Personalised Medicine Strategies For Patients With Neuroendocrine Tumours

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PERSONALISED MEDICINE STRATEGIES
FOR PATIENTS WITH NEUROENDOCRINE TUMOURS

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This dissertation is submitted for the degree of MD RES
April 2019
DECLARATION

This dissertation is the result of my own work and includes nothing, which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

Signed:

Date: 1st December 2018

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MB BCHir BA(Hons) MA MBA MRCP (Gastroenterology)
London
ABSTRACT

Personalised medicine strategies individually tailor the healthcare that a patient might receive on the basis of clinical and other diagnostic information. Neuroendocrine neoplasms (NENs), previously described as neuroendocrine tumours, commonly arise in the gastrointestinal tract or pancreas and are heterogeneous in nature, something that favours an individualised approach to healthcare from the onset of symptoms through to diagnosis and choice of therapies. The thesis explored clinical management, symptom patterns, screening programmes, as well as biomarkers for carcinoid heart disease and pancreatic NENs.

Analysis of a patient survey demonstrated that the median duration from the time of first symptoms to diagnosis was 36 months for small bowel NENs and 24 months for pancreatic NENs. Common first symptoms were pain (36%), flushing (24%) and diarrhoea (24%). 29% of small bowel NEN respondents were given an initial diagnosis of irritable bowel syndrome.

Rectal NENs are increasingly diagnosed at endoscopy with the majority (80-90%) being small and localised to the submucosa. Metastatic disease is infrequent (<20%) with risk factors including size, atypical appearance, grade, and depth of invasion. The primary resection modality influences complete resection rates and the need for secondary therapy. Lesions that are at higher risk of invasion and metastasis require a thorough diagnostic work up. Device-assisted endoscopic mucosal resection and endoscopic submucosal dissection are used to resect localised rectal NENs. The treatment of advanced disease is multimodal.

Data from the English bowel cancer-screening programme (BCSP), a double screening programme of faecal occult blood testing (FOBT) and colonoscopy, were analysed for NEN diagnoses. The incidence rates per 100,000 colonoscopies of NENs by anatomical site was 29 for rectal, 18 for colonic and 11 for ileal. The majority of rectal NENs had grade 1 (80%) and stage T1 (85%) disease. Over half of ileal NENs (54%) in this study had T3/4 invasive disease, with 85% having nodal and 36% having metastastatic disease.

Putative carcinoid heart disease (CHD) markers were studied in comparison to the recommended NT-proBNP in cohorts of patients with CHD, functional and non-
functional sbNENs. Calprotectin was elevated across all three groups while the remaining markers ST2, GAL3 and adrenomedullin were not elevated. NT-proBNP was significantly different in the CHD cohort when compared to the functional and non-functional groups. This supports the consensus guidance for NT-proBNP in assessing advanced CHD.

Quantitative proteomic studies of fresh frozen G1 pancreatic NEN (pNEN) tissue were performed. 187 significant proteins mapped to cancer pathways, in particular RAS and PI3K-Akt signalling pathways. Ten proteins of interest were identified that may be involved in cancer development; Neudesin, Tenascin-X, Actin-related protein 3, Fibulin-1, Moesin, Secretogranin-2, CD63 antigen, tropomyosin 3, 14-3-3 protein beta/alpha and Calnexin.

These results have added to our knowledge of symptoms that differentiate NEN patients from functional conditions, individualising rectal NEN patient care, NENs identified through BCSPs, NT-proBNP for screening for CHD and proteins of interest for future study in pNENs. These span the domains of personalised medicine from symptom onset through to diagnostic and biomarker strategies.
ACKNOWLEDGEMENTS

I would like to thank Dr. Raj Srirajaskanthan for giving me the opportunity to undertake this research. I am grateful for his supervision over the years and his encouragement to explore the different research themes. He helped reignite my interest in basic science and research in general.

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I am indebted to Catherine O’Donnell for her support and organisational skills that were crucial in the set up and data collection phases of the bowel cancer screening research.

I would like to thank the NET Patient Foundation for their support with the patient survey and for sharing the research details with their network, supporters and patients.
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PEER REVIEW PUBLICATIONS

