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Gastroenteropancreatic Neuroendocrine Neoplasms Grade 3: Biological and Clinical Aspects

ABIR SALWA ALI



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Abstract

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The aim of this thesis was to investigate biological and clinical aspects of G3 gastroenteropancreatic neuroendocrine neoplasms (G3 GEP-NENs).

In our first study, the expression of the tumor suppressor p53 was investigated. In a cohort of G3 GEP-NENs we found the expression of p53 protein to be present in 39% of 124 cases. Expression of p53 correlated to poorer progression-free survival (PFS) and overall survival (OS) for patients with G3 GEP-NENs originating from colon or rectum. In the next study, we aimed to demonstrate the prevalence of PD-L1 expression in G3 GEP-NENs and its possible clinical importance. Ten per cent of 136 tumor specimens were immunoreactive for PD-L1 in either tumor cells or immune cells. In contrast to p53 expression that could be correlated to PFS and OS in a subgroup of patients the expression of PD-L1 did not correlate to any clinicopathological variables and conclusively, PD-L1 may not have a vital role for the pathogenesis of G3 GEP-NENs. In a further study, we sought to identify new potential biomarkers and a panel of immuno-oncological proteins were measured in serum collected from pancreatic G3 NENs and healthy controls. Out of 87 proteins, 62% were significantly lower in serum concentration in healthy controls compared to patients. One protein, FasL, was present in significantly higher levels in healthy controls compared to patients. FasL may have a protective role in its ability to activate T cells in the immune system. Other proteins of interest were chemokine (c-c motif) ligand and interleukin 8 that both correlated to poorer prognosis in G3 pancreatic NEN patients. More studies are needed for further understanding of the roles and clinical relevance of immuno-oncological proteins in G3 pancreatic NENs.

Finally, we evaluated whether intravenous or oral administration of etoposide differed with regards to PFS and OS in patients with G3 GEP-NENs. There was no significant difference in PFS nor OS between patients receiving oral compared to intravenous etoposide; demonstrating that an oral option of etoposide is not inferior in its efficacy as compared to the more used intravenous formulation. These results suggest that considering oral options of etoposide is important since they are more often preferred by patients, increase the quality of life for the patients and reduce hospital costs.

This thesis has contributed to an understanding of the distribution and clinical relevance of p53 and PD-L1 in GEP-NENs. A potential role of FasL, chemokine and interleukin 8 as prognostic and/or diagnostic factors in pancreatic G3 NENs has been identified and should be further investigated. The thesis also gave some insight into the role of oral etoposide as an alternative option to intravenous formulation with regards to efficacy. Oral formulations are preferred by many patients and improve quality of life while decreasing hospital-related costs. Further studies are needed to compare the tolerability of oral formulation compared to the intravenous formulation.

Abir Salwa Ali, Endocrine Oncology, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

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To My Family



List of Papers

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

- I **Ali AS**, Grönberg M, Federspiel B, Scoazec JY, Hjortland GO, Grønbæk H, Ladekarl M, Langer SW, Welin S, Vestermark LW, Arola J, Österlund P, Knigge U, Sorbye H, Grimelius L, Janson ET (2017). Expression of p53 Protein in high-grade Gastroenteropancreatic Neuroendocrine Carcinoma. *PloSOne*, 12(11)
- II **Ali AS**, Langer SW, Federspiel B, Scoazec JY, Hjortland GO, Grønbæk H, Ladekarl M, Welin S, Vestermark LW, Arola J, Österlund P, Knigge U, Sorbye H, Micke P, Grimelius L, Grönberg M, Janson ET (2019). PD-L1 expression in G3 Gastroenteropancreatic Neuroendocrine Neoplasms. *Manuscript*
- III **Ali AS**, Perren A, Lindskog C, Welin S, Sorbye H, Grönberg M, Janson ET (2019). Serum biomarkers in Pancreatic G3 Neuroendocrine Neoplasms. *Manuscript*
- IV **Ali AS**, Grönberg M, Langer SW, Ladekarl M, Hjortland GO, Vestermark LW, Österlund P, Welin S, Grønbæk H, Knigge U, Sorbye H, Janson ET (2018). Intravenous versus oral etoposide: efficacy and correlation to clinical outcome in patients with high-grade metastatic gastroenteropancreatic neuroendocrine neoplasms (WHO G3). *Medical Oncology*, 6;35(4):47

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Additional Publications by the author

- ❖ Söderquist F, Janson ET, Rasmusson AJ, **Ali A**, Stridsberg M, Cunningham J L. (2016). Melatonin Immunoreactivity in Malignant Small Intestinal Neuroendocrine Tumours. *PloSOne*, 11(10)
- ❖ Dumanski, J., Rasi, C., Björklund, P., Davies, H., **Ali, A.**, Grönberg, M., Welin, S., Sorbye, H., Grønbæk, H., Cunningham, J., Forsberg, L., Lind, L., Ingelsson, E., Stålberg, P., Hellman, P., & Tiensuu Janson, E. (2017). A MUTYH germline mutation is associated with small intestinal neuroendocrine tumors. *Endocrine-Related Cancer*, 24(8), 427-443

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Abbreviations

ABC	Avidin-Biotin Complex
AST	Aspartate Aminotransferase
CCL4	Chemokine (c-c motif) ligand 4
CgA	Chromogranin A
CI	Confidence Interval
CUP	Cancer of Unknown Primary
DAB	3,3'-Diaminobenzidine
ENETS	The European Neuroendocrine Tumor Society
FasL	Fas Ligand
FFPE	Formalin Fixed Paraffin Embedded
GEP	Gastroenteropancreatic
GEP-NEN	Gastroenteropancreatic Neuroendocrine Neoplasm
GLP	Glucagon Like Peptide
HPF	High Power Field
HR	Hazard Ratio
IHC	Immunohistochemistry
IL8	Interleukin 8
IR	Immunoreactive
IV	Intravenous
LDH	Lactate Dehydrogenase
MEN 1	Multiple Endocrine Neoplasia-type 1
NEC	Neuroendocrine Carcinoma
NEN	Neuroendocrine Neoplasm
NET	Neuroendocrine Tumor
NSE	Neuron Specific Enolase
O.E.	Oral Etoposide
OS	Overall Survival
PD	Progressed Disease
PD-L1	Programmed Death Ligand 1
PEA	Proximity Extension Assay
PFS	Progression Free Survival
PLA	Proximity Ligation Assay
PR	Partial Response
PRRT	Peptide Receptor Radionuclide Therapy
SCLC	Small Cell Lung Cancer
SD	Stable Disease
Syn	Synaptophysin

TNF	Tumor Necrosis factor
TNM	Tumor Node Metastases
TP53	Tumor Protein 53
TS	Thymidylate Synthase
ULN	Upper Limit of Normal
VHL	von Hippel-Lindau
WHO	World Health Organization
WT	Wild Type

Introduction

The Endocrine system

The endocrine system is a collection of organs that are involved in several of the body's physiological processes. The cells of the endocrine system are known to produce and store different hormones specific for each of the organs that play a role in the regulation of the body's processes via feedback loops between each other [1].

These hormones are divided into two main categories, those that are built up from a cholesterol skeleton and those that are formed by amino acids, like amines and peptides [1, 2].

The Neuroendocrine cells

The neuroendocrine cells have their name because of their dual ability to act as endocrine cells while they also harbor similarities to neurons in the brain. These cells can secrete amines and peptide hormones upon depolarizing stimulus into the blood stream or surrounding cells [3].

Neuroendocrine cells include several different types of cells that can produce different types of hormones, e.g. insulin that is produced by beta-cells in the pancreas as a result of increasing blood levels of glucose, and somatostatin, produced by delta-cells, that is important for endocrine and exocrine digestive functions [4, 5].

