-value**	**HR**	**CI**	***p*-value**	**HR**	**CI**	***p*-value**
Female sex	0.93	0.46-1.92	0.854	0.91	0.44-1.86	0.789	1.01	0.49-2.08	0.989
Age at diagnosis	1.05	1.02-1.09	<0.001	1.05	1.02-1.09	<0.001	1.05	1.01-0.09	0.015
WHO PS									
0	1			1			1		
1	1.43	0.62-3.31	0.414	1.72	0.72-4.09	0.235	1.82	0.89-4.82	0.241
2	3.15	0.82-12.07	0.109	3.89	0.95-15.95	0.073	3.38	0.70-16.4	0.146
3	3.91	0.63-24.40	0.159	3.75	0.61-23.05	0.166	2.91	0.46-18.34	0.267
Grade									
1	1			1			1		
2	1.49	0.61-3.61	0.389	1.33	0.55-3.23	0.538	1.41	0.53-3.74	0.498
CgA									
<ULN	1			1			1		
<2x ULN	1.23	0.32-4.73	0.763	1.22	0.32-4.70	0.774	1.25	0.33-4.72	0.749
<6x ULN	0.40	0.10-1.52	0.191	0.43	0.11-1.65	0.230	0.36	0.09-1.36	0.145
<11x ULN	0.23	0.37-1.43	0.131	0.27	0.04-1.77	0.188	0.22	0.03-1.80	0.174
>11x ULN	0.79	0.17-3.65	0.761	1.17	0.23-5.50	0.848	0.65	0.13-3.30	0.611
SSA treatment	0.93	0.44-1.96	0.852	1.05	0.50-2.22	0.897	1.25	0.57-2.73	0.581
Exposure									
No PTR	1			n/a	n/a	n/a	1		
PTR	0.38	0.17-0.85	0.027				0.14	0.03-0.62	0.015
Institute									
AUH	n/a	n/a	n/a	1			n/a	n/a	n/a
NKI				0.33	0.15-0.73	0.008			

Supplementary Table S3. Per protocol analysis, intention-to-treat analysis and instrumental variable analysis for all-cause mortality. WHO PS: World Health Organization Performance Status, CgA: chromogranin A, ULN: upper limit of normal, SSA: somatostatin analogues, PTR: primary tumour resection, NKI: Netherlands Cancer Institute Amsterdam, AUH: Aintree University Hospital Liverpool.

6

Elevated serotonin and NT-proBNP levels predict and detect carcinoid heart disease in a large validation study

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Abstract

Carcinoid heart disease (CHD) is a rare fibrotic cardiac complication of neuroendocrine tumours. Besides known biomarkers N-Terminal pro-B-type natriuretic peptide (NT-proBNP) and serotonin; activin A, connective tissue growth factor (CTGF) and soluble suppression of tumorigenicity 2 (sST2) have been suggested as potential biomarkers for CHD. Here, we validated the predictive/diagnostic value of these biomarkers in a case-control study of 114 patients between 1990-2021. Two time-points were analysed: T0: liver metastasis without CHD for all patients. T1: confirmed CHD in cases (CHD+, n=57); confirmed absence of CHD five or more years after liver metastasis in controls (CHD-, n=57). Thirty-one (54%) and 25 (44%) females were included in CHD+ and CHD- patients, respectively. Median age was 57.9 years for CHD+, and 59.7 for CHD- patients (p=0.290). At T0: activin A was similar across both groups (p=0.724), NT-proBNP was higher in CHD+ patients (17 vs 6 pmol/L, P=0.016), area under the curve (AUC) 0.84 and most optimal cut-off at 6.5 pmol/L. At T1: activin A was higher in CHD+ patients (0.65 vs 0.38 ng/mL, p=0.045), AUC 0.62, without an optimal cut-off value. NT-pro-BNP was higher in CHD+ patients (63 vs 11 pmol/L, P<0.001), AUC 0.89, with optimal cut-off of 27 pmol/L. Serotonin (p=0.345), sST2 (p=0.867) and CTGF (p=0.232) levels were similar across groups. This large validation study identified NT-proBNP as the superior biomarker for CHD. Patients with elevated serotonin levels and NT-proBNP levels between 6.5 and 27 pmol/L, and specifically >27 pmol/L should be monitored closely for the development of CHD.

Introduction

Neuroendocrine tumours (NET) are rare, heterogeneous epithelial tumours, with an incidence of 1.09-5.25/100.000 persons per year, occurring primarily in the gastroenteropancreatic tract with the largest group of NET located in the small intestine (SI-NET).[1,2] In addition, NET can be found in – among others – the lungs and ovaries.[3] Patients with SI-NET often present with regionally advanced or metastatic disease.[1,4] These tumours can secrete vasoactive substances, in particular, 5-hydroxytryptamine (5-HT, also called serotonin).[5] In some rare occasions, ovarian and bronchopulmonary NET may secrete serotonin.[6] Elevated serotonin can lead to typical symptoms such as flushing, wheezing and diarrhoea and give rise to the carcinoid syndrome (CS), which occurs in 30-40% of patients with a SI-NET.[5, 7]

Serotonin is normally metabolised in the liver to the inactive 5-hydroxyindoleacteic acid (5-HIAA), however, the majority of CS patients have liver or retroperitoneal metastases that continuously produce serotonin which is directly released into circulation.[8] This exposes the heart to high circulating levels of serotonin and causes 20-40% of patients to develop carcinoid heart disease (CHD), as is also shown in a recent cohort of 139 patients with elevated urinary 5-HIAA, where 34.5% developed CHD.[7, 9-11] CHD is a complication of CS that is characterized by plaque-like deposits, composed of smooth muscle cells and myofibroblasts and extracellular matrix on the endocardium, leading to fixation and retraction of the heart valves.[7, 9, 10] Despite advances in therapeutic interventions, CHD is still associated with high mortality rates, especially in patients with advanced valve abnormalities,[12, 13] even after undergoing valve replacement surgery.[8,14]

Currently, as per European guidelines,[15] patients with elevated serotonin undergo frequent (1-2 yearly) echocardiography for the detection of CHD although CHD occurrence is highly variable between patients. Early CHD can be missed or progress to a fulminant form in between screenings, whereas other patients never develop CHD and undergo unnecessary visits to the outpatient clinic. In addition to echocardiographic screening, biomarkers are used to detect CHD. Currently, N-terminal pro B-type natriuretic peptide (NT-proBNP) is the best biomarker in diagnosing and assessing the severity of CHD, with levels of NT-proBNP being significantly higher in CHD patients.[12] NT-proBNP is secreted in response to stretching of the cardiac muscle due to increased pressure and thereby reflects the consequences of CHD, rather than predicting patients at risk for CHD. Serotonin has been identified as the key player in the development of CHD, both in human and animal studies.[16-18] Yet besides serotonin, it is assumed that CHD has a multifactorial pathogenesis.[7] Since fibrosis is an important feature of CHD, known mediators of fibrosis have been studied in relationship to CHD,[18-20] including activin A, connective tissue growth factor (CTGF) and soluble suppression of tumorigenicity2 (sST2) in several small studies. In these studies, activin A was associated with the presence of CHD with a sensitivity of 87% in a sample of 15 CHD patients;[18] CTGF was shown to be associated with RV dysfunction and valvular regurgitation in 33 patients with NET,[19] and lastly, sST2 levels that had been elevated at

CHD diagnosis, remained high during and after valve surgery, and only reduced after abdominal surgery for the primary NET.[20]

Here, we present the largest cohort of patients to date with blood samples and CHD to investigate the potential use of circulating activin A, CTGF and sST2 levels as biomarkers associated with the development or presence of CHD, which is confirmed by echocardiography. Our results will be compared to currently used biomarkers known to be associated with the presence of CHD, namely NT-proBNP and serotonin, to eventually identify the superior (combination of) biomarker(s).

Materials and Methods

Design

In a retrospective single centre case-control study serum samples of patients with CHD (cases) were compared to patients without CHD (controls) to find a classifier to predict and/or detect CHD.

Sample size

The primary endpoint for samples size calculation was based on previous literature and is the sensitivity of the classifier.[18] A power calculation was performed assuming an exact binomial distribution. It was calculated that if the true sensitivity of the classifier is 90%, then a sample of 30 condition positive patients (i.e., CHD patients) will be sufficient to reject the null hypothesis that the sensitivity is 65%, in favour of the alternative that it is higher, with 80% power at a significance level alpha of 0.05 (two-sided).

Patient selection

The institutional biobank and neuroendocrine neoplasia (NEN) database of the Netherlands Cancer Institute (NKI) stores patient material and clinical data, respectively, of consecutive patients referred to the NKI from 1990 (biobank) and from 2000 (NEN database) until 2021. From these resources, patients with available serum samples and accompanying clinical data were selected. This study was approved by the Institutional Review Board (IRB) under reference IRBm19-137. CHD (CHD+) was defined as the presence of CHD, determined by echocardiography. Controls were selected from the institutional population of patients with SI-NET. No CHD (CHD-) was defined as patients with a SI-NET, radiologically or histopathologically confirmed liver metastases and elevated serotonin, with no signs of tricuspid or pulmonic regurgitation or other CHD-related right-sided fibrosis of the heart, confirmed by echocardiography, after at least 5 years of follow-up from first occurrence of liver metastases.

Serum samples at two time-points were included, time-point T_0 and T_1. Patients were included if either or both time-points were available. For CHD+ patients, T_0 was defined as the presence of liver- or retroperitoneal metastases and elevated serotonin, with the echocardiographically

confirmed absence of CHD, after or at the moment of sample collection. T_1 was defined as the confirmed presence of CHD, before or simultaneous to sample collection.

For CHD- patients, T_0 was defined as the presence of liver metastases and elevated serotonin, with the proven absence of CHD, after or at the same moment of sample collection. T_1 was defined with similar criteria as T_0, with at least 5 years between the occurrence of liver metastases, before or simultaneous to sample collection. For the prediction of CHD, measurements at T_0 were compared between CHD- and CHD+ patients. For the detection of CHD, the association of included biomarkers with the presence of CHD was investigated by comparing measurements at T_1 between CHD- and CHD+ patients. Assays for sST2, CTGF and activin A were initially performed in selected patients with both T_0 and T_1 time-points available (see Figure 1). Based on results from these selected patients, biomarkers were selected for further analysis in all patients.

Echocardiography

Echocardiography reports were reviewed retrospectively, and information extracted to assess the presence of CHD. CHD was defined as at least moderate-to-severe tricuspid and/or pulmonic regurgitation or moderate tricuspid regurgitation identified by the screening cardiologist as related to the NET. Information from the reports was also recalculated to a CHD score by the standardized report recently defined by the European Neuroendocrine Tumour Society (ENETS) CHD Taskforce.[21] Echocardiography was a transthoracic echocardiography (TTE), performed by an experienced cardiologist as per clinical guidelines and standard operating procedure (SOP) for TTE in the Netherlands.[22]

Blood sampling

Peripheral blood from all patients selected for analysis were collected in serum separation tubes, BD Medical, SST™, BD Vacutainer®. Blood samples were spun down at 1700g for 10 minutes to recover the serum. All samples were stored at -80°C until further analysis.

Enzyme Immunoassays

Serum levels of sST2 and CTGF were analysed with enzyme-linked immunosorbent assay (ELISA) using the human ST2/IL-33R DuoSet ELISA by R&D Systems (Cat. No: DY523B-05; Minneapolis, MN, USA) and CTGF/CCN2 DuoSet by R&D Systems (Cat. No: DY9190-05; Minneapolis, MN, USA), respectively, according to the manufacturer's instructions. Activin A serum levels were assessed with an ELISA from RayBiotech (Cat. No: ELH-ActivinA-5, Norcross, GA, USA). sST2, CTGF and activin A were expressed in ng/mL. Serum serotonin levels were determined by a liquid chromatography tandem mass spectrometry (LC-MS/MS) based assay.[23] Platelet (plt) counts were determined routinely for clinical practice simultaneous to serotonin measurement and serotonin was expressed as nmol/10^9plt. Serum levels of NT-proBNP were determined in serum by an electrochemiluminescence immunoassay used on the Modular Analytics E170 (Roche Diagnostics, Mannheim, Germany) and expressed in pmol/L.[24]

Statistical Analysis

Descriptive statistics were used for baseline characteristics. Median and interquartile range (IQR) were used for continuous variables, frequencies and percentages were calculated for categorical variables. Fisher's exact test and Wilcoxon signed rank test was used for paired comparison within groups and Wilcoxon rank sum test for comparison between groups. Prior to presentation of the data, logarithmic transformation of sST2, CTGF and activin A serum samples was performed. The values were derived from linear regression analysis of the standard curve. For the analysis of NT-proBNP and serotonin, non-transformed values were used. Area under the receiver operator curve (AUC) was calculated with a 95% confidence interval (CI). Sensitivity and specificity were calculated for the relevant biomarkers. The case-control design prevented us from calculating the positive and negative predictive values (PPV and NPV, respectively). Statistical analysis was performed using SPSS 26.0.0.1 software (SPSS Inc., Chicago, IL.), GraphPad Prism software version 8.3.0 for Windows (GraphPad Software, San Diego, California USA) and R statistical software version 4.1.1. P-values were two-sided and considered statistically significant when $p<0.05$.

Disease specific survival (DSS) was defined as the time from initial diagnosis until NET-related death. Since all patients had stage IV disease at inclusion, patients that died of unknown causes were considered to have died of disease. Patients who were lost to follow-up or alive at end of follow-up were censored. Kaplan-Meier curves were used for analysis of survival. Since inclusion criteria for controls could possibly bias the comparison of survival between CHD and no CHD patients, survival analysis was performed in all consecutive patients with stage IV disease SI-NET referred to the NKI between 2000-2019.

Results

Patients

A total of 114 patients were included, of whom 57 CHD+ and 57 CHD- patients. No global differences between the CHD and non-CHD group could be found, except standard cardiac medication use in the CHD+ group (49% vs 28%, p=0.034). Baseline characteristics and comparison between groups are depicted in Table 1. Median time from NET diagnosis to CHD development was 13 months, ranging from 0 to 142 months. Forty-seven (82.5%) patients had underwent annual echocardiographic examination, nine (15.8%) patients bi-annually and one (1.8%) patient only had the first echocardiography three years after diagnosis of liver metastasis. Six (11%) patients developed CHD after ≥5 years. Tricuspid regurgitation was present in all CHD+ patients, being mild in one (2%) patient, moderate in 13 (23%), and severe in 43 (75%) patients. Pulmonic regurgitation was absent in 4 (7%), mild in 9 (16%), moderate in 11 (19%), severe in 14 (25%), and missing in 19 (33%) patients. Right ventricle dilation was assessed in 48 (84%) patients: cardiac dilation was absent in 11 (19%), mild in 8 (14%), moderate in 18 (32%) and severe in 11 (19%) cases. Echocardiographic characteristics can be found in Table 2. Median CHD score for all CHD patients was 10 (range 3-21), yet individual characteristics were often missing and could not be reported. An overview of CHD score per patient can be found in Supplementary

Table S1. Seven (12%) CHD+ patients and 13 (23%) CHD- patients had serum samples at two time-points. All patients had a sample at T_1. A flow diagram of all included patients and the time-points can be found in Figure 1.

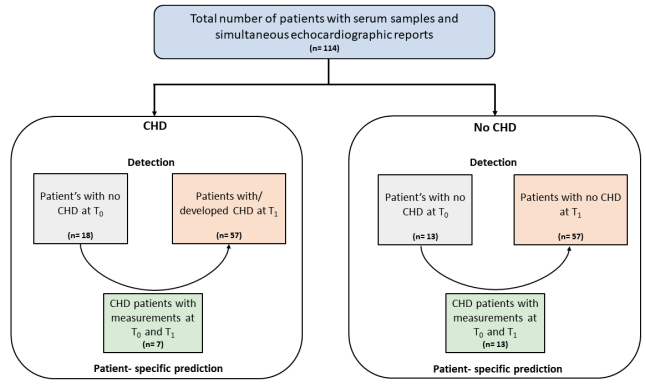

Figure 1. Flow diagram of the included study patients in the CHD or no CHD group at T_0 and T_1.

Characteristic	Group		
	No CHD (n= 57)	CHD (n= 57)	p-value
Sex, n (%)			
Male	26 (45.6)	32 (56.1)	
Female	31 (54.4)	25 (43.9)	0.349
Median age at diagnosis, years (range)	57.9 (32.3 - 76.9)	59.7 (26.8 - 81.7)	0.290
Primary tumour, n (%)			n/a†
Small intestine	56 (98.2)	37 (64.9)	
Ovarium	0	2 (3.5)	
Lung	0	2 (3.5)	
Unknown	1 (1.8)	16 (28.1)	
Patients receiving treatments, n (%)	16 (28.1)	28 (49.1)	0.034¥
Beta blockers	8 (14.0)	8 (14.0)	
ACE-inhibitor	2 (3.5)	5 (8.8)	
Calcium antagonist	7 (12.3)	5 (8.8)	
Nitrates	0	2 (3.5)	
ARB	0	5 (8.8)	
Diuretics	1 (1.8)	21 (36.8)	
Median CHD score (range)	n/a	10 (3-21)	n/a

Table 1. Baseline characteristics of all included patients. P-values show Fisher's exact test for comparison between the patient groups. Medication prescribed to patients included in the study at any moment during follow up. ACE: angiotensin converting enzyme, ARB: angiotensin receptor blocker.
† Comparison irrelevant since controls were selected from a cohort of patients with small intestinal NET.
¥ For comparison of cardiac medication yes/no between groups.

Biomarkers in the Prediction of Carcinoid Heart Disease

To predict the development of CHD, measurements at T_0 were compared between CHD+ and CHD- patients. The T_0 samples were taken a median of 1 (range 0-7) month after diagnosis of liver metastasis, and a median of 2 (0-6) months prior to echocardiographic absence of CHD for both CHD+ and CHD-. Serotonin levels were equally high in both CHD+ (35.3 nmol/10E^{9}plt [range 6.77- 57.2]) and CHD- patients (29.3 nmol/10E^{9}plt [range 8.79- 49.54]) at (P=0.488) (Figure2B). Median serum NT-proBNP were higher in CHD+ patients (17 pmol/L [range 7- 155]) compared to CHD- patients (6 pmol/L [2- 23]) (P=0.016) (Figure 2C). Moreover, the AUC for NT-proBNP was 0.84 (95% CI 0.63-1.0) with the most optimal cut-off for NT-proBNP being 6.5 pmol/L, with a sensitivity of 100% and a specificity of 71.4%. Median serum activin A levels in CHD+ patients (0.66 ng/mL [range 0.06- 3.75]) and CHD- (0.61 ng/mL [range 0.06- 4.93]) were not significantly different (P=0.724) (Figure 2A). Median serum sST2 levels (P=0.867) and CTGF levels (P=0.232) in CHD+ and CHD- patients were not significantly different (Supplementary Figure S1).

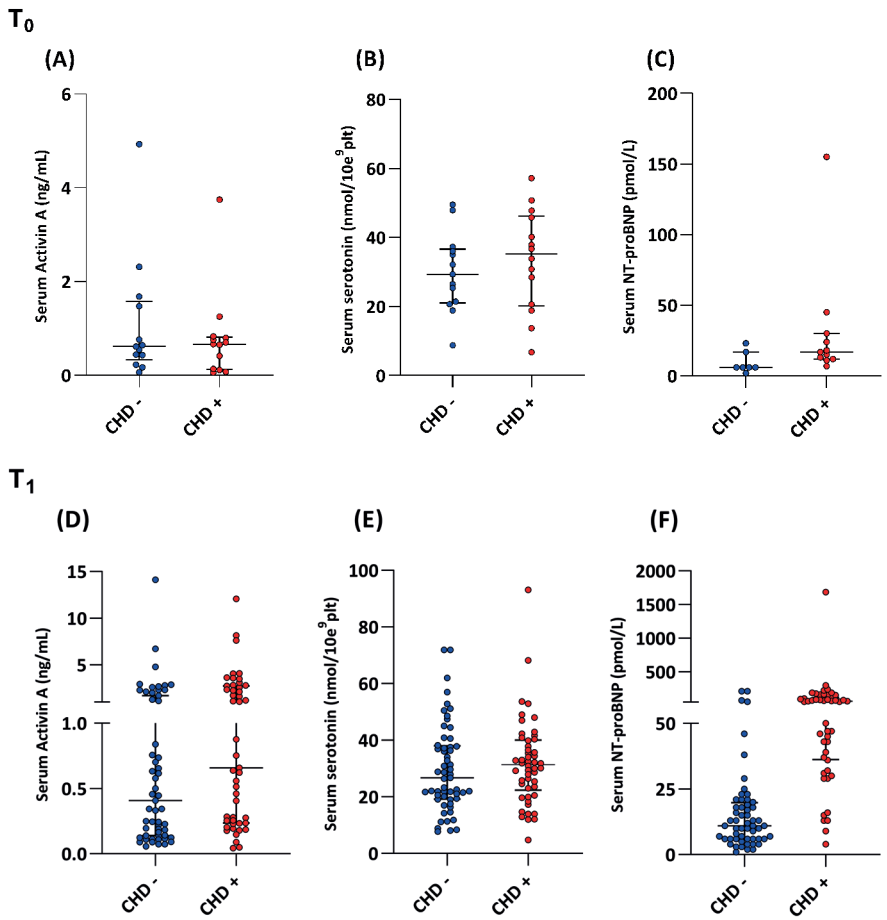

Figure 2. Serum Activin A (A), serotonin (B) and NT- pro BNP (C) levels in CHD+ patients and CHD- patients in the prediction (T_0) and detection of CHD (T_1) (D, E, F).

Biomarkers in the Detection of Carcinoid Heart Disease

For detection of CHD, measurements at T_1 were compared between CHD+ and CHD- patients. For CHD+ patients, T_1 samples were collected a median of 2 (0-4) months after echocardiographic evidence of CHD. For CHD- patients, T_1 samples were a median of 2 (range 0-9) months prior to echocardiographic confirmation of absence of CHD, but with a minimum of five years between first diagnosis of liver metastasis and the sample date. Serotonin levels were equally high in CHD+ patients (31.4 nmol/10E^{9}plt [range 4.79- 93.1]) and CHD- patients (26.7 nmol/10E^{9}plt [range 7.73- 71.9]) (P=0.345) (Figure 2E). Median serum NT-proBNP were higher in CHD+ patients (63 pmol/L [range 4- 1686]) compared to CHD- patients (11 pmol/L [range 1- 213]) (P<0.001) (Figure 2F). The AUC for NT-proBNP was 0.886 (95% CI 0.82 – 0.96) (Figure 3b). By using the current upper limit of normal (ULN) of NT-proBNP for the absence of cardiac conditions of 35 pmol/L,[25, 26] a sensitivity for detecting CHD of 77.1% and a specificity of 89.5% would be achieved. In our cohort, a cut-off of 27 pmol/L would provide the optimal threshold for CHD, with a sensitivity of 87.5% and a specificity of 87.7%. Median serum activin A levels between CHD+ (0.65 ng/mL [range 0.04-12.07]) and CHD+ patients (0.38 ng/mL [range 0.06- 14.12]) (P=0.0451) were significantly different (Figure 2D). The AUC for activin A was 0.616 (95% CI 0.51 – 0.72) (Figure 3a), and did not provide an optimal cut-off value for detection of CHD. Median serum sST2 (P=0.694) and CTGF (P=0.955) levels in CHD+ and CHD- patients were not significantly different (Supplementary Figure S1).

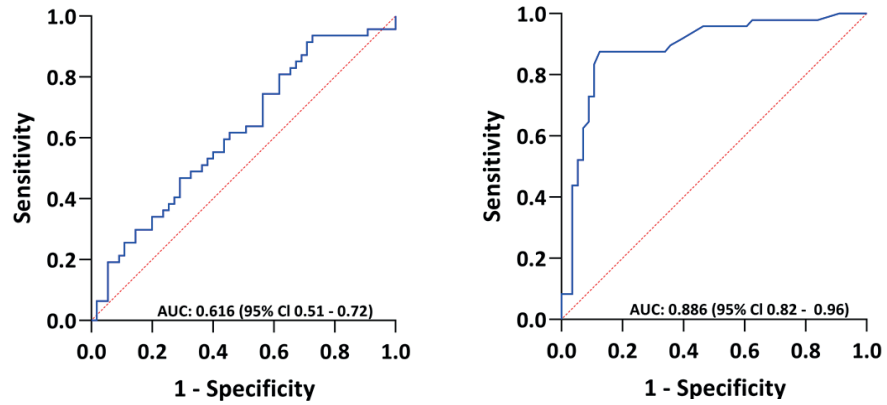

Figure 3A Receiver operator characteristic (ROC) curve of activin A in the detection of CHD at timepoint T_1, between patients with CHD and no CHD (AUC 0.616 [95%CI 0.51 – 0.72, P=0.0451); **3B** ROC-curve representing the ability of NT-pro BNP to detect CHD between patients with CHD and no CHD at T_1 (AUC 0.886 [95% CI 0.82 – 0.96], P<0.001). AUC: area under the curve. CI: confidence interval.

Follow up and survival

The median follow-up time for all 114 patients was 7.3 years (IQR 4.3-36.7). Twenty (35%) patients underwent valve replacement surgery. During follow up, 57 (50%) patients died of their NET, another nine patients (8%) died of unknown causes. In the CHD+ group, 40 (70%) patients died of NET-related causes, and eight (14%) patients died of unknown causes. The cause of death

in 11 (28%) CHD+ patients was directly attributable to CHD. Among CHD- patients, 20 (35%) patients died of NET-related causes one (2%) patient died of unknown causes. The median DSS in CHD+ patients reached 6.4 years (CI 4.2-8.5), this was 13.7 years (CI 11.7-15.6) in CHD- patients (p<0.001) (Figure 4). Similar results were found when including all consecutive patients with stage IV SI-NET as a control group. A total of 330 patients with stage IV SI- NET were included, with a medium DSS of 14.0 years (CI 8.0-20.0, p<0.001).

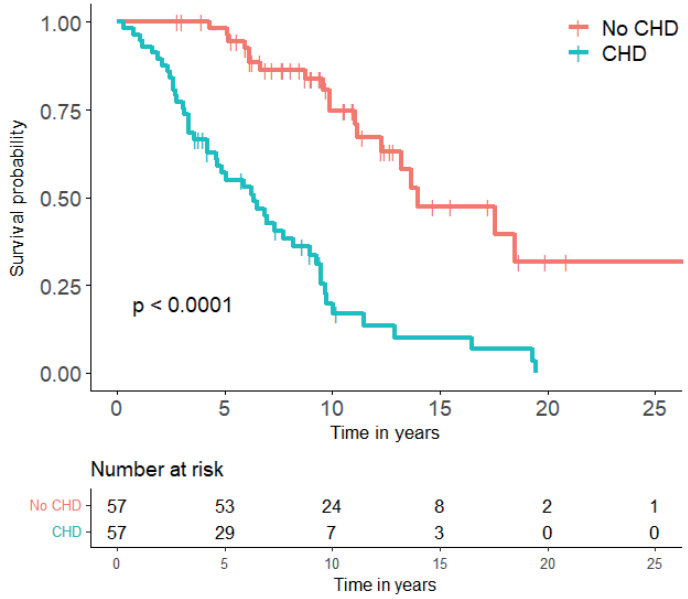

Figure 4. Kaplan-Meier curves for disease specific survival. Logrank test was performed for comparison between groups. CHD: carcinoid heart disease.

Discussion

In the present study, we aimed to validate if previously investigated circulating biomarkers could detect or predict carcinoid heart disease in the largest cohort of CHD+ patients with blood samples to date. We observed that sST2, CTGF and activin A did not show a superior association with CHD over currently used biomarkers. Moreover, NT-proBNP levels of 6.5 and 27 pmol/L showed high accuracy for the prediction and detection of CHD, respectively. Furthermore, survival in patients with CHD remains worse in comparison to patients without CHD.

Echocardiographic characteristic	**CHD patients (n=57)**
TV regurgitation, n (%)	
Mild	1 (1.8)
Moderate	12 (21.1)
Severe	44 (77.2)
TV leaflet thickening, n (%)	
None	5 (8.8)
Mild	10 (17.5)
Moderate	22 (38.6)
Severe	37 (64.9)
Missing	20 (35.1)
PV regurgitation, n (%)	
None	4 (7.0)
Mild	8 (14.0)
Moderate	11 (19.3)
Severe	13 (22.8)
Missing	21 (36.8)
RV dilation, n (%)	
None	10 (17.5)
Mild	7 (12.3)
Moderate	17 (29.8)
Severe	11 (19.3)
Missing	12 (21.1)
RV impairment, n (%)	
None	32 (56.1)
Mild	5 (8.8)
Moderate	2 (3.5)
Severe	1 (1.8)
Missing	17 (29.8)
MV regurgitation, n (%)	
None	6 (10.5)
Mild	22 (38.6)
Moderate	7 (12.3)
Severe	3 (5.3)
Missing	19 (33.3)
AV regurgitation, n (%)	
None	13 (22.8)
Mild	15 (26.3)
Moderate	3 (5.3)
Severe	0
Missing	26 (45.6)

Table 2. Echocardiographic characteristics of all patients with confirmed carcinoid heart disease (CHD). TV: tricuspid valve, PV: pulmonic valve, RV: right ventricle, MV: mitral valve, AV: aortic valve.

Regarding prediction of CHD, we are the first to identify NT-proBNP to be significantly higher in CHD+ patients, even before the onset of CHD, and can predict the development of CHD. These results suggest that mild to moderate strain on cardiomyocytes might release NT-proBNP before echocardiographic evidence of fibrosis of the right-sided heart can be identified. It is important to note that NT-proBNP is not a marker that shows the causal molecular pathway of the pathogenesis of CHD, and is therefore rather a sensible early diagnostic marker than a true predictor. Nevertheless, since NT-proBNP is elevated in patients that will develop echocardiographic CHD in the future, it has the capability to differentiate at baseline between patients are at risk of developing CHD and those that are not. Because of these strong predictive abilities, we have chosen to call it a predictor.

Regarding detection of CHD, NT-proBNP expression is significantly elevated in CHD+ patients and directly associates with CHD severity.[24, 27-29] For instance, in a cohort of 187 patients with NET and liver metastases, of whom 37 had CHD, NT-proBNP was found to be to have an AUC of 0.82.[28] Our results confirm that NT-proBNP outperforms other biomarkers for CHD, and further identify that a cut-off of 27 pmol/L has the best accuracy of detecting CHD. With these findings, we argue that clinicians could make a more accurate distinction of patients that would benefit from (more frequent) echocardiographic screening, and which would not. For instance, patients with NT-proBNP levels >6.5 pmol/L could undergo echocardiography 1-2 yearly as per current guidelines, whereas patients with NT-proBNP levels <6.5 pmol/L could be released from echocardiographic screening, but be followed only with active monitoring of NT-proBNP levels. Moreover, patients without echocardiographic signs of CHD, but with NT-proBNP levels >27 pmol/L could possibly benefit from more active screening than is currently advised by European guidelines,[15] for instance, by six monthly echocardiography.

Activin A levels differed significantly between CHD+ and CHD- patients at T_1. Despite this, we found that activin A was not able to provide an optimal cut-off level for CHD in our cohort. In the study by Bergestuen, et al., activin A levels ≥0.34 ng/ml were found to be associated with an increased risk of developing CHD in 15 patients.[18] The positive results for activin A in that study may have been caused by the small number of patients included. Most CHD patients included in this study cohort had moderate to severe or severe regurgitation and thickening of the tricuspid valves (TV) or pulmonary valves. It is hypothesised that activin A may reach a threshold value to initiate the molecular pathways associated with fibrosis, and not play a role in disease progression.[18] This may explain why, although elevated in CHD+ patients, we were not able to identify a cut-off value for detection of CHD, since this threshold may have been reached in moderately elevated levels of activin A. Nevertheless, it remains unknown why some patients would develop CHD above this threshold, and others do not.

Serotonin is still regarded as the best clinical tool in identifying patients at risk of CHD. However, it can be limited in providing optimal accuracy in diagnosing CHD since not all patients with elevated serotonin develop CHD. We did not find an association between higher serotonin levels and CHD, as has been identified previously.[28, 30, 31] A recent review concluded that elevated

5-HIAA levels were associated with CHD and with higher mortality.[32] Yet previous studies mostly compared CHD patients with NET patients, with or without elevated serotonin, whereas we refined our inclusion criteria and specifically selected controls with confirmed liver metastases and elevated serotonin. Consequently, this selection provided a more homogeneous group of patients to study, but it prevented us from comparing serotonin levels to patients with a NET in general. Nevertheless, the fact that we found equal groups of patients with and without CHD during the inclusion period, again suggests that elevated serotonin may not be the only factor that contributes to CHD, but an unknown causal factor is involved in the development of CHD. This implies that the management of CHD patients should not only be aimed at reducing serotonin levels by known methods such as somatostatin analogues or debulking surgery, but also at early detection and intervention for CHD.

We found that patients with CHD had a worse survival compared to patients without CHD. This has also been confirmed by other studies investigating the prognosis of patients with CHD.[32-34] Indeed the percentage of deaths directly attributable to CHD (27.5%) seem to make up the difference in survival between patients with and without CHD. This stresses the urge for early recognition and possible intervention for CHD in patients with elevated serotonin.

There are several limitations worth mentioning. Firstly, we used a cut-off value of five years as a criterion for the selection of controls. It is possible that patients in the CHD- group could yet develop CHD during follow-up. Nevertheless, in our CHD+ cohort, nearly 90% of patients developed CHD within five years of liver- or retroperitoneal metastases. Therefore, it is unlikely that the number of CHD- patients who could possibly still develop CHD would be large enough to bias our results. Secondly, our sample size calculations were based on the detection of CHD, and not on prediction of CHD. Also, a total of 31 patients had samples at T_0, which might be insufficient to adequately identify the optimal cut-off level of NT-proBNP for the prediction of CHD. Nonetheless, our results are the first to indicate a cut-off value for the detection of CHD, and provides evidence that NT-proBNP levels are significantly higher in patients with CHD, even before any abnormalities can be found by echocardiography. These results stress the need for adequate monitoring of patients with elevated serotonin, even with moderately elevated NT-proBNP levels.

A major strength of this study is the large sample size. This is the largest study to date to investigate patients with CHD and possible associated biomarkers. Moreover, we were able to nearly double the sample size initially calculated for this study, therefore increasing the statistical power.

Conclusions

In conclusion, in this largest validation study of biomarkers for CHD to date, we found that sST2, CTGF and activin A are not useful in predicting or detecting CHD over currently used biomarkers. NT-proBNP, in the presence of elevated serotonin, remains the best suited biomarker in clinical

practice. This is the first study that provides structured guidance in the management of patients with serotonin producing NET. Patients with NT-proBNP values below 6.5 pmol/L could likely be released from echocardiographic screening, whereas patients with NT-proBNP values above 6.5 pmol/L could undergo screening as per current guidelines. Moreover, patients with NT-proBNP above 27 pmol/L should be monitored even more closely for the development of CHD. Patients with CHD have a worse disease specific survival compared to patients without CHD. Future studies should focus on elucidating the molecular mechanisms of the development of CHD and further identify patients at risk thereof.