Directly arising from research


Other peer review publications arising from thesis


Oral presentations


Abstracts


Grants

Towards an earlier diagnosis of Neuroendocrine Tumours (NETs): Does Faecal occult blood testing identify ileo-colonic NET in the NHS Bowel Cancer Screening Programme (BCSP). Co-investigator: UKINETS TransNETS grant (2013).
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADM / AdrenoM</td>
<td>Adrenomedullin</td>
</tr>
<tr>
<td>BCSP</td>
<td>Bowel cancer screening programme</td>
</tr>
<tr>
<td>CHD</td>
<td>Carcinoid Heart Disease</td>
</tr>
<tr>
<td>CS</td>
<td>Carcinoid syndrome</td>
</tr>
<tr>
<td>FOBT</td>
<td>Faecal occult blood test</td>
</tr>
<tr>
<td>Gal-3 / GAL3</td>
<td>Galectin-3</td>
</tr>
<tr>
<td>GEP-NET</td>
<td>Gastroenteropancreatic neuroendocrine tumour</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>NEN</td>
<td>Neuroendocrine neoplasm (previously known as NET)</td>
</tr>
<tr>
<td>NET</td>
<td>Neuroendocrine tumour (now described as NEN)</td>
</tr>
<tr>
<td>NTP / NT-proBNP</td>
<td>N-terminal pro b-type natriuretic peptide</td>
</tr>
<tr>
<td>pNEN</td>
<td>Pancreatic neuroendocrine neoplasm</td>
</tr>
<tr>
<td>sbNEN</td>
<td>Small bowel neuroendocrine neoplasm</td>
</tr>
<tr>
<td>ST2</td>
<td>Suppression of tumorigenicity 2</td>
</tr>
<tr>
<td>TMT</td>
<td>Tandem Mass Tag</td>
</tr>
</tbody>
</table>
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1 INTRODUCTION

The concept of personalised medicine relates to the ‘customization of healthcare, with decisions and practices being tailored to the individual patient by use of genetic or other information’ (Redekop and Mladsi, 2013). Strategies focused at individualizing and improving the care of neuroendocrine neoplasm patients need to focus on all disease stages; from screening and earlier diagnosis through to personalised therapies and improving disease symptom control.

1.1 Neuroendocrine Neoplasms (NENs)

Neuroendocrine neoplasms (NENs), previously described as neuroendocrine tumours, are rare heterogeneous tumours that most commonly affect the gastroenteropancreatic tract (GEP) (Niederle et al., 2016, Delle Fave et al., 2016, Falconi et al., 2016). The incidence of all NENs is approximately 3-5 per 100 000 population per year but with a higher prevalence rate of 35 per 100 000 population because of the slow tumour growth associated with many NENs (Yao et al., 2008, Modlin et al., 2003, Dasari et al., 2017). All gastrointestinal NENs (apart from appendiceal) have increased in incidence over the last thirty years from analysis of the Surveillance, Epidemiology and End Results (SEER) database (Tsikitis et al., 2012, Modlin et al., 2008). Patients commonly present with advanced disease as many GEP NENs are indolent, slow growing malignancies. The prognosis is dependent on grade and stage as well as additional tumour related factors (Modlin et al., 2008).
Clinicians have a range of diagnostic tools, such as blood testing or ultrasound imaging, that are of value in advanced disease but of limited value in diagnosing early and localised NENs. The development of GEP malignancy screening tools, such as blood panels or biomarkers, which integrate a NEN specific panel may help with the earlier identification of patients that require further assessment and differentiate them from other patients with benign pathologies. However, at the present time there is no validated diagnostic tool for identifying patients who may have a NEN.

Personalising therapies in GEP NEN patients requires detailed knowledge of tumour biology to predict prognosis and to direct the therapeutic strategy over what can be a long disease history. Fundamentally, an understanding of the cancer pathways is needed at a molecular level to characterise the dominant pathogenic drivers and therapeutic targets.

1.2 Symptoms of neuroendocrine neoplasms

There may be a significant delay between onset of symptoms and diagnosis of between 5-7 years (Modlin et al., 2005, Singh et al., 2017). The duration from onset of symptoms to diagnosis varies considerably, from a few months for high grade (G3) NENs to years for low grade (G1) NENs (Ter-Minassian et al., 2013). Diarrhoea and flushing are known NEN-related symptoms but others, like fatigue and pain, are also reported. Patients may have hepatic metastases at diagnosis that can be ‘functionally’ active, secreting vasoactive substances or hormones that can cause systemic symptoms. Others may be diagnosed with advanced disease, including metastases, with minimal symptoms and no clear onset. The insidious nature of some GEP-NENs may cloud the development of worrying symptoms like a change in bowel habit and weight-loss.

1.2.1.1 Small Bowel NENs

Frequently reported symptoms from sbNENs are of abdominal pain and the classical carcinoid syndrome of diarrhoea, flushing, and palpitations, which develops in approximately a third of patients in the context of serotonin-secreting liver metastases (Halperin et al., 2017). Patients may experience abdominal pain from intermittent bowel obstruction, either from the intraluminal mechanical effect of the primary tumour or from extrinsic compression from mesenteric lymph node involvement and secondary desmoplasia, as well as from bowel ischaemia due to vessel involvement (Eckhauser et
Patients with carcinoid syndrome may develop right heart failure from carcinoid heart disease caused by fibrosis of the tricuspid and pulmonary valves (Lundin et al., 1988, Robiolio et al., 1995, Davar et al., 2017).

1.2.1.2 Pancreatic NENs

Less than half of pancreatic NENs release bioactive substances that can lead to symptoms, like hypoglycaemia with an insulinoma (Yao et al., 2008, Dasari et al., 2017). Non-functional pancreatic NENs are often incidental findings on cross-sectional imaging but a proportion of patients present with symptoms from mass effect, such as biliary obstruction, or from metastatic disease (Kulke et al., 2011).