Neuroendocrine neoplasms

Neuroendocrine neoplasms (NENs) are a disease entity that develop from neuroendocrine cells and may originate from a variety of organs. They are known to be a heterogeneous group of tumors differing in manifestations, pathology and degree of malignancy [6]. The discovery of NENs dates back to 1870 with pancreatic tumors being the first entity identified [7]. Neuroendocrine cells release hormones, stored in secretory granules, upon stimulation. Proteins contained in these granules include chromogranin A (CgA) and the membrane bound synaptophysin (Syn) which are the two general biomarkers used to determine if a cancer is of neuroendocrine origin [8, 9]. Considered a rare disease, the incidence of NENs account for 0.5% of all gastrointestinal and pulmonary tumors with an observed increase in incidence of these tumors, see Figure 1 [10]. Due to their heterogeneity, their diffuse symptoms and sometimes indolent biology, NENs still remain poorly understood more than a century after their discovery [11, 12].

NENs can be divided into functioning and non-functioning tumors. The functioning tumors produce specific hormones and can cause hypersecretion symptoms related to the hormone produced, e.g. VIPoma, insulinoma, glucagonoma and gastrinoma. [13]. Treating these tumors is a clinical challenge since both tumor growth and hormone production have to be managed.

Surgical resection is performed in patients who present with local disease. Patients with extensive disease may be treated with embolization (chemo- or radioembolization), peptide receptor radionuclide therapy (PRRT) and with drugs such as systemic chemotherapy or somatostatin analogues given as palliative treatment for hormone overproduction [14].

Heredity trait in NENs is uncommon with approximately 85% being sporadic tumors. Nevertheless, multiple endocrine neoplasia-type 1 (MEN1) and von Hippel-Lindau disease (VHL) are the two familial cancer syndromes known to be the cause of NENs [15-17]. Furthermore, a group of small intestinal NETs present with a family history of disease [18].

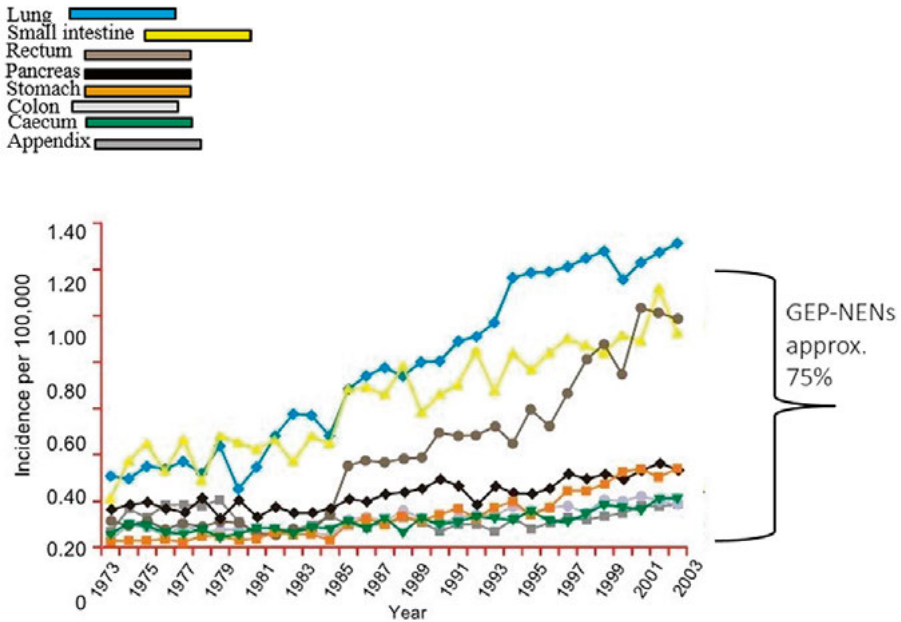


Figure 1. Increasing incidence of NETs, modified from Yao J, et al. *J Clin Oncol.* 2008;26:3063-3072.

Classification and histopathology

The classification of NENs is based on their histology and their expression of the general biomarkers CgA/Syn and a Ki67 index count [19]. In the year 2000, the World Health Organization (WHO) released a classification system for NETs, which was updated 2004 and 2010, with the later mentioned being the one mainly used today, Table 1 [20].

The 2010 WHO classification system divides NENs into three groups based on the rate of tumor proliferation (measured by Ki67 immunohistochemical expression). Tumors in the G1 group have Ki67 <2% and G2 tumors a Ki67 between 3-20%. G1 and G2 tumors are well differentiated and referred to as neuroendocrine tumors (NETs).

G3 tumors present with a Ki67 >20% and/or a mitotic count >20 per 2 mm² [21, 22] and are defined by WHO 2010 as poorly differentiated NENs, also known as neuroendocrine carcinomas (NECs). Gastroenteropancreatic NENs (GEP-NENs) refers to tumors with primaries in the GEP system and can be classified as G1, G2 or G3 [23].

Table 1. 2010 WHO classification system for GEP-NENs.

Grade	Mitotic count per 10 HPF*	Ki67 Index
G1	<2	≤ 2%
G2	2-20	3-20%
G3	>20	> 20%

*High power field

The WHO classification systems used before 2010 divided the GEP-NENs based on the organ of the primary tumor resulting in groups such as fore-gut, mid-gut and hind-gut. This tumor division did not consider differentiation and in the 2010 WHO classification, the tumors are divided according to their proliferation and to some extent differentiation [20, 24]. In recent publications, the role and importance of differentiation has been highlighted and many articles concerning differentiation and its relevance to clinicopathological features of the GEP-NENs have emerged [25-27].

Differentiation of a tumor refers to the resemblance of the cancer cell to cells of the host organ. When the cells become malignant and transform into tumor cells, their appearance may be different from normal cells of the organ in which the tumors are growing [28]. Differentiation has in recent years become a vital tool for classifying and treating NENs. In the 2010 WHO classification, all tumors with a Ki67 index above 20% were denoted poorly differentiated and hence seen as one disease entity. However, recent studies investigating differentiation of these tumors have revealed a new sub-group of GEP-NENs which has Ki67 >20% but morphologically look well differentiated [29, 30], Figure 2.

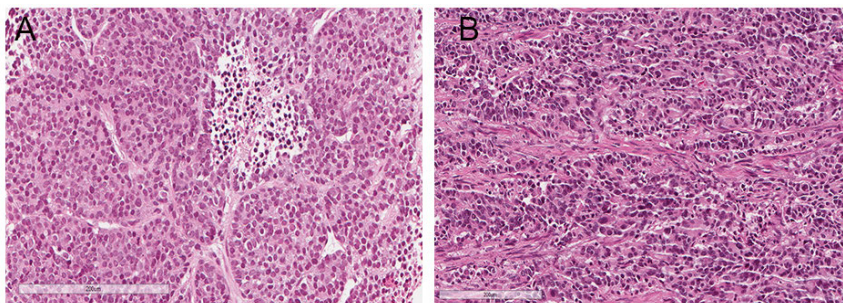


Figure 2. Hematoxylin staining of A) well differentiated neuroendocrine G3 tumor exhibiting growth in a trabecular organoid pattern B) a poorly differentiated pancreatic G 3 neuroendocrine carcinoma exhibiting diffuse growth pattern with barely visible cytoplasm and big distorted nucleoli.

In 2016, Tang *et al.* reported that a subset of pancreatic tumors with a Ki67 above 20% were not compliant with the WHO 2010 classification of GEP-NEN tumors. Thirty-three G3 pancreatic NENs, were assessed by three pathologists and among those, 35% showed well differentiated morphology. Furthermore, these tumors were divided into two groups by staining with antibodies targeting specific genetic mutations (*ATRX/DAXX* characteristic mutation for well differentiated tumors and *TP53* and *RB* for poorly differentiated tumors) [31]. These results were later reproduced in a publication by Konukiewicz *et al.* [32].