Author Contributions: Conceptualization, S.L, L.L., G.V. and M.T.; methodology, S.L, A.K., L.L, M.T.; software, S.L, A.K.; validation, S.L., A.K., L.L. and M.T.; formal analysis, S.L and A.K.; investigation, S.L and A.K.; resources, L.L, C.K. and M.T.; data curation, S.L and A.K.; writing—original draft preparation, S.L. and A.K..; writing—review and editing, S.L, A.K, C.K., M.O., J.S., L.L, G.V., M.T..; visualization, S.L. and A.K..; supervision, L.L., J.S., G.V., M.T..; project administration, S.L. and M.T..; funding acquisition, S.L, A.K., L.L., J.S., G.V., M.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of the Netherlands Cancer Institute (reference code IRBm19-137, date of approval 06 May 2019).

Informed Consent Statement Informed consent was obtained from all subjects involved in the study as per institutional protocol.

Data Availability Statement The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest The authors declare no conflict of interest.

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Supplementary material

Supplementary Table S1: Individual CHD-scores per patient.

Patient	TV - regurgitation	TV - stenosis	TV - leaflet thickening	TV - leaflet excursion	TV - leaflet retraction	PV - regurgitation	PV - stenosis	PV - cusp thick	PV - cusp excursion	PV - cusp retraction	RA -dilation	RV -dilation	RV -impairment	MV - regurgitation	MV -stenosis	MV - leaflet thickening	MV-leaflet excursion	MV - leaflet retraction	AV - regurgitation	AV - cusp thickening	AV - excursion	AV - retraction	CHD-score
1	3		3		3		0					3	3	0	0	0	0	0					18
2	3		3		3	2	0	2				3	3	0	0	0	0	0					18
3	3	3		3										1					1				11
4	3	0	3			2	1					3	0	1	1				1				14
5	3	0				3		2				2	1	1	1	1							14
6	3		3			3	1					2	0	1	0				1				14
7	2	0	2			1	0	0						3					1	1			9
8	2		2																				4
9	3		2			1						0	0	3					1	1			10
10	3		3			3	3					2	0	1					1				16
11	2		2			0	0					0	0	1					1	1			7
12	3	2	3		3	1	0					1	0	1		1			1				15
13	3	0	1			1	0					0	0	2		1							8
14	3		1									0	0	1					1				6
15	3		1																				4

Table S1; Continued.

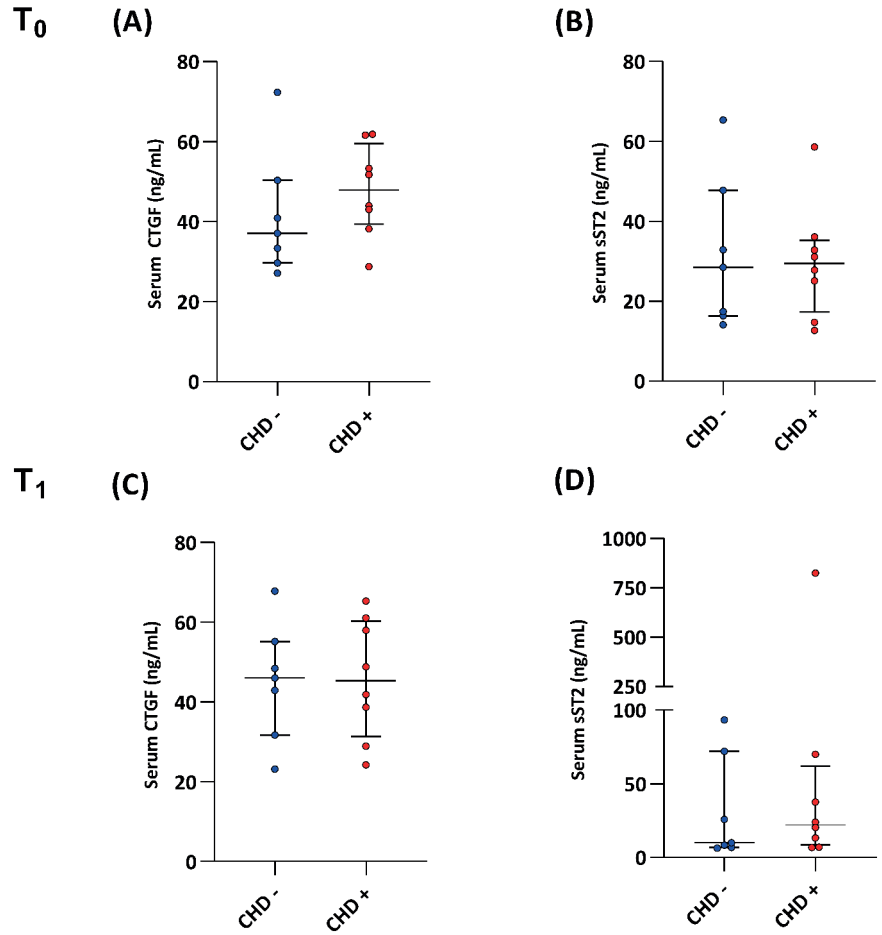
Patient	TV - regurgitation	TV - stenosis	TV - leaflet thickening	TV - leaflet excursion	TV - leaflet retraction	PV - regurgitation	PV - stenosis	PV - cusp thick	PV - cusp excursion	PV - cusp retraction	RA -dilation	RV -dilation	RV -impairment	MV - regurgitation	MV -stenosis	MV - leaflet thickening	MV-leaflet excursion	MV - leaflet retraction	AV - regurgitation	AV - stenosis	AV - cusp thickening	AV - excursion	AV - retraction	CHD-score
16	3											3												6
17	2		3			2		3				0	0	1										11
18	3											0	0						0		1			4
19	3		2									2	0	1					2					10
20	2	0	2			0	0						0	2					1					5
21	2		2									1	0	2					1		1			11
22	3		3			3						2	0	1					0					12
23	3					3						2	0	2					0					10
24	3											3												3
25	3	2				1	0					3		1					0					10
26	3	2	2			3	2					3	2	2					0					19
27	3											2	0	1					0					9
28	3		3			0	0					2	0	1					2					9
29	2		1									1	0	1					1					4
30	2					3						1	0	2					1					10
31	3		3									3	0	1					2					13
32	3					3						3	0	2					0					12
33	2					1						0		2					0					8

Table S1; Continued.

Patient	TV - regurgitation	TV - stenosis	TV - leaflet thickening	TV - leaflet excursion	TV - leaflet retraction	PV - regurgitation	PV - stenosis	PV - cusp thick	PV - cusp excursion	PV - cusp retraction	RA -dilation	RV -dilation	RV -impairment	MV - regurgitation	MV -stenosis	MV - leaflet thickening	MV-leaflet excursion	MV - leaflet retraction	AV - regurgitation	AV - stenosis	AV - cusp thickening	AV - excursion	AV - retraction	CHD-score
34	3				3							1	0	1										9
35	3		3			2	0					2	0	1										10
36	3	2			3							3	2	1					1					14
37	3		2									3												3
38	3				3	3						3	0	2					0					14
39	3		2		1	1						2	0	1					1					10
40	4					2						1	0	1					0					5
41	3		3			1		2				2	0	1					1					12
42	2		3			3		3				2	0	1					0					14
43	3		3		0							2	0	1					0					8
44	3		3		3	2						2	0	1					0					15
45	3											3												3
46	3					2						3	2	1					0					14
47	1					3		1				3	2	2					1					14
48	2											2	0	1					0					5
49	3		3		3	2						2	0	1		1			0					15
50	3		3		3	2						3	0	2					0					15
51	3		3		3	2						0	0	2					0					21

Patient	TV - regurgitation	TV - stenosis	TV - leaflet thickening	TV - leaflet excursion	TV - leaflet retraction	PV - regurgitation	PV - stenosis	PV - cusp thick	PV - cusp excursion	PV - cusp retraction	RA -dilation	RV -dilation	RV -impairment	MV - regurgitation	MV -stenosis	MV - leaflet thickening	MV-leaflet excursion	MV - leaflet retraction	AV - regurgitation	AV - stenosis	AV - cusp thickening	AV - excursion	AV - retraction	CHD-score
52	3		3		3							3	1											13
53	3		3			2						0	0											8
54	3		3		1							1	1											9
55	3		3		3	3	0					2	0	1					2					17
56	3											2	1											6
57	3		1			3	1					2	1	1					0					12

Table S1; Continued.



Supplementary Figure S1. Serum sST2 and CTGF levels at time-points T0 and T1. Both sST2 (P=0.867) (A) and CTGF (P=0.232) (B) are not significantly different at T0. This is similar at T1 for serum sST2 (P=0.694) (C) and CTGF (P=0.955) (D).

7

Four decades of experience with carcinoid heart disease: an analysis of 84 patients.

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Abstract

Background

Carcinoid heart disease (CHD) is a serious cardiac condition which is caused by elevated serotonin in the systemic circulation, secreted by neuroendocrine tumours (NET). It mostly affects the right-sided heart valves, where it causes fibrotic disturbances and is associated with worse survival. With this study, we describe a large cohort of patients with CHD and provide insight in the survival over the past decades.

Methods

All consecutive patients with a serotonin producing NET and CHD referred to the Netherlands Cancer Institute that presented with CHD or developed CHD during their follow up time were included from 1984 until 2021. Patients were divided in three time periods: 1984-2000, 2000-2010 and 2010-2018. Median N-terminal pro B-type natriuretic protein (NT-proBNP) and serum serotonin levels were stratified according to tricuspid regurgitation severity. Kaplan-Meier curves and logrank test were used for visualisation of survival. Cox regression was used for identification of characteristics associated with disease specific mortality (DSM).

Results

A total of 84 patients with CHD were included of whom 49 (58.3%) were male. Median age at NET diagnosis was 62.3 (range 23.9-81.7) years, and median time to development of CHD was 1.1 (range 0-24.2) years. NT-proBNP was significantly higher when more severe TR was present (p=0.027). Median survival from CHD diagnosis for 1984-2000, 200-2010 and 2010-2018 were 1.3 (confidence interval [CI] 0.9-1.6), 1.9 (CI 1.2-2.6) and 3.9 (CI 1.7-6.2) years (p=0.025). Valve replacement surgery (VRS) occurred more frequent in later time periods. VRS (hazard ratio [HR] 0.33, p=0.005) and NT-proBNP (HR 1.003, 1.00-1.005, p=0.036) were significantly associated with DSM.

Conclusion

The prognosis of patients with CHD has improved over the past decades, possibly caused by more VRS. NT-proBNP is a valuable biomarker in patients with CHD. Clinical practice should be aimed at timely diagnosis and intervention of CHD.

Introduction

Neuroendocrine tumours (NET) are an epithelial malignancy that occur in various parts of the body.[1, 2] NET, by definition, have a well-differentiated morphology and consist of grade 1-3 tumours, according to mitosis count and Ki67-index.[3] Although rare, the most common primary tumours arise in the gastroenteropancreatic tract, of which NET of the small intestine (SI-NET) comprise the largest group, with an overall incidence of 0.5-1.42/100.000 persons.[4-6] Since NET arise from cells with neuroendocrine abilities, these tumours are often accompanied by increased secretion of vasoactive peptides, of which the most common is 5-hydroxytryptamine, also called serotonin.[7] In the setting of localized disease, the excess of serotonin is metabolized by the liver to the inactive metabolite 5-hydroxyindoleacetic acid (5-HIAA).[8] Yet when liver or retroperitoneal metastasis is present, this process is bypassed and peptides secreted by the tumours may access the circulation, giving rise to systemic symptoms such as flushing, diarrhoea and wheezing, called the carcinoid syndrome (CS), which occurs in 30-40% of patients with SI-NET.[9, 10] In some cases, NET of the ovary or lung may also secrete serotonin and similar symptoms occur.[11, 12] In 30-50% of patients with CS, elevated systemic levels of serotonin cause carcinoid heart disease (CHD).[13, 14] CHD consists of fibrotic changes of the endocardium, which leads to thickening and retraction of the heart valves.[14-16] The mechanism with which serotonin leads to these fibrotic valve abnormalities has not been completely elucidated. The 5-hydroxytryptamine-2B ($5HT_{2B}$) serotonin receptor has been found to play a role in the pathogenesis cardiac valve disease associated with increased levels of serotonin.[17] It is thought that the interaction of serotonin with $5HT_{2B}$ leads to activation of the extracellular matrix which subsequently leads to the characteristic heart valve changes. Nevertheless, it is generally argued that the pathogenesis of CHD is a multifactorial process that most likely involves pathways that include members of the transforming growth factor beta (TGF-β) family, but these and other accompanying processes have yet to be identified.[15, 18, 19] When serotonin has passed through the right-sided heart, it enters the pulmonic circulation, where, similar to the liver, the same metabolization of serotonin to 5-HIAA occurs. Hence CHD occurs predominantly in the right-sided heart, with 90-100% of patients showing tricuspid valve regurgitation and 50% showing pulmonic valve regurgitation.[20, 21] The left-sided heart is may be affected in case of a patent foramen ovale or serotonin-secreting NET of the lung, and occurs in approximately 10% of patients with CHD.[22, 23] Since higher serotonin levels are associated with occurrence and progression of CHD, treatment is aimed at decreasing levels of serotonin, by the use of somatostatin analogues (SSA), or reducing tumour burden through debulking surgery, embolization procedures or peptide receptor radionuclide therapy (PRRT). Yet when fulminant CHD is present, patients undergo valve replacement of affected valves to prevent or improve right-sided heart failure.[24] Over the past decades, the screening for CHD has been implicated in the European Neuroendocrine Tumour Society (ENETS) guidelines and in clinical practice, including serotonin (or 5-HIAA) measurement, and measurement of cardiac damage by N-terminal pro B-type natriuretic peptide (NT-proBNP), combined with 1-2 yearly echocardiography for CHD.[25] This has led to increased diagnosis and more timely intervention for CHD. Nevertheless, despite advances in therapeutic interventions, CHD is still associated

with high mortality rates, especially in patients with advanced valve abnormalities, even after undergoing valve replacement surgery.[26,27]

In this study, we describe our experience with patients with CHD over the past four decades in the largest European cohort to date. We aim to show the current prognosis of patients with CHD when compared with previous time periods and to provide an indication of the areas in need of improvement in the management of patients with CHD.

Methods

Patients

In this retrospective cohort study all consecutive patients with a NET referred to the Netherlands Cancer Institute (NKI) ENETS Centre of Excellence between January 1st 1984 and March 1st 2021 were eligible for inclusion. Patients were selected when CHD was present either at presentation or developed during follow up. CHD was established by echocardiographic evaluation by an experienced cardiologist. Patients were followed up until death or end of follow up on December 31st 2021, whichever occurred first. Patient and tumour characteristics as well as information on treatment and surgical valve replacement were retrieved from patient records. Serum blood measurements of serotonin) and NT-proBNP at CHD diagnosis were collected routinely and extracted from laboratory records. For patients with missing serotonin or NT-proBNP values, but with available serum samples that were retrieved at the time of CHD diagnosis and were stored in the NKI biobank, serotonin and NT-proBNP measurements were performed on these samples. Serum serotonin levels were determined by a liquid chromatography tandem mass spectrometry (LC-MS/MS) based assay.[28] Platelet (plt) counts were determined routinely for clinical practice simultaneous to serotonin measurement and serotonin was expressed as nmol/10^9plt with an upper limit of normal [ULN] 5.8 nmol/10^9plt. Serum levels of NT-proBNP were determined in serum by an electrochemiluminescence immunoassay used on the Modular Analytics E170 (Roche Diagnostics, Mannheim, Germany) and expressed in pmol/L (ULN 35 pmol/L).[29] This study was approved by the institutional review board of the NKI and all patients gave consent for the use of their clinical and biological data as per institutional protocol.

Echocardiography

Echocardiography reports were reviewed and information extracted to assess the presence of CHD. CHD was defined as at least moderate-to-severe (II-III/IV) tricuspid and/or pulmonic regurgitation or moderate tricuspid regurgitation identified by the screening cardiologist as related to the NET. Information from the reports was also recalculated to a CHD score by the standardized report recently defined by the ENETS CHD Taskforce.[30] Echocardiography was a transthoracic echocardiography (TTE), performed as per clinical guidelines and standard operating procedure (SOP) for TTE in the Netherlands.[31]

Statistics

Descriptive statistics were used for patient characteristics: median with range for continuous and numbers with frequencies for categorical characteristics. For comparison between subgroups, Fisher's exact test was used for categorical variables and Wilcoxon rank sum or Kruskal-Wallis test when appropriate for continuous variables. Patients were divided in three time periods according to time of CHD diagnosis, to assess the change in prognosis over the past decades: 1985-2000, 2000-2010 and 2010-2018. To prevent biased results due to shorter follow up time, the last period was determined to end at 2018 to ensure at least three years of follow up after CHD diagnosis. Kaplan-Meier curves were used for visualisation of survival and logrank test was performed for univariable comparison between subgroups. Cox regression for proportional hazards was used for multivariable identification of characteristics associated with disease specific mortality (DSM). DSM was defined as time from diagnosis of CHD until documented death from NET. Variables included in the model were selected based on the prior assumption of their contribution to survival. To preserve statistical power, multiple imputation was performed for missing values. The number of imputations was determined by the largest proportion of missing data. Since all patients had stage IV disease, patients with an unknown cause of death were considered to have died of disease. Patients alive at end of follow up or who were lost to follow up were censored. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) Software version 26.0.0.1 (Chicago, IL.)) and R statistical software version 4.1.1. P-values were two-sided and considered statistically significant when $p<0.05$.

Results

Patients

A total of 84 patients with CHD were included, of whom 49 (58.3%) were male. Most patients (n=66, 78.5%) had a SI-NET as primary tumour, 3 (3.6%) had a primary ovarian NET, 2 (2.4%) had a primary lung NET and in 13 (15.5%) patients a primary tumour could not be identified. Median age at NET diagnosis was 62.3 (range 23.9-81.7) years, and median time to development of CHD from initial NET diagnosis was 1.1 (range 0-24.2) years, and median time to development of CHD from the first occurrence of liver metastasis was 1.0 (range 0-11.8) year. All but one (1.2%) patient developed CHD within ten years of the first occurrence of liver metastases, and 76 (83.9%) patients developed CHD within 5 years after liver metastasis. The majority of patients (n=56, 66.7%) had a I-II NYHA classification at CHD diagnosis. All patient characteristics are summarized in Table 1.

Biomarkers

Median NT-proBNP was 70.5 pmol/L (range 4.0-803.0 pmol/L) and median serotonin level was 35.5 nmol/10^9plt (4.8-97.0 nmol/10^9plt). When stratified by mild/moderate, moderate or severe tricuspid regurgitation (TR), NT-proBNP was increasingly higher in each subgroup (Kruskal-Wallis p=0.027). A similar association was seen for serotonin levels, although this did not reach statistical significance (Kruskal-Wallis p=0.054).

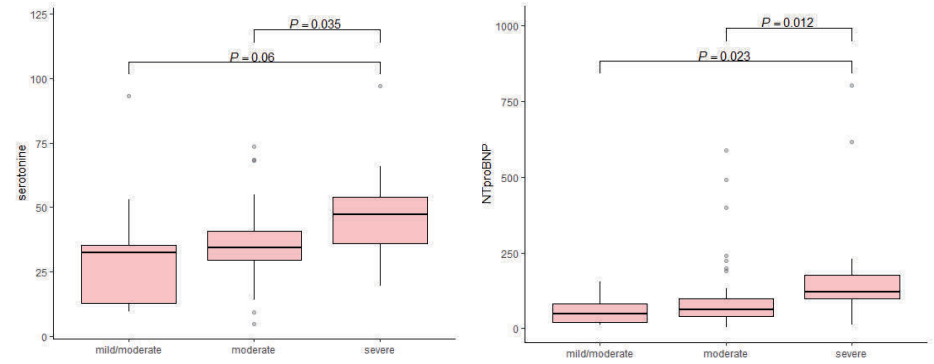
Patients with CHD	N (%)/median (range)
Total	84
Sex	
Male	49 (58.3)
Female	35 (41.7)
Age at diagnosis	62.3 (23.9-81.7)
Age at CHD	64.4 (23.9-85.3)
Primary tumour	
Small intestine	66 (78.5)
Ovary	3 (3.6)
Lung	2 (2.4)
Unknown	13 (15.5)
Time to CHD (years)	
From diagnosis	1.1 (0-24.2)
From liver/retroperitoneal metastasis	1.0 (0-11.8)
NYHA	
I	27 (32.1)
II	29 (34.5)
III	14 (16.7)
IV	6 (7.1)
Missing	8 (9.5)
NT-proBNP, pmol/L	70.5 (4.0-803.0)
Missing	10 (11.9)
Serotonin, nmol/10^9plt	35.5 (4.8-97.0)
Missing	20 (23.8)

Table 1. Patient characteristics of all patients with carcinoid heart disease (CHD). NYHA: New York Heart Association, NT-proBNP: N-terminal pro B-type natriuretic peptide.

Comparison of patients with severe TR to mild/moderate or moderate separately showed higher NT-proBNP levels in the most severe group (p=0.026 and p=0.013, respectively). For serotonin comparison between severe TR and mild/moderate TR was not significantly different (p=0.06), comparison between severe and moderate TR showed significantly higher serotonin levels (p=0.035). One patient developed CHD from a serotonin producing ovarian NET without metastases, and remained free of NET after a radical resection of the tumour. The CHD in this patient remained stable (moderately severe [III/IV]) with low serotonin levels (4.8 nmol/10^9plt). The values of NT-proBNP and serotonin stratified by severity of tricuspid regurgitation are shown in Table 2 and Figure 1A&B.

	Mild-to-moderate TR	Moderate TR	Severe TR	p-value
Total	**14**	**51**	**16**	
NT-proBNP, pmol/L				0.027
Available measurement N (%)	13 (92.9)	48 (92.3)	13 (81.3)	
Median (range)	66.0 (13.0-154.0)	61.0 (4.0-588.0)	121.0 (12.0-803.0)	
Serotonin, nmol/10^9plt				0.054
Available measurement N (%)	9 (64.3)	42 (80.8)	13 (81.3)	
Median (range)	32.5 (9.5-93.1)	34.2 (4.8-73.4)	47.3 (19.6-97.0)	

Table 2. Biomarkers stratified according to severity of tricuspid regurgitation (TR). NT-proBNP: N-terminal pro B-type natriuretic peptide.



A **B**

Figure 1. A Serotonin stratified stratified according to severity of tricuspid regurgitation (TR). **B.** NT-proBNP stratified stratified according to severity of tricuspid regurgitation (TR).

Valve replacement

More valve replacement surgeries were performed in more recent time periods. A total of 28 (33.3%) patients underwent valve replacement surgery: one (5.3%) patient in 1985-2000, ten (34.5%) in 2000-2010 and 17 (47.2%) in 2010-2018 (Table 3).

Time period	1985-2000	2000-2010	2010-2018
	N (%)/median (95% CI)	N (%)/median (95% CI)	N (%)/median (95% CI)
Total	**19**	**29**	**30**
Survival (years)	1.3 (0.9-1.6)	1.9 (1.2-2.6)	3.9 (1.7-6.2)
Valve replacement surgery	1 (5.3)	10 (34.5)	17 (47.2)

Table 3. Survival from carcinoid heart disease diagnosis until disease specific mortality of patients and frequency of valve replacement surgery stratified by time period. CI: confidence interval.

All patients underwent valve replacement surgery due to severe valvular dysfunction with either beginning right-sided heart failure or arguably the valve abnormalities would shortly lead to right-sided heart failure. Of these patients, 13 (39.3%) underwent a solitary replacement of the tricuspid valve, another 13 (29.3%) underwent replacement of both the tricuspid and the pulmonic valve. One (3.0%) patient underwent replacement of the tricuspid, pulmonic and mitral valve, and one (3.0%) patient underwent replacement of all four valves. Of the patients undergoing valve replacement, four (12.1%) had a patent foramen ovale that was closed surgically. The majority of patients (n=20, 60.6%) received a biological tissue valve. Of these, two (10.0%) patients had recurrent CHD of their biological tissue valves, and underwent a re-intervention for mechanical valve placement. The re-interventions occurred at 17 months post initial surgery with maximum pre-re-intervention serotonin levels of 42.6 nmol/10^9plt for the first patient and at 39 months post initial valve replacement with maximum pre-re-intervention serotonin levels of 52.4 nmol/10^9plt for the second patient. Seventeen (60.7%)

patients experienced grade 1-5 adverse events from the surgical procedure. Of these, six (35.3%) had a grade 3 adverse event (AE); and another six (35.9%) had a grade 4 AE; one (5.9%) patient died from complications 19 days after surgery. A summary of all adverse events can be found in supplementary Table S1.

Patients	No valve surgery N (%)/median (range)	Valve replacement surgery N (%)/median (range)	p-value
Total	**56**	**28**	
Sex			0.640
Male	34 (60.7)	15 (53.6)	
Female	22 (39.3)	13 (46.4)	
Age at CHD	68.9 (42.6-85.3)	58.5 (23.9-79.5)	0.005
NYHA			0.311
I	19 (38.8)	8 (28.6)	
II	17 (30.4)	12 (42.9)	
III	9 (16.1)	5 (17.9)	
IV	4 (7.1)	2 (7.1)	
Missing	7 (12.5)	1 (3.6)	
NT-proBNP, pmol/L	80.0 (11.3-803.0)	64.0 (4.0-198.0)	0.571
Serotonin, nmol/10^9plt	32.5 (4.8-93.1)	47.0 (13.9-97.0)	0.001

Table 4. Characteristics of patients with and without valve replacement surgery for carcinoid heart disease (CHD). NYHA: New York Heart Association, NT-proBNP: N-terminal pro B-type natriuretic peptide.

Patients that underwent valve replacement surgery were significantly younger (58.5 vs 68.9 years, p=0.005) and had higher serotonin levels (47.0 vs. 32.5 nmol/10^9plt) compared to patients that did not undergo a surgical procedure for CHD. Characteristics of patients that underwent cardiac valve replacement and the comparison with patients that did undergo surgical intervention can be found in Table 4. Survival curves for these groups can be found in Figure 2.

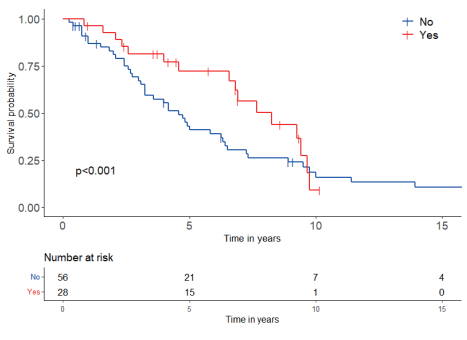


Figure 2. Kaplan-Meier curves for disease specific mortality according to valve replacement surgery.

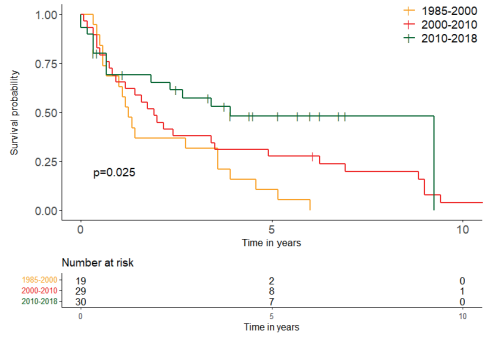


Figure 3. Kaplan-Meier curves for disease specific mortality according to time period.

Survival

After a median follow up time of 2.8 years for all patients, median disease specific survival was 2.2 (confidence interval [CI] 1.1-3.2) years from CHD diagnosis. A total of 55 (65.5%) died of their NET, five (9.5%) patients died of unknown causes and two (2.4%) patients died of comorbidities. In 20 (36.4%) patients who died of disease, the cause of death was directly attributable to CHD. Of the patients that underwent cardiac surgery, three (10.7%) died of CHD; of the patients that did not undergo cardiac surgery, 17 (30.4%) died of CHD directly. Survival times were significantly different across the three time periods: median survival time was 1.3 (CI 0.9-1.6) years for patients diagnosed in 1985-2000, this was 1.9 (CI 1.2-2.6) years for period 2000-2010, and 3.9 (CI 1.7-6.2) for the time period 2010-2018 (p=0.025) (Table 3). Survival curves for the three time periods are shown in Figure 3.

Variables included in the multivariable cox regression were age at CHD diagnosis, sex, NYHA classification, valve replacement surgery, NT-proBNP and serotonin levels. Only valve replacement surgery (HR 0.33, CI 0.15-0.71) and NT-proBNP levels (HR1.003 (1.00-1.005) were significantly associated with DSM. Since NT-proBNP was included in the model as a continuous variable, the HR of 1.003 for NT-proBNP is interpreted as follows: an increase of 10 pmol/L gives a HR of 1.003^10=1.03. Similarly, an increase of 100 pmol/L gives a HR of 1.003^100=1.3. Results of the multivariable analysis are shown in Table 5.

Patients	HR	95% CI	p-value
Sex	1	0.62-2.36	0.579
Male	1.21		
Female			
Age at CHD	1.00	0.97-1.03	0.998
NYHA			
I	1		
II	1.52	0.65-3.57	0.332
III	2.13	0.72-6.33	0.171
IV	2.33	0.68-7.99	0.176
Valve replacement surgery	0.33	0.15-0.71	0.005
NT-proBNP, pmol/L	1.003	1.00-1.005	0.036
Serotonin, nmol/10^9plt	1.00	0.98-1.02	0.920

Table 5. Cox regression for disease specific mortality. HR: hazard ratio, CI: confidence interval, CHD: carcinoid heart disease, NYHA: New York Heart Association, NT-proBNP: N-terminal pro B-type natriuretic peptide.

Discussion

In this large study of patients with CHD, covering a nearly four-decade time period, we found that the prognosis of patients with CHD has improved significantly over the past decades,

and that valve replacement surgery and NT-proBNP levels are associated with disease specific mortality. Further, we found that patients undergoing valve replacement are younger and have higher serotonin levels.

To the best of our knowledge, the last and only study that investigated the time-varying prognosis of patients with CHD, by Moller, et al. stems from 2005. There, 200 patients with CHD were included in three time periods: 1981-1989, 1989-1995 and 1995-2000, with a median overall survival of 1.5, 1.9 and 4.4 years, respectively.[32] Also, similar to our study, cardiac surgery was associated with improved survival (HR 0.48).[32] Interestingly, although the time periods in their study date from an earlier time, the survival times are quite similar to the survival times in our cohort. This is likely, at least partly, to be attributable to the increasing proportion of patients who received valve replacement surgery in more recent time periods. Cardiac valve replacement in the Netherlands found its way into standard of care for CHD in the first decade of this millennium, as can also be seen from our results, which is somewhat later than in the study by Moller, et al. Namely, in our cohort an increase in the proportion of patients is visible from 5.4% in 1985-2000, to nearly half of the patients in 2010-2018. Similarly, in the cohort from 2005, valvular replacement surgery occurred in 18% in 1981-1989, and increased to 64% in 1995-2000. Since both cohorts also found cardiac surgery to be associated with improved survival, the increased proportion of patients undergoing cardiac surgery per time period is highly likely to be associated with better survival outcomes. Moreover, we found that only 10.8% of the patients who underwent valve replacement surgery died of CHD directly, compared with nearly a third of patients who died of CHD directly in the group that did not undergo surgical intervention. These results further underscore the need for timely diagnosis and intervention of CHD.

An important finding in our study is that all but one patient developed CHD within a ten year period after first diagnosis of NET liver metastasis. Moreover, for 84% of patients this was within five years. This suggests that screening for CHD may be less stringent after a period of five years has passed after the onset of liver metastases, and may even be abandoned after ten years. Of course, such a decision should not be made lightly and should always be individualized based on the current NET status, both anatomically and biochemically, for instance by following up serotonin and NT-proBNP levels. Nevertheless, since it is not unlikely that patients with SI-NET live over ten years with metastatic disease,[33] releasing patients from echocardiographic controls may relieve the burden of hospital visits and costs for these patients.

Regarding biomarkers, we found that NT-proBNP levels increased significantly with increased severity of tricuspid valve regurgitation, and a similar situation was present for serum serotonin levels. Our results for NT-proBNP are similar to previous studies that have investigated the value of NT-proBNP for the diagnosis of CHD.[29, 34] For instance, Bhattacharyya, et al. found that NT-proBNP was significantly elevated in patients with CHD compared to patients with CS without CHD. Moreover, in their study, NT-proBNP positively correlated with more severe CHD and NYHA.[34] It is important to recognise that elevated NT-proBNP levels do not resemble

CHD specific cardiac damage, but is a marker that shows increased strain on cardiomyocytes. Nevertheless, in our cohort very few patients had other cardiac comorbidities, therefore it is unlikely that the elevation in NT-proBNP is caused by other diseases than CHD. Furthermore, we have recently identified NT-proBNP to be highly useful in both the prediction and detection of CHD, since it is elevated in patients who develop CHD during follow up, compared to patients who do not, even when echocardiography does not show any signs of tricuspid regurgitation yet. This further underscores the value of NT-proBNP in patients with elevated serotonin with or without accompanying CHD.[35]

Similar to NT-proBNP, our study resembles results of studies that have investigated either serum serotonin, serum 5-HIAA or urinary 5-HIAA, all markers of the presence of elevated serotonin, and its association with the development and progression of CHD. A recent review by Buchanan-Hughes, et al. identified 31 publications and summarized the results thereof. There, it was found that indeed measures of elevated serotonin are associated with CHD development, disease progression and increased risk of mortality.[36]

Our results thereby confirm the current practice that aims to reduce serotonin levels by either treatment with SSA, or reducing tumour burden. Also, newer management options such as teloristat ethyl, an inhibitor of tryptophan hydroxylase that is currently approved for the management of poorly controlled diarrhoea caused by the carcinoid syndrome, has shown promising preliminary results in lowering serum serotonin levels, and might therefore be useful in the future.[37] Nevertheless, it is important to emphasize that only 30-50% of patients with elevated serotonin develop CHD,[38] therefore serotonin or its metabolites can certainly identify patients with NET that are at risk of developing CHD, but actual development of (early) cardiac stress should be monitored closely, preferably by NT-proBNP.

Finally, the early mortality rate of patients undergoing valve replacement surgery in our cohort is similar to a study from the Mayo Clinic, where an early mortality rate of 5% (7/128) was seen for patients operated after 2005.[39] Although cardiac replacement surgery is not without risks, our results show that the mortality of patients undergoing cardiac surgery is much less than patients with CHD who did not receive valve replacement surgery. This highlights the fact that surgical intervention for CHD should be timed appropriately to improve survival.