1.2.1.3 Overlap with IBS symptoms

Patients with NENs complain of symptoms, such as loose bowel motions, that may be mistaken for other conditions like Irritable Bowel Syndrome (IBS). NEN patients anecdotally report that they describe florid symptoms to healthcare practitioners but are inappropriately reassured and managed with an IBS diagnostic label. There are no clear data if the symptoms described by NEN patients overlap with those of benign conditions like Irritable Bowel Syndrome (IBS) or if differentiating symptoms exist. It is also not clear how long symptomatic NEN patients are investigated and managed before their diagnosis by healthcare practitioners in both primary and secondary care and whether this contributes to an avoidable delay. Differentiating symptoms of NEN disease from more benign conditions remains key to an earlier diagnosis when therapeutic options are more likely to be curative in nature.

1.3 Screening for ileo-colonic neuroendocrine neoplasms

NENs affect the whole gastrointestinal tract with the SEER data highlighting the incidence of colorectal, appendiceal or small bowel NENs that may be diagnosed through colonoscopy (Tsikitis et al., 2012, Modlin et al., 2008). There are no age specific incidence data from the SEER database and the categorisation includes NENs that would not be visualised with colonoscopy, such as proximal small bowel NENs. However, it provides a guide as to the population incidence of ileo-colonic NENs.
1.3.1.1 Incidence of ileo-colonic and appendiceal NENs

The incidence in the SEER database is approximately 1 per 100,000 population per year for both rectal and small bowel NENs (Modlin et al., 2003). NENs of the rectum and small bowel represent 34% of all diagnosed NENs. Primary NENs from other colonic sites are of much lower incidence with little clinical data available (Modlin et al., 2008). The incidence of rectal NENs has increased rapidly to 17% of all NENs (Modlin et al., 2003, Tsikitis et al., 2012, Taghavi et al., 2013, Lawrence et al., 2011a). The rising incidence in rectal NENs when compared to small bowel NENs is likely to be multifactorial, most likely relating to improved endoscopic lesion recognition and reporting than a true rapid increase in incidence in the population. Over the last 20 years there has been improved endoscopic lesion characterisation, wider access to routine colonoscopy and colorectal cancer screening programmes as well as more robust coding in cancer and histological registries.

<table>
<thead>
<tr>
<th>Site</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small Intestine</td>
<td>0.9</td>
</tr>
<tr>
<td>Appendix</td>
<td>0.2</td>
</tr>
<tr>
<td>Colorectal</td>
<td>1.3</td>
</tr>
<tr>
<td>Caecum</td>
<td>0.2</td>
</tr>
<tr>
<td>Sigmoid</td>
<td>0.1</td>
</tr>
<tr>
<td>Rectosigmoid</td>
<td>0.1</td>
</tr>
<tr>
<td>Rectum</td>
<td>0.9</td>
</tr>
<tr>
<td>Ileo-colonic NET</td>
<td>2.4</td>
</tr>
<tr>
<td>(Incl. Appendix)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1-1. Incidence of ileo-colonic and appendiceal NENs from the SEER database

*Incidence is per 100,000 population per year from the Surveillance, Epidemiology and End Results (SEER) database (Modlin et al., 2008).*

1.3.1.2 Colorectal cancer screening programmes

Colorectal cancer represents 10% of the total cancer burden and is the second most common cancer in women after breast and the third most in men after lung and prostate cancer (Valle et al., 2015). It is the second most common cause of cancer deaths in UK, Germany, Japan, Australia, US and Canada (Honein-Abouhaidar et al., 2014). There are a number of different bowel cancer screening programme (BCSP) strategies that use single or double screening methods. A double screening BCSP strategy uses primary
screening faecal occult blood testing (FOBT) and invitations to attend for secondary screening colonoscopy if abnormal. Gastrointestinal lesions like colorectal cancer can cause occult blood loss that is detected with either guaiac or immunochemical FOBT. Screening with colonoscopy in those with abnormal guaiac FOBT results has been reported to reduce CRC mortality by 25% in targeted populations that complete screening (Mandel et al., 1993, Hardcastle et al., 1996, Kronborg et al., 1996, Brethauer, 2010). A single screening BCSP invites an age specific population to attend directly for endoscopy. There are limited data on rectal NENs diagnosed in bowel cancer screening programmes. Rectal NENs identified are smaller and at an earlier stage than those diagnosed from non-screening endoscopy (Kaminski et al., 2007b, Scherubl, 2009, Matsui et al., 1993). They are invariably 10mm or less, and of grade 1. There are no data on colonic or ileal NENs diagnosed through bowel cancer screening programmes.

1.4 Screening for Carcinoid Heart Disease

Carcinoid syndrome (CS) and carcinoid heart disease (CHD) both develop as a result of circulating vasoactive products (mainly serotonin) secreted by ‘functional’ metastatic small bowel NENs. Approximately a third of sbNEN patients with CS will develop CHD that results from fibrotic damage to predominantly the tricuspid and pulmonary valves that can lead to right heart failure (Fox and Khattar, 2004, Davar et al., 2017). Moreover, the development of CHD and ventricular dysfunction is associated with a markedly worse prognosis. Patients with carcinoid heart disease have a 3-year survival of 31% compared to 68% for patients without cardiac disease (Pellikka et al., 1993). Mortality from cardiac decompensation in untreated patients is high at 43% (Grozinsky-Glasberg et al., 2015). However, the prognosis has improved in recent years through advances in cardiac imaging, cardiothoracic surgery and therapies for functional sbNENs.