Additionally, Tang *et al.* published a follow-up study investigating well differentiated G3 tumors and their clinical presentation which differed from the poorly differentiated G3 tumors. The well differentiated G3 tumors were also positive on somatostatin receptor scintigraphy while poorly differentiated tumors rarely give a signal when scanned [32, 33].

In 2017, WHO presented a new classification based on the influx of new studies published with respect to the pathologically heterogeneous pancreatic G3 tumor group. In this new classification of pancreatic NENs, the groups are not solely based on the proliferation, but also on the presence of G3 tumors that are well differentiated as well as poorly differentiated. In the new 2017 classification system, pancreatic NENs in the G3 group are redefined as NET G3; well differentiated tumors with a high proliferative index $>20\%$, or NEC G3; poorly differentiated tumors with a high Ki67 $>20\%$ [34].

GEP-NENs may be of large cell or small cell type. These two types have different morphological appearances and may behave clinically different [19, 35]. Morphologically, large cell carcinomas are usually composed of cells that are rich in eosinophilic cytoplasm and with clearly visible nucleoli, while the small cell carcinomas comprised of smaller cells with scarce cytoplasm. Small cell carcinomas usually have rosette formations with cells that are semi round

[36, 37]. It is still unclear how small cell versus non-small cell carcinomas differ with respect to clinical outcome such as progression and survival.

The Tumor Node Metastases (TNM) staging is a system used for solid tumors to identify the extent of the disease. The European Neuroendocrine Tumor Society (ENETS) proposed in the year 2006 that TNM staging and grading system would be combined and that has been incorporated in the 2010 classification system. A study evaluated this combined TNM classification in comparison to older systems and concluded that it was a statistically powerful tool for the stratifications of these tumors [30, 38].

Another system that was used simultaneously was the TNM classification system developed by the American Joint Committee on Cancer and it was not until 2017 that, along with ENETs, an agreement on one consensus for TNM staging, was reached [39].

Gastroenteropancreatic neuroendocrine neoplasms, G3

G3 GEP-NENs are tumors with rapid progression and a bad prognosis and account for approximately 35-55% of all extra-pulmonary G3 NENs [40]. G3 GEP-NENs usually stain positive for Syn and less frequently for CgA, with a reported better prognosis when both CgA and Syn immunoreactivity is present [41, 42]. They are generally located in the esophagus, stomach, pancreas, colon and rectum, however in 30% of cases, they present as tumors of unknown primary location (CUP) [40].

Rapid disease progression leads to short 5-year survival for these G3 GEP-NEN patients with rather similar figures for different ethnic groups [43]. An observed median overall survival (OS) for chemotherapy treated patients in the Nordic countries was 11 months contra 1 month for untreated patients. The primary tumor location may also play a role in the OS of G3 GEP-NEN patients [40, 44]. Reported factors that have been proven to be of prognostic value are Ki67 index <55%, normal serum lactate dehydrogenase (LDH) and platelet count as well as good performance status [40].

Freis *et al.* showed in a recent report that elevated serum levels of LDH and aspartate aminotransferase (AST) were significantly correlated to poorer survival in G3 GEP-NEN patients [45]. Elevated CgA serum levels (>2 upper limit of normal, ULN) has also been suggested to be a significant prognostic factor [46]. Several publications have shown that GEP-NENs with a high grade Ki67 index and a well differentiated morphology seem to have a better prognosis [29, 31, 47].

Biomarkers

G3 GEP-NENs are rarely functioning tumors and most biomarkers for GEP-NENs are immunohistochemical markers such as CgA and Syn. CgA belongs to the granin family of proteins and is present in secretory granules in neuroendocrine cells. They are believed to play a role in production, maturation and exocytosis of vesicles containing hormones and neurotransmitters [48].

Syn is a glycoprotein that is present in presynaptic vesicles in the neuroendocrine cells of various organs. Syn is a membrane-bound protein that demonstrates neuroendocrine differentiation [49]. Neuron-specific enolase (NSE) is an enzyme expressed by cells of poorly differentiated tumors. Elevated NSE in blood reflect a high tumor burden of a poorly differentiated NEN. In small cell lung cancer (SCLC), NSE is seen as a poor prognostic factor and the same has been shown for pancreatic NETs [50, 51]. The above mentioned biomarkers have been known for several years but there is a need for new biomarkers for G3 GEP-NEN patients which can help to select treatment and predict survival.

Some of the recently discovered biomarkers are *ATRX/DAXX* and *TP53/RB*. Genetic alterations found in well differentiated and poorly differentiated G3 tumors differs where *TP53* and *RB* mutations are found in poorly differentiated tumors whereas *ATRX/DAXX* mutations are found in the well differentiated tumors [31, 33].

The enzyme thymidylate synthase (TS) is associated with distant metastases and poorly differentiated NECs of the colon, stomach and pancreas; an association that is statistically significant and that may be of use as a prognostic biomarker for poorly differentiated NENs [52]. In one study, TS was significantly higher in poorly differentiated GEP-NENs compared to well differentiated GEP-NENs and benign tumors [53] but more studies are needed to completely understand the full scope of TS role in GEP-NENs.

Genetics

Genetic profiling of NENs has been a challenge for many years. GEP-NECs show a relatively high chromosomal instability [54]. In GEP-NENs most genetic alterations are sporadic and seem to be harbored in cell-cycle related genes [51]. *TP53* mutations are amongst the most common in GEP-NENs that are poorly differentiated (90-95%), while they are rarely seen in GEP-NENs that are well differentiated (~3%) [55]. *RB* is another cell cycle regulator that is mutated in approximately 75% of poorly differentiated GEP-NENs [54]. Other reported sporadic gene mutations that occur in GEP-NENs are *CDKN1B*, *NF-1* and *TSC1/2* which are important in the mTOR pathway [56].

Tumor Protein 53

Tumor protein 53 (TP53) codes for the protein, p53 transcription factor, which has come to be known as a tumor suppressor involved in many processes and regulates various signaling pathways [57-59]. Some of the important roles in which the p53 protein is involved in are DNA repair, apoptosis and regulation of cell cycle arrest [60]. Wild type (WT) p53 is essential for genome stability and cell cycle arrest and is an important tool for tumor suppression. In the presence of DNA damage, WT p53 may induce cell repair and/or give rise to apoptosis through activating different cellular effector processes, Figure 3 [19, 61].

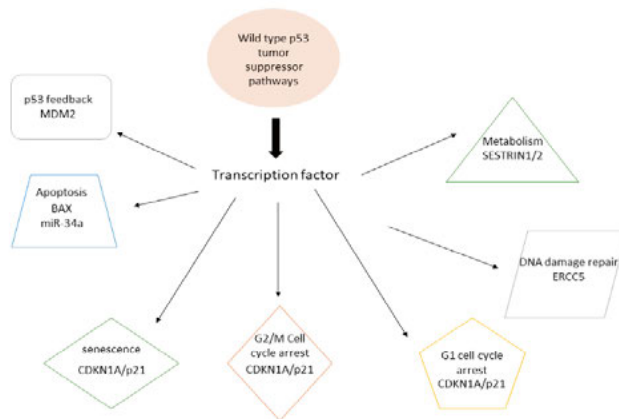


Figure 3. WT p53 activates several processes resulting in apoptosis, cell cycle arrest and DNA repair. Modified from Aubrey BJ et al., *Cell Death Differ* 2018; 25:104-113.

The p53 protein plays a role in most cancers and is often found to be mutated. A missense mutation frequently occurs that alters one single nucleotide by substituting it, leading to a defected p53 protein [62, 63]. *TP53* mutations have been seen to occur in 70-100% of NEC tumor cells and have been linked to poorer clinical outcome, treatment resistance and higher degree of metastases in different types of cancer [64-66]. Mutations in *TP53* in NECs have been confirmed in a recent report, in which next-generation sequencing of 50 cancer-related genes in 23 NEC tumors showed several different mutations, but with the presence of *TP53* mutation in the majority of them [67, 68]. In another study, *TP53* mutations were demonstrated in a high number of pancreatic NEC-patients [31].