Of course, the survival benefit of surgical intervention is likely to be – to some extent – caused by confounding by indication (i.e. more fit patients are more likely to receive surgical treatment). Also, besides improvements in surgical intervention, the management of SI-NET in general has improved greatly over recent decades, with the implementation of newer treatment strategies such as PRRT. Therefore, the survival benefit can also partly be explained by these advances. Nevertheless it is important to realize that most patients that died of CHD directly, without having cardiac surgery, could have benefitted from surgical intervention given an earlier diagnosis of CHD. Also, the obvious difference in survival from CHD directly between the

groups that did or did not receive surgery also indicates that in the group that did not receive surgical intervention mortality is largely driven by CHD.

Currently, no other screening options besides NT-proBNP and echocardiography exist. Other biomarkers (activin A, connective tissue growth factor and soluble suppression of tumorigenicity 2) have been investigated for CHD,[40-42] but we have recently found that these markers did not outperform NT-proBNP in the prediction or detection of CHD.[35] Yet, as mentioned previously, NT-proBNP is a marker of cardiac stress and shows the consequences of CHD, rather than the causes thereof. Ideally, a biomarker could be identified in the future that, at diagnosis, predicts which patients are at risk of developing CHD. Then, patients at risk of CHD could be monitored more closely, whereas patients that are classified as low risk for developing CHD would have to visit the hospital less frequently, hence reducing both patient and hospital burden. A recent review by Ciobanu, et al. has summarized available biomarkers for neuroendocrine neoplasms and possible future biomarkers.[43] Unfortunately, currently there are no candidate biomarkers that show promising perspectives for the screening for CHD. In other cancer types, cell-free DNA (cfDNA) or -RNA have been investigated for the screening of disease. Recently, Boons, et al. have shown that cfDNA could be detected in patients with NET and was associated with worse survival.[44] Although these are preliminary results, these outcomes may serve as a stepping stone for further development of biomarkers for CHD.

There are a number of limitations to this study worth mentioning. First, despite the fact that we present the largest European cohort to date of patients with CHD, the sample size remains relatively small. Nonetheless, this cohort gives a robust description of the prognosis of patients with CHD, and gives valuable insight in the changes in treatment and disease course over a period in time. Moreover, the cohort was large enough to be able to perform multivariable analysis of characteristics associated with DSM, identifying the most important factors for prognosis. Second, although the data storage in the NKI holds all patient and tumour characteristics needed for clinical practice as per current guidelines, the retrospective nature of this study inherently means that missing data was present. Nevertheless, the variables with missing values were limited, and by performing multiple imputation we were able to present the least biased results of our multivariable analysis in this cohort, whilst preserving statistical power.

The main strengths of this study are the very long time period over which the patients were included and the relatively large size of the cohort. This study is the first to describe changes in prognosis in patients with CHD over such a long time period, illustrating how practice and subsequent outcomes change. Such insight is highly valuable for further tailoring of screening, systemic or localized treatment and cardiac surgical intervention for CHD.

In conclusion, carcinoid heart disease is a serious cardiac complication of the carcinoid syndrome caused by neuroendocrine tumours. Its development, progression and severity is associated with increased serotonin and NT-proBNP levels. Cardiac valve replacement of

affected valves is the strongest predictor for reduced disease specific mortality. The mainstay of management of patients with elevated serotonin due to their NET is to reduce serotonin levels and adequate screening for CHD to provide timely surgical intervention, yet screening may become less stringent after five years of being diagnosed with NET liver metastases, and may even be abandoned altogether after ten years, after appropriate judgment. Future studies should aim to identify biomarkers that indicate patients at risk of developing CHD.

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Supplementary material

CTCAE	Description adverse event
5	High fever and asystoly days after surgery.
4	Thoracotomy for heart tamponade
4	Thoracotomy for suspicion of tamponade, clearing large amounts clotted blood and pleural effusion
4	AV-block, sinusbradycardia with accompanying sinus arrests. Re-admittance for pericardial effusion and drainage
4	Pneumothorax with drain placing
4	Heart tamponade, obstructive shock, re-thoracotomy, mediastitinis. At second surgery: again tamponade and re-thoracotomy
4	Thoracotomy for excessive thorax drain production, clearing large amounts of clotted blood
3	atrial flitter for which electrocardioversion was performed
3	Re-sternotomy for bleeding and pneumothorax
3	Pacemaker
3	Pacemaker for AV-block
3	Atrial flutter for which two electro-cardioversions were performed, pneumothorax with drain placing
3	Atrial flutter for which two electro-cardioversions were performed, pleural effusion with drain placing (3x)
2	Atrial fibrillation for which sotalol was prescribed
1	AV-block type Wenckebach, no pacemaker indication
1	AV-block, spontaneous recovery within 24 hours
1	Temporary AV-block and atrial fibrillation

Table S1. All adverse events after valve replacement surgery, scored by the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0. AV: atrio-ventricular.

8

Well-differentiated Bronchopulmonary Neuroendocrine Tumors: More Than One Entity

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Abstract

Background
Until now, well-differentiated bronchopulmonary neuroendocrine tumors (bpNET) occurring either sporadically (sp-bpNET) or in the context of Multiple Endocrine Neoplasia Type 1 (MEN1) and Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia (DIPNECH) are regarded as similar entities. However, in contrast to sp-bpNET: MEN1-related and DIPNECH-related bpNET rarely metastasize or lead to bpNET-related death.

Aims
To describe and compare the course of the disease of sp-bpNET, DIPNECH- and MEN1-related bpNET.

Methods
All patients with histologically confirmed MEN1-related bpNET from the DutchMEN Study Group database (1990-2017), patients with resected sp-bpNET and DIPNECH patients referred to a Dutch ENETS center between 2000-2018 were included. Fisher's exact test was used for comparison between groups. The primary endpoint was disease-specific mortality (DSM). Kaplan-Meier and logrank test were used to compare survival. Cox regression was used to identify risk factors for DSM in the sp-bpNET subgroup.

Results
We included 112 sp-bpNET, 29 MEN1 and 27 DIPNECH patients. Tumor classification was similar across subgroups. Twenty (18%) patients with sp-bpNET died because of bpNET, compared to none in the MEN1 group and DIPNECH group. Median disease-specific survival was 12.3 (CI 6.3-18.3) years for patients with sp-bpNET, and not estimable for the other subgroups ($p<0.001$). Differences in baseline characteristics did not explain worse survival in sp-bpNET. Tumor classification and age at diagnosis were independent risk factors for DSM in sp-bpNET.

Conclusion
Patients with sp-bpNET have a significantly higher DSM compared to MEN1 or DIPNECH-related bpNET, unexplained by differences in baseline characteristics. This implies that not all bpNET are similar entities.

Introduction

Bronchopulmonary neuroendocrine neoplasms comprise a heterogeneous group of malignancies of the lung, originating from neuroendocrine cells. These neoplasms can be classified as bronchopulmonary neuroendocrine tumors (bpNET), with a subdivision in typical carcinoid (TC) and atypical carcinoid (AC); small cell lung carcinoma (SCLC) or large cell neuroendocrine carcinoma (LCNEC). All these tumors have been grouped under 'bpNET' in the most recent World Health Organization (WHO) Classification of Lung Tumors in 2015.[1] Classification is based on histopathological features, including mitotic count, the presence or absence of necrosis and a variety of cytological and morphologic features.[1] TCs and ACs – historically called 'carcinoid' – account for 1-2% of all lung malignancies and are considered well-differentiated tumors with an overall favorable course.[2] Although grouped together with the poorly differentiated SCLC and LCNEC, the 2015 WHO classification recognizes the evident major clinical, epidemiological, histological and genetic differences between lung carcinoids and the high-grade SCLC and LCNEC.[1] For the purpose of this paper, we consider only the well-differentiated lung carcinoids, which we will refer to as bpNET. bpNET arise sporadically (sp-bpNET) or in the context of a hereditary predisposition, *e.g.* Multiple Endocrine Neoplasia type 1 (MEN1). Another context in which bpNET may arise, is Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia (DIPNECH), a proliferation of neuroendocrine cells.

The vast majority of bpNET develop sporadically. Sp-bpNET are classically diagnosed in the fifth and sixth decade of life, and prognosis largely depends on histological subtype: reported 5-year survival rates are 87-94% and 44-80% for TC and AC, respectively.[3–6] Furthermore, lymph node metastases, distant metastases and higher proliferation rate have been identified as adverse prognostic factors.[5,7]

Multiple Endocrine Neoplasia type 1 is a rare hereditary disease predisposing patients to the development of several endocrine tumors. The classic manifestations of MEN1 are parathyroid hyperplasia or adenomas, neuroendocrine tumors of the pancreas and duodenum and pituitary adenomas, which are caused by inactivation of the *MEN1* gene.[8] Next to other manifestations as gastric- and thymic NET, adrenal tumors and breast cancer, patients are also at risk of developing bpNET with a prevalence of 4.7-6.6% of MEN1 patients.[9–14] Clinical practice guidelines advise frequent thoracic imaging to detect and monitor these tumors. However, more recent studies have shown that MEN1-associated bpNET appear to have an indolent behavior and do not decrease overall survival in MEN1 patients, although a few aggressive cases with fatal outcome have been described.[11,12] Curative surgery is considered the first treatment of choice, but a watch-and-wait policy is suggested for small (<2 cm) and slow-growing MEN1-related bpNET.[15,16]

Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia, an uncommon pulmonary disease characterized by proliferation of pulmonary neuroendocrine cells restricted to the bronchial and bronchiolar epithelium and presence of tumorlets, is recognized by the WHO as a pre-invasive precursor lesion for bpNET.[1] This condition typically occurs in non-smoking,

middle-aged women and may cause a variety of symptoms (*e.g.* cough, dyspnea, wheezing) for which the term 'DIPNECH syndrome' has been coined.[17,18] Although the diagnosis of DIPNECH is currently not defined by stringent clinic-pathological and/or radiological criteria, Rossi *et al*. have proposed a comprehensive flow-chart for the diagnosis of either solely DIPNECH, or DIPNECH syndrome.[18] In most patients, DIPNECH is associated with a stable or slowly locally progressive disease, with only a few disease-related deaths reported to date.[19–24]

Until now, bpNET of any type are considered the same disease, which is also reflected in the recently updated international guidelines.[25,26] However, based on clinical experience and earlier reports on the natural course of sp-bpNET, MEN1-related bpNET and DIPNECH-related bpNET, the question arises whether these subtypes are in fact different entities; MEN1- and DIPNECH-related bpNET rarely metastasize or lead to bpNET-related death,[9,10,24,11–13,19–23] while the prognosis of sp-bpNET seems more heterogeneous – and perhaps worse than non-sporadic forms of bpNET.[3–7]

To our knowledge, head-to-head comparisons between sp-bpNET, MEN1-related bpNET and DIPNECH-related bpNET are lacking to date. Therefore, in this cohort study, we aimed to compare disease-specific mortality (DSM) of patients with sp-bpNET, MEN1- and DIPNECH-related bpNET. Additionally, since we describe a rather large cohort of sp-bpNET, we aimed to identify independent risk factors for DSM in patients with sp-bpNET.

Materials and methods

Study design and Patients

All patients with sp-bpNET referred to the Netherlands Cancer Institute (NKI)/University Medical Center Utrecht (UMCU) European Neuroendocrine Tumor Society Center of Excellence (ENETS CoE) between 2000-2019 who had undergone surgery with curative intent were included. Similarly, all patients with histopathologically confirmed bpNET in the context of DIPNECH referred to this ENETS CoE within the same time period were included. Patients were considered to have DIPNECH or DIPNECH syndrome based on the diagnostic flowchart that has been developed by Rossi *et al*., taking into account symptoms/lung function abnormalities, compatible radiological signs and histological features.[16] Patients with bpNET in the context of DIPNECH and DIPNECH syndrome were grouped in one subgroup and further named 'DIPNECH'.

Patients with bpNET in the context of MEN1 were all selected from the Dutch national MEN1 database of the DutchMEN1 Study Group (DMSG). This database covers over 90% of the adult Dutch MEN1 population and includes all MEN1 patients ≥16 years of age at the end of 2017, under treatment at one of the Dutch university medical centers between 1990 and 2017. Detailed information on the DMSG database methods have been described previously.[27] To avoid misclassification of lung metastasis from NET of a different origin in patients with MEN1, only patients with histopathologically confirmed bpNET were selected for analysis.

Patient and tumor characteristics were retrieved from the longitudinal institutional neuroendocrine neoplasia database, in which all patients treated in the joint center are included, and the DMSG database. Tumor staging at time of diagnosis was based on pathological reports and derived from the 8th edition of the Tumor-, Node-, Metastasis (TNM) staging for Non-Small Cell Lung Cancer, which is also used for bpNET.[28] Since no consensus exists on TNM staging for DIPNECH, this was not performed for the DIPNECH cohort. Tumor grading in typical and atypical carcinoid was based on mitotic count and the presence of necrosis. Ki67-index was also included in the analysis. When unusually high/low mitotic count or Ki67-index were found, consensus on typical or atypical classification was reached within a multidisciplinary tumor board, based on a combination of tumor morphology and the dis-/concordance of mitotic count and Ki67-index.

This study was conducted in agreement with the NKI/UMCU ethical guidelines and all patients gave consent for the use of their medical data as per institutional protocol.

Outcomes

For the three subgroups, primary outcome was disease-specific mortality. Secondary outcomes were identification of differences in patient characteristics between the subgroups that could influence survival. For patients with sp-bpNET, identification of independent risk factors for DSM was an additional outcome.

Statistics

Median with (interquartile) range was used to describe continuous variables, frequency and percentages were calculated for categorical variables. For comparison between groups Fisher's exact test was performed for categorical variables, and the Wilcoxon rank sum test for continuous variables. Disease-specific mortality (DSM) was defined as bpNET-related death. Patients who died of unknown causes were considered to have died of bpNET if recurrence or metastatic disease was present at last follow-up. Patients with no evidence of disease and death ≤6 months after last follow-up were considered to have died of other causes. Patients who died of other causes or were alive at end of follow-up were censored. For visualization and comparison of survival between subgroups Kaplan-Meier curves and the logrank test was used, respectively. Cox regression was performed for uni- and multivariable analysis of risk factors for DSM. Analysis were performed using IBM SPSS Statistics software, version 25.0, and R version 3.6.2, package 'survival'.

Results

Patients

A total of 168 patients were included, of which 112 were patients with sp-bpNET, 29 patients had histologically proven bpNET in the context of MEN1, and 27 patients had a bpNET in the context of DIPNECH. Baseline characteristics and comparisons for all three subgroups can be found in Table 1. Since pathological characteristics are inherently associated with tumor

classification, these were stratified according to typical and atypical carcinoid classification, and can be found in Table 2.

Survival

Median follow-up for all patients was 4.8 years (interquartile range (IQR) 2.2-7.5).

For patients with sp-bpNET, this was 4.4 years (IQR 2.0-7.2), for patients with MEN1-related bpNET this was 6.7 years (IQR 4.9-12.0) and for patients with DIPNECH median follow-up was 2.9 years (IQR 1.3-6.7). Twenty patients (17.8%) died because of their bpNET in the sp-bpNET group. Six (5.3%) of them had an unknown cause of death but were considered to have died of bpNET due to the presence of metastatic disease at last follow-up and occurrence of death ≤6 months afterwards. Taking censoring of patients into account, most patients with sp-bpNET die of bpNET (50% at 10 years of follow-up, 70% at 25 years). In both the MEN1 and DIPNECH group no patients had died of bpNET. Four patients (3.6%) in the sp-bpNET group and 4 patients (13.8%) in the MEN1 group died of other causes. In the MEN1-group, only one of the patients died of a MEN1-related cancer (thymic NET), all other causes of death were non-MEN1-related cancers or the complications thereof. No deaths occurred in the DIPNECH group. Median disease-specific survival was shorter for patients with sp-bpNET, namely 12.3 years (95% confidence interval 7.4-17.1), whereas this was not estimable for patients with MEN1 or DIPNECH. The logrank test showed a significantly different survival distribution between subgroups (p<0.001). Survival curves for all subgroups are shown in Figure 1.

In the sp-bpNET group, patients with AC had a significantly worse survival than patients with TC (p=0.003). Survival curves for TC and AC in sp-bpNET are shown in Figure 2.

Comparison between subgroups

sp-bpNET with MEN1. Patients with sp-bpNET were significantly older at time of diagnosis (54 vs. 44 years in the MEN1 group). Patients with MEN1 more often had T1 (72.4% vs. 53.6%) or T3 tumors (13.8% vs. 4.5%). Histological classification (typical/atypical) and N-stage was comparable between the two groups. Tumor necrosis occurred more frequently atypical carcinoids of patients with sp-bpNET (39.4% vs. 0%),. No metastatic disease was present in patients with sp-bpNET, compared to 1 patient (3.4%) with M1 disease in the MEN1 group; this was a histologically confirmed contralateral pulmonary lesion. In patients with sp-bpNET, significantly more anatomical resections (85.7% vs. 51.7%) and more lymph node dissections (50.9% vs. 14.2%) were performed.

sp-bpNET with DIPNECH. Patients in the DIPNECH group had a significantly higher age at diagnosis (64 years vs. 54 years) and female predominance was more pronounced in this group (100% vs. 58.9% females). Also, similar to MEN1 patients, DIPNECH patients had significantly less anatomical resections (14.8% vs. 85.7%) and lymph node dissections (18.5% vs. 50.9%), compared to patients with sp-bpNET.

Characteristics N (%)/median (range)	Sporadic bpNET	MEN1	Sporadic vs. MEN1 p-value	DIPNECH	Sporadic vs. DIPNECH p-value	MEN1 vs. DIPNECH p-value
Total	**112**	**29**		**27**		
Age at diagnosis	54 (18-76)	44 (23-66)	0.008	63 (34-85)	0.004	<0.001
Gender			0.671		<0.001	0.001
Male	46 (41.1)	10 (34.5)		0		
Female	66 (58.9)	19 (65.5)		27 (100)		
WHO PS			n/a		0.351	n/a
0	45 (40.2)			8 (29.6)		
1	45 (40.2)			16 (59.3)		
2	2 (1.8)			0		
Unknown	20 (17.9)			3 (11.1)		
Tumor classification			0.863		0.096	0.209
Typical	73 (65.2)	20 (69.0)		23 (85.2)		
Atypical	38 (33.9)	9 (31.0)		4 (14.8)		
Unknown	1 (0.9)	0		0		
T stage						
1	60 (53.6)	21 (72.4)	0.009			
2	27 (24.1)	1 (3.4)				
3	5 (4.5)	4 (13.8)				
4	2 (1.8)	1 (3.4)				
Unknown	18 (16.1)	2 (6.9)				
N stage			0.949			
N0	52 (46.4)	18 (62.1)				
N1	16 (14.3)	5 (17.2)				
N2	17 (15.2)	4 (13.8)				
Unknown	27 (24.1)	2 (6.9)				
M stage			0.206			n/a
M0	112 (100)	28 (96.6)				
M1	0	1 (3.4)				
Resection			<0.001		<0.001	0.001
No resection	0	1 (3.4)		9 (33.3)		
Lobectomy	64 (57.1)	14 (48.3)		4 (14.8)		
Sleeve lobectomy	7 (6.3)	0		0		
Pneumonectomy	9 (8.0)	0		0		
Wedge resection	11 (9.8)	8 (27.6)		13 (48.1)		
Segmental resection	2 (1.8)	5 (17.2)		1 (3.7)		
Bilobectomy	8 (7.1)	1 (3.4)		0		
Endobronchial approach	8 (7.1)	0		0		
Lymph node dissection	57 (50.9)	5 (17.2)	0.001	5 (18.5)	0.002	1.00

Table 1. Baseline characteristics for the three subgroups
WHO PS: World Health Organization Performance Status, T: tumor, N: nodal, M: metastasis, n/a: not applicable.

Characteristics N (%)/median (range)	Sporadic bpNET	MEN1	Sporadic vs. MEN1 p-value	DIPNECH	Sporadic vs. DIPNECH p-value	MEN1 vs. DIPNECH p-value
Typical Carcinoid	**73**	**20**		**23**		
Ki67-index (%)	3 (0-16)	2 (1-5)	0.948	1 (0-5)	0.077	0.462
Mitotic count/2mm^2	1 (0-8)	1 (0-2)	0.623	1 (0-1)	0.231	0.253
Atypical Carcinoid	**38**	**9**		**4**		
Ki67-index (%)	7.5 (0-30)	10 (1-20)	0.704	2.5 (2-3)	0.089	0.250
Mitotic count/2mm^2	3 (0-27)	4 (2-10)	0.762	2 (2-2)	0.414	0.418
Necrosis *			0.029		0.104	0.119
Not present	20 (52.6)	7 (77.8)		1 (25.0)		
Present	15 (39.4)	0		1 (25.0)		
Unknown	3 (7.8)	2 (22.2)		2 (50.0)		

Table 2. Pathological characteristics for the three subgroups, according to TC and AC classification.
* Since the presence of necrosis is a characteristic in the definition the tumor classification for atypical carcinoids, this was only assessed for ACs.

MEN1 with DIPNECH. Patients with MEN1 were younger at time of diagnosis compared to patients with DIPNECH (44 years vs. 64 years), and more MEN1 patients were male (34.5% vs. 0%). Finally, less patients underwent resection in the DIPNECH group (66.7% vs. 96.5%).

Risk factors for disease-specific mortality in sp-bpNET

Univariable survival analysis for patients with sp-bpNET identified age at diagnosis (HR 1.09), atypical carcinoid (HR 4.70), Ki67-index (HR 1.17), mitotic count (HR 1.07) and lymph node dissection (HR 2.52) as risk factors for DSM. Since the number of disease-specific deaths was limited, multivariable cox regression was performed with selected variables that were deemed most contributing to DSM, according to prior clinical knowledge. Hence, age at diagnosis and tumor classification (typical vs. atypical) were included in the model. Both variables were identified as independent risk factors for DSM; a HR of 1.09 (p=0.001) was found for age at diagnosis, and HR 3.61 (p=0.009) for atypical carcinoids. Results of uni- and multivariable analysis can be found in Table 3.

Discussion

Results from this head-to-head comparison study showed that patients with sp-bpNET had a higher DSM than patients with MEN1-related bpNET, despite similar histological classification and a more aggressive surgical approach in patients with sp-bpNET. Furthermore, patients with DIPNECH-related and MEN1-related bpNET were found to have a similar outcome. Finally, age at diagnosis and histological classification showed to be an independent prognostic factor for survival in sp-bpNET.

	Univariable			Multivariable		
Characteristic	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Age at diagnosis	1.09	1.04-1.14	<0.001	1.09	1.04-1.14	0.001
Gender						
Male	1					
Female	0.52	0.22-1.25	0.143			
WHO PS						
0	1					
1	2.24	0.85-5.90	0.104			
2	1.9	0.18-12.36	0.711			
Tumor classification						
Typical	1			1		
Atypical	4.70	1.81-12.18	0.001	3.61	1.38-9.44	0.014
Ki67-index (%)	1.17	1.10-1.26	<0.001			
Mitotic count/2mm^2	1.07	1.002-1.13	0.044			
T stage						
1	1					
2	0.89	0.32-2.47	0.692			
3	3.73	0.44-31.83	0.148			
N stage						
0	1					
1	1.94	0.58-6.52	0.283			
2	2.72	0.70-10.51	0.147			
Lymph node dissection	2.52	1.02-6.22	0.045			

Table 3. Univariable and multivariable analysis for disease-specific mortality in sporadic bp-NETs
HR: hazard ratio, CI: confidence interval, WHO PS: World Health Organization Performance Status, T: tumor, N: nodal.

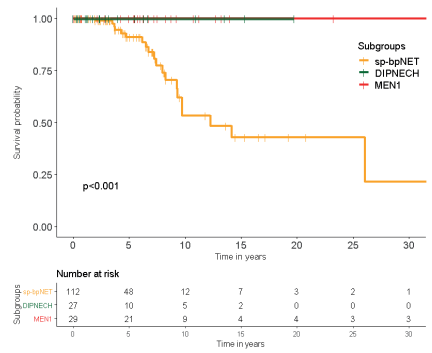

Figure 1. Kaplan-Meier curves for disease-specific survival. Sp-bpNET: sporadic bpNET, DIPNECH: Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia, MEN1: Multiple Endocrine Neoplasia type I. P-value shows logrank test for comparison between disease-specific survival.

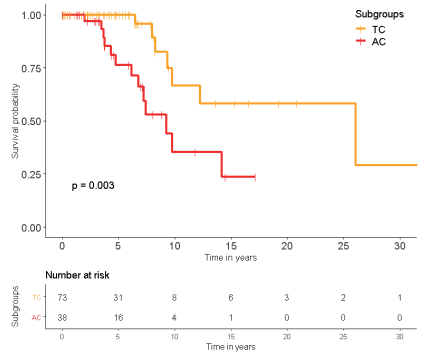

Figure 2. Kaplan-Meier curves for disease-specific survival for sp-bpNET, according to tumor classification. TC: typical carcinoid, AC: Atypical carcinoid.

The relatively good prognosis of MEN1-related bpNET in this study is in line with earlier findings in other MEN1 cohorts.[9–12] To our knowledge, only eight bpNET-related deaths in patients with MEN1 have been reported to date. In the largest cohort of histologically proven MEN1-related bpNET (n = 51), median overall survival was 20.2 years and not significantly different from the rest of the cohort.[12] Likewise, the absence of bpNET-related deaths in patients with DIPNECH in our cohort underlines the excellent prognosis of patients with DIPNECH described by others previously.[19–24] Also, the female predominance and high age at diagnosis (median 63 years) in our cohort of patients with DIPNECH are comparable with other cohorts.[23]

In line with previous research, patients with sporadic atypical lung carcinoid and older patients had significantly worse survival than patients with a typical carcinoid.[3–6] Others have identified additional prognostic factors associated with adverse prognosis for sp-bpNET, which – among others – were male gender, peripheral tumors and TNM stage.[5,7] Although survival was worse for patients with sp-bpNET as compared to patients with MEN1-related bpNET or DIPNECH, the number of disease-specific events was modest. This prevented us to accurately investigate additional prognostic parameters in our study.

The question arises what could explain the difference in survival between patients with sporadic and MEN1-related bpNET. Although the limited power prevents us to draw firm conclusions, the similarities in tumor classification, Ki67% count and mitotic count between both groups suggest that these histopathological prognostic factors are not responsible for the striking differences in mortality. This is also underscored by the decreasing survival in both TC and AC in sp-bpNET, compared to MEN1-related bpNET. This shows that even the more favorable typical carcinoids behave much more aggressive in sp-bpNET, compared to MEN1-related bpNET. Interestingly, several factors could arguably have led to a better survival in patients with sp-bpNET: firstly, patients with sp-bpNET were treated more aggressively, with more anatomical resections and lymph node dissections. Secondly, the lack of lymph node involvement was based on imaging studies in 12 out of 18 (67%) MEN1 patients, while N-status in sp-bpNET was based on pathology in all cases. This could have resulted in an underestimation of the number of patients with lymph node involvement in the MEN1 group. Patients with sp-bpNET showed a significantly higher DSM nonetheless, underscoring the different course of disease between these two groups. Thirdly, indication bias could have led to the inclusion of more aggressive MEN1-related lung NET: large tumor size and high growth rate frequently are indications for surgery in MEN1 patients with thoracic nodules suspect of bpNET.[16] Nevertheless, distribution of tumor sizes was quite heterogeneous across the subgroups of MEN1 and sp-bpNET. Although patients with MEN1 had more T1 tumors compared to sp-bpNET patients, they also had a larger proportion of T3 or higher tumors, whereas patients with sp-bpNET had more intermediate (T2) tumors. This can be explained by the often multifocal occurrence of MEN1-related bpNET: the T3 classification of all MEN1-related tumors were based on the presence of a second tumor in the same lobe, while the only MEN1 patient with T4 suffered from two tumors in the same lobe and tumor spread into a major vein. Obviously, patients with sp-bpNET have to develop tumors large enough to cause symptoms before they are recognized, whilst MEN1-related bpNET

are usually identified as a small asymptomatic nodule during periodic thoracic surveillance. This latter situation might prompt earlier intervention compared to the sp-bpNET group, thereby possibly explaining the difference in prognosis between groups. However, we saw no differences in N-stage between the two subgroups, which implies that the difference in T-stage did not lead to difference in metastatic disease. Taking into account the aforementioned factors, we still saw a lower DSM in patients with MEN1-related bpNET than in their sporadic counterparts, underlining the true different nature of sporadic bpNET when compared to MEN1-related bpNET.

Possibly, unidentified underlying molecular processes are responsible for the difference in outcome. This hypothesis is supported by recent data from Simbolo *et al*.[29] In their study, next-generation sequencing (NGS) in atypical carcinoids and LCNECs distinguished three transcriptional clusters; patients with a bpNET in the cluster characterized by frequent somatic *MEN1* mutations had a longer cancer-specific survival compared to a cluster with concurrent inactivation of *tumor protein p53* gene and *retinoblastoma 1* gene. However, this seems to contradict previous findings by the same research group: in a subset of 35 atypical lung carcinoids, the presence of a somatic *MEN1* mutation was associated with worse disease-specific survival (p=0.0045).[30] Additionally, lung carcinoids and high-grade neuroendocrine carcinomas with inactivation of *MEN1* had shorter survival and low *MEN1* mRNA levels correlated with distant metastasis and shorter survival.[31] Therefore, the precise role of *MEN1* mutations in the natural course and prognosis of bpNET is yet to be determined and requires further research into the molecular background of these tumors.

As for patients with DIPNECH, we showed that the clinical behavior is highly comparable with that of MEN1-related bpNET. Interestingly, although the proportion of atypical and typical carcinoids was similar across all subgroups, there seems to be a trend towards a significantly lower mitotic count and Ki-67-index range for patients with DIPNECH compared to the other two subgroups. Especially, there is a notable difference in the ranges of mitotic count and Ki-67-index, with a maximum mitotic count of 2 and a maximum Ki67-index of 5. Arguably, patients who develop DIPNECH-related bpNET might be on an even more favorable end of the lung carcinoid spectrum. This suggests that the subtypes of bpNET in some ways parallel those in gastric NET; type 1 gastric NET is associated with (auto-immune) chronic atrophic gastritis and is characterized by multiple lesions but has an excellent prognosis, illustrated by a very low frequency of submucosal invasion or metastasis (like DIPNECH-related bpNET). Type 2 gastric NETs are usually detected in patients with MEN1-related gastrinomas, invade into the underlying tissue somewhat more commonly than type 1 gastric NET but still have a very good prognosis with only a small risk of disease-related death (like MEN1-related bpNET). On the contrary, type 3 gastric NETs – which arise sporadically – show a more aggressive course with frequent metastasis to lymph nodes (50-100%) and liver (22-75%), resulting in a prognosis similar to gastric adenocarcinoma (which seems to mirror characteristics of sp-bpNET).[32]

Some limitations must be considered when interpreting these results. Firstly, the retrospective nature of this study could have influenced the results due to the dependency on accurate record keeping. However, we did not encounter large issues with missing data. Data concerning WHO performance status (WHO PS) of patients with MEN1-related bpNET could not be retrieved. Although WHO PS might be associated with survival, since this parameter was already quite favorable in patients with sp-bpNET – with most patients having WHO PS 0-1 – we do not expect that differences might have contributed to a worse survival for patients with sp-bpNET. Furthermore, tumor T- and N-stage at time of diagnosis were unknown in a considerable proportion of patients with sporadic bpNET (16% and 24%, respectively), presumably due to the aspect of the NKI/UMCU functioning as a tertiary referral center: patients with sp-bpNET were often referred to our center years after initial resection, leading to missing data in some cases. However, we have no reason to believe that the distribution of T- and N- stage of sp-bpNET has been significantly affected by these missing data.

Secondly, pathological samples of MEN1-related bpNET did not undergo revision. Since DIPNECH is a novel diagnosis, it might be possible that (some) MEN1-related bpNET fall in the DIPNECH category if material were to be revised. Nevertheless, this study is the first step in acknowledgement that MEN1-related bpNET are a truly different entity than sp-bpNET, and future research should be aimed in more in-depth comparison of MEN1-related bpNET and DIPNECH-related bpNET.

Thirdly, despite the relatively large cohort of patients with bpNET, the number of deaths was limited. This prevented us from analyzing survival in bpNET in more detail. Ideally, we would have liked to compare DSM between groups while adjusting for prognostic factors, like age at diagnosis. However, the lack of bpNET-related death in patients with MEN1- and DIPNECH-related bpNET already underscore the true divergent nature of these entities compared to sp-bpNET. Furthermore, we were able to identify the two most important prognostic factors for DSM in sp-bpNET, *i.e.* age at diagnosis and histological classification (typical vs. atypical carcinoid). A follow-up study with even longer follow-up and more patients might result in sufficient events to analyze prognosis in these subgroups in more detail.

Finally, the predisposition to develop multiple neuroendocrine tumors (NET) in MEN1 patients could have led to a selection of MEN1 patients included in this analysis, thereby affecting comparability between groups: among other manifestations, MEN1 patients are prone to the occurrence of duodenopancreatic NET, one of the major causes of MEN1-related death. Events like these earlier in life might have prevented the diagnosis of bpNET in a significant part of the MEN population, due to (1) MEN1-related death, or (2) a lack of histological diagnosis of bpNET due to refraining from biopsy or lung surgery due to (presumed) metastatic disease or poor WHO PS. Theoretically, this might have caused us to miss patients that would have developed bpNET later in life, and perhaps would have shown a more aggressive disease course. Nevertheless, our selection of patients – by including only those patients with histologically

confirmed bpNET – was done in such a manner to ensure comparability with sp-bpNET. Also, this selection remains a true representation of clinical practice over a long time period.

To the best of our knowledge, this study is the first to directly compare the outcome of patients with bpNET in the context of MEN1, DIPNECH and the sporadic variant. Despite the rarity of these entities, we were able to include a relatively large cohort by using data from the NKI/UMCU combined ENETS CoE and the population-based Dutch MEN1 Study Group cohort of MEN1 patients. Furthermore, all participating institutions have a team of specialists dedicated to neuroendocrine tumors, including thoracic radiologists and pathologists, which has strengthened the quality of data. Lastly, the standardized and comprehensive data collection ensured precise and detailed information about relevant patient and tumor characteristics.

Conclusion

Sporadic and MEN1-related bpNET are currently considered the same disease, but results from this study show that there is a significant difference in survival between these groups despite similar histopathological features. Paradoxically, several factors (such as the more aggressive surgical approach in sp-bpNET, possible underestimation of proportion of MEN1-related bpNET with lymph node involvement and the probable indication bias leading to a selection of aggressive MEN1-related bpNET) arguably could have led to a better survival in patients with sp-bpNET compared to MEN1-related bpNET, underscoring the true different nature of these two entities. A possible effect of earlier detection of MEN1-related bpNET cannot be excluded entirely, although potential differences in tumor size at time of surgical resection had not resulted in a difference in locoregional or distal spread. The remarkable difference in survival suggests that these are truly distinctive entities. Furthermore, patients with MEN1- and DIPNECH-related bpNET showed similar survival, suggesting that these entities are more alike, with no bpNET-related death in our study despite the presence of atypical carcinoid in a significant part of these groups. These findings call for verification in other large cohort studies and further research into underlying explanatory (molecular) mechanisms, potentially leading to prognostic guidelines for different subgroups of bpNET.