1.4.1.1 Serotonin and the development of CHD

Gastrointestinal NENs of an enterochromaffin (EC) or enterochromaffin-like (ECL) origin predominantly secrete serotonin (5-hydoxytryptamine, 5-HT) as well as tachykinins, kallikrein and prostaglandins. The liver normally inactivates products that are secreted into the mesenteric and portal venous systems from primary sbNENs. Carcinoid syndrome and CHD can develop if vasoactive products enter the systemic circulation via the hepatic veins and inferior vena cava when hepatic degradation of vasoactive products is reduced and overwhelmed. This commonly occurs in the presence
of a significant volume of hepatic metastases, particularly with a bilobar distribution or a dominant large metastasis. 30-40% of patients with hepatic metastases develop classical CS (Grozinsky-Glasberg et al., 2015, Kvols, 1994). Once secretory products enter the systemic venous circulation they pass through the right side of the heart and into the pulmonary arterial circulation where they are inactivated in the lungs. Rarely, a patent foramen ovale (PFO) may be present that allows right to left cardiac shunting of blood and secretory products to the left cardiac chambers and valves, as well as systemic arterial circulation. In these instances, the aortic and mitral valves may be affected by CHD. Less common routes for developing CS, without the presence of hepatic metastases and that bypass hepatic degradation, occur in extensive retroperitoneal NET disease through retroperitoneal venous collaterals and lymphatic drainage to the thoracic duct.

The role of 5-HT in the development of CHD is based on evidence ranging from cellular models through to associations with pharmacological agents. In cell cultures, serotonin is thought to cause cell division of smooth muscle cells, fibroblasts, endothelial cells and mesangial cells (Takuwa et al., 1989, Seuwen et al., 1988, Pakala et al., 1994, Nemecek et al., 1986). However, its role in cell division through mitogenesis is still debated (Ruiz-Perez et al., 2011). The treatment of Parkinson’s disease, obesity and migraine with serotonergic drugs has been associated with the development of valvular fibrosis (Feldman, 1987, Khan et al., 1998). Repeated stimulation of the 5HT2B receptor results in uncontrolled valve cell division (Fitzgerald et al., 2000, Rothman et al., 2000). TGF-β is up regulated via 5-HT2 G-protein receptor signal transduction and stimulates collagen synthesis in sheep heart valve interstitial cells (Jian et al., 2002, Xu et al., 2002). Valvular dysfunction is caused by carcinoid plaques consisting of both cellular proliferation and extracellular deposition of collagen, myxoid substances and elastin on leaflets and endocardial surfaces (Simula et al., 2002).

1.4.1.2 Diagnosis of CHD
The diagnosis of carcinoid heart disease is from transthoracic echocardiography (TTE), although other modalities like cardiac magnetic resonance are useful (Pellikka et al., 1993, Fox and Khattar, 2004, Moerman et al., 2012). The tricuspid valve, the closest to the hepatic venous outflow of secreted products from liver metastases, is affected in 97% of patients with CHD with the pulmonary valve and left sided cardiac valves affected in 88% and 7% respectively (Pellikka et al., 1993). Pathognomic echocardiographic features
of the tricuspid valve in CHD are ‘thickened, shortened, retracted and hypomobile leaflets’, particularly of the septal and anterior leaflets, resulting in regurgitation that can be moderate to severe in 90% of patients. In contrast the pulmonary valve in CHD can develop regurgitation (81%) and stenosis (53%). This valvular disease leads to pressure and volume overload in the right atrium and ventricle resulting in chamber enlargement in 90% of CHD patients. Intractable right heart failure develops if left untreated.

Consensus guidance recommends screening for carcinoid heart disease in patients with small bowel NENs, especially in those with elevated CgA and urinary 5-HIAA, with N-terminal pro-brain natriuretic peptide (NT-proBNP) (Ramage et al., 2012, Bhattacharyya et al., 2008, Korse et al., 2009, Davar et al., 2017). NT-proBNP is a neurohormone secreted from cardiac muscle in atrial and ventricular overload with clinical utility in differentiating congestive heart failure from other causes of dyspnea (Morrison et al., 2002, Nakagawa et al., 1995, Maeda et al., 1998, Bhattacharyya et al., 2008). NT-proBNP is associated with volume overload and natriuresis in heart failure. The marker has also been demonstrated to have high specificity (0.91) and sensitivity (0.92) at a cut off of 260pg/ml for screening for CHD diagnosed by TTE in NEN patients with CS (Bhattacharyya et al., 2008). An increase in NT-proBNP (also referred to as NTP in a later chapter of this thesis) is associated with increased risk of death but not with CHD progression (Dobson et al., 2014). Serial measures of NT-proBNP are recommended, particularly in symptomatic patients with CS. The clinical utility of NT-proBNP in early CHD, in the absence of symptomatic and significant valve disease or right ventricular strain, is not clear. Early CHD is characterised by the development of fibrotic carcinoid plaques that can lead to progressive valve damage. A number of other biomarkers are associated with inflammation and fibrosis in heart failure that have not been studied in CHD.