Mutations in the *TP53* gene may result in an immunohistochemically detectable expression of the p53 protein that accumulates in the tumor cell nuclei [69]. However, most studies that have investigated p53 immunohistochemically have shown heterogeneity in the outcome of p53 expression [19, 70, 71].

Programmed death-ligand 1

Programmed cell death ligand 1 (PD-L1) is a protein that is encoded by the CD274 gene [72]. PD-L1 is an immune checkpoint protein, which, upon binding to its receptor programmed cell death protein (PD1), can modify T cell activity, inhibit their proliferation and hence, stop the immune system from recognizing tumors [73]. The mechanism of action that is proposed includes that PD-L1 which is found on the tumor cell binds to its receptor PD1, that is expressed on activated T cells, and this interaction inhibits the T cell activation [74]. This inhibitory activity makes it possible for tumors to escape recognition by the immune system and is linked to predict poorer outcome [75-77].

Fas ligand

Fas ligand (FasL) is a 40 kDa protein that is expressed mainly on activated T cells [78]. FasL binds to its receptor, Fas, mediating cell apoptosis in vulnerable cells. This interaction is vital for maintaining tissue development, homeostasis and regulation of the immune system. Disruptions in the Fas-FasL interaction can lead to tissue destructions and in some instances, accelerate autoimmune disorders [79, 80].

Although mainly being a protective protein, studies have also shown that the Fas-FasL pathway may have a pathological role for tumors in developing resistance to apoptosis via loss of Fas function [81-83].

Evasion of the immune system by tumors may be facilitated with the help of FasL. Tumors may express FasL on their surfaces to induce apoptosis of lymphocytes and via this mechanism escape the immune system, which in turn leads to disease progression [84].

Treatment

Treating GEP-NENs often needs a multidisciplinary approach. Somatostatin analogues are used for treatment of NETs but is not recommended in G3 NENs as the response has been seen best in tumors with Ki67 <10%. Somatostatin analogues are also efficient for the control of hormone overproduction and hormonal symptoms that are very rare in G3 GEP-NENs [85, 86].

Surgery is the first choice of therapy in NEN patients, especially if the operation can be radical. However, even if a radical operation is not possible, it may be discussed depending on symptoms from the tumor or severe hormone-related symptoms which may be reduced by reduction of tumor mass. Efficacy of surgery in G3 NEN patients has been debated. Studies of surgery in cohorts of these patients have been conflicting in their results, Brennan *et al.* concludes that surgery is not recommended for G3 NEN patients while other studies of a similar population recommends the combination of surgery and local therapy for patients with limited stage disease [87-89].

Chemotherapy is the most commonly used treatment in for G3-NEN patients and platinum-based combination chemotherapy of cisplatin/carboplatin and etoposide is considered to be first-line treatment [90, 91]. This first-line regimen is agreed on by leading NET societies in the western world [22, 92, 93]. PPRT is another method of treating cancers. This method is based on radiation to damage or kill cancer cells in a specific way. Peptides are radiolabeled and are used as targeting vectors that deliver the radioactive nucleotide to a tumor site [94]. The use of PPRT with radiolabeled somatostatin analogues in G2 and G2 GEP-NENs is well established but its use in G3 GEP-NENs is still to be fully evaluated, although there are promising results with a 69% disease control rate in a recent study by Carlsen *et al.* [95].

Administering chemotherapy intravenously to cancer patients has been a standard clinical routine. Etoposide can be given intravenously or orally. The effect of etoposide, which is inhibition of topoisomerase, is directly correlated to the concentration and duration of etoposide in the blood [96]. There may be an advantage to use intravenous (IV) formulations that allow for higher blood concentrations, although this also comes with disadvantages such as long hospital stays and elevated costs for hospitals as well as for patients.

Administration of oral etoposide (O.E) has been linked to decreased bioavailability and there are studies that show a bioavailability of etoposide ranging between 30% and 76% with a nonlinear absorption that decreases in bioavailability with increased dosage [97, 98].

As with all cancer treatments, there is a subset of patients that fail to respond adequately to first-line treatments. Welin *et al.* demonstrated that temozolomide, alone or combined with capecitabine had a 71% objective response rate in poorly differentiated NECs and may serve as a second-line option for patients who fail first-line treatment [41]. There is still no consensus

on what strategies to use for second-line treatment for patients who fail responding to first-line therapy. Studies have shown a variety of possible options that vary in responses and toxicity, but these have so far not led to any really preferred second-line chemotherapy. In the Nordic NEC study, patients with a Ki67 >55% were those who benefited most from the suggested first-line chemotherapy [40, 99, 100]. Furthermore, the results suggested that patients with a Ki67 <55% may benefit from other types of medical treatments. Everolimus is an mTOR kinase inhibitor that has been used for treatment of NETs of well differentiated character and low proliferation (G1 and G2 tumors). It has been suggested to be a possible option for G3 patients with Ki67 index in the lower range. A study revealed that a cell line from a poorly differentiated G3 tumor showed expression of mutated mTOR pathway components and that everolimus reduced tumor volumes in animal experiments. This suggest that mTOR inhibition may be a valid option for G3 GEP-NEN patients that do not respond to first-line treatment [101].

Techniques

Immunohistochemistry (IHC)

One technique used for diagnosing and classifying GEP-NENs is immunohistochemistry (IHC). IHC is based on the process of binding between an antibody to its specific antigen where sensitivity and specificity are parameters sought out to be optimized for each antibody used.

IHC is widely used in diagnostics and has several advantages. It is a fast and rather robust technique that has proven to be easy to use in a clinical setting. Limitations of the technique are possible issues with antibodies, sometimes time-consuming validation and optimization of the antibody and risk of mistakes due to manual handling of several steps in staining protocols [102]. In histological examinations, the hematoxylin-eosin staining is a vital part of IHC evaluation of patient samples. This simple staining, that was introduced over a century ago, is a very good way of distinguishing cell types. Hematoxylin, oxidized into hematein, is a basic dye, and eosin is acidic. These different properties are useful in histology where for example extracellular matrix is eosinophilic and the nucleus is basophilic [103].

Another way of using IHC is through staining target proteins with antibodies. Antibodies may be monoclonal or polyclonal. Monoclonal antibodies are produced by one immune cell that produces identical antibodies that bind to one single epitope. These are usually commercially produced in mice via a process called the Köhler technique where mice are immunized with purified antigen to generate antibody production that is collected through the harvesting of B lymphocytes. B cells do not have the ability to survive in medium and are fused with myeloma cells to produce hybrids that later can be cultured to extract the desired antibodies [104, 105]. For polyclonal antibodies, rabbits are immunized with chosen antigen intravenously or subcutaneously. Blood is then drawn from the rabbit and from its serum the polyclonal antibody is purified [106].

There have been different detection systems used for IHC and one of them is the avidin-biotin complex (ABC) method. In the ABC method, a biotin conjugated secondary antibody binds to primary antibodies that are bound to targeted proteins on tissues. There is a major drawback with this method which is that the ABC complex also will bind to endogenous biotin in tissues and may result in unspecific background staining [107].

A recently developed system, EnVision, does not have this limitation. The EnVision system is based on blocking endogenous peroxidase activity after which specimens are incubated with the primary antibody. For detection, a labeled polymer, with up to 20 molecules of secondary antibodies is used to detect the primary antibody binding to the designated epitope in the tissue [108, 109].

In the final step, incubation with 3,3'-diaminobenzidine (DAB)+ substrate-chromogen is used, which results in a brown-colored precipitate on sites where primary antibody and antigen are bound which in turn makes this detectable using a light microscope.