Author contribution statement WB, MT, GV: Conceptualization; MB, SL, KD: Data curation; SL: Formal analysis; MB, SL, MT, GV: Investigation; MB, SL, KH, RL, MT, GV: Methodology; MB, SL: Project administration; MB, SL, MT, GV: Resources; MB, SL, MT, GV: Software; MT, GV: Supervision; MB, SL: Visualization; MB, SL: Roles/Writing - original draft; All authors: Writing - review & editing.

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Part II

Tailoring Treatment in Neuroendocrine Neoplasia

9

Postoperative Radiotherapy in Stage I-III Merkel Cell Carcinoma

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Abstract

Background

Postoperative radiotherapy (PORT) is currently recommended for the treatment of Merkel cell carcinoma. Nevertheless, deviations occur frequently due to the generally elderly and frail patient population. We aimed to evaluate the influence of PORT on survival in stage I-III MCC patients treated in the Netherlands.

Methods

Patients were included retrospectively between 2013 and 2018. Fine-Gray method was used for cumulative incidence of recurrence and MCC-related survival, cox regression was performed for overall mortality. Analyses were performed in patients with clinical (sentinel node biopsy [SN] not performed) stage I/II (c-I/II-MCC), pathologic (SN negative) stage I/II (p-I/II-MCC) and stage III MCC (III-MCC), separately. Propensity score matching (PSM) was performed to assess confounding by indication.

Results

In total 182 patients were included, 35 had p-I/II-MCC, 69 had c-I/II-MCC and 78 had III-MCC. Median follow up time was 53.5 (IQR 33.4-67.4), 30.5 (13.0-43.6) and 29.3 (19.3-51.0) months, respectively. Multivariable analysis showed PORT to be associated with less recurrences and redued overall mortality, but not with MCC-related mortality. In stage III-MCC, extracapsular extension (sub-distribution hazard [SDH] 4.09, p=0.012) and PORT (SDH 0.45, p=0.044) were associated with recurrence, and ≥4 positive lymph nodes (SDH 3.24, p=0.024) were associated with MCC-related survival.

Conclusions

PORT was associated with less recurrences and reduced overall mortality in patients with stage I-III MCC, but not with improved MCC-related mortality. Trends in OS benefit are likely to be caused by selection bias suggesting further refinement of criteria for PORT is warranted, for instance by taking life expectancy into account.

Background

Merkel cell carcinoma (MCC) is a rare and aggressive neuroendocrine malignancy of the skin. The incidence of MCC is 0.5-0.8/100.000, but has been rising over the past decades.[1, 2] With a median age of 75 years at diagnosis, it is predominantly a disease of the elderly.[3-5] The prognosis of patients strongly correlates with disease stage. Five-year overall survival (OS) for localized disease (stage I and II) was reported to be between 35-63%.[5-7] Up to 37% of patients present with nodal disease, which is associated with a five-year OS of 26.8-46.0%.[5, 6, 8]

The mainstay of treatment for MCC consists of locoregional surgery. A wide local excision (WLE) of the primary tumor, accompanied by a sentinel lymph node biopsy (SN) in clinically node-negative disease, is recommended.[9] The National Comprehensive Cancer Network (NCCN) guidelines recommend – if appropriate after consultation within a multidisciplinary tumor board – a completion or therapeutic lymph node dissection (LND) (neck-dissection in case of MCC arising from the head and neck region), in case of microscopic or macroscopic nodal involvement, respectively.[9] Despite surgical efforts, the risk of recurrence is high.[10, 11] Since MCC is generally considered to be very sensitive to radiotherapy, postoperative radiotherapy (PORT) has been implemented in the standard of care.[12] The NCCN guidelines recommend PORT in all primary MCC, although observation can be considered in widely excised, small primary tumors (<1 cm), and in the absence of other risk factors. PORT is also recommended when macro- or microscopically nodal disease led to LND, and more than 3 positive lymph nodes or extracapsular extension are found on pathological examination.[9] These guidelines are based on a retrospective analysis of 6908 cases, which showed an OS benefit in stage I and II treated with PORT, but not stage III MCC patients.[13] In the Netherlands, most patients with MCC are treated in specialized referral centers. These centers work in close collaboration and treatment decisions are based on established evidence and guidelines.

Nonetheless, clinicians often deviate from the treatment protocols. This occurs because of physicians' or patients' preference, because patients are too frail to undergo treatment, or because the patients prognosis is defined by other comorbidities.[14, 15] SN procedure is often omitted for similar reasons, which may lead to incomplete staging of disease and under-informed decision making.[16] For PORT, this has been illustrated in a recent study that investigated the concordance of PORT guidelines and treatment in MCC patients. The authors found that 57% of patients with a PORT indication were actually treated with radiotherapy. In these patients, PORT was associated with improved OS.[15]

The guideline discordance in regard to staging and treatment, together with an often elder and frail population, makes it difficult to assess whether patients would benefit from adjuvant therapies, especially in retrospective analyses. In this study, we will evaluate the influence of PORT in patients with both clinically and pathologically defined stage I-III MCC, whilst controlling for confounding by indication in our analysis. Further, we will investigate the effect of PORT on different outcomes, namely recurrence, MCC-related mortality and overall survival.

By doing so, we will be able to assess whether the frailty and non-MCC-related prognosis of patients are likely influencing treatment decision and survival outcomes.

Materials and Methods

Patient selection

Patients from three referral centers were included in this retrospective multicenter observational study, covering over three quarters of the Dutch MCC population. All patients with histologically proven stage I-III MCC, diagnosed between 2013 and 2018, and with an indication for PORT, were eligible for inclusion. This study was conducted according to the principles of the Declaration of Helsinki and approved by local Institutional Review Boards (IRBs). All patients gave consent for the use of their pseudo-anonymized medical data.

Objectives

The primary objective of this study was to investigate the influence of PORT on recurrence, MCC-related mortality and overall mortality of patients with stage I-III MCC. Recurrence was defined as time in months from initial histopathological diagnosis until documented first recurrence or death from MCC. MCC-related mortality was defined as time in months from the same initial time point until death from MCC. Patients that died from unknown causes but had stage IV disease at last follow up were considered to have died from MCC. Patients that died of comorbidities were considered as competing risks for both RFS and MCC-related mortality. Patients who were alive at the end of study were censored.

The secondary objective of this study was to identify predictors for recurrence, MCC-related mortality and overall mortality in stage I-III MCC patients.

Statistical analyses

Descriptive statistics were used to describe baseline characteristics: frequencies and percentages for categorical variables, medians with interquartile ranges (IQR) for continuous variables. Characteristics of patients who did and did not receive PORT were compared using the Fisher's exact test in categorical variables and Wilcoxon rank sum test in continuous variables.

Due to the observational nature of this study, the choice for PORT could be subject to differences in patient characteristics. Therefore, patients were matched using propensity score matching (PSM) to ensure two groups with equal characteristics associated with receiving PORT. One-to-*n* matching with replacement with the nearest Mahalanobis metrics matching was performed. The PORT group was used as reference group for matching. Propensity score was estimated by a logistic regression and covariates included in the propensity score were selected based on their contribution to PORT treatment decision. Covariates were gender, age, World Health Organization (WHO) performance score (PS), T stage, N stage, head and neck tumors (as binary variable, yes/no), radical excision (yes/no) and lymph-/angioinvasion (yes/no). The standardized mean difference was used to assess the balance of covariates after matching, a value of >0.1 was used as a cut-off for imbalance of covariates.

For recurrence and MCC-related mortality competing risk analyses using the Fine-Gray method were performed. Cumulative incidence and Kaplan-Meier curves were plotted for visualization of recurrence, MCC-related and overall mortality. A multivariable Fine-Gray model was constructed for identification of independent predictors of recurrence and MCC-related mortality. Multivariable cox regression was performed for overall mortality. Predictors were selected according to clinical knowledge regarding their influence on survival, radiotherapy was included as predictor of interest. The sub-distribution hazards (SDH) were shown and can be interpreted in similar manner to hazard ratios (HRs) in a cox proportional hazards model. To preserve statistical power, we included patients with missing values as 'unknown' categories in our multivariable analysis. A statistical probability (p-value) of <0.05 was considered significant.

Analyses were performed using IBM SPSS, version 25 and R version 3.6.2. R packages 'survival', 'MatchIt', 'cmprsk' were used.

Results

A total of 218 patients with stage I-III were referred to the three expert centers in the study period. Of these, 182 had an indication for PORT according to current guidelines. Of the patients without a PORT indication, 2 (5.5%) received PORT. In contrast, 94 (51.6%) patients with a PORT indication did not receive PORT.

All further analysis were performed in the patients with a PORT indication. Median age was 73.8 years (IQR 66.8-81.1) and 80 patients (44.0%) were female. Thirty-five patients (19.2%) had pathological (SN negative) stage I/II MCC (p-I/II-MCC), 69 patients (37.9%) had clinical (SN not performed) stage I/II MCC (c-I/II-MCC), and 78 patients (42.9%) had stage III MCC (III-MCC). Baseline characteristics for all patients, the distribution according to disease stage and PORT are summarized in Table 1. Significant differences were found in the following characteristics: p-I/II-MCC patients treated with PORT more often had primary tumors of the head & neck (45.5% vs. 8.3%), whereas patients with a MCC of the extremity were less frequently treated with PORT (27.3 vs. 66.3, p=0.023). In c-I/II-MCC, patients treated with PORT were older (81.9 years vs 77.1 years, p=0.029). In patients with III-MCC, significant differences were mostly seen in pathological characteristics: patients treated with PORT had more unknown primary tumors (Tx) (24.4% vs. 12.1%) and larger tumors (T2 and T3) (37.8% vs. 21.2% and 11.1% vs. 6.1%, p=0.034, respectively). Free excision margins had been achieved less frequently In the patients receiving PORT (86.7% vs. 100%, p=0.032), SN-procedure was performed less often (26.7% vs. 54.5%, p=0.018), but when additional lymph node dissection was performed, positive lymph nodes were found more frequently: 44.4% vs. 22.2% for 2-3 lymph nodes, and 29.9% vs 9.1% for ≥4 lymph nodes (p=0.011). Compared to patients who did not receive PORT, lymph-/angioinvasion was found in less patients treated with PORT (15.6% vs 42.2%), yet this was unknown in a larger proportion of patients with PORT (57.8% vs. 39.4%, p=0.032). Patients without PORT more often had unknown extracapsular invasion status (57.6% vs. 31.1%, p=0.063). Median follow up time for all patients was 34.6 months (IQR 18.3-55.5). For patients with p-I/II-MCC median follow up

time was 53.5 months (IQR 33.4-67.4), for patients with c-I/II-MCC this was 30.5 months (IQR 13.0-43.6) and for patients with III-MCC this was 29.3 months (IQR 19.3-51.0).

PSM yielded 51:84 matched treated:control units. The PSM cohort showed highly similar effects for PORT compared to the unmatched cohort. Further analyses were therefore executed in the unmatched cohort to maximize power. Results of PSM and survival curves for original and matched cohort can be found in the supplementary material, Figure S1 and S2, respectively.

Considering all patients, 80 (44.0%) had recurrent disease. Of the 44 patients that received PORT of only the primary tumor (and not the nodal basin), recurrences were local in one (2.4%), regional in 14 (31.8%) and distant in three (6.8%) patients. In patients that received PORT of both the primary tumor and the nodal basin, all recurrences were distant (n=12, 38.7%). Thirteen patients received PORT of nodal basin only, these were all patients with unknown primary tumors. Of these, one patient (7.7%) had a regional recurrence, and 5 (38.5%) had distant recurrences. Recurrences across local and/or regional PORT are summarized in Table 2. For illustration of local, regional or distant recurrences, cumulative incidence curves stratified by PORT are shown in Figure 1.

Univariable competing risk analysis showed no difference in disease recurrence for patients treated or not treated with PORT in p-I/II-MCC (p=0.590) and in c-I/II-MCC (p=0.260). In patients with III-MCC, PORT was associated with less recurrences (p=0.030). Cumulative incidence of recurrence curves are shown in Figure 2a. Multivariable analysis of recurrence identified a higher disease stage: c-I/II-MCC had a SDH of 3.05 (p=0.025), III-MCC had a SDH of 6.24 (p<0.001) and PORT (SDH 0.59, p=0.039) as independent predictors. Also, an unknown PS (SDH 5.33, p<0.001) was significantly associated with recurrence, but this group only included 3 patients. To assess the influence of known nodal pathological characteristics on recurrence, multivariable analysis was repeated in III-MCC with inclusion of known risk factors for recurrence. Here, unknown lymph-/angioinvasion (SDH 0.29, p=0.012), the presence of extracapsular extension (SDH 4.09, 0=0.012) and PORT (SDH 0.45, p=0.044) were found to be independent predictors for recurrence (Table 4).

Regarding MCC-related mortality, Fine-Gray analysis did not show a significant difference for PORT in any of the three subgroups: p=0.530, p=0.430 and p=0.980 for p-I/II-MCC, c-I/II-MCC and III-MCC, respectively. In the complete cohort, 13 (7.1%) patients died from other causes than MCC, two of whom died from a malignancy other than MCC. Cumulative incidence curves are found in Figure 2b. Multivariable analysis identified male gender (SDH 2.21, p=0.033), and disease stages c-I/II-MCC (SDH 4.84, p=0.017) and III-MCC (SDH 7.12, p<0.001) to be associated with MCC-related mortality (Table 3). Similar to the analysis of recurrence, unknown PS (SDH 6.13, p=0.033) showed significant results. In multivariable analysis for III-MCC, only the presence of ≥4 lymph nodes was associated with MCC-related mortality (Table 4).

			Pathological Stage I/II				Clinical Stage I/II				Stage III			
Characteristic		**All N (%)**	**All**	**No PORT**	**PORT**	**P-value**	**All**	**No PORT**	**PORT**	**P-value**	**All**	**No PORT**	**PORT**	**P-value**
Total		**182**	**35**	**24**	**11**		**69**	**37**	**32**		**78**	**33**	**45**	
Gender						0.146				0.811				0.346
	Female	80 (44.0)	17 (48.6)	14 (58.3)	3 (27.3)		34 (49.3)	19 (51.4)	15 (46.9)		29 (37.2)	10 (30.3)	19 (42.2)	
	Male	102 (56.0)	18 (51.4)	10 (41.7)	8 (72.7)		35 (50.7)	18 (48.6)	17 (53.1)		49 (62.8)	23 (69.7)	26 (57.8)	
Age	(median, range)		69.9 (17.3-81.5)	70.7 (17.3-81.5)	66.8 (48.9-81.2)	0.456	80.3 (54.3-94.8)	81.9 (59.8-84.7)	77.1 (54.3-94.6)	0.029	71.0 (41.9-89.6)	73.6 (41.9-85.4)	70.5 (44.4-89.6)	0.125
CCS						0.086				0.721				0.418
	0	76 (41.8)	14 (40.0)	12 (50.0)	2 (18.2)		30 (43.5)	15 (40.5)	15 (46.9)		32 (41.0)	11 (33.3)	21 (46.7)	
	1	53 (29.1)	14 (40.0)	9 (37.5)	5 (45.5)		16 (23.2)	8 (21.6)	8 (25.0)		23 (29.5)	10 (30.3)	13 (28.9)	
	2	34 (18.7)	6 (17.1)	2 (8.3)	4 (36.4)		11 (15.9)	8 (21.6)	3 (9.4)		17 (21.8)	10 (30.3)	7 (15.6)	
	3	15 (8.2)	1 (2.9)	1 (4.2)	0		8 (11.6)	4 (10.8)	4 (12.5)		6 (7.7)	2 (6.1)	4 (8.9)	
	4	3 (1.6)	0	0	0		3 (4.3)	2 (5.4)	1 (3.1)		0	0	0	
	5	0	0	0	0		0	0	0		0	0	0	
	6	0	0	0	0		0	0	0		0	0	0	
	7	0	0	0	0		0	0	0		0	0	0	
	8	1 (0.5)	0	0	0		1 (1.4)	0	1 (3.1)		0	0	0	
PS						0.460				0.598				0.634
	0	85 (46.7)	16 (45.7)	10 (41.7)	6 (54.5)		24 (35.1)	13 (35.1)	11 (34.4)		45 (57.7)	18 (54.5)	27 (60.0)	
	1	60 (33.0)	15 (42.9)	12 (50.0)	3 (27.3)		27 (39.1)	14 (37.8)	13 (40.6)		18 (23.1)	9 (27.3)	9 (20.0)	
	2	27 (14.8)	4 (11.4)	2 (8.3)	2 (18.2)		9 (13.0)	4 (10.8)	5 (15.6)		14 (17.9)	5 (15.2)	9 (20.0)	
	3	7 (3.8)	0	0	0		6 (8.7)	5 (13.5)	1 (3.1)		1 (1.3)	1 (3.0)	0	
	Unknown	3 (1.6)	0	0	0		3 (4.3)	1 (2.7)	2 (6.3)		0	0	0	

Table 1. Baseline characteristics of all patients across all three subgroups and PORT. PORT: postoperative radiotherapy, CCS: Charlson comorbidity score, PS: World Health Organization performance score, T: tumor, SN: sentinel node, LND: lymph node dissection.* One patient was suspected of metastasis to parotid gland on imaging, sentinel node procedure not performed. Resection showed no metastasis. For analysis purposes this patient was further included in SN-negative group. † Only applicable for stage III-MCC.

		Pathological Stage I/II				Clinical Stage I/II				Stage III			
Characteristic	**All N (%)**	**All**	**No PORT**	**PORT**	**P-value**	**All**	**No PORT**	**PORT**	**P-value**	**All**	**No PORT**	**PORT**	**P-value**
Total	**182**	**35**	**24**	**11**		**69**	**37**	**32**		**78**	**33**	**45**	
T-stage					0.550				0.116				0.034
T0	15 (8.2)	n/a	n/a	n/a		n/a	n/a	n/a		15 (19.2)	4 (12.1)	11 (24.4)	
T1: ≤1cm	27 (14.8)	8 (22.9)	6 (25.0)	2 (18.2)		14 (20.3)	7 (18.9)	7 (21.9)		5 (6.4)	2 (6.1)	3 (6.7)	
T1: >1 cm & ≤ 2cm	62 (34.1)	11 (31.4)	8 (33.3)	3 (27.3)		29 (42.0)	16 (43.2)	13 (40.6)		22 (28.2)	16 (48.5)	6 (13.3)	
T2: >2 cm & ≤ 5cm	52 (28.6)	10 (28.6)	7 (29.2)	3 (27.3)		18 (26.1)	13 (35.1)	6 (15.6)		24 (30.8)	7 (21.2)	17 (37.8)	
T3: >5cm	9 (4.9)	1 (2.9)	0	1 (9.1)		1 (1.4)	0	1 (3.1)		7 (9.0)	1 (6.1)	5 (11.1)	
T4	6 (3.3)	1 (2.9)	0	1 (9.1)		3 (4.3)	0	3 (9.4)		2 (2.6)	1 (3.0)	1 (2.2)	
Unknown	11 (6.0)	4 (11.4)	0	1 (9.1)		4 (5.8)	1 (2.7)	3 (9.4)		3 (3.8)	1 (3.0)	2 (4.4)	
Location primary					0.023				0.241				0.469
Head & Neck	78 (42.9)	7 (20.0)	2 (8.3)	5 (45.5)		49 (71.0)	23 (62.2)	26 (81.3)		22 (28.2)	9 (27.3)	13 (28.9)	
Trunk	30 (16.5)	9 (25.7)	6 (25.0)	3 (27.3)		9 (13.0)	6 (16.2)	3 (9.4)		12 (15.4)	5 (15.2)	7 (15.6)	
Extremity	59 (32.4)	19 (54.3)	16 (66.7)	3 (27.3)		11 (15.9)	8 (21.6)	3 (9.4)		29 (37.2)	15 (45.5)	14 (31.1)	
Unknown primary	15 (8.2)	n/a	n/a	n/a		n/a	n/a	n/a		15 (19.2)	4 (12.1)	11 (24.4)	
Excision margins					n/a				0.743				0.032
Margins free	165 (90.7)	35	24 (100)	11 (100)		58 (84.1)	32 (86.5)	26 (81.3)		72 (92.3)	33 (100)	39 (86.7)	
Margins not free	17 (9.3)	0	0	0		11 (15.9)	5 (13.5)	6 (18.8)		6 (7.7)	0	6 (13.3)	
SN-procedure					0.314				n/a				0.018
Negative	34 (18.6)	34 (97.1)	24 (100)	10 (90.1)						0	0	0	
Positive	30 (16.5)	0	0	0						30 (38.5)	18 (54.5)	12 (26.7)	
Not performed	118 (64.8)	1 (2.9)	0	1 (9.1)*		69	37 (100)	32 (100)		48 (61.5)	15 (45.5)	33 (73.3)	

Table 1. *continued*

		Pathological Stage I/II				Clinical Stage I/II				Stage III			
Characteristic	**All N (%)**	**All**	**No PORT**	**PORT**	**P-value**	**All**	**No PORT**	**PORT**	**P-value**	**All**	**No PORT**	**PORT**	**P-value**
Nodal status†													0.011
1 node positive	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7 (9.0)	6 (18.2)	1 (2.2)	
2-3 nodes positive	n/a	n/a	n/a	n/a		n/a	n/a	n/a		31 (39.7)	11 (33.3)	20 (44.4)	
>3 nodes positive	n/a	n/a	n/a	n/a		n/a	n/a	n/a		16 (20.5)	3 (9.1)	13 (29.9)	
SN positive, LND not performed	n/a	n/a	n/a	n/a		n/a	n/a	n/a		20 (25.6)	12 (36.4)	8 (17.8)	
SN and LND not performed	n/a	n/a	n/a	n/a		n/a	n/a	n/a		4 (5.1)	1 (3.0)	3 (6.7)	
Extracapsular extension†													0.063
No	n/a	n/a	n/a	n/a		n/a	n/a	n/a		26 (33.3)	9 (27.3)	17 (37.8)	
Yes	n/a	n/a	n/a	n/a		n/a	n/a	n/a		19 (34.4)	5 (15.2)	14 (31.1)	
Unknown	n/a	n/a	n/a	n/a		n/a	n/a	n/a		33 (42.3)	19 (57.6)	14 (31.1)	
Lymph-/ angioinvasion					0.422				0.264				0.032
No	50 (27.5)	14 (40.0)	8 (33.3)	6 (54.5)		18 (26.1)	7 (18.9)	11 (34.4)		18 (23.1)	6 (18.2)	12 (26.7)	
Yes	43 (23.6)	9 (25.7)	6 (25.0)	3 (27.3)		13 (18.8)	9 (24.3)	4 (12.5)		21 (26.9)	14 (42.2)	7 (15.6)	
Unknown	89 (48.9)	12 (34.3)	10 (41.7)	2 (18.2)		38 (55.1)	21 (56.8)	17 (53.1)		39 (50.0)	13 (39.4)	26 (57.8)	

Table 1. *continued*

	PORT			
	No PORT, n (%)	**Primary tumor only, n (%)**	**Lymph nodes only, n (%)**	**Primary and lymph nodes, n (%)**
No recurrence	50 (53.2)	26 (59.1)	7 (53.8)	19 (61.3)
Local	5 (5.3)	1 (2.3)	0	0
Regional	27 (28.7)	14 (31.8)	1 (7.7)	0
Distant	12 (12.7)	3 (6.8)	5 (38.5)	12 (38.7)
Total	94 (100)	44 (100)	13 (100)	31 (100)

Table 2. Recurrences across local and/or regional postoperative radiotherapy (PORT).

Kaplan-Meier curves and cox regression were performed for overall mortality. In patients treated with PORT a trend was seen towards improved survival in c-I/II-MCC (p=0.076), and no difference was seen in p-I/II-MCC (p=0.990) and III-MCC (p=0.200). Kaplan-Meier curves for overall mortality are shown in Figure 2c. Multivariable cox regression identified a PS of 2 (HR 2.23, p=0.039), PS 3 (HR 3.36, p=0.044) and an unknown PS (HR 8.84, p=0.002), primary tumor location on the trunk (HR 2.21, p=0.039), more advanced disease stage: c-I/II-MCC (HR 4.92, p=0.008) and III-MCC (HR 7.81, p<0.001) and treatment with PORT (HR 0.52, p=0.035) as significant predictors for overall mortality (Table 3). In III-MCC, a PS of 2 (HR 5.64, p=0.003) and PORT (HR 0.37, p=0.031) were associated with overall mortality (Table 4).

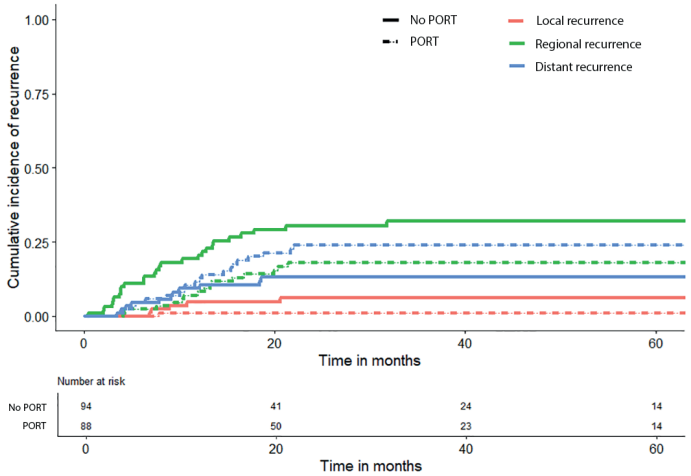
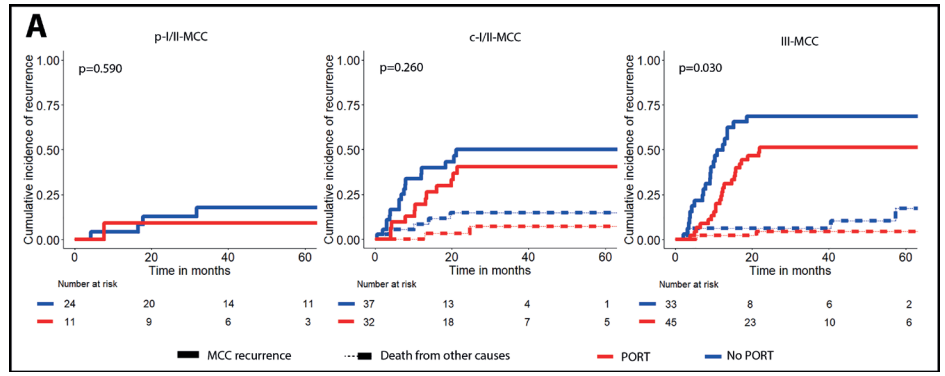
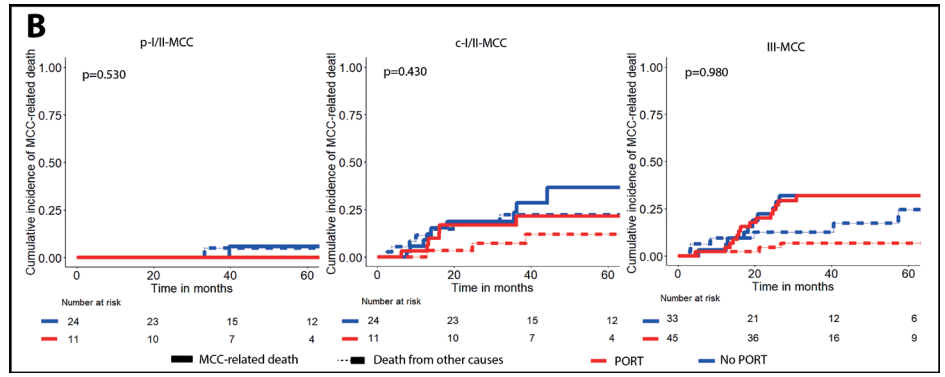


Figure 1. Cumulative incidence curves for local, regional and distant recurrence. PORT: postoperative radiotherapy.





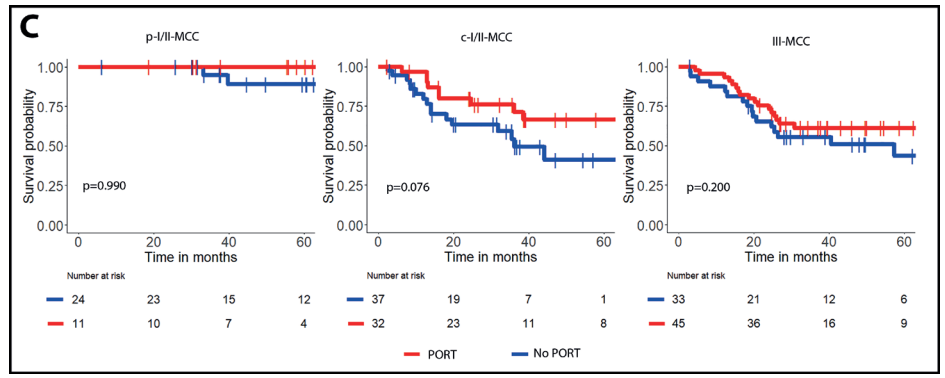


Figure 2. A: cumulative incidence of recurrence curves. **B:** Cumulative incidence curves for MCC-related death. **C:** Kaplan-Meier curves for overall survival. PORT: postoperative radiotherapy, MCC: Merkel cell carcinoma, p-I/II: pathological stage I/II, c-I/II: clinical stage, III: stage III.

		Recurrence free survival			MCC-related survival			Overall survival		
Characteristic		**SDH**	**CI**	**P-value**	**SDH**	**CI**	**P-value**	**HR**	**CI**	**P-value**
Gender										
	Female	**1**	**0.86-2.27**	**0.180**	**1**	**1.07-4.56**	**0.033**	**1**	**0.99-3.26**	**0.056**
	Male	**1.40**			**2.21**			**1.79**		
Age		1.0	0.98-1.03	0.970	1.00	0.96-1.04	0.980	1.01	0.99-1.05	0.252
Performance status										
	0	1	0.68-2.04	0.550	1	0.30-1.67	0.430	1	0.66-2.49	0.470
	1	1.18	0.321.70	0.480	0.71	0.27-2.09	0.580	1.28	1.04-4.77	0.039
	2	0.74	0.40-8.99	0.420	0.75	0.21-6.25	0.893	2.23	1.03-1094	0.044
	3	1.90	2.02-14.09	<0.001	1.13	1.16-32.40	0.033	3.36	2.21-35.43	0.002
	Unknown	5.33			6.13			8.84		
Primary location										
	H&N	1	0.59-2.33	0.650	1	0.92-5.20	0.075	1	1.04-4.70	0.039
	Trunk	1.18	0.38-1.26	0.230	2.19	0.45-2.38	0.930	2.21	0.39-1.58	0.496
	Extremity	0.69	0.18-1.08	0.074	1.03	0.06-1.69	0.180	0.78	0.13-1.38	0.153
	Unknown	0.44			0.33			0.42		
Radical exision										
	No	1	0.28-1.63	0.390	1	0.11-2.50	0.420	1	0.29-2.43	0.741
	Yes	0.68			0.53			0.83		
Lymph-/angioinvasion										
	No	1	0.81-3.19	0.180	1	0.26-1.72	0.400	1	0.44-2.13	0.950
	Yes	1.61	0.71-2.48	0.370	0.67	0.40-1.74	0.610	0.96	0.53-2.04	0.899
	Unknown	1.33			0.82			1.04		
Stage										
	Path I/II	1	1.15-8.10	0.025	1	1.31-17.82	0.017	1	1.51-16.07	0.008
	Clin I/II	3.05	2.68-14.54	<0.001	4.84	2.31-21.97	<0.001	4.92	2.60-23.50	<0.001
	III	6.24			7.12			7.81		
PORT										
	No	1	0.37-0.95	0.039	1	0.43-1.62	0.580	1	1.28-0.95	0.035
	Yes	0.59			0.83			0.52		

Table 3. Multivariable analysis for all patients. PORT: postoperative radiotherapy. SDH: sub-distribution hazard, HR: hazard ratio, CI: 95% confidence interval.

		Recurrence free survival			MCC-related survival			Overall survival		
Characteristic		**SDH**	**CI**	**P-value**	**SDH**	**CI**	**P-value**	**HR**	**CI**	**P-value**
Gender										
	Female	1			1			1		0.718
	Male	1.48	0.62-3.56	0.380	1.60	0.30-2.21	0.490	1.19	0.46-3.09	
Age		0.96	0.91-1.00	0.070	0.98	0.93-1.04	0.500	0.99	0.94-1.03	0.592
Performance status										
	0	1			1			1		
	1	1.30	0.54-3.12	0.560	1.10	0.28-4.03	0.890	1.25	0.46-3.42	
	2	1.17	0.30-4.52	0.820	1.23	0.25-6.33	0.780	5.64	1.80-17.64	0.663
	3*	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.003
Primary location										
	H&N	1			1			1		
	Trunk	1.18	0.37-3.78	0.780	1.72	0.39-7.52	0.471	1.72	0.55-5.32	0.349
	Extremity	1.10	0.42-2.90	0.840	0.86	0.22-3.34	0.820	0.42	0.15-1.14	0.090
	Unknown	0.74	0.23-2.34	0.600	0.36	0.05-2.53	0.300	0.44	0.11-1.72	0.239
Radical excision										
	No	1			1			1		
	Yes	0.57	0.16-1.96	0.370	0.84	0.22-3.34	0.890	0.61	0.07-5.12	0.649
Lymph-/angioinvasion										
	No	1			1			1		
	Yes	0.78	0.31-1.95	0.600	0.36	0.07-1.88	0.220	0.66	0.18-2.48	0.543
	Unknown	0.29	0.11-0.76	0.012	0.35	0.07-1.61	0.180	0.45	0.13-1.56	0.207
Nodal status										
	1-3 nodes positive	1			1			1		
	≥4 nodes positive	1.56	0.61-4.02	0.350	3.24	1.16-8.97	0.024	2.77	0.93-8.27	0.067
	Unknown	0.49	0.15-1.57	0.230	0.57	0.09-3.66	0.550	1.96	0.49-7.87	0.343

Table 3. Multivariable analysis for all patients. PORT: postoperative radiotherapy. SDH: sub-distribution hazard, HR: hazard ratio, CI: 95% confidence interval. (continued)

		Recurrence free survival			MCC-related survival			Overall survival		
Characteristic		**SDH**	**CI**	**P-value**	**SDH**	**CI**	**P-value**	**HR**	**CI**	**P-value**
Extracapsular extension										
	No	1			1			1		
	Yes	4.09	1.43-11.65	0.012	1.61	0.38-6.91	0.520	1.44	0.51-4.05	0.487
	Unknown	2.60	0.80-8.44	0.370	2.79	0.44-16.84	0.280	0.84	0.51-3.43	0.806
PORT										
	No	1			1			1		
	Yes	0.45	0.20-0.98	0.044	0.81	0.30-2.21	0.680	0.37	0.15-0.91	0.031

Table 4. Multivariable analysis for stage III MCC patients. MCC: Merkel cell carcinoma, PORT: postoperative radiotherapy. SDH: sub-distribution hazard, HR: hazard ratio, CI: 95% confidence interval, H&N: head and neck.