### 1.5 Biomarkers and proteomics in NENs

A number of biomarkers exist for NENs that are assessed by histology, biochemistry, radiology or nuclear medicine investigations. Biomarkers have a role as a diagnostic tool to help identify patients with a specific disease as well as staging and classifying the extent of that disease. A biological marker (‘biomarker’) is defined as a ‘characteristic that is objectively measured and evaluated as an indicator of normal biological processes,
pathogenic processes, or pharmacologic responses to a therapeutic intervention’ (Biomarkers Definitions Working, 2001). A biomarker can also be used as an indicator of prognosis and in predicting and monitoring response to intervention as well as identifying relapse.

1.5.1.1 Histological markers
The mainstay of assessing prognosis is the histological and immunohistochemical analysis of tumour tissue on the basis of grade (G1-3) and morphological appearances (Bosman, 2010a, Rindi et al., 2006, Rindi et al., 2007). Grade 3 NENs, also described as neuroendocrine carcinomas (NECs), are either well or poorly differentiated NENs with a higher mitotic count or Ki67 proliferation index rate that are associated with a worse prognosis (Panzuto et al., 2005, La Rosa et al., 2009). The Ki67 index is significantly associated with prognosis, although the thresholds that define grades are debated (Khan et al., 2013b). A Ki-67 >9% correlated with increased risk of disease recurrence and decreased OS in patients with resected pancreatic NENs (Hamilton et al., 2012).

1.5.1.2 Nuclear Medicine markers
Somatostatin receptor subtypes (SSTR 1-5) are differentially expressed in NENs (Fjallskog et al., 2003). Functional imaging modalities, such Somatostatin receptor scintigraphy (SRS) with 111Indium-octreotide (OctreoScan) or PET-CT with 68Ga-DOTATATE, assess for SSTR2 expression in NENs, providing staging and prognostic information as well as indicating the utility of therapy with somatostatin analogues (SSAs) and peptide receptor radionuclide therapy (PRRT) (Teunissen et al., 2011). These modalities are useful in the detection and staging of well-differentiated G1 or G2 GEP-NENs. In particular, 68Ga-DOTATATE PET has a high sensitivity (93%) and specificity (96%) (Geijer and Breimer, 2013). In contrast, poorly differentiated G3 NENs with a higher proliferative index have low sensitivity on SSTR functional assessment but are highly metabolic tumours that take up the glucose analogue 18FDG on PET imaging (Binderup et al., 2010). Well-differentiated G1 or 2 NENs do not have increased glucose metabolism and 18FDG-PET has low sensitivity for lesions. 18FDG-PET imaging provides prognostic information with positivity associated with reduced survival (Bahri et al., 2014, Johnbeck et al., 2016). A NETPET grading system incorporating both the FDG and somatostatin receptor imaging results correlated with overall survival and has been
proposed as a single parameter that may support individualised patient care (Chan et al., 2017, Hindie, 2017).

1.5.1.3 Circulating markers
Circulating biomarkers in NENs can be general or specific tumour markers (Vinik et al., 2009). General tumour markers include chromogranin A (CgA), Neuron-specific enolase (NSE), pancreatic polypeptide (PP). CgA represents the best routinely used general biomarker in NEN disease with diagnostic, prognostic and predictive value (Lawrence et al., 2011b). CgA is a sensitive marker for NEN from a variety of sites; gastrinoma (100%), gastric NENs (>95%), ileal NEN (80%) and non-functional pancreatic NENs (70%) (Modlin et al., 2010). However, CgA can be elevated in other conditions like chronic atrophic gastritis, renal impairment, chronic heart failure and PPI usage (Hsiao et al., 1990, Giusti et al., 2004, Spadaro et al., 2005). CgA correlates with tumour burden and biological activity (Turner et al., 2006, Grossmann et al., 1994). CgA is of diagnostic value in G1 and G2 NENs as well as large cell G3 NEC but of less value in small cell G3 NEC (Korse et al., 2012). There is variability in commercial assays with sensitivities varying from 67-93% and specificities between with 85-96% (Stridsberg et al., 2003). An elevated CgA in patients with metastatic neuroendocrine neoplasms correlates with hepatic tumour burden and is predictive for shorter survival in both small bowel and pancreatic NENs (Arnold et al., 2008, Bergestuen et al., 2009, Pavel et al., 2011, Turner et al., 2010, Durante et al., 2009). CgA can be used as a marker for response prediction and survival in patients treated with Everolimus and somatostatin analogue therapy (Massironi et al., 2010, Yao et al., 2011, Yao et al., 2010). As a corollary, an increasing CgA from baseline may indicate progression (Chou et al., 2012).