Proximity extension assay (PEA)

Proximity extension assay (PEA) is a recently developed technique aiming to screen for proteins in serum or other body fluids. The technique is a further improvement of the technique that preceded it, proximity ligation assay (PLA). In PLA, DNA oligonucleotides are used to bind to different epitopes on specific targets [110, 111].

In the PLA method, probes are bound to oligonucleotides that are bound to streptavidin that in turn binds to biotinylated antibodies. In PEA, the oligonucleotides are bound directly on the 3' or 5' of streptavidin which allows for direct binding to biotinylated antibodies [112].

The PEA method is based on oligonucleotides (PEA probes) that are bound to paired antibodies. Upon antibody binding to targeted protein, the PEA probes are brought in close proximity of each other and hybridize. A DNA polymerization reaction starts with a new sequence that is unique for the targeted antigen. Detection is made either through direct antibody binding to target protein or through a secondary antibody attached to DNA [113] and detection of targeted proteins is obtained through qPCR [114]. This technique is quick and efficient in that it can test up to 96 proteins in one single sample of 1 μ l of body fluid.

Aims of the current investigation

The aims of the research in this thesis were:

- To investigate the immunohistochemical expression of p53 protein in tumor specimens from patients diagnosed with G3 GEP-NEN and the clinical relevance of this expression.
- To describe the expression of the immune checkpoint protein PD-L1 in G3 GEP-NENs.
- To identify potential new serum biomarkers in patients with pancreatic G3 NENs.
- To evaluate the effects on progression-free survival and overall survival in G3 GEP-NEN patients receiving etoposide as infusions compared to oral administrations.

Materials and methods

Patient and tumor characteristics (Paper I-IV)

The tumors tissues in Paper I, II and III were collected from the Nordic NEC study, which includes patients, diagnosed with G3 GEP-NEN with a primary tumor located in the gastrointestinal tract or a CUP.

Paper I included 124 G3 GEP-NEN patients treated with a platinum-based chemotherapy at the Nordic Centers, and diagnosed 1999-2011. Tumor specimens for IHC were obtained, based on availability, from the Nordic NEC study and clinical data was obtained from the registry of the Nordic NEC study [37]. Formalin-fixed paraffin-embedded (FFPE) material included: 40 needle biopsies, 20 surgical biopsies and 64 surgical specimens.

Exclusion criteria were Ki67 index $<20\%$ and the diagnosis of a mixed adenocarcinoma-NECs based on the WHO definition [110].

Patients were divided into groups depending on location of the primary tumor and included four tumors located in the esophagus, 11 in the stomach, 28 in the pancreas, 31 in colon and 17 in rectum. In 33 cases, the primary tumor was unknown.

Paper II included 136 patients from the Nordic NEC study and inclusion of patients was based on availability of tumor tissue. Tumor tissues were embedded in formalin-fixed paraffin. All the tumors were immunoreactive (IR) for CgA and Syn, Ki67 $> 20\%$ in all specimens. Clinical data was collected from the Nordic NEC study registry. All the patients were treated with chemotherapy and they were all metastatic at time of diagnosis, with liver and lymph node metastasis dominating at 64% and 59% respectively.

Paper III included patients diagnosed with a G3 pancreatic NEN. Serum from 42 patients and 42 healthy controls was collected and analyzed with the PEA technique through a commercially available platform from Olink Proteomics®. An immuno-oncology protein panel was chosen to be used for the serum samples (Olink® IMMUNO-ONCOLOGY). Statistical comparisons were made between the patients and healthy controls. Sixteen tumor specimens from patients diagnosed with G3 Pan-NENs were also included for IHC staining.

For **Paper IV**, 236 patients, diagnosed 1995-2012 were studied. Clinical data was obtained from the Nordic NEC registry [37]. The cohort included patients that received platinum-based combination (cisplatin/carboplatin + etoposide) chemotherapy; cisplatin was given as an infusion for all 236 patients. Etoposide was given as <5h infusion, 24h infusion, or O.E administration.

Immunohistochemistry (Paper I-III)

Paper I included 124 tumor specimens placed on Superfrost Plus glass (Menzel Gläser, Braunschweig, Germany). Tumor specimens were prepared by deparaffinization in alcohols of decreasing concentrations after which sections were treated in a pressure cooker with retrieval buffer Tris-HCl, pH 9.0 in 121°C. Following retrieval was incubation with a primary monoclonal antibody (anti-p53, clone DO-7, Dako, Glostrup, Denmark) at room temperature for 30 minutes (dilution 1:100). A polymer-detection system was used (EnVision Plus-HRP, Dako, Glostrup, Denmark) according to manufacturer's instructions. DAB was used as chromogen. Tissue specimen from adenocarcinoma was used as a positive control.

Paper II included 136 tumor specimens to be analyzed immunohistochemically. Tumor sections on glass slides (Superfrost Plus, Menzel Gläser, and Braunschweig, Germany) were baked overnight and stained with a commercially available PD-L1 antibody (PD-L1 IHC clone 22C3 pharm Dx, Agilent, USA). Staining was performed in an autostainer (Link 48 from Agilent Dako, Thermo Shanon LTD, United Kingdom) according to manufacturer instructions. The PD-L1 antibody kit included positive and negative controls in the form of two cell lines (NCI-H226 and MCF-7).

Paper III included 16 tumor specimens, cut and place on Superfrost Plus glass (Menzel Gläser, Braunschweig, Germany) that were collected according to availability. Tumors were deparaffinized in xylene, hydrated in graded alcohols and blocked for endogenous peroxidase in 0.3% hydrogen peroxide diluted in 95% ethanol. For antigen retrieval, a decloaking chamber (Biocare Medical, Walnut Creek, CA) was used. Tumor tissues were boiled in citrate buffer and then left to cool down before being stained in an Autostainer 480 instrument (Thermo Fischer Scientific, Waltham, MA). The polyclonal FasL antibody was manufactured by Human Protein Atlas (HPA054959, Atlas Antibodies, Stockholm, Sweden) and human tonsil tissues was used as a positive control.

Specimens were evaluated with the aid of experienced pathologist under a microscope (Axioskop 40, Zeiss, Germany) at 20x and 40x magnifications.

Statistical analysis (Paper I-IV)

Progression-free survival (PFS) was defined as the time between first treatment and first progression, and OS was defined as time from diagnosis of metastatic disease until date of death; or if event was not found, censored at date of last observation.

Kaplan-Meier plots were used for PFS and OS analysis, and the log-rank test was used to compare curves separated according to expression of p53 (**Paper I**) or according to type of etoposide administration (**Paper IV**).

In both papers, cox proportional regression was performed for the estimation of hazard ratios (HRs) and confidence intervals (CIs).

Spearman's rank correlation was used to assess correlations between variables in **Paper I**. Calculations were performed using IBM SPSS statistics software (v22/25, USA).

In **Paper II**, analysis was done with regards to PFS and OS. Correlations of PD-L1 expression to clinical variables was also an endpoint in this study and was analyzed through Chi-2 test (correlations of categorical variables) and correlations for continuous variables were analyzed with cox regression. PFS and OS were analyzed with the help of Kaplan-Meier analysis and Mann-Whitney test.

All statistical analyses were performed using IBM SPSS statistics software (v25, USA).

In **Paper III**, a statistical analysis package was performed by Olink Biostatistics. The aim with the statistical analysis was to identify differences in protein concentrations between patients and healthy controls (T-test). Furthermore, we sought to correlate levels of proteins to Ki67 index in patient samples (regression analysis). An ANOVA test was done to compare differences of serum proteins in different response groups (partial response, stable disease or progressed disease) and Kaplan-Meier analysis was performed to determine if any proteins were associated to survival.

All p-values were adjusted for multiple testing within each test using the Benjamini-Hochberg approach [115].