* Since this group only included one patient, results were left out of the analysis.

Discussion

In this large multicenter cohort of patients with stage I–III Merkel cell carcinoma we found that PORT was associated with less recurrences and reduced overall mortality across all stages, yet we found no difference for PORT in MCC-related mortality. Further, in stage III-MCC, we found that known prognostic factors such as extracapsular extension were associated with recurrence, and ≥4 positive lymph nodes with MCC-related death, respectively.

The benefit of PORT in the treatment of MCC has long been the subject of debate. In 2019, a meta-analysis summed available evidence of 29 studies that included PORT in MCC patients. Similar to our study, this study indicated that PORT seemed to be associated with improved disease free and overall survival.[17] We found that PORT was associated with reduced overall mortality, but not with MCC-related mortality. This can be explained by the guideline-discordance that has been mentioned previously. In our cohort, 51.6% of patients did not receive PORT when this was indicated. Although the reasons for this are unknown, the reluctance to treat patients with PORT could be based on a pre-existent shorter life expectancy. This would explain the difference in overall and MCC-related mortality, indicating that patients who did not receive PORT were deemed more likely to die of other causes. If so, PORT was correctly withheld from these patients. A similar bias could have been present in the large study on which the current guidelines are based, and in other studies reported in aforementioned review.[13, 17] Interestingly, we did not find an overall mortality benefit for PORT in patients with p-I/II-MCC, whereas Bhatia, et al. did.[13] Since this benefit potentially rises from an association with causes of death unrelated to MCC, it is possible that in their cohort more patients with stage I/II MCC died from other causes than MCC, compared the cohort in the current study.

Our results are similar to a number of studies that have investigated both MCC-specific survival and OS. For instance, after analyzing 269 propensity score matched pairs of patients with MCC, Kim, et al. concluded that the survival benefit of PORT may be due to selection bias or unmeasured confounders, and not PORT.[18] Similarly, this phenomenon was demonstrated in a recent analysis, where PORT was identified as a significant contributor to a nomogram for OS, but not to a nomogram for MCC-related survival, again suggesting possible selection bias.[19] Finally, in an analysis of patients with MCC >65 years old, treatment with PORT was found to be associated with improved OS, but not MCC-specific survival.[20] The discrepancy between MCC-related survival and OS in these studies indicate that selection criteria for PORT could be refined, for instance by taking life expectancy into account. The use of composite endpoints (combinations of multiple endpoints into one primary endpoint) is common in medical research, especially in clinical trials.[21-23] Yet the use of such endpoints should be judged critically, and when an outcome is (partly) associated with the exposure – as is the case with PORT and OS in patients with MCC – serious selection bias may occur.[24] Therefore studies investigating survival in patients with MCC, should include MCC-related survival, with or without OS outcomes.

An important finding of our study is that c-I/II-MCC stage was associated with worse outcomes for all endpoints. This suggests that an important proportion of these, clinically node-negative patients, most likely had unidentified nodal disease. Analogous to the guideline discordance regarding PORT, deviations from protocol for SN biopsy or imaging were mostly due to patients' and clinicians' preference, comorbidities or patients' frailty. Similarly, in a study of patients with MCC of the head and neck, over half of the patients (52.2%) did not receive guideline-compliant regional lymph node evaluation. There, lymph-node evaluation was associated with improved OS in an inverse probability weighted multivariable regression.[25] These results underscore the need for SN biopsy in patients with c-I/II-MCC, since adequate staging leads to more appropriate treatment decision.

There are a number of limitations to the present study. First, although a fairly large group of patients for this rare disease were included, the cohort size was still relatively small. This might have led to our cohort being underpowered for assessment of treatment outcome associated with PORT. Nevertheless, we found distinctive differences between overall and MCC-specific mortality, which are unlikely to change with an increased sample size. Second, for some characteristics we encountered large proportions of missing values, such as lymph-/angioinvasion. For these, we were unable to draw conclusions regarding their association with prognosis, but by including missing values as 'unknown' categories in multivariable analysis, we were able to preserve statistical power and assess the value of other known prognostic characteristics such as nodal status or disease stage. Third, similar to nearly all studies involving patients with MCC, the retrospective observational nature is prone to bias. By performing PSM, we were able to create a cohort of treated and untreated patients that was balanced according to known characteristics associated with treatment decision. Of course, some relevant parameters, such as margin width, were missing in the majority of patients, and could not be included in the PSM analysis. Nonetheless, this inherently means that PSM analysis was conducted with knowledge highly similar to real clinical decisions, therefore we believe the analysis performed was an adequate representation of real-world practice. Interestingly, our matched cohort showed the same results as our unmatched cohort, suggesting that confounding by indication did not play a significant role in our cohort and our data for analysis of treatment outcome is robust.

The management of MCC has changed substantially over the past years: immune-checkpoint-inhibitors (ICI) have been introduced in the treatment of MCC and have changed the prognosis of patients tremendously.[26-30] ICI have been incorporated in the standard of care in the Netherlands since 2017, which means that a proportion of the patients included in this study did not yet have the opportunity to be treated with ICI.[31] Although this implies that the median survival for all patients nowadays might be longer than in this cohort, we do not expect any differences in the effect of PORT. Moreover, we have recently shown that there are no differences in response to ICI in patients with advanced MCC, with or without prior PORT.[32] The role of ICI in the adjuvant treatment of MCC is currently being explored prospectively in the ADMEC-O (NCT02196961), I-MAT (NCT04291885) and ADAM (NCT03271372) trials, including

patients with or without prior PORT. The results from these studies will help further tailor the role for PORT in MCC.

In conclusion, this study is the first to directly address the probable influence of selection bias in the management and research of Merkel cell carcinoma. We have shown that PORT was associated with less recurrences in patients with stage III MCC, but was not with improved MCC-specific mortality in patients with stage I-III MCC. Trends in overall survival benefit are likely to be caused by selection bias suggesting further refinement of criteria for PORT are warranted, for instance by taking life expectancy into account.

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Conflicts of interest We have no conflicts of interest to disclose for this work.

Data availability statement Data are available upon reasonable request.

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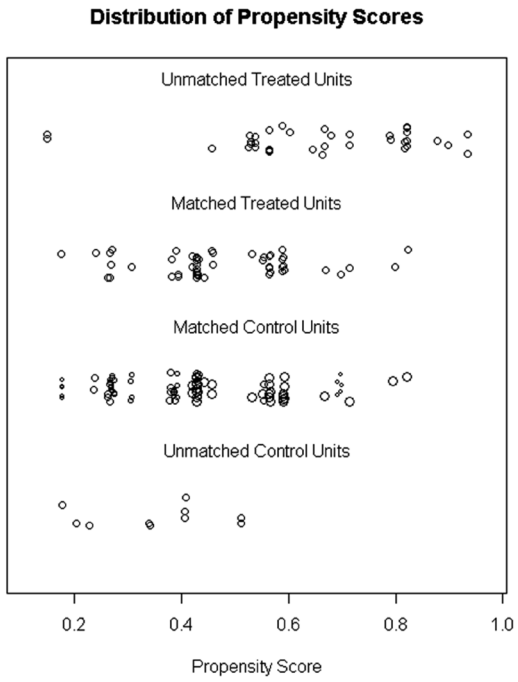
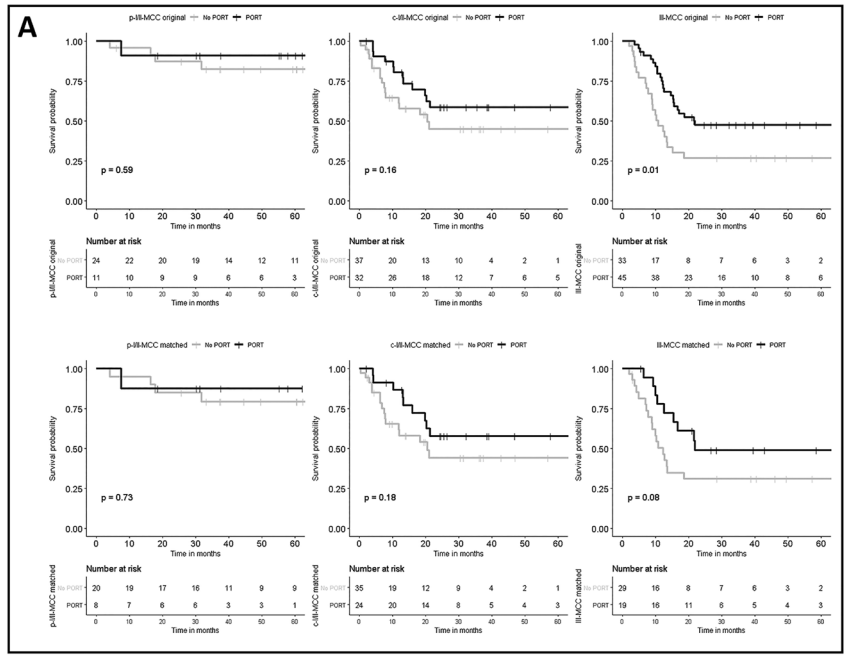
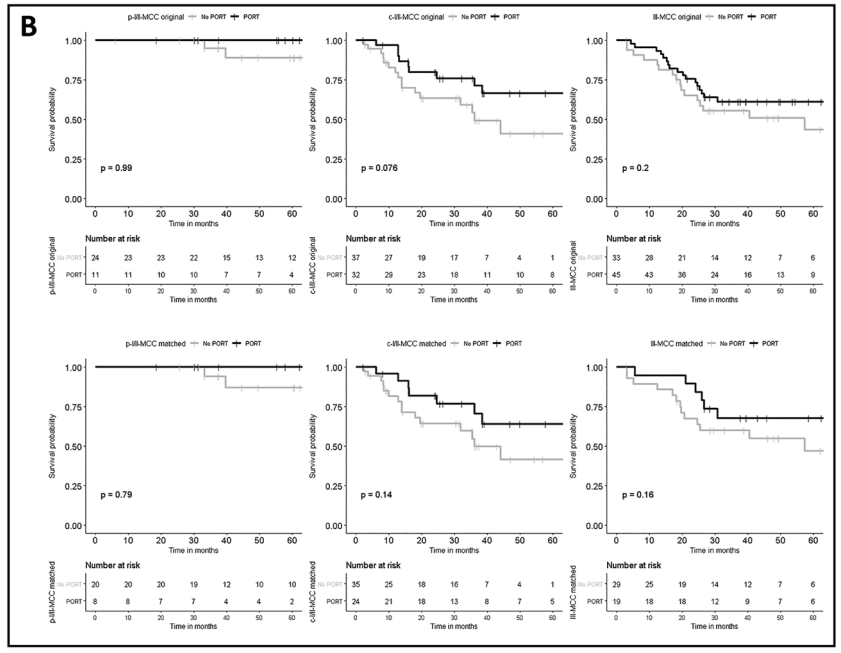
Supplemementary Material



Supplementary figure 1. Results from propensity score matching. Circles represent patients.





Supplementary figure 2. A: Kaplan-Meier curves for recurrence free survival between original (top) and matched (bottom) cohort. B: Kaplan-Meier curves for overall survival between original (top) and matched (bottom) cohort. PORT: postoperative radiotherapy, MCC: Merkel cell carcinoma, p-I/II: pathological stage I/II, c-I/II: clinical stage I/II, III: stage III.

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Avelumab for advanced Merkel cell carcinoma; a nationwide study

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Abstract

Background

Merkel cell carcinoma (MCC) is associated with high recurrence rates and poor survival when metastatic disease is present. The immune checkpoint inhibitor avelumab has shown high response rates and durable responses in patients with advanced MCC (aMCC) in clinical trials. To date, only results from clinical trials, patients treated in an expanded access program and very small numbers of patients have been reported. In this study detailed real-world efficacy and toxicity data of avelumab in patients with aMCC are reported.

Methods

Patients with aMCC treated in 4 dedicated referral centres in the Netherlands were analysed from February 2017 until December 2019. Patients were included if they had received at least one administration of avelumab, regardless of previous lines of therapy. Patient data were collected retrospectively from patient records. Primary endpoints were response rate (RR) and duration of response (DOR). Secondary endpoints were progression free survival (PFS), overall survival (OS) and toxicity.

Results

Fifty-four patients received avelumab. Eight (15%) patients had locally advanced disease (laMCC). In 40 (74%) patients avelumab was first-line treatment, these included all patients with laMCC. Median follow up was 8.9 (range 0.5-35.9) months. RR was 57% (n=31) with 24% (n=13) of patients achieving a complete response. Median DOR was 8.4 (range 1.3-22.1) months and 23 (43%) patients had ongoing response at end of study. Median PFS was 8.6 (CI 1.6-15.5) months, median OS was 25.8 (CI 9.1-42.4) months. Six (11%) patients experienced grade 3 toxicity. No grade 4-5 toxicity was seen.

Conclusions

In this real-world cohort, clinical efficacy and toxicity outcomes in clinical practice were in line with results from clinical trials and show relatively high response rates and durable responses in patients with aMCC.

Introduction

Merkel cell carcinoma (MCC) is a rare and potentially aggressive neuroendocrine carcinoma of the skin with an incidence of around 0.5-0.8/100,000.[1-4] Incidence has been rising over the last few decades.[2 3] This is thought to be due to improved diagnostics, better awareness of this illness, but also increasing sun exposure and an aging population. Median age of presentation is 75 years and in approximately 7-27% of patients regional or distant metastases are present at diagnosis.[1 2] Prior to the introduction of immunotherapy for this disease, patients who no longer had curative, surgical treatment options due to metastatic MCC (mMCC) or locally advanced MCC (laMCC), had 5-year overall survival rates of only 7-12%.[5 6] Treatment strategies for advanced MCC (aMCC), including both laMCC and mMCC, were historically based on those for other small cell malignancies, such as small cell lung cancer, and mostly consisted of polychemotherapy. Although initial response rates of aMCC to platinum-based chemotherapy were high, patients rapidly relapsed and no durable responses or survival benefit have been reported.[1 7]

MCC is associated with two different pathways of pathogenesis. The first route involves the Merkel cell polyomavirus (MCV). MCV is present in up to 80% of patients with MCC in the Northern hemisphere and integrates into the genome of cells driving oncogenic processes such as expression of T-antigen oncoproteins.[8-11] In MCV-negative MCC exposure to ultraviolet (UV)-radiation appears important in the pathogenesis. MCV-negative tumours mostly arise from sun-exposed areas of the skin and show a high mutational burden and adaptive immune responses that are associated with chronic exposure to UV-radiation.[12 13] These alternative pathways of pathogenesis both provide a good rationale for treatment with immune-checkpoint inhibitors (ICI).[14 15]

Several clinical trials showed beneficial results of ICI, such as programmed cell death-1/programmed cell death ligand-1 (PD-(L)1) inhibitors, in the treatment of aMCC.[8 16 17] Pembrolizumab was shown to have an objective response rate (ORR) of 56%, with progression free survival (PFS) at six months of 67% in 26 patients with aMCC.[17]

In 2016 the JAVELIN study, a phase-2 clinical trial that investigated avelumab treatment in patients with aMCC, showed significant and durable responses.[8] In this study, 88 patients who had progressed after chemotherapy were treated with avelumab and an ORR of 31.8% was seen, with 8 patients achieving a complete response (CR) and 20 patients a partial response (PR). Median follow-up was 10.4 months. Based on this study, patients in the Netherlands were able to receive avelumab within an expanded access program (EAP). Avelumab was granted accelerated approval for aMCC by the Food and Drug Administration (FDA) in North America in March 2017, which was followed by the European Medicines Agency (EMA) in September of 2017. In November of 2017 reimbursement in the Netherlands followed and avelumab was integrated into routine management of patients with aMCC.[18]

The population of patients with aMCC is frequently elderly and frail, making it essential to determine whether the results described in a clinical trial population can be replicated in a real-world setting. In 2019 Knepper, et al. performed a large genomic analysis of patients with MCC, and investigated the response to various ICI in 36 patients with aMCC, of which 10 were treated with avelumab. There, a response rate of 44% was seen in all 36 patients.[13] More recently, a large study was performed in an elegant attempt to evaluate the clinical efficacy and safety of avelumab in the real-world population. There, the authors included patients with aMCC that had received avelumab in the EAP. They found that ORR was 47%, with 23% of patients achieving a CR. Unfortunately, although a large number of patients was evaluable for response (n=240, 46% of total), data were limited since evaluation of progression and toxicity were not documented according to a study- or clinical protocol, but was at the discretion of the treating physician to document in the EAP system. Also, duration of response was merely based on resupply of avelumab and data on the medical history of patients included were sparse.[19]

Both the clinical trials and the results from the expanded access program indicate an auspicious effect of avelumab in treatment with aMCC, but detailed data on patients with aMCC treated with avelumab in routine clinical practice are still lacking.

In the Netherlands, patients with aMCC are treated in four dedicated tertiary referral centres across the country. In this nationwide study we aimed to evaluate efficacy and toxicity of avelumab in a large real-world cohort of patients with aMCC treated in routine clinical practice in the Netherlands.

Methods

Patients

Patients with aMCC treated with avelumab since the introduction of the EAP in the Netherlands were included from all four MCC referral centres from February 2017 until December 2019. Data were collected retrospectively and patients were followed up until death or end of follow up. Patients were excluded if they had received other types of ICI prior to avelumab. Histopathological analyses were performed during the diagnostic work-up according to standard of care for these tumours. MCV positivity was determined immunohistochemically using CM2B4 monoclonal antibody as described previously.[20 21]

Avelumab was administered in a two weekly interval as per institutional protocol. Premedication consisting of 2mg clemastine and 1000mg paracetamol was administered intravenously during the first three cycles and continued thereafter only if infusion reactions occurred.
Patient characteristics, response to avelumab, adverse effects and toxicity were gathered from electronic patient records. All patients gave consent to use their medical data according to institutional protocols.

Outcomes

Primary endpoints were response rate (RR) and duration of response (DOR). Response evaluation by computer tomography (CT) or positron emission tomography (PET-)CT was performed at approximately 12 week intervals. Since this study was not conducted within a trial setting, response was reported in radiological records according to routine diagnostic practice. When radiological evaluation was not possible clinical parameters such as changes in visible skin lesions that were measured with a calliper or other evaluable parameters such as performance status were used. For biochemical response measurements, all centres used lactate dehydrogenase (LDH) with upper limit of normal 248 U/L, and additionally, neuron-specific enolase (NSE) with upper limit of normal 18.2 ug/L was used in the largest referral centre.[22] Responses were extracted retrospectively from patient records and radiology reports. The measurements in the reports initially described by a radiologist were reassessed according to RECIST criteria. Partial response (PR) was defined as radiographic shrinkage of tumours ≥30%. In the absence radiological response evaluation, visible and/or palpable shrinkage ≥30% of skin tumours and/or lymph nodes were evaluated. Complete response (CR) was defined as complete metabolic and radiological response on (PET-)CT. When mixed response (MR) was present at ≥2 consecutive response evaluations, defined as ≥30% tumour shrinkage, with simultaneous growth ≥20% of other lesions and/or occurrence of new lesions, consensus on continuation or cessation of avelumab was reached in a multidisciplinary team (MDT). The decision to perform salvage treatment including surgery or radiotherapy was also reached in an MDT. Progressive disease (PD) was defined as radiographic tumour growth ≥20% and/or growth of visible skin tumours, and/or increase of biochemical markers such as LDH and/or NSE above the upper limit of normal, and/or deterioration of a patients' performance status due to aMCC. DOR was defined as the time from first documented PR or CR until documented PD, death or end of follow up.

Secondary endpoints were progression free survival (PFS), overall survival (OS) and toxicity. PFS and OS were defined as the time from first administration of avelumab until documented progression or death, respectively. Patients were censored at end of study. Toxicity was evaluated according to CTCAE version 5.0, grades 1-5 were included.

Statistical analysis

Descriptive statistics with median and ranges were used for continuous variables and frequency and percentages for categorical variables. Pearson's Chi-square test was used to compare response between groups and for the forest plot, univariable Clopper-Pearson calculations were performed to establish confidence intervals for proportions of patients that responded to avelumab. Kaplan-Meier method was used to evaluate OS and PFS and the logrank test was performed for comparison between first- and second-line treatment. IBM SPSS version 25 (SPSS Inc., Chicago, IL) was used to perform all statistical analysis.

Results

Baseline characteristics

We identified 55 patients with aMCC who had received at least one dose of avelumab, 54 of these patients fulfilled the inclusion criteria. One patient was excluded due to prior treatment with ICI (nivolumab). Two patients received avelumab in the EAP. The first administration of avelumab was in February 2017, the last patient started treatment in September 2019.

Patients were first diagnosed with MCC at a median age of 71 (range 50-86), and had a median age of 73 (range 53-88) years at the start of avelumab. Thirty-four (63%) patients were male. Primary tumour localisations were head & neck, trunk, extremities or unknown primary tumour (UPMCC) in 13 (24%), 8 (15%), 25 (46%) and 8 (15%) patients, respectively. Eight (15%) patients had locally advanced (stage IIIB/laMCC) disease and 46 (85%) had distant disease (stage IV/mMCC) at the start of avelumab administration. Of the latter, 35 (65%) patients had distant nodal and/or (sub)cutaneous disease, and in 19 (30%) visceral and/or peritoneal/mesenterial metastasis were present. In 12 (22%) patients one organ site was involved, and in 4 (7%) patients disease was present in two organs. Seven (13%) patients had a history of immunosuppression, including chronic lymphatic leukaemia, Waldenström's macroglobulinemia, human immunodeficiency virus, idiopathic pulmonary fibrosis and a kidney transplant recipient. Merkel cell polyomavirus status was determined in 21 patients. Of these, 15 (71%) was positive. PD-(L)1 expression was not determined in routine clinical practice, hence no data on PD-(L)1 expression were available. LDH levels were available for 50 patients at the start of avelumab. Of these, 29 (58%) had elevated LDH levels above ULN. Baseline characteristics are shown Table 1. Avelumab was first-line treatment for all patients with laMCC (n=8, 15%) and in 32 (59%)patients with mMCC, the remaining 14 (26%) patients with mMCC received avelumab as second-line treatment. Prior therapy in all patients consisted of platinum-based chemotherapy. Additionally, 31 (57%) patients had received radiotherapy on either primary tumour area or metastasis prior to avelumab initiation. All patients had a performance score (PS) ≤2. Patients with a performance score of 2 (n=5, 9%) were all in the mMCC group. In patients with laMCC, 3 (38%) had PS 0 and 5 (63%) had PS 1, compared to 14 (30%) and 27 (58%) in patients with mMCC, respectively. Patients received a median of ten (range 1-39) doses of avelumab and median follow up time was 8.9 (range 0.5-35.9) months.

Response to avelumab

Three patients (6%) were not evaluable for response: two died due to comorbidities before response evaluation. Comorbidities included rapidly progressive dementia and a superinfection due to pre-existent idiopathic pulmonary fibrosis shortly after avelumab administration. One patient discontinued avelumab after one infusion due to an allergic reaction and was referred to the general practitioner for palliative care.

Characteristics	All patients (n=54)
Sex, n (%)	
Male	34 (63)
Female	20 (37)
Immunosuppression history, n (%)	
CLL	3 (6)
WM	1 (2)
HIV	1 (2)
IPF	1 (2)
KT recipient	1 (2)
Age in years, median (range)	
At diagnosis	71.1 (50.2-86.3)
At start IT	73.0 (53.0-88.0)
Primary tumour site, n (%)	
Head and Neck	13 (24)
Trunk	8 (15)
Extremity	25 (46)
Unknown Primary	8 (15)
WHO Performance Status, n (%)	
0	17 (32)
1	32 (59)
2	5 (9)
Disease status, n (%)	
Locally advanced	8 (15)
Distant disease	46 (85)
Metastasis, n (%)	
Visceral metastases *	19 (35)
Nodal or (sub)cutaneous metastases	35 (65)
Number of organ sites involved, n (%) #	
1	12 (22)
2	4 (8)
Line of therapy, n (%)	
First	40 (74)
Second	14 (26)
Radiotherapy	
Yes	31 (57)
No	23 (43)
MCV, n (%)	
Yes	15/21 (71)
No	6/21 (29)
LDH n (%)	
Yes	21/50 (39)
No	29/50 (54)
Missing	4/50 (7)

Table 1. Baseline characteristics of all included patients. * Also including distant mesenterial or peritoneal metastasis. # Organ sites included liver, bone, adrenal cortex, pancreas, intestine, pleura. CLL: chronic lymphatic leukaemia, WM: Waldenström's macroclobulinemia, HIV: Human immunodeficiency virus, IPF: idiopathic pulmonary fibrosis, KT: kidney transplant, IT: immunotherapy, WHO: World Health Organization. MCV: Merkel cell polyomavirus, LDH: lactate dehydrogenase.

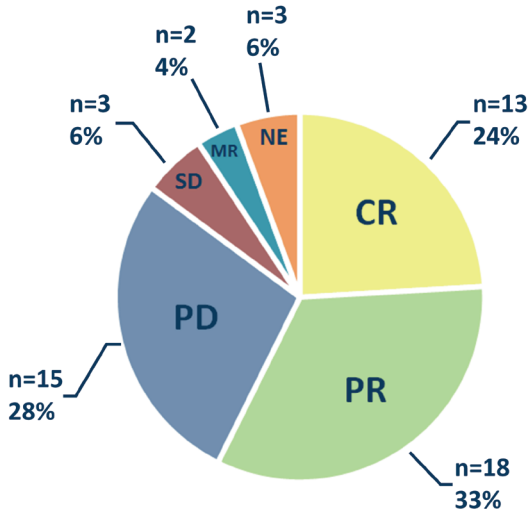

Figure. 1 Best overall response during follow up for all 54 patients. CR: complete response, PR: partial response, PD: progressive disease, SD: stable disease, MR: mixed response, NE: not evaluable.

Out of all 54 patients, objective response to avelumab was seen in 57% (n=31). In 24% (n=13) of patients best overall response (BOR) was a CR, PR was seen in 33% (n=18), stable disease in 6% (n=3), MR in 4% (n=2) and PD in 28% (n=15) of patients. Best overall responses are shown in Figure 1. Response to avelumab therapy in all patients divided and analysed by subgroups are shown in Figure 2. In patients with laMCC RR was 50% (n=4), compared to 59% (n=27) in patients with mMCC (p=0.646). Regarding complete responses: in patients with laMCC 25% (n=2) achieved a CR, and in patients with mMCC, 24% (n=11) patients achieved a CR (p=0.947). In five (9%) patients salvage surgery or radiotherapy was performed for either residual tumour lesions (n=3) or new solitary lesions (n=2). All salvage interventions were in patients with mMCC. Response rate was 41% (n=7), 63% (n=20) and 80% (n=4) in patients with PS 0, 1 and 2, respectively (p=0.200). Of these, CR was achieved in 12% (n=2), 28% (n=9) and 40% (n=2), respectively (p=0.303).

In patients with unknown primary tumour locations (UPMCC), 63% (n=5) had an objective response, whereas in patients with known primary locations 56% (n=26) had a response (p=0.752). Complete responses occurred in 25% (n=2) of patients with UPMCC, and in 24% (n=11) of patients with known primary tumours (p=0.947).

Out of the 19 patients with visceral metastases (including peritoneal or mesenteric metastases) 63% (n=12) had a response, compared to 54% (n=19) out of the remaining 35 patients that had nodal or subcutaneous metastases only (p=0.529). Complete responses were achieved in 37% (n=7) of patients with visceral metastases, and in 17% (n=6) of patients without visceral metastases (p=0.106).

In the 50 patients for which LDH levels were known, no differences in response to avelumab between elevated LDH levels and normal LDH levels were seen. In patients with elevated LDH levels response was seen in 62% (n=13) of patients, compared to 55% (n=16) of patients who had normal LDH levels (p=0.634).

Interestingly, in patients that had received avelumab as second-line treatment 11/14 (79%) had response, compared to patients that received avelumab as first-line treatment, of which 20/40 (50%) patients had a response (0.063). On the other hand, CRs were present in 28% (n=11) of patients receiving first-line avelumab, compared to 14% (n=2) of patients treated with second-line avelumab (p=0.302). We saw no difference in response to avelumab for patients who had received radiotherapy prior to avelumab initiation: of the 31 patients who underwent radiotherapy 52% (n=16) had a response, compared to 65% (n=15) out of 23 patients who did not (p=0.317).
In patients with a history of immunosuppression 29% (n=2) had a PR, no CRs occurred in this group.

Toxicities	**Patients N (%)**
Grade 1, n (%)	
Fatigue	5 (9)
Hypothyroidism	1 (2)
Grade 2, n (%)	
Hepatitis	1 (2)
Allergic/infusion reaction	1 (2)
Hypothyroidism	2 (4)
Grade 3, n (%)	
Allergic	3 (6)
Nausea/vomiting	1 (2)
Renal insufficiency	1 (2)
Grade 4, n (%)	0 (0)
Grade 5, n (%)	0 (0)
Total	15 (28)

Table 2. Avelumab associated toxicities.

Median DOR was 8.4 (range 1.3-22.1) months, and median PFS was 8.6 (CI 1.6-15.5) months. Estimated median OS was 25.8 (CI 9.1-42.4) months. We saw no significant differences in PFS and OS between patients treated with avelumab in first- or second-line setting, p=0.337 and p=0.548, respectively. PFS and OS are shown in Figure 3a and 3b. Responses were ongoing in 23 (43%) patients at end of follow up. Progressive disease occurred in 7/19 patients for whom BOR was PR. All 7 patients were on active therapy at time of progression. For patients who achieved CR: at end of study, 12/13 patients remained free of disease, with a median DOR of 12.8 (range 3.6-22.1) months.

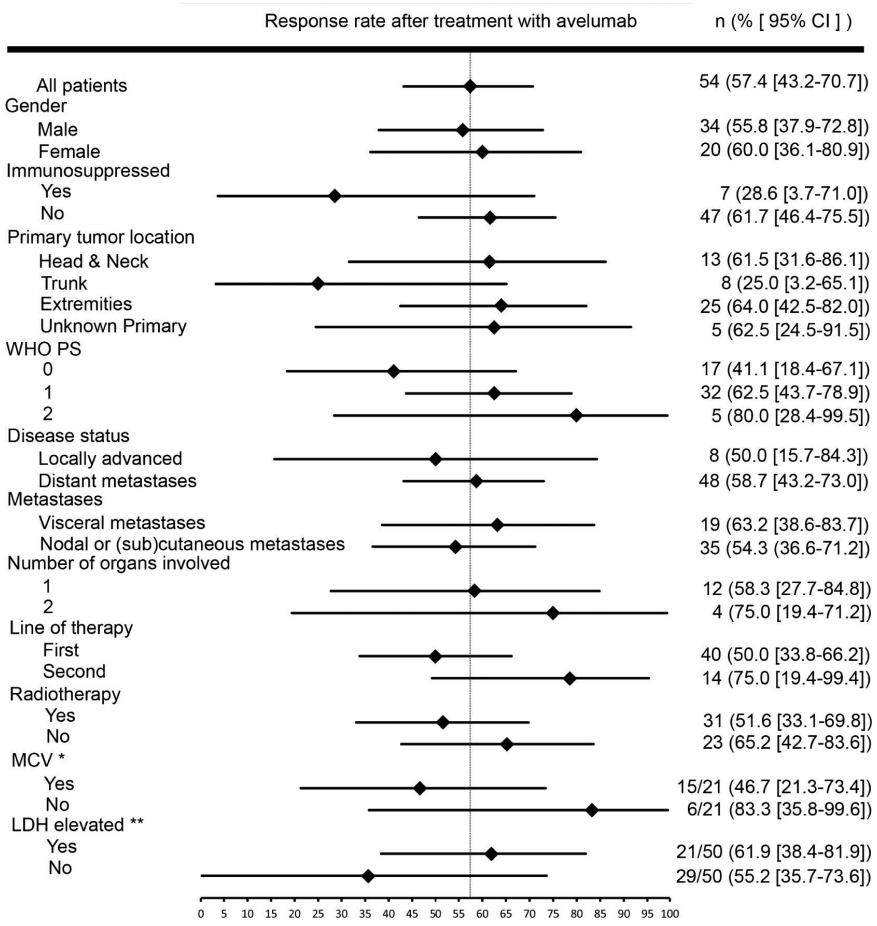

Figure 2. Response rates by subgroups. *Response rates calculated for patient with known viral status. **Response rates calculated for patient with known LDH levels at the start of avelumab. LDH, lactate dehydrogenase; MCV, Merkel cell polyomavirus; WHO PS, World Health Organization performance status.

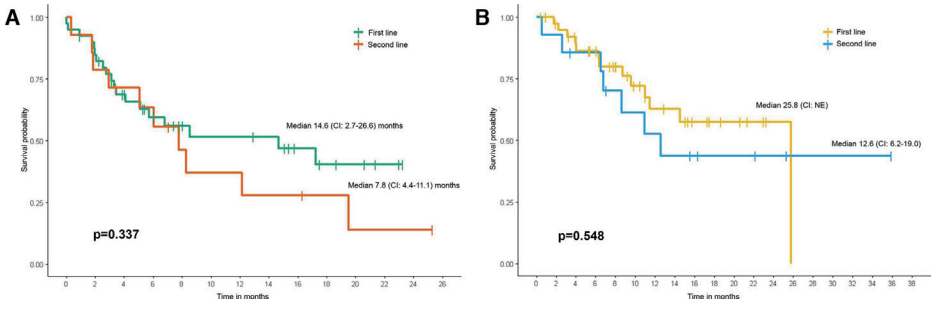

Figure 3. A. Progression free survival all patients, divided by subgroups of patients receiving avelumab in first-line and second-line treatment. **B.** Overall survival of all patients, divided by subgroups of patients receiving avelumab in first-line and second-line treatment. NE, not estimable.

The remaining patient had achieved a CR after 2.6 months and had discontinued avelumab treatment after 19 cycles (8.8 months). Recurrence occurred 17.2 months after start of therapy. During follow up no rechallenges with avelumab were initiated for patients who had initially responded but then progressed. Clinical activity of avelumab in all patients evaluable for response are shown in Figure 4.

Out of all 54 patients, six (11%) experienced grade 1 toxicity, which was mostly fatigue. Four (7%) patients experienced grade 2 toxicity, which constituted of allergic reactions requiring oral intervention, hypothyroidism, and hepatitis. Grade 3 toxicity including allergic reactions and renal insufficiency resulting in clinical admission were seen in five (9%) patients. No grade 4 or 5 toxicities were seen. Toxicities are shown in Table 2.