A number of other circulating markers have also been evaluated in NENs. Plasma neurokinin A is an independent predictor of outcomes that also supports a survival advantage in patients in whom the level is altered by somatostatin analogues (Turner et al., 2006). Circulating tumour cells (CTCs) have been reported in NEN patients to be associated with increased tumour burden, increased tumour grade, and elevated CgA as well as poorer PFS and OS (Khan et al., 2013a). A panel of 51 circulating neuroendocrine neoplasia (NEN) transcripts (NETest, mRNA) for GEP NENs has been identified from a putative analysis of 75 genes (blood & tissue from NET patients, adenocarcinoma control, published data) by PCR analysis (Modlin et al., 2013). The panel has been described to
differentiate both pancreatic and GI NETs from controls with a high positive and negative predictive value, including in instances when CgA is low. However, consensus guidance on biomarkers, including CTCs and NETest, highlights the challenges of their use in routine practice (Oberg et al., 2015).

1.5.1.4 Proteomics
Proteomic studies in cancer offers an opportunity to systematically study the dynamic expression and modification of proteins in cells and tissue when compared to the static genome (Maes et al., 2015). The relative increase in protein diversity when compared to the number of protein coding genes results from splicing and post translation modification as well as differential expression at any given snapshot (Dove, 1999). Moreover the changes seen in mRNA are not often linearly correlated with protein expression (Pan et al., 2013). Advances in mass spectrometry and bioinformatics are supporting the development of proteomics for clinical use through understanding the pathophysiology of disease, characterizing drug targets for intervention, and identifying biomarkers (Baker et al., 2012, Boja and Rodriguez, 2011). Proteome changes may reflect processes in tumourigenesis through abundance, post-translational modifications, protein complex and pathway interaction (Pan et al., 2013).

1.5.1.4.1 Proteomic studies of neuroendocrine neoplasms
There have been limited studies on proteomics with NENs, predominantly in pancreatic insulinomas and lung NENs (Song et al., 2017, Tanca et al., 2011, Nomura et al., 2011). A recent study with paired benign insulinoma and pancreatic tissue described 3476 proteins with differential expression in over 40% (1455/3476 proteins) (Song et al., 2017). A quarter of the differentially expressed proteins were from the cytoplasm, while 15% and 12% were from the membrane and nucleus respectively. The proteins were associated with signalling pathways that included cytoskeleton signaling, protein ubiquitination pathway, VEGF signaling, mTOR and PI3K/AKT pathway. Overall there were significant differences in 62 down-regulated and 219 up-regulated proteins in insulinomas when compared to pancreatic tissue. The authors validated the expression of UCH-L1, MAP1B, MAP2, VCAN, PDX-1, CDK4 and α-internexin proteins with immunohistochemistry in a range of pNENs, including both functional and non-functional neoplasms (Song et al., 2017). MAP1B, MAP2 and α-internexin are associated with cytoskeleton organization, UCHL-1 and CDK4 with cell proliferation, VCAN with cell adhesion, and PDX-1 with insulin expression. Concurrent expression of UCH-L1 and
alpha-internexin were associated with significantly better overall survival and progression free survival and were hypothesiszed as prognostic biomarkers. A recent study of paired pNEN and liver metastases focused on ANXA6, CNPY2, RAB11B, TUBB3 proteins and reported significantly poorer recurrence free survival in CNPY2 positive patients (Shimura et al., 2018). Published studies to date used formalin-fixed, paraffin-embedded (FFPE) samples for proteomic analysis with no published data from fresh frozen (FF) samples. Proteins in FFPE can undergo chemical modification with cross-linking during archival processing when compared to fresh frozen samples. A greater number of proteins can be identified from FF samples than FFPE samples with a 40–90% overlap in identified proteins across FF and FFPE tissues (Giusti and Lucacchini, 2013). In particular, there are no published proteomic studies of non-functional pNENs.

1.6 Rationale and aims

The rationale was to explore personalised medicine strategies for patients with neuroendocrine neoplasms by describing ways that their healthcare can be tailored from initial symptom assessment, screening and diagnostics, and exploratory biomarker development. The first part of the thesis explores the patterns of patient symptoms prior to diagnosis, individualised care of rectal NEN patients, and whether NENs are identified through bowel cancer screening programme in England. The second part of the thesis explores a panel of circulatory markers for carcinoid heart disease and identifies proteins of interest from non-functional pancreatic NENs through quantitative proteomics. The following are titles and aims for each chapter:

1.6.1.1 Symptoms before diagnosis: a national survey of neuroendocrine neoplasm patients

Conduct a patient questionnaire to establish pre-diagnosis patterns of NEN symptoms and to understand their interaction with primary and secondary healthcare providers.

1.6.1.2 Investigation and management of rectal neuroendocrine neoplasms

Perform a literature review on rectal NENs was performed to explore how diagnostics and therapeutics can be tailored to individualise patient care to assist clinicians in managing patients.
1.6.1.3 Ileo-colonic neuroendocrine neoplasms identified in the English bowel cancer screening programme (BCSP)

Identify and characterise NENs diagnosed from the English BCSP, a double screen programme using guaiac FOBT and colonoscopy, by analysing the national colorectal screening database and validating the findings with individual BCSP centers.