Results and Discussion

Expression of p53 in GEP-NENs (Paper I)

The aim of this paper was to investigate the expression of p53 protein in tumor samples obtained from G3 GEP-NENs. We evaluated all the specimens with regards to neuroendocrine differentiation. All the tumor specimens were positive for CgA and/or Syn verifying neuroendocrine differentiation. Furthermore, all specimens had Ki67 >20%, i.e., all tumors belonged to the G3 subgroup. Results from the immunohistochemical staining showed that 39% of the NEN patients had p53 IR tumors while 61% showed no immunoreactivity (Paper I, Table 1). Tumors that were IR for p53 expression showed a range of immunoreactivity between 20-100%. All tumor specimens included were confirmed to be poorly differentiated by an experienced endocrine pathologist.

Primary tumors were located in esophagus, stomach, pancreas, colon, rectum and 27% of the patients presented with CUP. Patients with colon primaries constituted the tumor group with most frequently p53 IR cells followed by pancreas, CUP, rectum, stomach and esophagus. In both the p53 IR and p53 non-IR groups, approximately 80% had distant metastases and Ki67 was >55% in a majority of patients in both groups. Two staining patterns were observed for IR tumors, but these showed no statistically relevant association to clinical parameters.

In the whole cohort, IR tumors with Ki67 index above 55% presented with higher frequency of IR cells and this was statistically significant (Paper I, Table 2). For patients with colorectal tumors, a positive correlation was found between p53 immunoreactivity and performance status, showing that those with p53 IR tumors had a poorer performance status compared to those with non-IR tumors. For patients with colorectal tumors and distant metastases, p53 immunoreactivity correlated negatively with treatment response. Spearman's correlations are presented in Paper I.

Patients with colorectal G3 NENs expressing p53 protein had a shorter PFS compared to patients with non-IR tumors. In the group of patients presenting with colorectal tumors with distant metastases, both PFS and OS were shorter for patients with tumors IR for p53 compared to non-IR tumors (Paper I, Table 2).

In the group of colorectal patients with distant metastases, these associations remained significant in multivariate analysis and a positive correlation was found between p53 immunoreactivity and disease control by chemotherapy (Paper I, Table 3).

These results show that colorectal NENs with distant metastases, expressing p53, had a significantly shorter PFS and OS, than those lacking p53 expression. Furthermore, the positive correlation between p53 expression and Ki67 indicates that p53 may be a marker for poorer prognosis [68].

We observed that p53 IR tumors exhibited two different staining patterns, one scattered pattern with up to 40% IR tumor cells and the second pattern was a more densely packed pattern with higher range of IR tumor cells (60-100%). Results similar to ours were seen in a study of p53 in gastric cancer and like in our cohort, no correlations were found between staining pattern and clinicopathological parameters [116]. An important fact is also, that although an expression of p53 suggest the presence of a *TP53* mutation, there are mutations in *TP53* that do not result in a stable, detectable protein [117].

TP53 mutations are common occurrences thought to have a role in NEN development [67, 68]. In a study done on ovarian carcinoma, the use of IHC to detect p53 mutation was successful in showing that it was a tool that may be used to stratify patients for treatment choices [70].

IHC may be a useful tool in detecting mutations in NENs. However, more studies are needed to ensure consensus on pathological evaluation and the clinical meaning of such mutations [118].

PD-L1 is rarely expressed in G3 GEP-NENs (Paper II)

In this study we aimed to examine the rate of PD-L1 protein expression in our cohort of G3 GEP-NENs. The study enrolled patients from whom tumor tissue was available from the Nordic NEC cohort and that resulted in 136 included patients. IHC with an antibody against PD-L1 was used to stain tumor tissues in an autostainer according to the manufacturer's instructions

Evaluation and annotation of the PD-L1 expression was done by an experienced pathologist. Tumor cells and immune cells were assessed for PD-L1 expression and immunoreactivity was defined as positive staining (>1%) in either tumor cells or immune cells or both.

In total, 14 out of 136 (10%) specimens were IR for PD-L1 either in tumor cells or immune cells, and there were no specimens in which immunoreactivity was seen in *both* tumor cells and immune cells.

Statistical analysis was performed to explore if PD-L1 expression correlated to any clinicopathological variables or other variables. The variables used for testing included sex, age, performance status, Ki67 and morphology of tumor. We could not find any statistical correlation between PD-L1 and any of the tested variables and immunoreactivity was not associated to a specific primary tumor site. Median PFS and OS were not statically different between the patients IR for PD-L1 (in tumor cells and immune cells) compared to non-IR patients. Furthermore, there was no difference in PFS and OS between patients whose tumors were IR compared to those where immunoreactivity was exclusively seen in immune cells. Survival data presented in Figure 3, Paper II.

In this cohort, we found that 10% of GEP-NENs expressed PD-L1 in either tumor cells or immune cells, with the latter being the dominant one. Studies on PD-L1 expression in GEP-NENs are scarce and often performed on small cohorts where all grades of GEP-NENs are included (G1, G2 and G3). Studies have reported immunoreactivity in 30% of the patients and presence of immunoreactivity has been correlated to higher grade and poorer PFS and OS [119, 120]. Treatment with chemotherapy is a factor that can affect the PD-L1 expression and hence effect conclusions drawn from studies done on a mix of patients treated differently or untreated. Cisplatin has been reported to result in overexpression of PD1 and PD-L1 in hepatoma H22 cells when administered below IC₅₀ [121].

In our study, there was no correlation between PD-L1 expression and clinical aspects. The tumors where PD-L1 was expressed in tumor cells were predominately from colon primaries, which may suggest that PD-L1 may be expressed by the more aggressive subtype of G3 GEP-NENs, but our data could not confirm this to be statistically significant.

The lack of statically significant correlation (which may be due to the low number of IR samples) and the lack of consensus on whether tumor cells and/or immune cells should be taken into consideration when assessing the clinical importance of PD-L1 expression, makes it difficult to understand the true clinical role of PD-L1 in GEP-NENs. In conclusion, more prospective studies with larger and more homogeneous cohorts are needed to fully understand if PD-L1 expression has a clinical role in GEP-NENs [122].

Serum biomarkers for Pancreatic G3 GEP-NENs (Paper III)

In this study we aimed to search for possible biomarkers in serum from patients diagnosed with pancreatic G3 GEP-NENs. We included serum from 42 patients and 42 healthy controls and they were tested for 96 immuno-oncological proteins. The panel was used to measure the relative serum concentrations of these 96 proteins in patients and healthy controls. In total, 79 out of the 84 serum samples were analyzed (five samples did not meet the quality control criteria). Eighty-seven proteins out of 96 were analyzed since nine proteins did not reach the detectability limit of 15%.

There was a large number of proteins (62%) that differed in measurable levels between patient's serum samples and samples from the healthy controls. All these proteins, except one (FasL) had significantly higher concentrations in patients compared to healthy controls (adjusted p-value <0.05). There was an observed negative association for six proteins to Ki67 index. The higher Ki67 index, the lower concentrations of these six proteins was detected (unadjusted p-value <0.05). A similar correlation was seen with other proteins that seemed to differ between patients when comparing different response groups (response according to RECIST criteria), unadjusted p-value <0.05. There were no proteins that were associated to survival in this group of patients with pancreatic G3 GEP-NENs. For visual representation and charts, see Paper IV.

FasL was the only protein that had lower levels in patients than in the control samples. Immunohistochemical staining for FasL was performed with a commercially available antibody, HPA054959, from Atlas Antibodies. Sixteen tumor specimens were stained and evaluated with regards to immunoreactivity to FasL. Two tumor samples were excluded due to poor quality. Out of 14 tumor samples, ten had poorly differentiated morphology and four had well differentiated morphology.

Immunoreactivity for FasL was present in eight tumor specimens while six did not exhibit immunoreactivity, see representative figures of the immunostainings in Paper IV.