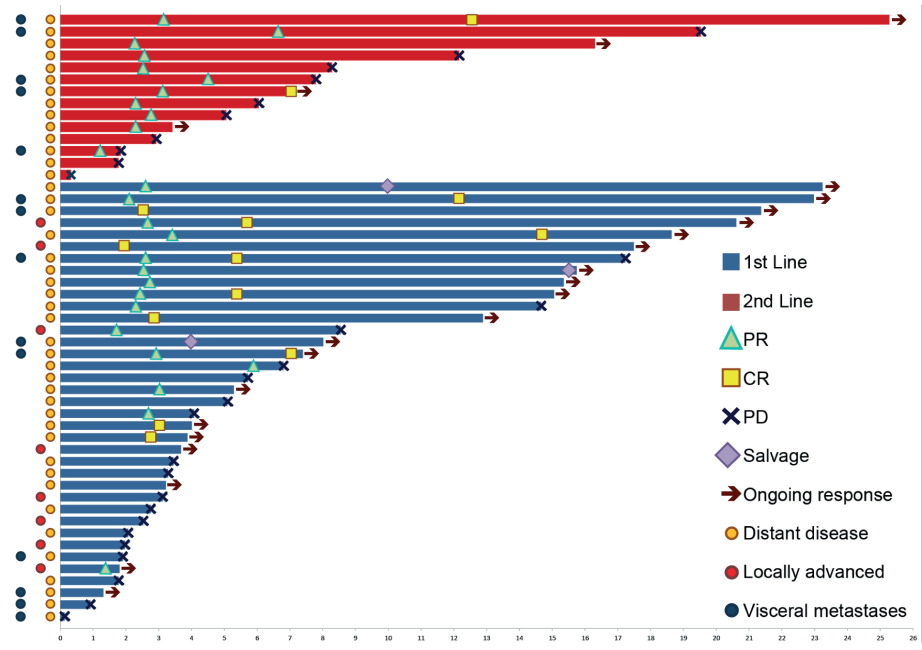

Figure 4. Clinical activity of avelumab in all evaluable patients, (n=51). CR: complete response, PD: progressive disease, PR: partial response.

Discussion

In this real-world cohort of patients with aMCC, avelumab treatment resulted in high response rates, durable responses and manageable toxicities. This study describes a detailed and relatively large cohort of patients with aMCC patients outside the setting of a clinical trial or an expanded access program, showing a true representation of the real-world clinical practice for patients with aMCC. We demonstrate that avelumab now has a prominent role in both first-, and second-line treatment for aMCC.

In the 40 patients in our study that received avelumab in first-line setting, we found a RR of 50% with 28% achieving a CR. The EAP study found similar results in 15 patients that were treated with first-line avelumab, where 47% of patients had a reponse.[19] This is also in concordance with results seen in clinical trials involving pembrolizumab or avelumab as first-line treatment for aMCC.[17 23 24]

Regarding previous lines of therapy, in the cohort of 36 patients treated with various ICI that were investigated by Knepper, et al. a dramatic decrease in response was seen in patients treated with higher therapy lines. There, a response rate of 75% was found for patient treated with ICI as first-line therapy, 39% as second-line therapy and 18% in third- or higher-line therapy.[13] Similarly, in our cohort patients treated with first-line avelumab also had a more favourable outcome. We found that nearly all CRs arose in patients treated with first-line avelumab, and responses were similar in both laMCC and mMCC, indicating that tumours in patients without prior lines of chemotherapy might be more sensitive to PD-(L)1 blockade. This supports the clinical practice that we see evolving in the Netherlands in which avelumab is increasingly being used as first-line therapy for mMCC.

Although only 14 patients in our cohort were treated with avelumab as second-line therapy for aMCC, still interesting results were seen. When comparing our patients receiving avelumab as second-line treatment for aMCC to those from clinical trials, we saw similar rates of complete responses: 14% in our cohort compared to 9% in the JAVELIN trial.[8] In contrast, the overall ORRs were quite different, with 79% in our second-line patients, compared to 32% in the trial. There, over half of the patients were treated with more than one prior line of chemotherapy, and the authors suggest that this could have led to more immunologically depleted patients, resulting in worse response rates. This is substantiated by the updated results of the JAVELIN trial after a median follow up of 41 months, where a trend towards a higher overall RR in patients with fewer prior lines of therapy was seen.[24] This could explain the higher RR in our cohort, since we had no patients with more than one prior line of therapy.

In earlier years when polychemotherapy was the treatment of choice for aMCC, a more advanced disease stage was associated with worse prognosis. Several epidemiological studies have found that a more advanced disease stage is an independent predictor for survival.[1 25-30] Interestingly, in studies where patients with aMCC were treated with ICI, no significant difference in response to treatment were seen between stage IIIb or IV disease, but a trend towards lower response rates remained.[8 17] In our cohort, 35% of patients had visceral metastases at treatment initiation. This is a smaller percentage than was shown in the clinical trials, were visceral metastases have been reported to be present in 53-67% of patients.[8 16] However, older studies that focussed on chemotherapy for aMCC, found similar or even lower percentages of visceral metastases than in our cohort, suggesting that the percentage of visceral metastases varies greatly between different study populations.[7 31] Besides this, although we saw no statistically different responses, we saw a trend towards higher response rates in patients with visceral metastases. This seems contradictory to the results from clinical trials.[8 32] Finding these differences may be attributable

to the small number of patients with visceral metastases (n=19). Another explanation might be that because of the retrospective nature of this study, we did not perform other measurements of disease burden, such as sum of lesion diameter (SLD) parameters. This might underestimate the disease burden in patients with nodal and/or subcutaneous disease only, subsequently overestimating disease burden in patients with visceral metastases. Nevertheless, our cohort represents an accurate representation of radiological documentation and response to avelumab in patients with aMCC in routine clinical practices. Therefore these results remain generalizable to clinical practices elsewhere.

Patients with UPMCC have been shown to have a longer OS and a higher tumour mutational load than patients with known primary tumour locations.[33 34] It has therefore been implied that patients with UPMCC are more likely to respond to immunotherapy.[33] However, both clinical trials involving ICI and this real-world cohort did not show different RRs in patients with an UPMCC.[8] Potential differences may have been missed due the fact that we had only eight (15%) patients with UPMCC.[8] Yet our findings are in concordance with results shown in a Dutch cohort of 351 patients, where UPMCC was not associated with survival benefit in multivariable analysis.[35]

Since we did not perform additional genomic sequencing, data on mutational burden were not available. To determine whether mutational burden indeed plays a role in response, larger studies are needed. Recently, several potential strategies to involve and study larger numbers of patients with aMCC have been described.[36] Future studies should aim to elucidate which patients would benefit from ICI by investigating which possible biomarkers or genomic characteristics of the tumour are associated with response to PD-(L)1 blockade.

Conclusion

In the Netherlands avelumab is increasingly being used as first- and second-line systemic treatment for aMCC. In this real-world cohort, response rates, duration of response, progression free survival and toxicity results are promising and essentially in line with results found in clinical trials. Although higher response rates were seen in patients treated in second line, more complete and durable responses were seen in patients that received avelumab as first-line treatment.

Declarations

Ethics approval and consent to participate This study was conducted in accordance with the Declaration of Helsinki and the International Council for Harmonisation Good Clinical Practice Guidelines. All patients gave consent for the use of their data.

Patient consent for publication Not required.

Availability of data and material Data is available upon reasonable request.

Competing interests MA, FE, KK, LB, DG, AK, MJ and MT report to hold a position on an advisory board for Merck.

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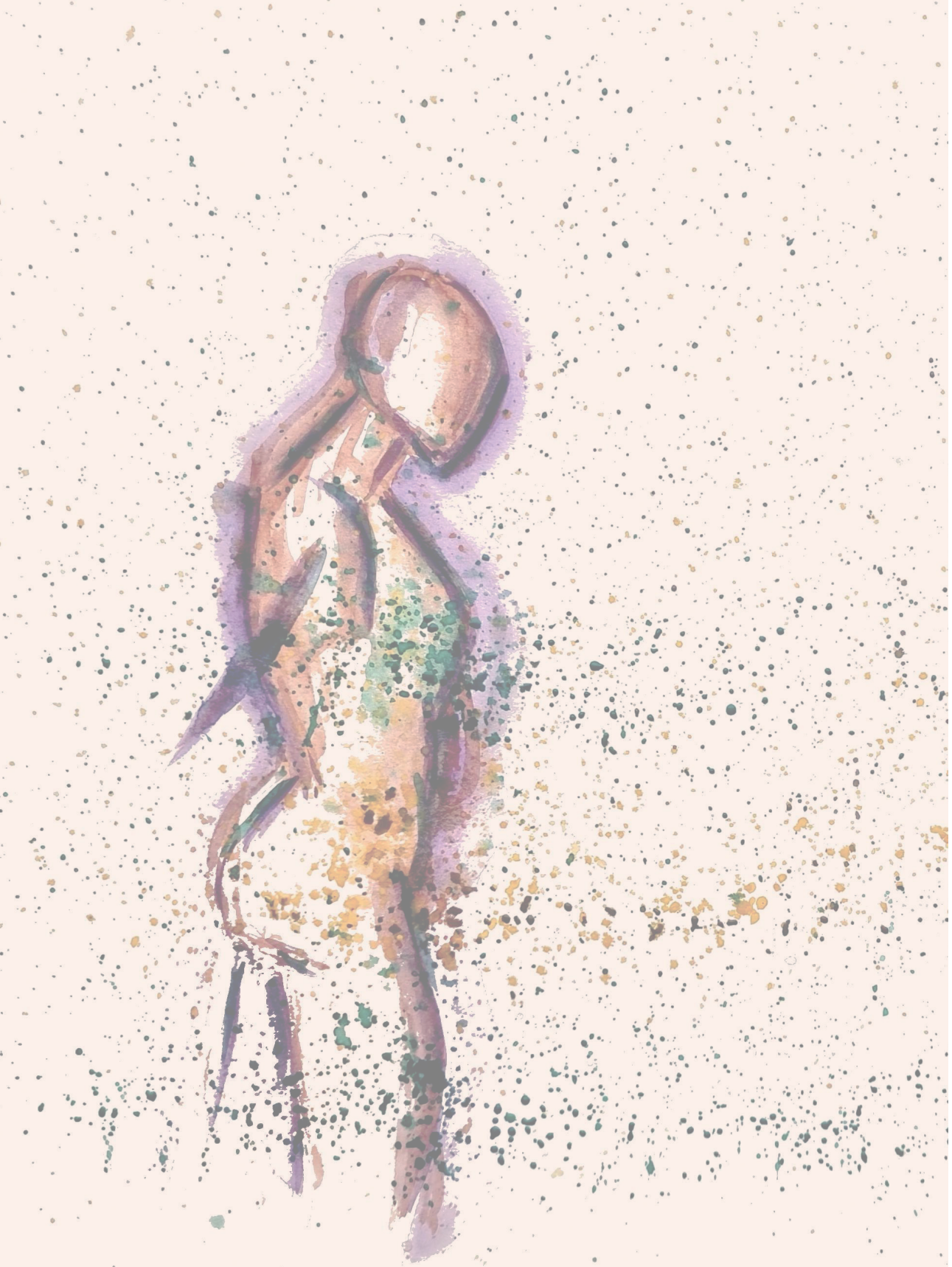

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First-line Everolimus and Cisplatin in Patients with Advanced Extrapulmonary Neuroendocrine Carcinoma

A Nationwide Phase 2 Single-Arm Clinical Trial

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Abstract

Background

Extrapulmonary neuroendocrine carcinoma (EP-NEC) are an aggressive subgroup of neuroendocrine neoplasms (NEN). Advanced EP-NEC is generally treated with platinum-based cytotoxic regimens, but progressive disease occurs rapidly, resulting in a poor prognosis. Genetic alterations in the mammalian target for rapamycin (mTOR) pathway have been identified in NEN, providing a rationale for treatment with the mTOR-inhibitor everolimus.

Methods

A prospective phase 2 single-arm study included patients with advanced EP-NEC from three Dutch NEN expertise centres between March 2016 and January 2020. Treatment consisted of cisplatin 75mg/m2 every 3 weeks in combination with daily everolimus 7.5 mg for a maximum of 6 cycles, followed by maintenance everolimus until disease progression. Primary endpoint was disease control rate (DCR), defined as the sum of overall response rate (ORR) plus the rate of stable disease according to RECIST 1.1, assessed at a 9 week intervals. Toxicity was evaluated according to CTCAE version 5.0.

Results

Thirty-nine patients, with a median age of 64 years (range 28-74), of whom 20 (51%) were male, were enrolled. DCR was 82.1% (95% confidence interval [CI] 66.4-92.4), with an ORR of 58.9% (CI 42.1-74.4). Median duration of response was 6.4 (CI 5.8-7.0) months and median progression free survival was 6.0 (CI 4.3-7.8) months. Three patients (8%) had durable responses lasting >12 months. Median overall survival was 8.7 (CI 7.8-9.6) months. Most common grade 3/4 toxicities were haematological (36%) and renal (21%).

Conclusion

Everolimus in combination with cisplatin is an effective first-line treatment option for advanced EP-NEC, especially in highly selected patients.

Introduction

Neuroendocrine neoplasms (NEN) are a heterogeneous class of malignancies that may arise in various localizations throughout the body. NEN have been classified according to morphological properties and biological behaviour into well-differentiated neuroendocrine tumours (NET) and poorly-differentiated neuroendocrine carcinomas (NEC).[1] This classification and hence the nomenclature of various NEN has been altered several times over the past decades.[2-4] The most recent update in 2017 has identified three grades (G1-G3) of NET, and a solitary high-grade (G3) NEC.[1] By definition, NEC show a mitotic count of >20 per 2 mm2 and/or a Ki67-proliferation index of >20%, although most NEC have a Ki67-index of >55%.[5, 6] The majority of NEC are of pulmonary origin in the form of either small cell lung carcinoma (SCLC) or large cell neuroendocrine carcinoma (LCNEC).[4, 7, 8] Extrapulmonary NEC (EP-NEC) mostly originate from the gastroenteropancreatic tract (GEP-NEC), accounting for around 35-55% of all NEC.[9] Other primary sites of EP-NEC include the genitourinary tract and the skin (i.e. Merkel cell carcinoma).[10, 11]

EP-NEC usually display very aggressive behaviour with up to 85% of patients presenting with locally advanced or metastatic disease at diagnosis.[9, 12, 13] Due to the rarity of the disease, evidence regarding systemic treatment is scarce and is often based on guidelines for treating SCLC.[8, 9, 14] At the time of initiation of this study, a few retrospective studies were available regarding cytotoxic therapies in EP-NEC.[13, 15] A large retrospective study of 305 patients with EP-NEC indicated that cytotoxic treatment offers survival benefit over a best supportive care (BSC) approach. They observed a median overall survival (OS) of 11 months with palliative chemotherapy, including first- to third treatment lines, and a mere 1 month OS in patients who received BSC only. In this series, first-line chemotherapy resulted in an overall response (OR) of 31% and disease stabilization of 33%, summing to a disease control rate (DCR) of 64%. Also, a Ki67-index of >55% cut-off was found to be significantly associated with worse OS (10 months vs 14 months).[13] More recently, a prospective study of patients with GEP-NEC or unknown primary NEC showed an OR of 50% and SD of 23% for first-line treatment and a progression free survival (PFS) and OS of 6.2 and 11.6 months, respectively.[16] Most cytotoxic regimens used in the treatment of EP-NEC consist of a platinum backbone, often combined with etoposide or irinotecan.[13, 16] Currently, the European Society for Medical Oncology (ESMO) guidelines recommend cisplatin/etoposide or carboplatin/etoposide as first-line treatment for advanced EP-NEC.[14] Although highly responsive to these therapies, progression occurs rapidly, often with a strikingly poor prognosis due to the lack of treatment options. To illustrate this, a recent meta-analysis in patients with advanced EP-NEC showed that second-line treatment had very limited efficacy, with a pooled PFS of 2.5 months.[17]

Mutations in the mammalian target of rapamycin (mTOR) are present in various cancers, including well-differentiated NET.[18, 19] mTOR is a serine/threonine kinase that regulates cell growth and proliferation, metabolism, and angiogenesis. It has also been implicated in the pathogenesis of neuroendocrine tumours. Inhibition of the mTOR signalling pathway has

shown antiproliferative effects in cell lines and primary cultures of human neuroendocrine tumours.[18] A therapeutic intervention that specifically targets this mTOR pathway is everolimus. This one-of-a kind anticancer drug has been extensively studied in randomized trials of NET of the pancreas, lung and small intestine.[20-22] Based upon superior PFS data, albeit with somewhat disappointing effects on OS, everolimus has become part of standard of care for patients with these tumours.[23-25] Preclinical studies have demonstrated synergistic anti-tumour activity of everolimus in combination with cisplatin, which prompted the necessity to further investigate this combination.[26, 27] The mechanism underlying this synergistic activity has not been fully elucidated, but it has been suggested that reducing cellular levels of p21, thereby impairing DNA repair, could be an underlying mechanism.[26]

The poor efficacy of current treatment options for EP-NEC combined with the abovementioned interactions, as well as the widely accepted anticancer activity of everolimus in patients with NEN, provided the rationale to perform this multicentre phase 2 clinical trial to evaluate the efficacy and safety of everolimus in combination with cisplatin in patients with advanced EP-NEC.

Methods

Study design

A single-arm, open-label, three-centre, national phase 2 clinical trial was designed to assess antitumor activity and safety of cisplatin in combination with everolimus as first-line treatment in patients with advanced EP-NEC. Between March 2016 and January 2020, patients were included from three referral centres in the Netherlands (Erasmus Medical Centre in Rotterdam, Netherlands Cancer Institute (NKI) in Amsterdam and the University Medical Centre Groningen). The study complies with the Declaration of Helsinki rules and the principles of Good Clinical Practice guidelines. This study was approved by the institutional review board (IRB) of the NKI (organizing institute) under IRB-identification number: NL50842.031.15. Written informed consent was obtained from all patients. This trial is registered at clinicaltrials.gov (NCT02695459) on March 1st, 2016. The first patient was included in the study on March 21st, 2016.

Patients

Eligible patients were adults with histopathologically confirmed locally advanced or metastatic EP-NEC. All pathological samples were classified by a NEN expert pathologist. Inclusion criteria were a World Health Organization (WHO) performance status (PS) of 0-2, adequate bone marrow, liver and renal function (creatinine clearance >60ml/min); and an estimated life expectancy of >3 months.

Patients were excluded if they had received previous chemotherapy for advanced or metastatic EP-NEC or had previously been exposed to everolimus. Neo-adjuvant and peri-operative chemotherapy or chemoradiation with curative intent was allowed if at least 6 months had elapsed between completion of therapy and enrolment in the study.

Study treatment

Study treatment consisted of daily everolimus 7.5 mg/day combined with cisplatin 75 mg/m2 every 21 days, up to a maximum of six cycles, unless withdrawn earlier due to unacceptable toxicity or progressive disease. Everolimus and cisplatin dosage was established based on a phase I study in patients with advanced head and neck tumours.[28] After 6 cycles of cisplatin and everolimus were completed, patients continued with single-agent everolimus 7.5 mg/day until disease progression or unacceptable toxicity. On day 1 of every cycle, patients were evaluated for renal function, myelosuppression, ototoxicity and peripheral neuropathy. Cisplatin was switched to carboplatin with an AUC=4 if creatinine clearance had dropped below 50ml/min, or when grade 3 or higher ototoxicity or peripheral neuropathy occurred. When creatinine clearance had decreased to 50-60 ml/min, cisplatin was reduced by 20% in the next cycle. Cisplatin was only administered when neutrophils were ≥1.5 10^9/L and thrombocytes ≥100 10^9/L. When these were below 1.5 and 100, respectively, cisplatin was postponed for one week. Similar cut-offs were used if carboplatin was given. After recovery of bone marrow toxicity, cisplatin or carboplatin was given with a 20% dose reduction.

Everolimus dosages were adjusted to 5mg/day (-1 dose level) or 5mg every other day (-2 dose level) when tolerability issues occurred, including cytopenias, hepatotoxicity's, infection, skin toxicity , oral mucositis, pneumonitis, hyperlipidaemia or hyperglycaemia. Dose reductions below -2 dose level were not allowed and patients would go off study.

After disease progression, patients were treated at the discretion of the treating physician and were monitored for survival.

Study Endpoints

Primary endpoint of this study was DCR, defined as the sum of overall response rate (ORR) consisting of complete (CR) and partial response rate (PR) plus the rate of SD, according to RECIST 1.1, assessed at a 9 week intervals. Patients were evaluable for response if at least one follow-up examination was performed.

Secondary endpoints were PFS, according to RECIST 1.1; duration of response (DOR); OS, defined as death from any cause; and safety of everolimus in combination with cisplatin/carboplatin.

Adverse events (AE) were defined as any undesirable experience occurring to a subject during the clinical trial, whether or not considered to be related to the investigational drug. AEs were graded according to National Cancer Institute Common Toxicity Terminology (version 5.0).

Statistical analysis

A two-stage phase 2 design was set up with response as outcome allowing for early termination should the response rate appear to be (unacceptably) low. Given the evidence in advanced EP-NEC at study initiation, a DCR of 50% or more would warrant further investigation and continuation of the study. Applying the 'Simon 2-stage minimax' design, with an α of 0.10

(the probability of rejecting the null hypothesis when it is in fact true) and a power of 90% (the probability of rejecting the alternative hypothesis), 28 patients had to be enrolled in the first stage, with an additional 11 patients to be recruited in the second stage if DCR proved acceptable (to a total of 39 evaluable patients). The combination would be deemed to be effective if the total number of responses exceeded 16.

The primary endpoint DCR was calculated using the Clopper-Pearson method. Patients who experienced early death (within 8 weeks of enrolment in the study) were considered as non-responders.

Descriptive statistics were used for baseline characteristics: median with ranges and numbers with frequencies for continuous and categorical characteristics, respectively. Kaplan-Meier method was used for time-to-event analysis. DOR was presented for all patients who presented with an objective (complete or partial) response, and was measured from the date of treatment initiation until date of documented progression. If a new treatment was started before progression, DOR was censored on the date of new treatment. Analyses were performed using R statistical software version 4.1.1.

Results

Patients

The predefined interim analysis showed a DCR of 78.6% (CI 59.0-91.7), so both steps of the 'Simon 2-stage minimax' design were completed. A total of 39 patients were included with a median age of 64 (28-74) years. Gender was equally distributed with 20 (51.3%) male patients. Most patients (n=25, 64.1%) had their primary tumour arising from the gastroenteropancreatic tract; 11 patients (28.2%) had colorectal, six patients (15.4%) pancreatic, four patients (10.3%) oesophageal, three patients (7.7%) gastric and one patient (2.6%) had an appendiceal NEC. Five patients had gynaecological primary tumours, of whom four (10.3%) had a NEC of the cervix and one patient (2.6%) had an ovarian NEC. Three patients (7.7%) had a Merkel cell carcinoma (MCC) and six patients (15.4%) had a NEC of unknown primary location. Most common metastatic sites were liver in 32 (82.1%) and lymph nodes in 27 (69.2%) patients. Nine (23.1%) patients had undergone previous surgery for their primary tumour, 2 (5.1%) of which had received postoperative radiotherapy. No prior neo-adjuvant or peri-operative chemotherapy treatment was given. All patients had a poorly differentiated morphology. For one patient, Ki67-index could not be reliably determined. For patients with known Ki67-index (n=38, 97.4%), median Ki67-index was 80% (40-100%). One (2.6%) patient had a Ki67-index below 55%, 24 (61.5%) patients had a Ki67-index of 55-80% and 13 (33.3%) had a Ki67-index >80%. All patients had a WHO performance score (PS) ≤1. Baseline characteristics are summarized in Table 1.

Efficacy

Best overall response was a CR in one (2.6%) patient, PR in 22 (56.4%) patients and SD in 9 (23.1%) patients, with a DCR of 82.1% (CI 66.4-92.5) and an ORR of 58.9% (CI 42.1-47.4). For all

patients Median PFS for all patients was 6.0 (4.3-7.8) months and median OS was 8.7 (7.8-9.6) months. PFS and OS are shown in Figure 2. with a response, median DOR was 6.4 (CI 5.8-7.0) months. Three (7.7%) patients had a DOR of >12 months. Of these, two patients had an unknown primary NEC, and one had a colorectal NEC. All three patients had liver metastasis at baseline and one patient also had a metastatic lesion in the pancreas, the sum of lesions were 41, 123 and 144 mm. The Ki67-index for these patients was 70-80%. Duration of response and survival after progression are shown in Figure 1. No differences in responses were found for subgroups, including gender, PS, previous therapies or primary tumour origin. DCR according to subgroups with corresponding confidence intervals can be found in Table 2.

Regarding the patients with MCC: during the conduct of the study approval for the immune checkpoint inhibitor avelumab was granted for the treatment of MCC. One patient received avelumab prior to study inclusion, two patients received avelumab following progression on the current study treatment. Of these, one patient was alive with disease at time of study analysis (45.9 months after study initiation).

Patients	**No./median**	**%/range**
Total	**39**	**100**
Gender		
Male	20	51.3
Female	19	49.7
Age	64	28-74
Primary tumour type		
GEP-NEC	25	64.1
Colorectum	11	28.1
Pancreas	6	15.4
Oesophagus	4	10.3
Stomach	3	7.7
Appendix	1	2.6
Other	8	20.5
Cervix	4	10.2
Merkel cell carcinoma	3	7.7
Ovary	1	2.6
Unknown Primary	6	15.4
Ki67-index[a]		
Median	80	40-100
20-55%	1	2.6
55-80%	24	61.5
>80%	13	33.3
Missing	1	2.6

Distant disease at diagnosis		
Yes	38	97.4
No	1	2.6
Distant disease at start therapy	39	100
Metastatic sites at start therapy		
Liver	32	82.1
Lymph nodes	27	69.2
Lung	7	17.9
Bone	4	10.2
Peritoneum	4	10.2
Adrenal gland	2	5.1
Pancreas	1	2.6
Gall bladder	1	2.6
Ovary	1	2.6
Omentum	1	2.6
Soft tissue	1	2.6
Sum of lesions (mm)	85	15-328
Previous therapies		
Surgery	9	23.1
Radiotherapy	3	7.7
Chemotherapy	0	0
Immunotherapy	1	2.6
WHO PS		
0	22	56.4
1	17	44.6

Table 1. Baseline characteristics of all included patients. WHO PS: World Health Organization Performance Score. [a]Ki67-index for 38 out of 39 patients with known Ki67-index.

Safety

Patients received a median of 4 (1-6) cycles of cisplatin. Thirteen (33.3%) patients switched to carboplatin, after a median of 2 cycles (1-5) and received an additional median of 3 (1-5) cycles (totalling up to a maximum of 6 platinum-based cycles). Dose reductions of cisplatin occurred in 28 (71.8%) patients. Median everolimus exposure in the entire cohort was 19 (3-57) weeks, and 7 (17.9%) patients had dose reductions for everolimus. Thirty-five (89.7%) patients experienced grade 1-4 adverse events of any kind, related or unrelated to the study medication. Of these, most common grade 1-2 events were nausea in 18 (46.1%) patients, pain in 15 (38.6%) patients, and haematological adverse events: anaemia in 17 (43.5%); thrombopenia in 16 (41.0%) and neutropenia in 11 (28.2%) patients. Regarding grade-3/4 adverse events: thirty-three (84.6%) patients experienced grade-3/4 adverse events of any kind. Most treatment-unrelated AEs consisted of pain (abdominal or other) and was present in 6 (15.4%) patients. A total of 22

(56.4%) patients experienced treatment-associated grade-3/4 adverse events. Haematological toxicity was most common with a total of 14 (35.9%) patients, including 5 (12.9%) patients with anaemia, 7 (17.9%) with neutropenia and 2 (5.1%) with thrombopenia. Renal toxicity occurred in 8 (20.5%) patients, treatment-associated gastrointestinal toxicity occurred in 5 (12.8%) patients and electrolyte imbalances in 4 (10.3%) patients. No grade 5 adverse events occurred. All grade-1/4 adverse events are summarized in Table 3.

	DCR in %	**95% Confidence interval**
Total	**82.1**	
Gender		
Male	75.0	50.9-91.3
Female	89.5	66.9-98.7
WHO PS		
0	86.4	65.1-97.1
1	76.5	50.1-93.2
Surgery		
No	80.0	61.4-92.3
Yes	88.9	51.8-99.7
Radiotherapy		
No	83.3	67.2-93.6
Yes	66.7	9.4-99.2
Primary tumour type		
GEP-NEC	84.0	63.9-95.5
Colorectum	72.7	34.8-93.3
Pancreas	83.3	35.9-99.6
Oesophagus	100	39.8-100
Stomach	100	29.2-100
Appendix	100	2.5-100
Other	83.3	35.9-99.6
Cervix	75.0	19.4-99.4
Merkel cell carcinoma	100	9.4-99.2
Ovary	100	2.5-100
Unknown Primary	66.7	9.4-99.2

Table 2. Disease control rate according to subgroups, with corresponding Clopper-Pearson 95% confidence intervals. DCR: disease control rate, the sum of complete responses, partial responses and stable disease, WHO PS: World Health Organization Performance Score.

Discussion

In this phase 2 clinical trial, everolimus in combination with cisplatin showed to be an effective first-line treatment in patients with advanced EP-NEC.

This study was the first to investigate the combination of targeted therapy with conventional, platinum-based cytotoxic therapy in patients with advanced EP-NEC. Interestingly, the combination of everolimus with cisplatin/carboplatin used in this study showed comparable response rates, duration of responses, survival and adverse events compared to studies investigating platinum based cytotoxic combination therapies.[16, 29] For instance, regarding ORR, Walter, et al. performed a prospective observational study in patients with advanced EP-NEC and found an ORR of 50% for patients treated with cisplatin and etoposide, comparable to the ORR observed in this study.[16] Other, retrospective studies found ORRs ranging from 28 to 52%.[5, 30-32]

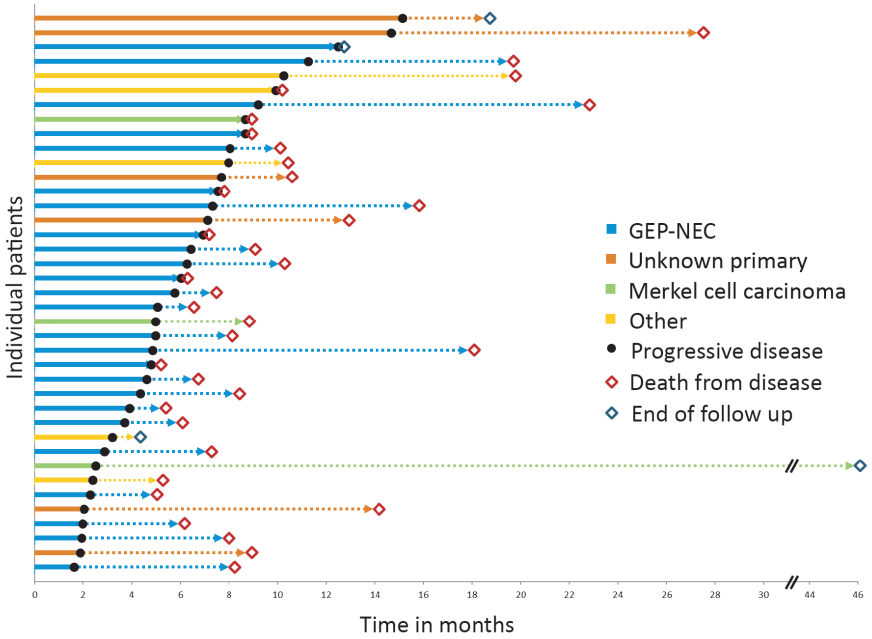

Figure 1. Swimmersplot showing all patients with time to progression and to death or end of follow up. One patient received immunotherapy after study termination and was alive at end of analysis, 45.9 months after study treatment initiation. Time-axis is interrupted due to the relatively long survival of this patient compared to other study participants. GEP-NEC: gastro-entero-pancreatic neuroendocrine carcinoma.

Duration of response and survival in our study was similar to that observed in the study of Zhang, et al. in which patients with advanced GEP-NEC were randomly assigned to receive cisplatin with etoposide (EP) or cisplatin with irinotecan (IP). The study was terminated early due to slow accrual (66 patients of planned 144 included). At premature analysis PFS of 6.4 months and 5.8 months for EP and IP, respectively, was noted.[33] The OS in our cohort was slightly shorter than found in the aforementioned study by Walter, et al. (8.7 vs 11.6 months). This might be explained by the fact that a third of the patients included in our study had a Ki67-index >80%. Previous reports identified a Ki67-index cut-off of 55% to be associated with worse survival.[13]

Although Walter, et al. found no difference in survival between patients with a Ki67-index <55% and >55%, their cohort only included 18% of patients with a Ki67-index >80.[16] Therefore the somewhat less favourable outcome in our study might still be due to the higher proportion of more aggressive cancers.

Adverse events mainly consisted of haematological and renal toxicities, and the percentage of patients that experienced grade-3/4 AEs was comparable with that from studies of cisplatin combined with etoposide.

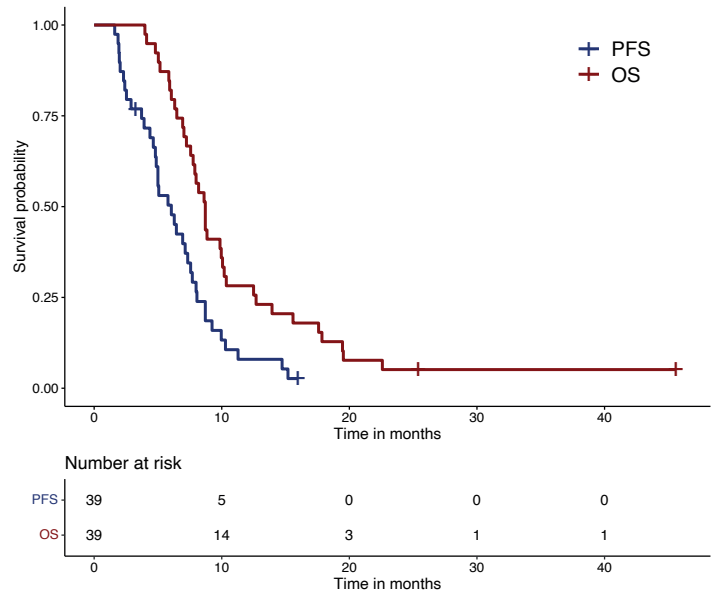

Figure 2. Progression free survival (PFS) and overall survival (OS) for all patients.

The most striking difference is the absence of alopecia in our study population, since this is a side effect caused by etoposide, and does not occur in cis-/carboplatin or everolimus. Alopecia is known to have a significant impact on the quality of life and wellbeing of cancer patients.[34, 35] Since efficacy of the cisplatin and everolimus combination seems similar to current recommended treatment of cisplatin and etoposide, but an important quality of life related side-effect is avoided, the choice of treatment by both clinicians and patients is likely to favour everolimus with cisplatin.