1.6.1.4 Markers of carcinoid heart disease in small bowel neuroendocrine neoplasms

Explore potential carcinoid heart disease circulatory markers in a comparative study with NT-proBNP in three clinically distinct subpopulations of sbNEN patients, who were biochemically non-functional, functional, or with established carcinoid heart disease. Four potential markers for CHD (adrenomedullin, calprotecin, galectin-3, and soluble ST2) were studied.

1.6.1.5 Proteomic signatures of pancreatic neoplasms

Identify proteins that are of interest in low-grade non-functional pancreatic NENs through quantitative proteomics of fresh frozen tissue samples with bioinformatics analysis.
2 SYMPTOMS BEFORE DIAGNOSIS: A NATIONAL SURVEY OF NEUROENDOCRINE NEOPLASM PATIENTS

2.1 Abstract

Background: The gastrointestinal tract and pancreas are common primary sites for neuroendocrine neoplasms (NENs). Patients often report long duration of non-specific symptoms in the year prior to diagnosis that are often attributed to functional conditions.

Aims: The aims of this study were to establish pre-diagnosis patterns of symptoms. A secondary aim was to determine the time from onset of symptoms to NEN diagnosis and understand the interaction with primary and secondary healthcare providers.

Methods: A survey was designed on a web-based survey platform with the focus on patient symptoms prior to diagnosis and a screen for functional diarrhoea (Rome III criteria (C4)).

Results: A total of 303 responses were received. The median duration from the time of first symptoms to diagnosis was 36 months for small bowel NENs and 24 months for pancreatic NENs. Common first symptoms were pain (36%), flushing (24%) and diarrhoea (24%). 29% of small bowel NEN respondents were given an initial diagnosis
of irritable bowel syndrome. Dyspepsia was the second most common initial incorrect diagnosis. Respondents saw their GP on a median of 5 occasions over a median 18-month period for their symptoms. 31% of patients were diagnosed following unplanned emergency admission.

Conclusion: This survey demonstrates a median time to diagnosis of 36 months for patients with small bowel NENs. Incorrect initial diagnosis appears to be very common, with a high number of attendances in primary and secondary care prior to a correct diagnosis being made. An earlier diagnosis may improve patients’ quality of life and possibly survival.

2.2 Introduction

Patients with neuroendocrine neoplasms (NENs) complain of symptoms, such as loose bowel motions, that may be mistaken for other conditions like Irritable Bowel Syndrome (IBS). NEN patients anecdotally report that they describe florid symptoms to healthcare practitioners but are reassured and managed with an IBS diagnostic label. There are no clear data if the symptoms described by NEN patients overlap with those of benign conditions like Irritable Bowel Syndrome (IBS) or if differentiating symptoms co-exist. It is also not clear how long symptomatic NEN patients are investigated and managed before diagnosis by healthcare practitioners in both primary and secondary care and whether this contributes to an avoidable delay.

The Rome criteria for adult functional GI disorders describes IBS (functional bowel disorder C1) as recurrent abdominal pain at least 3 days/month in the last three months associated with at least two of an improvement with defecation, onset associated with a change in frequency of stool, onset associated with a change in form (appearance) of stool (Drossman et al., 2006). Functional diarrhoea (functional bowel disorder C4) is separately described as loose (mushy) or watery stools without pain occurring in at least 75% of stools at least 75% of the time without pain or discomfort for at least 3 months. The presence of pain differentiates IBS (C1) from functional diarrhoea (C4) in the Rome Criteria. Furthermore, a diagnosis of a functional gastrointestinal disorder is dependent on the exclusion of alarm symptoms like weight loss of >4.5kg, age over 50 years with a change in bowel habit, blood loss or anaemia and significant family history.
The aim of this study was to establish pre-diagnosis patterns of symptoms, including overlap with functional diarrhoea criteria, and healthcare interactions through an online survey of NEN patients.

2.3 Methods

The survey was designed on SurveyMonkey, a web-based survey solution, in collaboration with the NET patient foundation (NPF); a UK based neuroendocrine patient organisation. The survey was designed to cover patient symptoms prior to diagnosis. The survey incorporated a question subset screen for functional diarrhoea (Rome III criteria (C4); loose stool for at least 75% of stools occurring 75% of time without pain for over 3 months duration). An introductory section with agreement to proceed via click through was incorporated on the survey-landing page. No personal data questions, including contact details, were incorporated into the survey. An online HRA decision aids tool form was completed that confirmed that NHS REC approval was not required. The survey was entirely voluntary, and respondents were fully informed on the website on the aim of the survey and that their responses were anonymised.

2.3.1 Survey design

Free text open questions were used at the start of the survey to avoid influencing respondents with closed symptom questions. Mandatory questions acted as filters to proceed through a related subset or to skip to the next filter question (skip logic). Previous answers (free text or categorical) were piped where possible into later questions to help with continuity and framing for respondents. Questions were developed based on multiple choice, 5-point scale or free text responses. Question and block randomization was not incorporated due to the designed flow of questions and the use of skip logic and piping. Durations and timelines questions were anchored on the baseline of the patient’s current age and survey completion date.