In total, 54 out of 87 proteins differed between patients and controls with a very strong statistical significance. These proteins may be interesting and important for further studies but in this small cohort there was no protein that emerged with regards to its clinical relevance. One protein sparked a special interest. The FasL protein was the only protein that was present in higher levels in serum of healthy controls compared to the patient serum. FasL is a protein expressed on the surface of cells that belong to the tumor necrosis factor (TNF) family of proteins, which binds to the TNF receptor Fas on different cells and tissues [123]. FasL is one of the key components seen on cytotoxic

T lymphocytes [124, 125] and it is predominantly seen on activated T lymphocytes and natural killer cells [78]. When FasL binds to its receptor it triggers death of various cells and is very important in cancer cell immunity as the interaction seems to have both tumorigenic and tumor suppressive roles [124, 126, 127]. There is a theory of tumor cells developing a counter attack mechanism through expressing FasL on their cell surfaces to escape the immune system [128]. Such a mechanism has been reported in a study on ovarian carcinomas [129]. In our cohort, FasL correlated negatively to higher Ki67 index and was also present in significantly higher levels in healthy controls compared to patients. The clinical relevance of FasL in G3 PanNENs is yet to be fully understood.

Chemokine (c-c motif) ligand 4 (CCL4) and interleukin 8 (IL8) were two other proteins of interest that correlated to response, both proteins were present in significantly higher levels in patients with progressive disease. This is interesting since elevated serum concentration of IL8 has been seen to have a direct link to disease progression in melanoma, colon and ovarian cancer [130]. In other studies, elevated serum IL8 was seen to alter composition of immune cells in the tumor microenvironment [131] and elevated CCL4 in serum from patients with colon cancer was seen to induce pro-tumor macrophages which in turn help tumors to escape the immune system [132].

In conclusion, we found many proteins that are of potential interest from a clinical as well as pathophysiological point of view. However, the complexity of the expression of these proteins needs more studies to determine each protein's clinical relevance for these pancreatic G3 GEP-NENs.

Etoposide: efficacy unaffected by administration route (Paper IV)

In this study, we aimed to evaluate whether the route by which etoposide is administered to patients with G3 GEP-NENs could have an effect on the efficacy of the treatment. We included 236 patients in a retrospective manner, gathered data on their disease progression and survival, and correlated this to the administration route by which etoposide was given to the patients. The route of administration as well as the duration of infusion was different at different departments included in the Nordic NEC study.

The 236 patients were divided according to which etoposide formulation that was given. The largest group received etoposide as long-time infusions, 24h (n=170) while 33 patients were given short-time infusions (≥ 5 h) or O.E., respectively. Ki67 was above 55% in more than half of the included patients (54%). All included patients died due to their malignant disease.

PFS and OS showed no differences between the three groups. To assess the effect of administration on survival, cox regression models for univariate and multivariate analyses was used. We found no differences in progression and OS on the basis of administration when comparing outcome in the group given oral administration to the groups with short- and long-term infusion.

In this study, the main finding is that there were no statistically significant differences between the three administration groups, which suggest that none of these administration routes is superior to another.

Some studies suggest that O.E. is better or at least equally effective as IV etoposide and some studies report the opposite. O.E. given to patients with ovarian cancer and prostate cancer as second-line treatment, showed the same or better efficacy and safety when compared to IV administration. As an example, in a study including patients with castration resistant prostate cancer, the efficacy, compliance and safety profile did not differ between etoposide given IV versus orally [133]. These results suggest, in accordance with our results, that O.E. may be a valuable option to consider when treating patients with etoposide.

A study on patients with non-SCLC demonstrated that the safety profile for O.E. is significantly better compared to that of IV etoposide. In that cohort, there was a significantly higher need for hospitalization due to neutropenia in the IV group compared to the oral group [134].

Etoposide has been reported to have a higher intra-patient and inter-patient variability when given orally compared with IV formulations [98]. These observed variabilities may not have a clinical relevance but drug-drug interactions are a real concern for O.E. An example is the interaction with the commonly used antifungal agent, ketoconazole, resulting in an increase of the etoposide concentration systemically [135].

Concerns aside, O.E. is a preferred option for patients when they are asked (89% preferring O.E. over IV administration was reported in a study from 1997) and this is a trend that has continued for almost two decades (an 85% preference for O.E. was reported in a meta-analysis in 2016) [136, 137]. Thus, we suggest that prospective studies should be performed to evaluate the efficacy and safety of O.E. in comparison with IV administration along with patient reported quality-of-life.

Major Findings

In this thesis, our aim was to explore the complexity of G3 GEP-NENs. We performed studies with the goal to better understand the disease and to identify possible important biomarkers that can be of diagnostic and prognostic value. We also evaluated whether the route of administration of etoposide had an impact on PFS and OS.

One major finding was the correlation of the expression of p53 protein to poorer PFS and survival for patients with a colorectal G3 GEP-NEN. This finding could be useful for more careful monitoring of these patients.

In contrast to this, we were not able to establish a correlation between PD-L1 expression and any clinical parameters in our cohort. Immune checkpoint inhibitors have become a hot topic in oncology in recent years and investigating the expression of PD-L1 may be important to explore if new treatment options should be considered.

There are very few clinically relevant biomarkers for G3 EP-NEN patients that can be measured in blood and used to monitor the disease. In our search for such biomarkers we focused on G3 GEP-NENs from the pancreas. We performed protein analysis of serum from patients and compared these with protein analysis from healthy controls. A large subset of proteins were higher in serum concentration from patients compared to healthy controls. FasL was the one protein that was present in higher concentration in healthy controls. This protein is involved in tumor necrosis and apoptosis and may have a protective role. However, further studies are needed to understand the importance of this protein in pancreatic NENs.

In these three studies, our attempt was to analyze the expression of a number of proteins in tumor specimens from G3 GEP-NEN patients in order to find new diagnostic and prognostic markers. In Figure 4, a tumor sample from one patient with a pancreatic G3 NEN is shown, stained for the three proteins included in this thesis. In summary, we found that p53 expression may indicate a worse prognosis, while the importance of PD-L1 and FasL expression still remains to be elucidated.

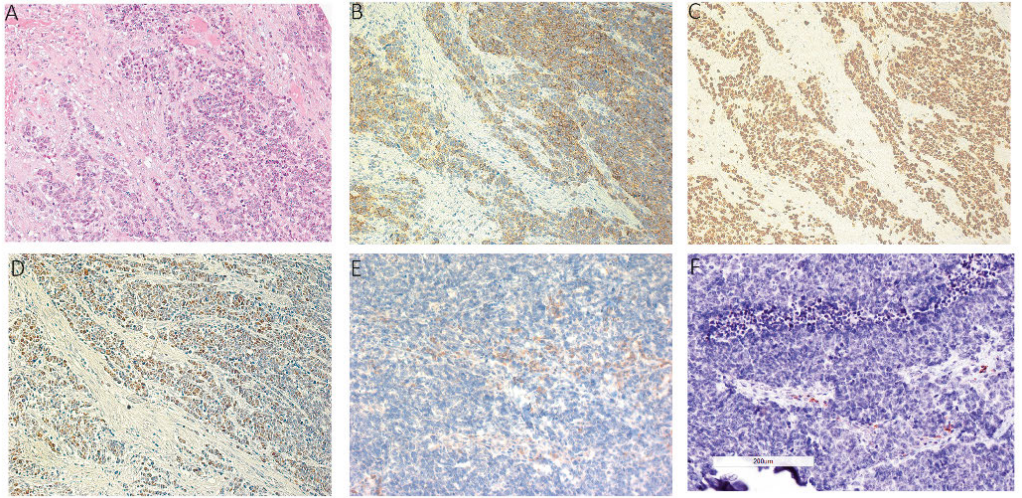


Figure 4. Stainings from a pancreatic G3 GEP-NEN. **A:** Hematoxylin &Eosin, **B:** CgA, **C:** Ki67, **D:** p53 **E:** PD-L1 and **F:** FasL, *10x magnification*.