The added survival benefit of everolimus to cisplatin in EP-NEC remains unclear. Although everolimus was able to improve cisplatin-resistance in vitro,[36] our study results show that everolimus was unable to uphold such a resistance for a longer duration than current therapies. Nevertheless, our cohort included three patients that had a DOR of >12 months, suggesting that highly selected patients might have particular benefit from this combination. Similar results

were found a study that investigated everolimus in combination with cisplatin and paclitaxel in LCNEC, with a median PFS of 4.4 months and OS of 9.9 months. There, 34% of 49 patients were alive at 1 year after treatment initiation, again suggesting the possible benefit for highly selected patients with NEC.[37] Unfortunately, since molecular and mutational tumour analyses were not performed in their study as well as in ours, mechanisms underlying these notable effects remain to be elucidated.

Adverse event	Grade 3-4 No. (%)	Grade 1-2 No. (%)
Haematological		
Anaemia	5 (12.8)	17 (43.5)
Thrombopenia	2 (5.1)	16 (41.0)
Neutropenia	7 (17.9)	11 (28.2)
Renal toxicity		
Acute kidney injury	6 (15.4)	9 (23.1)
Chronic kidney disease	2 (5.1)	8 (20.5)
Gastrointestinal toxicity		
Nausea	3 (7.7)	18 (46.1)
Constipation	0	5 (12.8)
Diarrhoea	2 (5.1)	4 (10.3)
Pancreatitis	2 (5.1)	0
Paralytic ileus	1 (2.6)	0
Ascites	1 (2.6)	0
Bile duct stenosis	1 (2.6)	1 (2.6)
Cholangitis	1 (2.6)	1 (2.6)
Cholecystitis	0	1 (2.6)
Haemorrhoids	0	1 (2.6)
Anus cracks	0	1 (2.6)
Pyrosis	0	1 (2.6)
Electrolyte imbalances		
Hyponatremia	2 (5.1)	1 (2.6)
Hypernatremia	1 (2.6)	0
Hypokalaemia	1 (2.6)	1 (2.6)
Hypomagnesemia	0	3 (7.7)
Hypocalcaemia	0	1 (2.6)
Hepatotoxicity		
Increased liver enzymes	1 (2.6)	3 (7.7)
Other		
Pain	6 (15.4)	15 (38.6)
Fatigue	0	10 (25.6)
Mucositis	2 (5.1)	8 (20.5)
Rash	2 (5.1)	8 (20.5)
Dyspnoea	1 (2.6)	6 (15.4)
Malaise	1 (2.6)	3 (7.7)
Hypertension	1 (2.6)	3 (7.7)
Hyperglycaemia	1 (2.6)	2 (5.1)
Dizziness	1 (2.6)	2 (5.1)
Anxiety	1 (2.6)	1 (2.6)
Urinary tract infection	1 (2.6)	1 (2.6)
Dehydration	1 (2.6)	0
Peripheral neuropathy	0	5 (12.8)
Cough	0	4 (10.3)
Taste alteration	0	2 (5.1)
Oedema	0	2 (5.1)
Weight loss	0	2 (5.1)
Infection	0	1 (2.6)
INR increased	0	1 (2.6)
Nose bleed	0	1 (2.6)
Anorexia	0	1 (2.6)
Palmar-plantar erythrodysesthesia	0	1 (2.6)
Fever	0	1 (2.6)
Conjunctivitis	0	1 (2.6)
Insomnia	0	1 (2.6)
Dysgeusia	0	1 (2.6)
Impaired hearing	0	1 (2.6)
Tinnitus	0	1 (2.6)
Vaginal dryness	0	1 (2.6)
Flushes	0	1 (2.6)
Dysesthesia	0	1 (2.6)
Pneumonitis	0	1 (2.6)

Table 3. All grade-1/4 adverse events of any kind, related or unrelated to the study medication.

Besides acting as a synergistic component to platinum, everolimus has also been investigated as a single-agent after progression on platinum-containing chemotherapy in patients with pancreatic NEC (pNEC) or in SCLC.[38, 39] This resulted in a disappointing PFS of 1.2 months and an OS of 7.5 months for pNEC,[38] and similarly, a PFS of 1.3 months and OS of 6.9 months for SCLC.[39]

Currently, there are a few randomized trials that involve different treatment combinations registered for first-line treatment of patients with EP-NEC, (such as NCT04325425 and

NCT02595424). These will hopefully further contribute to unveiling the tumour resistance mechanisms and improvement of survival if patients with EP-NEC.

A possible limitation of the current study is that patients with MCC were also included in the EP-NEC cohort. This might have increased the heterogeneity of the study cohort, since MCC has a slightly different pathogenesis, including the oncogenic Merkel cell polyomavirus and ultraviolet exposure.[40, 41] Nonetheless, at the time of study initiation in 2016, no other systemic treatments for advanced MCC were approved, and MCC had a similar treatment regimen and prognosis as all other EP-NEC. And although one patient had received avelumab prior to study initiation, Figure 1 shows that patients with MCC were randomly distributed across the cohort and responses to therapy, hence were very unlikely to influence the results.

A major strength of this study is the prospective, multicentre study design, which is challenging when studying such rare diseases. This study is one of very few that managed to complete predefined accrual and hence is adequately powered. By including patients from three large referral centres in the Netherlands, the current study provides insight into the nationwide approach in the treatment of EP-NEC, as well as epidemiological aspects such as the occurrence and survival of patients with EP-NEC.

In conclusion, the combination of everolimus with cisplatin is considered to be an effective treatment option for patients with advanced extrapulmonary neuroendocrine carcinoma. While, in general, treatment-related adverse events are in line with those observed in more classic cytotoxic regimens, absence of alopecia could favour this regimen. The observation of some patients obtaining DOR exceeding twelve months, urges for additional in-depth analysis of so-far unknown predictive biomarkers in this highly aggressive disease. If this research leads to real patient selection, this could be considered to be nothing less than a giant leap forwards.

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12

General Discussion

Introduction

The landscape of neuroendocrine neoplasms has changed greatly over the past ~120 years. First, the nomenclature, grading and staging have evolved to well-equipped systems, but continue to grow and mature further on. This thesis has provided evidence, knowledge and tools for further tailoring of various of such systems. But most importantly, this thesis has shown the utmost value of collaboration of various disciplines and institutes to reach higher levels of power of studies, larger cohorts, stronger evidence and more in-depth knowledge of this difficult to study entity. Therefore the landscape of neuroendocrine neoplasms has not only changed for the disease itself, but also the research and researchers have grown closer to each other, and will evolve and expand further for increasingly interesting joint efforts and collaborations.

Tailoring prognosis in neuroendocrine neoplasia

In **Chapter 2**, we attempted to validate a previously established nomogram with the largest cohort of SI-NET to date and found that the nomogram was able to differentiate between patients with low-, intermediate- or high risk of dying from their NET, but unfortunately underestimated disease specific survival in our cohort. This meant the survival predicted by the nomogram was significantly lower than the observed survival in our cohort. We found this this difference in survival to be present across all subgroups, namely patients that had underwent curative surgery, patients with palliative surgery, and patients that did not undergo surgery at all. The difference that was found between the observed and predicted survival may partly be explained by the time periods over which patients were included in both cohorts. The nomogram was based on a cohort of patients that were diagnosed between 1977 and 2007, whereas our cohort constituted of patients diagnosed between 2000 and 2018. Issues regarding different outcomes in different time periods are unavoidable in all diseases, but are an even bigger challenge when the disease is rare, as is the case with SI-NET. The rarity of disease causes the inclusion time to be much longer to reach sufficient numbers for adequately powered studies, thereby preventing researchers from capturing an image of the current situation. Nevertheless, our study showed that the nomogram in its current form could not be directly implemented in clinical practice, but that there remains value in the use of clinical parameters in predicting the disease course of patients with SI-NET disease. This result is reassuring to all clinicians treating patients with NET, since it underscores the importance of clinical judgment in patient management. Also, to challenge the difficulty of arranging large cohorts within the same periods, the answers seems to lie in (inter)national collaborations between institutes and researchers. With joint (inter)national forces, larger numbers of patients can be included over shorter periods of time, thereby not only providing a more accurate reflection of the current situation, but also speeding research in a so dramatically understudied field such as that of neuroendocrine tumours.

The results of **Chapter 2** showed that there is room for improvement of prognostication of patients with SI-NET. In recent years, research in NET as well as in other oncological disciplines

has been aimed at developing alternative tools that delineate the biological characteristics of malignancies. A number of blood-based biomarkers that can be used in clinical practice to predict disease presence or prognosis have been investigated. In a recent study of 152 patients with GEP-NETs, circulating tumour transcripts (NETest®), using a cut-off of 33%, have been shown to be the strongest predictor for disease progression.[1] This prompted the question whether addition of such a strong predictor for progression could be auxiliary in the prognostication of patients with SI-NET. In **Chapter 3**, we have investigated whether addition of the NETest® would benefit the prediction of disease specific survival or progression free survival to the clinical parameters already present in the nomogram. The most important predictors for disease specific survival from the nomogram were selected, and the activity score of the NETest® was added to a multivariable cox model. We found that indeed the NETest® remained the strongest predictor of progression free survival, whereas it had no added value in the prediction of disease specific survival. These findings could initiate a discussion regarding which endpoints are most valuable for patients with SI-NET, and whether progression should be used as a surrogate endpoint for survival? The use of progression for overall survival is increasingly being implemented in oncologic research, but the studies that do so are heterogeneous and clear substantiation is often lacking.[2] Similar situations have occurred in trials including patients with NET, where progression free survival was used as a surrogate endpoint for overall survival, but updates of these trials did not show overall survival benefit.[3] In NET patients however, the value of progression free survival may not only be important because of the association with overall survival, but may also be dominated by the hormonal syndromes that are unique for NET, as these may cause significant decreases in quality of life and progression thereof is preferably deterred.[4] From this it follows that being able to predict which patients are more likely to show more rapid progression than others may still be valuable, as this may also indicate an increase in functional symptoms caused by hormonal secretion. This knowledge may help to tailor treatment decisions such as changing dosage of SSA or starting a work-up for PRRT.

In **Chapter 4**, we made an attempt to unravel the genome of SI-NET. We investigated the presence of driver mutations in metastatic SI-NET and explored the clinicopathological significance thereof. We found that although SI-NET are mutationally quiet tumours, SI-NET harbour a number of driver mutations in known cancer genes. Despite these mutations however, the disease course of patients with driver mutations was highly similar compared to those that did not have driver mutations. This suggests that even though these mutations are present, they are insufficient to alter the clinical behaviour of SI-NET. Nevertheless, the sole presence of these mutations, for some of which targeted treatments exist, opens the way for new therapy options when progressive disease inevitably occurs.

The landscape of clinicians treating patients with NET could possibly be as heterogeneous as the tumours themselves. Although European and North American guidelines exist for different tumour types, localizations and stages of disease, some question have simply remained unanswered thus far, and room for clinical judgement remains. Certainly, when dealing with a rare entity, the opinions and vantage points of experienced clinicians can be extremely valuable

when large trials or validation studies have not been, or will never be undertaken. One such matter is the resection of the asymptomatic primary tumour in SI-NET, when inoperable distant metastatic (stage IV) disease is present. On the one hand, patients would no longer be at risk of developing symptoms of the primary tumour or MF, on the other hand, patients might never develop these symptoms, and undergo unnecessary invasive surgery and possible complications thereof. This room for interpretation has resulted in different institutes adopting different approaches in this matter. The Netherlands Cancer Institute (NKI) in Amsterdam has chosen to resect the primary tumour whenever technically possible, whereas the Aintree University Hospital (AUH) in Liverpool has chosen to only resect the primary tumour when symptoms such as obstruction or ischemia become apparent. Intuitively, one might argue that removal of the primary tumour in stage IV SI-NET would have no influence on survival of patients, since metastatic disease is already present. Yet contrastingly, several retrospective studies have identified primary tumour resection to be associated with improved survival. Unavoidably, confounding by indication (i.e. more fit patients or with less burden of disease are more likely to receive surgical intervention) has played a role in these studies. Nevertheless, the results remain intriguing and beg for more in-depth studies regarding this issue. In **Chapter 5** we were able to perform an unique study where we compared the opposite treatment approaches of the two institutes through various contemporary statistical methods, with the aim to maximally control for confounding. We found that, with every method applied, resection of the primary tumour was associated with reduced disease specific mortality. Our results suggest that there might be a signalling interaction between the primary tumour and metastatic disease, such as slowing progression of disease or the occurrence of CHD when the primary tumour is removed, although such mechanisms have not currently been identified. Nevertheless, the implications of this study may be of great value for future patients. First, this was the first study to make use of such institutional differences in treatment approaches, and thereby attempting to control for confounding by indication. Although the retrospective nature of this study prevented us from executing a truly randomized comparison between treatments, this study approaches a randomized setting as best as retrospectively possible. Since the results are so robust, this study might cause treating physicians and surgeons to be more inclined to resect the primary tumour over a watch-and-wait approach. Second, the most valuable contribution of this study would be to act as a kickstarter for a (inter)national prospective study in which patients with SI-NET will be randomised to undergo resection of the primary tumour or a watch-and-wait approach. This study should not only investigate the influence of primary tumour resection on survival, but also on progression, quality of life, symptoms of the primary tumour or MF, and complications of surgery. Besides this, blood and tissue based analysis should be performed to identify possible molecular signalling mechanisms or prognostic factors.

In **Chapter 6** and **Chapter 7**, we concentrated our focus on patients with carcinoid heart disease (CHD). CHD is a serious fibrotic cardiac complication of metastatic SI-NET, that is caused by serotonin secreted by the tumour and predominantly consists of regurgitation of the tricuspid and pulmonic valve.[5] If CHD is present and the patient is sufficiently fit to undergo highly

invasive cardiac surgery, replacement of the damaged valve(s) is the treatment of choice.[6] Yet even with more advanced surgical possibilities, the survival of patients with CHD is significantly worse than patients who do not develop CHD. Unfortunately it is quite difficult to predict which patients are at higher risk of developing CHD than others. Currently, as per European guidelines,[7] patients with elevated serotonin undergo frequent (1-2 yearly) echocardiography for the detection of CHD although CHD occurrence is highly variable between patients. Early CHD can be missed or progress to a fulminant form in between screenings, whereas other patients never develop CHD and undergo unnecessary visits to the outpatient clinic. In **Chapter 6**, we hence set out to validate various known biomarkers, as well as new promising biomarkers in the prediction and detection of CHD. We found that the new biomarkers activin A, soluble suppression of tumorigenicity 2 (sST2) and connective tissue growth factor (CTGF) were not helpful in diagnosing CHD. More importantly, we found that N-terminal pro-B-type natriuretic peptide (NT-pro-BNP) was significantly associated with the prediction and detection of CHD. With our findings, we were able to identify patients that are unlikely to develop CHD, patients that have an increased chance of developing CHD, and patients that are likely to have CHD at time of measurement. These results translate directly into clinical practice, since patients at low risk of CHD according to their NT-pro-BNP levels, may be – as we suggested – released from echocardiographic screening, with active monitoring of NT-pro-BNP. Similarly, patients with moderate-to-high levels of NT-pro-BNP could possibly benefit from more frequent screening than is currently advised. From this, it follows that patients may be diagnosed with CHD at an earlier stage, leading to more timely intervention, i.e. surgical valve replacement, hopefully improving the survival of these patients.

Regarding survival of patients with CHD, in **Chapter 7** we investigated how this has developed over a nearly four-decade time period, in the largest European cohort of patients with CHD to date. We found that the survival of patients with CHD has improved significantly over the past years, but that it remains worse compared to patients without CHD. The improvement in survival may have a multifactorial cause. First, surgical interventions for CHD have improved and increased in frequency, leading to markedly more patients that undergo surgical valve replacement procedure for CHD, leading to an improved survival. However, some caution is necessary when interpreting these results, as also here confounding by indication may play an important role in the selection of patients undergoing surgery. Nevertheless, this further underscores the need for timely intervention to prevent patients' condition to progress until surgery is no longer an option. Second, as has also been argued in Chapter 2,[8] the treatment options for patients with NET have improved, and survival of patients with SI-NET has subsequently improved. A major finding of this study was that nearly all patients developed CHD within a timeframe of a maximum of 10 years after the first occurrence of liver metastases. From this, it can be derived that patients may be released from screening for CHD after a decade of living with liver metastases, as the chance to develop CHD slims to nil. In addition to the results of **Chapter 6**, this further thins the subgroup of patients at risk of CHD and reduces patient and hospital burden by lowering unnecessary screening. Although we are still long from

completely obliterating this serious complication of SI-NET, our studies have aided in further tailoring the screening of those patients that need to be watched more carefully.

The objective of this thesis has mainly involved NEN that arise sporadically. Yet there are a few conditions of which NEN may be a manifestation of an underlying disease. Two of these are the Multiple Endocrine Neoplasia type 1 (MEN1) syndrome, and Diffuse Idiopathic NeuroEndocrine Cell Hyperplasia (DIPNECH).[9, 10] MEN1 is a hereditary disease predisposing patients to the development of various endocrine tumours. Around 10% of patients with MEN1 may develop neuroendocrine tumours of the bronchopulmonary tract (bpNET). DIPNECH is a rare pulmonary condition characterised by proliferation of neuroendocrine cells restricted to the bronchial and bronchiolar epithelium, in which also bpNET may arise. As with other NET, bpNET may also develop sporadically, i.e. without any underlying condition predisposing to the occurrence of these tumours. Results from earlier publications showed that MEN1-related bpNET have a relatively indolent disease course and rarely lead to bpNET-associated death.[11] Contrastingly, data on sporadic bpNET showed that patients survival is mainly dominated by their NET, rather than by other comorbidities. These results, as well as the clinical observation of vastly different outcomes led to the execution of **Chapter 8**, where we compared disease specific mortality between patients with sporadic bpNET to patients with bpNET in the context of MEN1 or DIPNECH. We found that no patients in the MEN1 or DIPNECH cohort died of their bpNET, whereas tumour progression was the main cause of death in the sporadic bpNET, leading to a median survival of 12.3 years after first diagnosis. Interestingly, the difference in survival between these cohort could not be explained by known prognostic factors such as mitotic count, Ki67-index or the presence of necrosis, as this was similar across all subgroups. Paradoxically, the probable selection bias that occurred in this study would favour the sporadic bpNET group, as these patients all had more aggressive anatomical resections, with curative intent, whereas patients with bpNET with MEN1 only underwent surgery when rapid tumour growth or large tumour size would prompt surgical intervention, underscoring the true different nature of these tumour entities. Since the macro- and microscopic features of these tumours are highly similar, the underlying cause of the different malignant behaviour could possibly be explained by molecular differences. Although research has shown that, for instance, MEN1 related mutations or inactivations may be associated with survival, results are contrasting in whether the association provides reduced or improved survival.[12, 13] This study was the first head-to-head comparison of these entities and provides clues for management of bpNET in various contexts. It is clear that in sporadic bpNET the survival is dominated by the NET diagnosis and follow up should be constituted of close monitoring for recurrence of disease after elaborate, radical surgical intervention. In contrast, bpNET in the context of MEN1 or DIPNECH may be released from all too stringent controls, thereby reducing radiation exposure, patient distress and hospital burden and costs.

Tailoring treatment in neuroendocrine neoplasia

In this thesis, we evaluated a number of treatment strategies for extra-pulmonary neuroendocrine carcinoma (EP-NEC), to which the Merkel cell carcinoma (MCC) also belongs.

First, in **Chapter 9**, we investigated the influence of postoperative radiotherapy (PORT) in stage I-III MCC. We found that PORT has a beneficial influence on recurrence rates in stage III MCC, but fails to provide survival benefit of disease specific mortality in all stages. Contrastingly, PORT was associated with reduced overall mortality. These contradictory results of overall and disease specific mortality find their explanation in the characteristics of the patients with MCC. Since MCC mainly occurs at a median age midway the eighth decade, the population of patients with MCC is generally elderly and frail, and often have multiple comorbidities.[14] This situation influences the results of PORT on overall mortality in two ways. The first is that patients who are indeed frail and likely to die of other causes than MCC, are more likely to opt out of adjuvant treatment for MCC, such as PORT. This is a decision that is often made by patients and clinicians together in a shared decision making process, yet thereby deviating from guidelines for adjuvant treatment of this disease, as occurs in over half of the patients.[15] The second issue is that these patients indeed often die of other causes than MCC, as predicted or assumed by the treating physician and/or patient. From this, it follow that patients that do not receive PORT, are more likely to have a shorter estimated lifespan than patients that do, leading to serious selection bias when PORT is studied in relation to overall mortality. **Chapter 9** is the first study to address the bias that is likely present in both the management and research of MCC and confront the reader with the importance thereof. This study is therefore a stepping stone that can be used to improve current guidelines, for instance, by taking factors such as life expectancy into account.

Second, in **Chapter 10**, we presented the largest cohort of patients with advanced MCC that were treated with immunotherapy outside of a clinical trial or pharmaceutical expanded access programme to date, thereby displaying the very important, but often abandoned phase 4 of clinical research for pharmaceutical agents. We found that response rates, duration of response and progression free survival were high and essentially in line with results found in clinical trials.[16, 17] Our findings are important, since they give a representation of the true benefit of a newly introduced systemic therapy in actual clinical practice. Often in clinical trials, results may be over- or underestimated due to the selection of patients. For instance, patients need to be fit enough to be included in the trial, hence a selection bias of patients likely to have a more favourable outcome occurs. In routine clinical practice, clinicians are likely to offer a new treatment regimen to patients that would normally not be allowed to enter a clinical trial, thereby broadening the patient population, often leading to less impressive results initially found in a trial setting. In our study, we found that the response rates and durations of response were even somewhat higher than found in the trials. This could partly be explained by the fact that more patients received immunotherapy as first line treatment, rather than second line as was executed in the initial trials, at the time. These results further confirmed and underscored results seen in other malignancies treated with immunotherapy, that first line treatment offered survival benefit over second line treatment.[18]

Furthermore, **Chapter 9** and **Chapter 10** show how valuable the national collaboration of referral centres for MCC in the Netherlands has proven to be. When dealing with rare diseases,

joint efforts are of utmost importance to fuel scientific research and clinical practice to progress more rapidly and with greater power. Such endeavours are the first steps into a future where larger, collaborative cohorts will finally provide adequate power and speed to study rare entities, so these will no longer have to lag behind in the scientific and clinical progress that is accomplished in other regions of healthcare.

Similarly, in **Chapter 11**, the collaboration of three referral centres for NEC ensured the execution of a phase 2 study of everolimus in combination with cisplatin in patients with advanced extrapulmonary NEC (EP-NEC). This study was one of the very few, if not only prospective study that was able to finish predefined accrual for this extremely uncommon malignancy. We found that the combination of everolimus with cisplatin yielded similar response rates and durations of response compared to the conventional combination of a platinum agent with etoposide or irinotecan,[19] albeit without an important adverse effect, namely alopecia. Since median survival for these patients is around one year, quality of life is highly important, and the absence of alopecia might very well favour the choice for everolimus with cisplatin over current treatments. Moreover, we found that three patients in our cohort had a duration of response that lasted over year. This raises the question what distinguishes these patients from the others? Everolimus is an inhibitor of the mammalian target of rapamycin (mTOR). Mutations in the mTOR pathway have been identified in NEN and hence provided the rationale for this study. Although unfortunately we had not performed sequencing on all included patients, it is possible that patients with a longer duration of response had mTOR mutations for which everolimus could have provided a beneficial effect. Future studies should therefore be aimed at unravelling the genome of EP-NEC to reveal possible targets for treatment.

Future directions

This thesis has attempted to unravel the landscape of neuroendocrine neoplasms and improve the understanding thereof. We have provided an oversight in the disease course of various NET and have studied treatment effect in NEC. Yet future efforts and studies are needed to further improve the management of NEN. But what should these future efforts look like, and how will the field progress? Here, we present some thoughts and considerations for future decades to come.

Collaborations

As mentioned a number of times in this thesis, the field of NEN has, and will greatly benefit from international or national collaborations between expert centres. In recent times, globalization has grown exponentially and the means and practice of digital communications have been exceeding everybody's expectations. Although the latter have been firmly driven by extraneous influences such as a worldwide pandemic, this should be regarded as a gift rather than a penalty. As such, collaborations are being initiated and they spark future research investigations and knowledge exchanges. There are a number of examples already worth mentioning within the field of NEN. For instance, on a Dutch national level, a collaboration between all referral centres for MCC has been initiated and from 2019 on, a database has been instated in which all

patients referred to any of the expert centres are being included prospectively. This database contains all patient, tumour, radiological and treatment characteristics part of regular clinical practice. With this resource, high quality, evidence based cohort studies can be performed with sufficient numbers and therefore scientific power. Additionally, in the Netherlands Cancer Institute an institutional biobank has been instated for blood and tumour material of patients with MCC, which can be directly linked with the nationwide database. Chapters 9 and Chapter 10 are already an example of the scientific output this collaboration will potentiate. This thesis also already provides an example of fruitful collaborations that cross borders. In Chapter 5, the joint output of a Dutch and a British referral centre for NET shows powerful results that could not have been put together in a one-nation project at this time. These, and other studies may serve as an inspiration for future researchers to work together and provide robust outcomes. Further, the ENETS has also embarked on shifting the focus to international collaborations by setting up specific taskforces that include experts from various ENETS Centres of Excellences across Europe and therefore combining very valuable expertise into uniform reporting, guidelines and formulating currently unmet needs in the epidemiology and management of NEN. An example of such an assemblage of experts is the CHD Taskforce that has recently been instated by the ENETS. One of the first products hereof was the publication of a CHD-score, to be used for uniform reporting of CHD in the occurrence, follow up and response to treatment of CHD.[20] Even more intriguing, recently a large European study that was initiated by the ENETS included data from a staggering number of 40 hospitals in 15 European countries, and could thereby show that additional hemicolectomy after prior appendectomy could be abandoned in T1-2 appendiceal neuroendocrine tumours.[21] These, and many more taskforces, partnerships, consortiums or other groupings will likely shape the future scientific and clinical future of NEN and will prove highly valuable in improving diagnosis, treatment, survival, quality of life and health costs for patients with NEN. Yet hopeful as this may sound, setting up large collaborations as such entails more than just working together, one has to strive to be united and mutually driven to collaborate to create new and valuable research output. Ideally large databases are built that are able to include multiple important variables, in which all relevant institutes may include their data, thereby forming large high-quality databanks that can then be used for different study outputs. Building such a database is not easy and requires full cooperation from every participating institute, uniform data input and management, not to mention the informed consent of all included patients and the time and resources to include data in the database. In the past years, newer forms of informed consent have emerged, including for instance institutional informed consents, where patients provide or decline consent for the use of their data or material directly at hospital registration. Another addition are statistical methods, some of which used in this thesis, such as propensity score matching, that provide possibilities to abandon the classical but often impossible to execute randomized controlled trial, and still provide robust results and stepping stones to further unravel the neuroendocrine field.

Personalized cancer care in neuroendocrine neoplasia

The world of cancer has changed dramatically over the past decades. Diagnostics and treatment have shifted from histopathological determination and one-size-fits-all cytotoxic therapies to highly specific molecular characteristics of malignancies and the possibility of targeting certain, patient- or tumour specific mutations or alterations by precision medicine, respectively.[22] Although initially certain mutations were determined specifically for certain tumour types or localizations, nowadays whole genome sequencing is starting to make an entrance in routine clinical practice for all patients.[23] With this, it will be possible to identify targetable mutations in the initial phases of disease, thereby providing a map of possibilities in the treatment and follow up of patients. We have addressed these possibilities in Chapter 4 of this thesis, but many more developments are likely to change the future course of patients with NEN. For instance, an initiative to study the genomics of NEN more closely, investigated the genomic spectrum of bronchopulmonary NEN.[24] It is widely accepted that bronchopulmonary NET (bpNET) are a different entity from bronchopulmonary NEC (bpNEC), but it has been suggested that a more aggressive bpNET exists, that has possibly gained mutations associated with bpNEC. Within this initiative, multi-omics factor analysis of a large cohort of 116 pulmonary bpNET, 75 large-cell NEC (LCNEC) and 66 small cell lung cancers (SCLC) indeed identified a group of atypical carcinoids that showed bpNET morphology but the molecular and clinical features that resembled the much more aggressive LCNEC. These tumours were characterised as 'supra-carcinoids' and are identified as an intermediate tumour entity between bpNET and LCNEC.[25] Moreover, it was found that supra-carcinoids had higher levels of neutrophil infiltration, which is known to be associated with favourable responses to immunotherapy,[26] opening a treatment pathway that was previously considered to be closed.

In the diagnostics of NEN, blood-based analyses are starting to make their entrance in tailoring the detection of various tumours. Besides the NETest® mentioned in this thesis, more tumour-specific biomarkers such as circulating tumour cells, or circulating genetic material of tumours, such as cell-free RNA (cf-RNA) or cell-free DNA (cf-DNA) have appeared in the diagnostics and follow up of NEN.[27, 28] For instance, bespoke cfDNA has been identified through a personalized and tumour-informed assay and was able to predict treatment response in a patient with MCC.[29] Although such, truly personalised diagnostics and follow up markers are only just making an appearance, they show very promising insight in the future of NEN. To investigate such promising markers of disease further, large validation studies will have to be set up to assess the true, practical value for of new research outputs. Here, collaborative initiatives mentioned in the previous section will again play a major role in propagating scientific harvests.

Conclusions

The disease course of NET is driven by various prognostic components of patients' personal characteristics as well as by tumour- or disease associated factors, but may change direction through timely diagnosis and various treatment interventions. The combination of intuitive patient and tumour characteristics as well as new, emerging biomarkers or genomic factors

have formed the knowledge on which we now base our clinical decisions. This thesis has contributed to further understanding the natural development and prognosis of NET and associated syndromes, as well as defined determinants for the prediction and diagnosis thereof.

Similarly, the treatment in NEC and the success thereof is based not only on the treatment characteristics, but also on factors that, at first sight, seem to lie outside the scope of the treatment. These determinants may be the comorbidities of a patient, but also the specific molecular tumour alterations or immunogenic profile that guide the response to treatment.

Future research outputs will surely rely on national and international collaborations to ensure sufficient patient numbers, and will provide in-depth epidemiological occurrences and influences over time, as well as changes in the management of NEN. Molecular methods will subsequently aid in understanding the true physics of the development and progression of these malignancies and likely alter the manner in which we treat patients to a more personalised approach.

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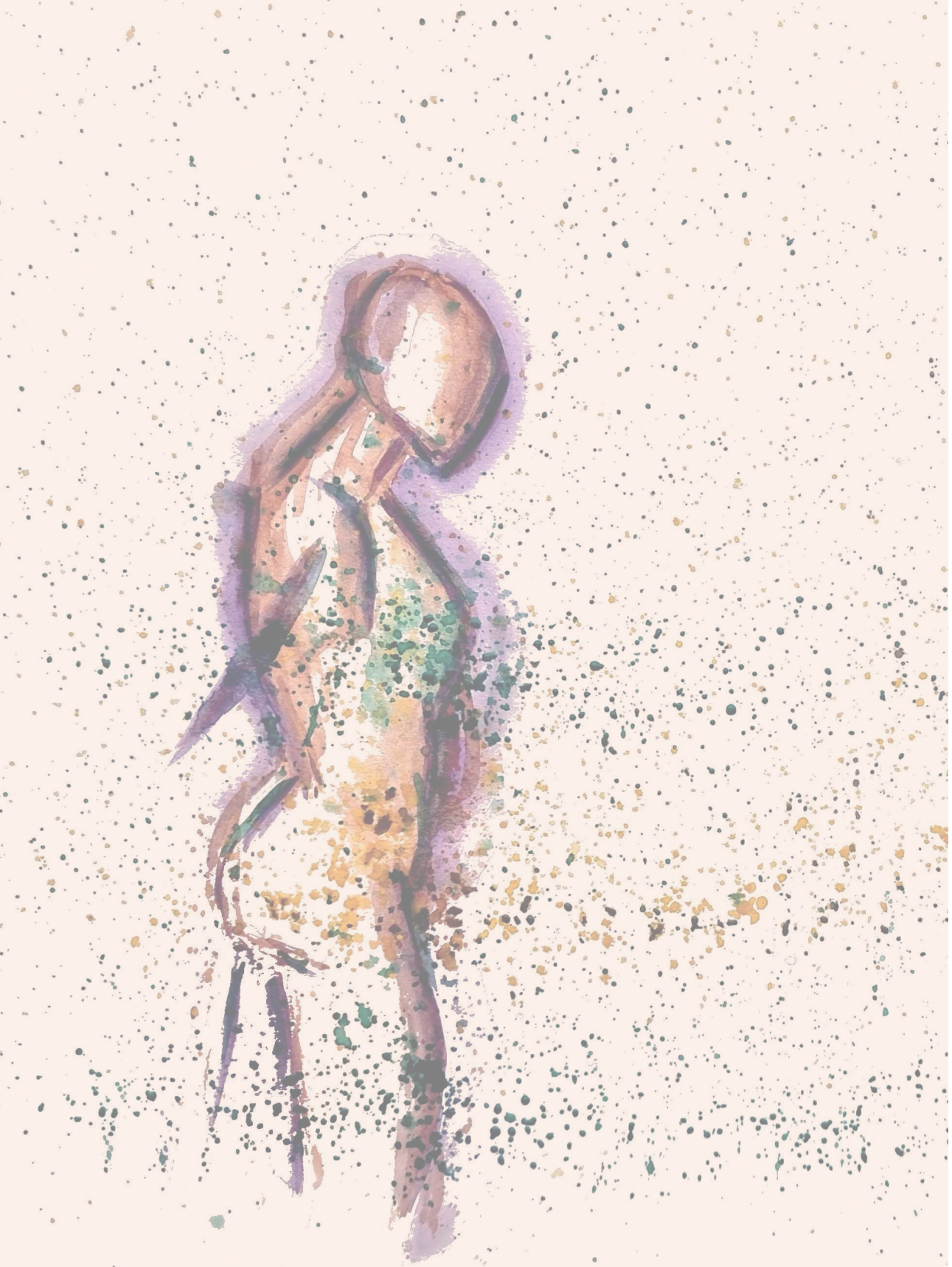

13

Summary

Samenvatting
(summary in Dutch)

Neuroendocrine neoplasia

Neuroendocrine neoplasia (NEN) are complex and heterogeneous malignancies that may arise in various parts of the body. Their commonality is that they all arise from neuroendocrine cells. In 2000 the World Health Organization (WHO) has established a new grading system for NEN, in which for the first time the distinction was made between well-differentiated neuroendocrine tumours (NET), and poorly differentiated neuroendocrine carcinoma (NEC). Currently, this classification still holds, with the following divisions. NET are, as the well-differentiated nature already implies, highly similar to their cells of origin, meaning they show a high resemblance to their originators under the microscope. NET are further classified in grade 1-3 by their mitotic count and Ki67-index, both measures of proliferation. In contrast, poorly differentiated NEC may hardly be distinguished from their cells of origin, and are classified by having high proliferation indices. NET and NEC, although they arise from the same origin, are considered different entities, and have a highly different therapeutic management and prognosis. NET have an overall favourable prognosis, and patients may live many years even when metastatic disease is present. Contrastingly, NEC have a very poor prognosis, with a median survival of approximately one year after the first occurrence of metastasis. Similarly, the treatment of both entities is highly different: NET are treated with treatment regimens that focus on binding to proteins expressed by the tumours, such as somatostatin analogues (SSA) or peptide-receptor-radionuclide therapy (PRRT). NEC on the other hand are treated with conventional cytotoxic regimens, that have been used in many other cancers for decades.

Current thesis

In the past years, clinicians have improved the classification and treatment of patients with NEN, but a number of unmet needs remain. This thesis has sought out to fulfil a number of these needs. First, the heterogeneous nature of NET has made it challenging to provide an accurate prognosis for progression and survival of this disease. This is knowledge that is highly important for both the patients, as well as the doctors treating patients with this disease. The current thesis provides various answers for a more tailored prognostication, especially which variables are important for an adequate prediction of disease course (prognostication), or which patients may be released from stringent controls, or on the other hand should be monitored more closely.

Second, the treatment of NEC has made some progress in the past decades, but room for improvement remained. We have shown that real world data and the outcomes thereof are extremely valuable in achieving high quality evidence for the treatment of local disease, as well as metastatic disease in Merkel cell carcinoma (MCC), an extra-pulmonary NEC that arises from the skin. Further, this thesis describes the first large multicentre study that investigated a new treatment regimen for all metastatic EP-NEC.

Tailoring prognosis

The most common primary site for NET is the gastroenteropancreatic site, of which NET of the small intestine (SI-NET) are the most common, with an incidence of around 1.2/100 000 persons. Although aforementioned classification of NET into three different grades aids in predicting the prognosis of patients with SI-NET, the actual disease course remains difficult to predict. In 2010, a nomogram was developed that was based on 3450 patients, and included various clinical and biochemical parameters as predictors for disease specific survival. In **Chapter 2**, we attempted to validate this nomogram in the largest cohort of patients with SI-NET to date, for the use in clinical practice. We found that although the nomogram was able to differentiate between patients at low-, intermediate- or high risk of dying from their NET, it was not able to make an adequate prediction for disease specific survival in our cohort. We found that the nomogram underestimated survival in all subgroups. This was most likely due to the overall improvement of management of patients with SI-NET in more recent years. Nevertheless, with this study we showed that clinical and biochemical parameters remain of utmost value in the prediction of disease course in patients with SI-NET. In **Chapter 3**, we hypothesized that perhaps the addition of a new, blood-based analysis of circulating tumour transcripts, namely the NETest©, could improve the nomogram to further tailor the previously established predictive capacities of joint variables. We found that the NETest© was indeed helpful in predicting progression of disease, but failed to add any value in the prediction of disease specific survival. Although disappointing, this was not a complete surprise as the NETEst© is a measure of disease activity rather than bulk of disease. This paper hast mostly underscored the difference in results and associations when investigating different outcomes, and has initiated the discussion whether progression free survival is an adequate surrogate endpoint for disease specific survival. In NET, measuring progression could be highly valuable since progression is not only driven by radiological or biochemical burden of disease, but may also be driven by progression of clinical syndromes such as the carcinoid syndrome (including symptoms of diarrhoea, flushing and wheezing), yet progression free survival should perhaps not be considered as an intermediate endpoint for the more definite disease specific survival.

In the past decades of oncology research, unravelling the tumour genome has been an area of immense growth and progress. More and more malignancies have had their DNA sequenced and many targetable mutations (i.e. a drug has been developed that targets this specific mutation) have been found, often with impressive results. Unfortunately, the genome of NET has largely remained a mystery, mostly because of the overall low mutational burden of NET. In **Chapter 4** however, we described a relatively large cohort of SI-NET for which mutational panels – either Next Generation Sequencing (NGS) or Whole Genome Sequencing (WGS) were performed. We found that indeed the mutational burden of these tumours was low, but that some known, targetable mutations were still present. In our cohort, these mutations did not lead to a significantly different disease course, but the sole presence of these mutations might in the future open the way for new treatment strategies.

An important aspect of the pathophysiological occurrence of SI-NET is that the primary tumour is often accompanied by some extend of fibrosis. In the abdomen, this occurrence may lead to extensive mesenteric fibrosis (MF), and subsequently cause serious symptoms of bowel ischemia or obstruction. In unfortunate cases, this may even lead to life-threatening situations. Disappointingly, clinicians to date are not able to predict accurately which patients will indeed develop symptoms of MF, and which will not. This has led to the important question: whether to resect the primary tumour when distant metastatic disease is already present, or whether to postpone the resection of the primary tumour until symptoms thereof become apparent. Evidence regarding this subject mostly consisted of retrospective, confounded studies, and European and American guidelines only offered quite the indecisive advice. Due to this ambiguity, different institutes across Europe had adopted different treatment strategies for patients who present with metastatic SI-NET at diagnosis. In **Chapter 5**, we were able to compare two treatment strategies, namely that of the Netherlands Cancer Institute (to resect the primary tumour whenever technically achievable) to that of the Aintree University Hospital (to postpone resection of the primary tumour until symptoms of the primary tumour or MF arose). We conducted various statistical analyses and methods to maximally control for confounding in this study. Surprisingly, we found that the upfront approach provided disease specific survival benefit across all applied methods. These results are the first to strongly confirm that resection of the primary tumour in patients with metastatic SI-NET has a survival benefit. This study will likely aid in decision forming of clinicians treating NET and will hopefully be a stepping stone to initiate large (inter)national prospective studies.

In **Chapter 6** and **Chapter 7** we focussed on carcinoid heart disease (CHD). CHD is a serious cardiac of SI-NET. Since SI-NET originate from cells in the gut that have the ability to secrete serotonin, when these cells become a malignancy, they retain this ability, which may lead to excessive efflux of serotonin. Elevated serotonin may cause the carcinoid syndrome in 30-40% of patients, which constitutes of diarrhoea, flushes and wheezing. Normally, this excess of serotonin passes through the liver, where a metabolization to the inactive 5-hydroxyindoleacetic acid (5-HIAA) occurs. Yet when liver metastasis of SI-NET are present, this excess of serotonin may enter the systemic circulation directly, bypassing the hepatic metabolization, and enter the heart. In around 30% of patients with carcinoid syndrome, CHD occurs. Although the mechanisms are not fully understood, serotonin causes fibrosis of the right-sided heart, affecting mostly the tricuspid and pulmonic valve – causing valve regurgitation and eventually right-sided heart failure. Currently, all patients with SI-NET undergo frequent (1-2 yearly) echocardiography to screen for CHD. In **Chapter 6**, we have investigated whether we could find biomarkers that may predict which patients are at risk of developing CHD. We found that three biomarkers that had previously been shown to be associated with CHD (Activin A, connective tissue growth factor and soluble suppressor of tumorigenicity-2) did not play a role in the prediction or diagnosing of CHD. We did find however that the N-terminal pro-B-type natriuretic peptide (NT-pro-BNP) was an excellent marker to predict and diagnose CHD. The main finding of this study was that we were able to provide cut-off values for NT-proBNP that are associated with low risk of CHD, intermediate risk of CHD and high risk of CHD. This directly provides a guide for clinicians to

know which patients may be released from echocardiographic screening, which may continue with the screening as previously, and which should be monitored more closely.

In **Chapter 7**, we have performed an epidemiological study to describe how the survival of patients with CHD has changed over the past four decades. We found that the survival of patients with CHD has improved significantly over the past years, but that it remains worse compared to patients without CHD. Possibly the improvement in survival is due to a larger proportion of patients undergoing surgical valve replacement, although general improvement in the treatment of patients with SI-NET is likely to also have played a role. This study has underscored the importance of adequate screening and timely diagnosis of patients with CHD.

NET may also arise in the bronchopulmonary tract (bpNET). These tumours have a somewhat different classification, and are divided in the lower grade typical and higher grade atypical carcinoids according to their mitotic count and Ki67-index. There are three contexts in which bpNET occur: bpNET may arise sporadically, in the context of Multiple Endocrine Neoplasia type 1 (MEN1) syndrome or in the context of Diffuse Idiopathic NeuroEndocrine Cell Hyperplasia (DIPNECH). Previously, all bpNET arising in all contexts were considered the same entity, yet some case series and clinical observations led to the hypothesis that bpNET that arise sporadically have a more poor prognosis than bpNET arising from the other contexts. In **Chapter 8**, we performed the first head-to-head comparison of bpNET in all three contexts. We found that indeed bpNET in the context of MEN1 or DIPNECH have a highly favourable prognosis, and almost no bpNET-related deaths occur in those groups, whereas the survival of patients with sporadic bpNET is dominated by bpNET related deaths. This study was the first to show that bpNET in the context of MEN1 or DIPNECH may be followed up much less stringently than bpNET that arise sporadically.

Tailoring treatment

Merkel cell carcinoma (MCC) is an extra-pulmonary neuroendocrine carcinoma (EP-NEC) that arises from the skin, that occurs predominantly at ages above 60 years old. It is a highly aggressive malignancy with a unique pathogenesis in most cases: in 80% of patients on the Northern hemisphere it is caused by the oncogenic Merkel cell polyomavirus, in all other cases it is associated with exposure to ultraviolet light. Since MCC is considered highly sensitive to radiotherapy, this treatment option is firmly incorporated in the management of MCC. International guidelines therefore advice to treat patient with postoperative radiotherapy (PORT) after initial resection of the tumour with curative intent. However, these guidelines were based on large studies that investigated overall survival, rather than disease specific survival. In **Chapter 9**, we investigated the survival benefit of PORT in stage I-III MCC for recurrence free survival, disease specific survival and overall survival. Interestingly, we found that PORT provided survival benefit for recurrence free- and overall survival, but not for disease specific survival. This result is probably caused by the fact that patients who are already likely to die from other causes often will not receive additional adjuvant therapies, such as PORT. Hereby the overall survival benefit of patients that did receive PORT is highly influenced by confounding

by indication. In this study, we were the first to address this issue and have therefore initiated the debate whether all patients with stage I-III MCC should be treated with PORT, or whether the guidelines could be improved, for instance by taking life expectancy into account.

In the past decade, immunotherapy has made an entrance into the world of MCC, and has greatly changed the outcomes for patients with metastatic disease. For comparison: before immunotherapy the five-years survival of patients with stage IV MCC was around 10%, after the introduction of immunotherapy this had undergone a 2.5 fold increase. Since immunotherapy had only been introduced so recently, all data in MCC patients came from results of clinical trials. In **Chapter 10**, we described the largest group of patients with stage IV MCC that was treated with immunotherapy, outside a clinical trial or pharmaceutical expanded access program. We found that the responses to treatment and durations of responses were indeed highly similar to what was found in clinical trials, further confirming how much of a game-changer the introduction of immunotherapy for MCC is.

Besides MCC, other EP-NEC have unfortunately not shown such impressive responses to immunotherapy, and the treatment of this, also highly aggressive malignancy still consist of cytotoxic regimens that are mostly based on regimens from other small cell malignancies. Since in NET, the mammalian target of rapamycin (mTOR) pathway is often upregulated, we hypothesized in **Chapter 11**, that the addition of everolimus (an mTOR inhibitor) might by a good addition to known treatments. We found that the responses and duration thereof were similar to previously established regimens, with the important exception that alopecia is not part of the array of adverse effects, which may be valuable to the quality of life of patients. Also, we found that a selection of patients had a response that lasted longer than one year, which prompts the follow up question: what distinguishes these patients from the others? With this finding, this study will inspire future studies investigate the genome of these tumours and patients for the quest for other treatment options.

In **Chapter 12**, we have provided a general overview of the results of the previous chapters, reflected on the clinical, scientific and societal implications thereof and provided suggestions for the direction in which future research should be aimed.

Nederlandse samenvatting

Neuro-endocriene neoplasma

Neuro-endocriene neoplasma (NEN) zijn een complexe en heterogene groep maligniteiten die door het gehele lichaam kunnen voorkomen. Hun overeenkomstigheid is dat ze allemaal ontstaan uit neuro-endocriene cellen. In 2000 heeft de Wereldgezondheids Organisatie (WHO) een nieuw graderingssysteem gedefinieerd, waarin voor het eerst het onderscheid werd gemaakt tussen goed-gedifferentieerde neuro-endocriene tumoren (NET) en slecht gedifferentieerde neuro-endocriene carcinomen (NEC). Tegenwoordig houdt dit onderscheid nog steeds stand, met de volgende subgroepen gedefinieerd. NET lijken, zoals de goed-gedifferentieerde definitie al doet vermoeden, het meest op de cellen waaruit zij ontstaan. Dit betekent dat zij bij microscopische beoordeling de grootste gelijkenis laten zien met de originele niet-tumoreuze cel. NET zijn verder ingedeeld in graad 1-3, afhankelijk van de delingsgraad, namelijk mitose telling en Ki-67 index, beiden maten die weergeven hoeveel van de cellen in een tumor zich in een actieve delingscyclus bevinden. NEC zijn, ondanks dat ze ontstaan uit dezelfde cel als NET, een geheel andere entiteit en hebben dan ook een geheel andere benaderingswijze, behandeling en prognose.

NET hebben over het algemeen een gunstiger prognose en patiënten leven doorgaans nog vele jaren in de aanwezigheid van gemetastaseerde ziekte. NEC daarentegen hebben een zeer beperkte prognose, waarbij de mediane overleving na diagnose korter dan 1 jaar is, zelfs bij een actieve behandeling. De behandeling van NET en NEC is dan ook geheel verschillend. Gezien NET een lage delingsgraad hebben, hebben conventionele chemotherapieën geen invloed op de progressie van NET. Hier worden andere middelen gebruikt, die de eigenschap hebben zich op eiwitten te binden die door de tumor tot expressie worden gebracht, zoals somatostatine analogen (SSA) en peptide-receptor radionuclide therapie (PRRT). Het tegenovergestelde is weer het geval bij NEC, daar zij wel een hoge delingsgraad hebben, worden zij behandeld met cytotoxische medicatie die onder andere ingrijpt op de delingscyclus.

Huidig proefschrift

In de afgelopen jaren hebben clinici en onderzoekers gepoogd de indeling en behandeling van patiënten met NEN te verbeteren, maar er persisteren nog lacunes waarin verdere verbetering te behalen valt. Dit proefschrift heeft tot doel om deze lacunes te vullen. Allereerst, maakt de eigenschap van NET dat zij zo heterogeen zijn het ingewikkeld om een accurate voorspelling voor de progressie van ziekte en overleving van patiënten te schetsen. Kennis hierover is van het hoogste belang voor patiënten, maar ook voor de artsen die deze patiënten behandelen. Dit proefschrift biedt een aantal antwoorden op vragen betreffende een meer passende prognose vorming, door onder meer aan te geven welke variabelen het meest bijdragen aan het ziekte beloop. Andere antwoorden betreffen welke patiënten minder streng gecontroleerd hoeven te worden en derhalve meer vrijheid kunnen beleven, of juist welke patiënten beter en frequenter in de gaten gehouden zouden moeten worden.

Ten tweede, is er in de behandeling van NEC wel enige vooruitgang geweest in de afgelopen decennia, maar is er zeker ruimte voor verbetering gebleven. We hebben in dit proefschrift laten zien dat het gebruik van real world data (gegevens uit de klinische praktijk) een enorme bijdrage kan leveren aan het bereiken van hoogwaardige resultaten voor de behandeling van zowel locoregionale ziekte als gemetastaseerde ziekte in patiënten met Merkel cel carcinoom, een neuro-endocrien carcinoom van de huid. Voorts heeft dit proefschrift ook de eerste grote multi-centrum studie beschreven waarin een nieuwe behandelingscombinatie wordt gebruik voor patiënten met gemetastaseerde extra-pulmonale neuro-endocriene carcinomen (EP-NEC).

Het vormen van prognose

De meest voorkomende primaire tumorlokalisatie van NET is de gastro-entero-pancreatische neuro-endocriene tumor (GEP-NET), hiervan maken NET van de dunne darm (small intestine, SI-NET) het grootste deel uit, met een gemiddelde incidentie van 1.2/100 000 personen. Hoewel eerder genoemde indeling van NET waarin ze worden ingedeeld in 3 verschillende graden wel behulpzaam is in het voorspellen van prognose, blijft het daadwerkelijke ziekte beloop dikwijls gissen. In 2010 werd er een nomogram ontwikkeld dat gebaseerd was op 3450 patiënten, en waarin verscheidene klinische en biochemische parameters werden meegenomen als voorspellers van ziekte-specifieke overleving. In **Hoofdstuk 2**, hebben we gepoogd dit nomogram te valideren voor gebruik in de klinische praktijk in, tot op heden, de grootste groep van patiënten met SI-NET. We vonden dat hoewel het nomogram zeker geschikt was om te differentiëren tussen een laag, intermediair en hoog risico voor het overlijden aan NET, de voorspelde overlevingsduur niet overeenkwam met de daadwerkelijke overlevingsduur in ons cohort. Het nomogram gaf in onze data een structurele onderschatting van de overlevingsduur. Dit heeft er hoogstwaarschijnlijk mee te maken dat de diagnose en behandeling van patiënten met SI-NET in recenter jaren is verbeterd, waardoor de getallen waarop het nomogram gebaseerd was achterliepen. Desalniettemin, liet deze studie mooi zien dat de gebruikte klinische en biochemische variabelen hoogst belangrijk blijven in de voorspelling van het ziektebeloop van patiënten met SI-NET. In **Hoofdstuk 3**, formuleerden we de hypothese dat wellicht het toevoegen van een nieuwe, op bloedafname gebaseerde, analyse van circulerende tumor transcripten, namelijk de NETest©, aan het nomogram, zou kunnen bijdragen om voorspelling van ziektebeloop accurater te maken. We vonden dat de NETest© inderdaad een goede aanvulling was op het voorspellen van progressie van ziekte, maar helaas niet geschikt was voor het voorspellen van ziekte-specifieke overleving. Hoewel dit teleurstellend was, was dit niet geheel onverwacht aangezien de NETest© een maat is voor ziekteactiviteit, en niet zozeer voor hoeveelheid van ziekte. Dit stuk heeft met name laten zien dat het van groot belang is dat men zich realiseert welke uitkomstmaat het meest geassocieerd is met hetgeen dat men onderzoekt, en ook of progressie vrije overleving wel een geschikte surrogaat-uitkomst maat is voor ziekte-specifieke overleving. In patiënten met NET is het meten van progressie zeer belangrijk aangezien progressie niet alleen hoeft te bestaan uit tumor groei, maar ook juist kan bestaan uit progressie van functionele klachten van bijvoorbeeld het carcinoid syndroom (wat bestaat uit diarree, opvliegers en bronchospasme – veroorzaakt door een uitstroom van serotonine uit de SI-NET). Ondanks de waarde van het meten van deze

progressie, valt het sterk te betwijfelen of progressie vrije overleving wel beschouwd moet worden als intermediair voor uiteindelijke ziekte-specifieke overleving.

Het ontrafelen van het tumor genoom is een gebied dat in de afgelopen decennia enorme groei en vooruitgang heeft doorgemaakt. Van steeds meer maligniteiten is het DNA in kaart gebracht (gesequenced) en er zijn vele mutaties aangetoond waar specifieke gerichte medicatie voor is gevonden (targeted therapy), vaak met indrukwekkende resultaten. Helaas is het genoom van de NET tot op heden grotendeels onontdekt gebleven, voornamelijk omdat NET geen hoge mutatie last hebben. Met andere woorden: in het genoom van NET zitten weinig tot geen mutaties waarvan we op dit moment weten dat zij eigenschappen hebben die de tumorgenese drijven. In **Hoofdstuk 4** hebben we een relatief groot cohort van SI-NET beschreven waarvan bepaalde mutatie panels – Next Generation Sequencing (NGS) of Whole Genome Sequencing (WGS) – was verricht. In onze resultaten vonden we inderdaad dat de mutatie last van deze tumoren laag was, maar dat er toch sommige mutaties waar gerichte behandelingen voor bestaan aanwezig waren in de tumoren. We zagen in ons cohort niet terug dat de deze mutaties ook daadwerkelijk leidden tot ernstiger ziekte beloop, maar de aanwezigheid van deze mutaties alleen al zou in de toekomst kunnen betekenen dat er nieuwe behandelstrategieën voor patiënten met NET gevormd zouden kunnen worden.

Een belangrijk aspect van de pathofysiologie van SI-NET is dat de primaire tumor vaak, zo niet altijd, gepaard gaat met enige mate van fibrose. In het abdomen leidt dit tot uitgebreide mesenteriale fibrose (MF), met dientengevolge serieuze symptomen van darmischemie of –obstructie. In ongelukkige gevallen kan het zelfs leiden tot levensbedreigende situaties. Helaas slagen clinici er tot op heden nog niet in om te voorspellen welke patiënten klachten en symptomen van MF zullen ontwikkelen, en welke niet. Dit probleem heeft geleid tot de belangrijke vraag: moet de primaire tumor in de dunne darm gereseceerd worden in patiënten die reeds afstandsmetastasen hebben, of moet er gewacht worden tot patiënten klachten krijgen die passen bij ischemie of obstructie? De kennis die beschikbaar is over dit onderwerp bestaat voornamelijk uit retrospectieve studies, waarbij 'confounding by indication' (patiënten met een betere uitgangsfitheid en a priori kans op overleving hebben een grotere kans om geopereerd te worden) een grote rol speelt. Daarnaast bieden de Europese en Amerikaanse richtlijnen slechts beperkt directieve adviezen. Gedreven door deze ambiguïteit hebben verschillende NET-centra verschillende behandelstrategieën afgesproken voor de behandeling van patiënten met SI-NET. In **Hoofstuk 5** hebben we twee behandelstrategieën uit twee NET-centra, namelijk het Antoni van Leeuwenhoek ziekenhuis (AvL) in Amsterdam en het Aintree University Hospital (AUH) in Liverpool met elkaar kunnen vergelijken. Het AvL had besloten bij elke patiënt de tumor te reseceren indien dit technisch haalbaar was, ook in de aanwezigheid van gemetastaseerde ziekte. Het AUH daarentegen, had juist besloten alleen te opereren in geval van symptomen van ischemie of obstructie. We hebben verscheidene, zeer uitgebreide statistische analyses uitgevoerd om in dit cohort maximaal te kunnen corrigeren voor confounding. Enigszins verrassend, vonden we dat het tijdig reseceren van de primaire tumor, dus in de afwezigheid van symptomen, ziekte-specifieke overlevingswinst gaf in alle

toegepaste methoden. Deze resultaten zijn de eerste die met sterke zekerheid kunnen zeggen dat het reseceren van de primaire tumor in de aanwezigheid van gemetastaseerde ziekte overlevingswinst geeft in patiënten met SI-NET. Deze studie zal derhalve ondersteunend zijn in de besluitvorming omtrent de behandeling van patiënten met NET en zal hopelijk ook fungeren als een opstapje om grotere (inter)nationale, prospectieve studies op te zetten.

In **Hoofdstuk 6** en **Hoofdstuk 7** stond carcinoid hartziekte (carcinoid heart disease – CHD) in het middelpunt. CHD is een ernstige complicatie van SI-NET. Aangezien SI-NET ontstaan uit neuro-endocriene cellen die serotonine uitscheiden, ontwikkelt zich een enorme uitstroom aan serotonine wanneer deze cellen maligne ontaarden en nog ernstiger wanneer zij metastaseren. Een verhoogd serotonine leidt tot het carcinoid syndroom in 30-40% van patiënten met SI-NET. Het carcinoid syndroom bestaat uit diarree, opvliegers en bronchospasme. Normaal gesproken, wanneer serotonine wordt aangemaakt in de darm, passeert dit door de lever waar het wordt omgezet naar de inactieve metaboliet 5-hydroxyindoleacetic acid (5-HIAA). Wanneer er echter levermetastasen aanwezig zijn, wordt deze metabolisering omzeild en kan serotonine in de systemische circulatie terecht komen en daarmee ook in het hart. In ongeveer 30% van patiënten met het carcinoid syndroom, ontstaat er ook CHD. Hoewel het mechanisme hiervan nog niet volledig bekend is, weten we dat de aanwezigheid van serotonine leidt tot fibrosering van de rechter harthelft, waarbij voornamelijk de tricuspidalis en pulmonalis klep worden aangedaan, leidend tot klep-insufficiënties. Tegenwoordig worden alle patiënten met een SI-NET volgens de richtlijnen iedere 1-2 jaar gescreend op CHD middels een trans-thoracale echocardiografie (TTE). In **Hoofdstuk 6** hebben we onderzocht of we biomarkers konden vinden die voorspelden welke patiënten al dan niet CHD ontwikkelden. Drie biomarkers die vanuit de literatuur geassocieerd leken met CHD (Activin A, connective tissue growth factor en soluble suppressor of tumorigenicity-2), bleken dit niet te zijn in ons cohort. Wel vonden we dat N-terminal pro-B-type natriuretic peptide (NT-proBNP) een uitstekende marker was in de voorspelling en diagnostisering van CHD. De belangrijkste bevinding in onze studie was tevens dat we afkappunten hebben kunnen formuleren, welke onderscheid maakten tussen patiënten die een laag risico hadden op het ontwikkelen van CHD, een intermediair risico en een hoog risico. Hiermee konden we een direct voorschrift geven aan clinici die werken met patiënten met NET, waarbij patiënten met een laag risico geen screening middels TTE meer hoefden te krijgen, patiënten met een intermediar risico konden volgens de huidige richtlijn worden gescreend, en patiënten met een hoog risico konden nog frequenter worden gescreend dan nu wordt geadviseerd.

In **Hoofdstuk 7** hebben we een epidemiologische studie uitgevoerd om te beschrijven hoe de overleving van patiënten met CHD over de laatste vier decennia is veranderd. We vonden dat de overleving van patiënten met CHD significant is verbeterd over de afgelopen jaren, maar dat het nog steeds slechter is in vergelijking met patiënten die geen CHD ontwikkelen. Mogelijk is de verbetering in overleving goeddeels te wijten aan het feit dat tegenwoordig een grotere proportie patiënten chirurgische klepvervanging ondergaat. Echter is ook de algemene diagnostisering en behandeling van patiënten met NET in de afgelopen jaren verbeterd, wat

ook bijdraagt aan onze gevonden uitkomsten. Wat deze studie met name heeft laten zien is dat de tijdige diagnostisering en behandeling van groot belang is in de prognose van patiënten met CHD.

NET kunnen naast het gastro-entero-pancreatische stelsel, ook voorkomen in de bronchopulmonale tractus (bpNET). Deze tumoren hebben een enigszins andere indeling, waarbij zij zijn ingedeeld in de laaggradige typische carcinoiden, aan hooggradige atypische carcinoiden op basis van de mitose telling en Ki-67 index. Er zijn drie verschillende contexten waarbinnen bpNET kunnen ontstaan: bpNET kunnen sporadisch voorkomen, in de context van het Multipele Endocriene Neoplasma type 1 (MEN1) syndroom, of in de context van Diffuse Idiopathic NeuroEndocrine Cell Hyperplasia (DIPNECH). Voorheen werd gedacht dat bpNET zich in alle gevallen vergelijkbaar gedroegen, onafhankelijk van in welke context zij zijn ontstaan. Sommige case series en klinische observaties lieten echter de hypothese ontstaan dat sporadische bpNET een veel slechter ziekte beloop hadden dan bpNET die vanuit een andere context ontstonden. In **Hoofdstuk 8** hebben we de eerste rechtstreekse vergelijking uit kunnen voeren waarbij we bpNET uit alle drie de contexten mee konden nemen. Onze uitkomsten lieten zien dat inderdaad bpNET die ontstaan in de context van MEN1 of DIPNECH een hele gunstige prognose hebben, en er bijna geen bpNET gerelateerde overlijdens voorkomen in die groepen. Dit terwijl de overleving van patiënten met sporadische bpNET gedomineerd wordt door bpNET-gedreven overlijdens. Deze studie geeft ook gelijk handvatten voor de klinische praktijk, namelijk dat bpNET in de context van MEN1 of DIPNECH veel minder streng vervolgd hoeven te worden dan patiënten met sporadische bpNET.

Het vormen van behandeling

Merkel cel carcinoom (MCC) is een extra-pulmonaal neuro-endocrien carcinoom (EP-NEC) dat ontstaat in de huid. MCC komt voornamelijk voor boven een leeftijd van 60 jaar en is een hoogst agressieve maligniteit met een bijzondere pathogenese. Op het noordelijk halfrond wordt 80% van de MCC veroorzaakt door het oncogene Merkel cel polyomavirus, in de overige 20% van de gevallen is zon-expositie de oorzaak voor het ontstaan van MCC. Aangezien MCC een maligniteit is die sterk gevoelig is voor radiotherapie, is dit een modaliteit die een ferme plaats heeft in de behandeling van patiënten met MCC. Internationale richtlijnen adviseren dan ook om patiënten met adjuvante radiotherapie (postoperatieve radiotherapie – PORT) te behandelen, nadat zij een resectie in curatieve opzet hebben ondergaan. De richtlijnen baseren echter hun advies op grote studies die met name hebben gekeken naar algemene overleving, en niet naar ziekte-specifieke overleving. In **Hoofdstuk 9** hebben we onderzocht of PORT overlevingswinst geeft in stadium I-III MCC in ziekte-vrije overleving, ziekte-specifieke overleving en algemene overleving. We vonden dat PORT wel overlevingswinst geeft op ziekte-vrije en algemene overleving, maar niet op ziekte-specifieke overleving. Deze uitkomst wordt hoogstwaarschijnlijk veroorzaakt doordat patiënten doorgaans van een oudere leeftijd zijn, en dat zij die waarschijnlijk al aan een andere oorzaak zullen overlijden, vaak geen additionele behandelingen krijgen zoals PORT voor het MCC. De algemene overlevingswinst van patiënten die PORT hebben ondergaan wordt dus voor een groot deel beïnvloed door confounding by

indication – fittere patiënten krijgen meer behandeling. Wij zijn met deze studie de eersten die dit aspect van de behandeling benoemen en hebben derhalve de discussie geopend of patiënten met stadium I-III MCC wel PORT moeten krijgen. Wij stellen voor dat de indicatie voor het al dan niet behandelen met PORT zou moeten worden aangepast, bijvoorbeeld door levensverwachting mee te nemen in de indicatiestelling.

In het afgelopen decennium heeft immuuntherapie zijn intrede genomen in de wereld van MCC, en heeft enorme veranderingen teweeg gebracht in de uitkomsten van patiënten met gemetastaseerde ziekte. Ter vergelijking: voordat immuuntherapie een mogelijkheid was, was de vijfjaarsoverleving van patiënten met stadium IV MCC ongeveer 10%. Sinds de introductie van immuuntherapie is dit met 2.5 maal vermenigvuldigd. Aangezien immuuntherapie nog maar zo kort geleden geïntroduceerd is, waren alle tot op heden bekende data gegevens uit klinische studies. In **Hoofdstuk 10** beschrijven we de grootste groep patiënten met stadium IV MCC die behandeld zijn met immuuntherapie buiten studie verband of farmaceutische toegang programma's. Onze uitkomsten lieten zien dat de responsen en duur van respons inderdaad vergelijkbaar waren met de klinische studies, hiermee daadwerkelijk bevestigend wat een omslag in de prognose van patiënten met MCC dit heeft betekend.

Buiten MCC, hebben andere EP-NEC helaas niet zulke indrukwekkende resultaten van immuuntherapie laten zien. De behandeling van deze agressieve maligniteiten is derhalve nog steeds gebaseerd op klassieke, conventionele cytotoxische behandel regimes, die grotendeels hun oorsprong kennen in de behandeling van kleincellig longcarcinoom. Aangezien er eerder in NET was aangetoond dat de mammalian target of rapamycin (mTOR) traject vaak is opgereguleerd, hadden we de hypothese gevormd dat de toevoeging van een mTOR remmer, namelijk everolimus, een goede toevoeging zou zijn aan huidige behandelstrategieën. We beschrijven de resultaten hiervan in **Hoofdstuk 11**. We vonden in ons multi-centrum cohort dat de overlevingsduur van patiënten vergelijkbaar was met die van eerder vastgestelde behandel regimes. Het belangrijke verschil echter was dat alopecia niet optrad bij het nieuw getoetste regime, wat een belangrijke factor is in de kwaliteit van leven van patiënten. Daarnaast vonden we ook dat er een selectieve groep was van patiënten die een overlevingsduur had langer dan 1 jaar, wat de volgende vraag liet rijzen: wat zorgt er nou voor dat deze patiënten anders zijn dan de rest? We hopen met deze bevindingen toekomstige studies te inspireren om het genoom van deze kanker verder uit te pluizen om voorts andere behandelstrategieën te kunnen ontwikkelen.

In **Hoofdstuk 12** hebben we een algemeen overzicht van alle uitkomsten van de eerder genoemde hoofdstukken, en reflecteren we op de klinische, wetenschappelijke en sociale implicaties hiervan. Voorts geven we ook suggesties voor de richting waarin toekomstig onderzoek plaats zal moeten vinden.

Appendices

List of publications

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We zien wel.

Curriculum Vitae

Sonja Levy was born in Moscow, Soviet-Union on the 31st of May, 1989. After spending a year in Israel until 1991, she moved to the Netherlands with her parents where she grew up in Amsterdam and Bodegraven, a village near Gouda.

She went to high school at the Coornhert Gymnasium, where she enjoyed various extracurricular activities, including the founding of and participating in the debate club.

After graduating high school in 2008, she moved back to Amsterdam to study Biomedical Sciences, but her heart never left the aim to become a physician, and in 2011 she was admitted to medical school at the University of Amsterdam. During medical school she had various other interest, such as participating in research projects, working on the set-up of a new electronic patient record system between two large academic institutes, and chairing the interns council (CoRaad UvA). Within the interns council, she worked together with other interns to improve the education programme of the interns. Another project she was part of was the implementation of a compensation for the work interns do, which is now instated nationally. She performed her final internship as well as her research internship in the Antoni van Leeuwenhoek hospital – the Netherlands Cancer Institute (NKI). The former took place in the medical oncology ward, the latter was a project on the subject of neuroendocrine tumours, which eventually led to the publication of chapter 2 of this thesis.

She graduated with honours in 2018, after which she continued her research endeavours at the NKI in a PhD trajectory, on the subject of neuroendocrine tumours, under the supervision of dr. Margot Tesselaar, prof. dr. Gerlof Valk and prof. dr. Monique van Leerdam. She performed various epidemiological studies which investigated the prognosis and treatment of patients with neuroendocrine malignancies. She finished her PhD in 2024. During her PhD trajectory, she was admitted to the Postgraduate Masters' Programme of clinical epidemiology, and finished all courses.

In 2022 she started as a resident-not-in-training in internal medicine at the Diakonessenhuis in Utrecht. From there, she was admitted to the internal medicine specialist training programme at the University Medical Centre in Utrecht, which she is currently still enrolled in.

Besides working, Sonja enjoys spending time with family and friends and being creative. The latter mostly entails working with ceramics, which consists of spending time at her potters' wheel making various pots and cups. Her favourite sports are cycling and kickboxing.

She lives in Utrecht in a cosy home with her husband Sebas and her son Nemo.