The route of administration of etoposide did not seem to have an impact on how the patients survived, or how they responded to the chemotherapy. These results suggest that giving patients oral formulation of etoposide may be a viable option that increases the quality of life and decreases adverse events and hospital related costs as compared to IV formulations.

Future perspectives

The thesis has provided us with answers on the significance of expression of p53 in G3 GEP-NENs that probably is the result of mutations in the TP53 gene. This finding is very interesting and should be studied further in larger, prospective studies where genetic profiling, through sequencing of TP53 to identify mutations, could be correlated to protein expression in tumor tissue to fully understand the mechanism of these mutations in G3 GEP-NENs.

In the study of pancreatic G3 NENs, we were able to demonstrate many proteins that differ in serum levels between healthy controls and patients. We are planning to evaluate more of these proteins to fully understand their potential clinical relevance and whether they may have a diagnostic or prognostic value in pancreatic G3 NENs.

Different administration routes of etoposide did not affect the PFS or OS of patients in our cohort. This was a study done with solely clinical information and therefore it would be of interest to look at this phenomena with regards to blood concentrations of etoposide following different administration schedules as well. For this purpose, a prospective study would be used, to evaluate and compare the adverse events profiles of each administration route to see if there is any difference in response and tolerability which can be coupled to serum concentrations of etoposide. Furthermore, patient reported quality-of-life should be evaluated in such a study.

Summary of the Thesis in Swedish

Populärvetenskaplig sammanfattning på svenska

Neuroendokrina neoplasier (NENs) är ovanliga solida tumörer som uppstår från neuroendokrina celler som finns på många platser i kroppen och kan drabba organ som bland annat lunga, matstrupe, magsäck och tarmar. NEN kännetecknas av att de kan producera hormoner och ibland kan de ge fysiologiska problem som är kopplade till överproduktion av ett visst hormon. Diagnos av NENs ställs via en teknik som kallas för immunhistokemi där man använder antikroppar för olika proteiner som man färgar tumörvävnaden med. Kromogranin A och synaptofysin är två proteiner som används som biomarkörer för att säkerställa att det är en tumör med neuroendokrin karaktär. NENs delas även in i tre grupper baserat på deras tillväxthastighet. Ki67 är ett protein som finns i delande cellkärnor och går att upptäcka via immunhistokemi. Antikroppar mot detta protein används för att färga tumörvävnader tagna från patienter för att avgöra hur snabbt tumören växer. Ki67 används för att se vilken grupp tumören tillhör enligt World Health Organisation (WHO)-klassifikationen. De tre grupperna är baserade på gränsvärden för Ki67 som är framtagna av WHO där G3 gruppen är definierad som NEN med ett Ki67 index på >20%.

Gastroenteropankreatiska NEN (GEP-NEN) är ett samlingsbegrepp för de tumörer som drabbar mag-tarm kanalen och bukspottkörteln.

Behandling av dessa tumörer beror på vilken grupp de tillhör. Denna avhandling berör GEP-NEN som tillhör grupp 3 (G3 GEP-NENs) där behandling i första hand är en kombination av cisplatin/carboplatin och etoposid.

Syftet med de fyra delarbetena i denna avhandling är att få en bättre förståelse för G3 GEP-NENs med avseende på biomarkörer och behandling och korrelera detta till tumörens progression och patientens överlevnad.

Delarbete 1

I första delarbetet använde vi tumörvävnad från 125 patienter som samlats in i ett samarbete mellan de nordiska länderna. Dessa var retroaktivt insamlade och skickades till vårt laboratorium i Uppsala. Immunhistokemi användes för att färga tumörcellerna för ett protein, p53, som är känt att vara involverad i progression av olika typer av cancer. Av de 125 tumörvävnaderna som ingick i studien kunde vi visa att 39 % av dem hade en positiv färgning för p53 proteinet. Denna positiva färgning kan betyda att det finns ett muterat p53 protein

i tumörerna. De patienter som hade tumörer i tjocktarmen och svarade signifikant sämre på cytostatikabehandling och en sämre överlevnad.

Sammanfattningsvis kan vi från detta arbete dra slutsatsen att patienter med ökad infärgning för p53 protein i deras tumörer har en sämre prognos och sämre överlevnad än patienter i samma grupp utan en ökad infärgning för p53 i tumören.

Delarbete 2

I detta delarbete undersökte vi proteinuttrycket av PD-L1 i 136 G3 GEP-NEN tumörer. PD-L1 är ett protein som används som markör för immunoterapi vid olika typer av cancer. PD-L1-uttrycket undersöktes via immunhistokemi och i vårt material var 10 % av tumörvävnaderna positiva för PD-L1, antingen i tumörcellerna eller i immuncellerna i tumörstromat.

PD-L1-positivitet var inte korrelerat till några kliniska parametrar. Sammanfattningsvis behövs flera studier för att förstå om PD-L1 har en klinisk roll i G3 GEP-NENs och vad betydelsen av dess uttryck skulle vara.

Delarbete 3

I detta delarbete var syftet att screena proteiner i serum från patienter med G3 pankreas NENs. Serum från 42 patienter och 42 friska kontroller analyserades för förekomst av 87 olika proteiner via ett chip som framställts hos Olink Proteomics AB i Uppsala. Vi fann att 54 av 87 proteiner skiljde sig signifikant mellan de sjuka och de friska kontrollerna. Dessa proteiner är involverade i inflammation och cancerutveckling. Inga proteiners uttryck kunde korreleras till överlevnad men 6 proteiner korrelerade till hur patienten svarade på kemo-terapi.

Sammanfattningsvis fann vi i detta arbete många proteiner som skilde sig mellan G3 pankreas NENs och friska kontroller. Alla dessa proteiner kan ha en roll i tumörutvecklingen och det är därför viktigt med fortsatta studier för att försöka förstå deras funktion i pankreas G3 NENs.

Delarbete 4

I delarbete 4 undersökte vi behandlingen av patienter med G3 GEP-NENs. Dessa patienter behandlas i första hand med kemoterapi i en kombination av cisplatin/carboplatin och etoposid. Cisplatin/carboplatin ges intravenöst (IV) till patienterna medan etoposid kan ges antingen IV eller oralt. Vårt syfte var att undersöka om etoposid givet oralt hade samma effektivitet för patienterna som om det gavs IV. Vi inkluderade data från 136 patienter, alla behandlade med cisplatin/carboplatin och etoposid i de nordiska länderna. Administrationen delades upp i tre grupper, IV 24h, IV \geq 5h och oralt etoposid.

Vi undersökte om tiden till progression under behandlingen och överlevnaden skilde sig åt mellan de tre grupperna. Vi fann vi inga skillnader i effektivitet mellan de olika beredningsformerna som gavs till patienterna.

Sammanfattningsvis har vi visat att oralt administrerat etoposid verkar vara lika effektivt som etoposid givet IV. Detta ger patienten en bättre livskvalité och kan minska sjukhuskostnaderna som uppkommer när patienten måste läggas in på sjukhuset för intravenös administrering av läkemedlet.

Slutsats

Denna avhandling innehåller studier som leder till ökad förståelse för G3 GEP-NENs. Vi påvisar att ökat uttryck av p53 korrelerar till sämre prognos för patienter med GEP-NEN i tjocktarmen och ändtarmen och att 10 % av G3 GEP-NENs uttrycker PD-L1.

Vi har hittat 54 proteiner som skiljer sig mellan patienter med pankreas G3 GEP-NEN och friska kontroller. Några av dessa proteiner kan visa sig ha en diagnostisk eller prognostisk roll för GEP-NEN och fortsatta studier för att kartlägga detta är nödvändiga. Vidare har vi kunnat visa att oral administrering av etoposid är lika effektiv som intravenös samtidigt som den är mer bekväm och önskad av patienter.

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