The question set was tested and adapted from patient and clinician input from the Kings College Hospital neuroendocrine neoplasm service and from subject matter experts at NPF. The finalized survey contained 130 questions and was estimated to take a maximum of 30 minutes to complete online. Subsets of questions included first and worst symptoms,
Chapter 2: Symptoms before diagnosis: a national survey of neuroendocrine neoplasm patients

weight and appetite change, pain and discomfort, nausea and vomiting, change in bowel habit, alarm signs, GP and hospital interactions, and diagnosis. Respondents were asked if they knew the primary site or origin of their NEN, which was used for subgroup analysis of different patterns of symptoms, duration and healthcare interactions.

2.3.2 Survey distribution and data analysis
The survey was promoted and distributed via clinical nurse specialists at NHS NEN centers using business cards containing the URL and via the NPF using their regular email newsletter and Twitter update. The survey was available from September 2014 and was closed after a predetermined number of responses (300) to ensure that the data would be sufficiently robust to describe patterns in symptoms and healthcare interactions. Data coding and analysis was performed with Microsoft® Excel® for Mac (2011). Free text response data was searched and categorised with key word searches. Descriptive statistics were used given the lack of counterfactual. Key primary site subgroups for analysis were small bowel and pancreatic NENs respondents. Mean values were calculated for age-related analysis, both overall and for NEN primary site subgroups, given the number of predetermined responses. Median values were calculation for duration-related analysis given the smaller subgroups by primary site and possibility of outlier responses given the retrospective nature of the survey based on recall.

2.4 Results
There were a total of 303 responses of which 229 completed the whole survey (75.6%) between 25 September 2014 and 2 May 2015. The mean time to complete the survey was 34 minutes. The survey was predominantly accessed by click-through soon after promotions in the NPF newsletter and Twitter feed. The majority of respondents accessing the survey were female patients (205/303, 67.7%), including completing the whole survey (154/229, 67.2%). Over 75% of respondents (231/303) completed the ethnicity question (grouped as per UK Office for National Statistics) with the overwhelming majority describing themselves as of white ethnicity (94%). The mean age of respondents completing the survey was 55.7 years old with the mean ages for female and male respondents of 53.3 and 60.8 years old respectively.
The majority of responses (214/303) were from patients with small bowel (n=99), pancreatic (n=64) and lung (n=51) primary sites (Table 2-1). Overall, respondents completed the survey a median of 48.1 months after their initial NEN diagnosis (IQR 41-57.6 months). The median time after diagnosis to survey response for small bowel NEN and pancreatic NEN respondents was 36.7 months (IQR 42.9-58.6 months) and 29.2 months (IQR 11-50.7 months) respectively. The interval between diagnosis and survey responses may lead to recall and reporting biases. The mean age at diagnosis was 51.6 years old for all respondents and 55.2, 49.2 and 50.7 years for sbNENs, pNENs and lung NEN respondents respectively. The responses for pancreatic, small bowel and lung NENs are discussed in the following results sections, given the limited data for other primary sites.
Chapter 2: Symptoms before diagnosis: a national survey of neuroendocrine neoplasm patients

Table 2-1. Survey respondents categorised by site of NEN with age at diagnosis, % with symptoms and duration, and percentage over 50 years old.

Respondents were asked the anatomical site of their NEN that supported the analysis of NEN subtypes. Age at diagnosis and duration of symptoms were calculated from the benchmark response of age at survey completion with additional timeline questions.

2.4.1 Symptoms prior to diagnosis

80% of respondents reported that they had symptoms prior to diagnosis (243/303). Most of these respondents reported that their symptoms led to the NEN diagnosis (73%, 221/303), in particular those with pancreatic (73%, 47/64) and small bowel NENs (83%, 82/99). Over half of respondents reported that their symptoms led to a scan (124/221, 56%) that diagnosed their NEN, with the remainder reporting that a biopsy or surgery led to the NEN diagnosis. 66% of pNEN respondents (31/47) and 57% of sbNEN respondents (47/82) had symptoms that led to a scan and diagnosis. Over half of all respondents (31/58, 53%) without symptoms report a scan led to the NEN diagnosis.
2.4.1.1 First Symptom

81% of symptomatic respondents (180/243) described their first symptom in free text that was categorised into seven main areas; diarrhoea, pain, flushing, cough, wheeze, tiredness/ fatigue and other. The majority of respondents described a single symptom (70%, 126/180) while the remainder described overlap with two or more symptoms in free text from the seven main areas. The most frequently described first symptom prior to diagnosis was pain (33%) followed by diarrhoea (22%), flushing (17%) and cough (10%) (Figure 2-1). Respondents with small bowel NENs (n=77) described pain (36%), flushing (26%) and diarrhoea (24%) as the most common first symptoms. Respondents with pancreatic NENs (n=22) described pain (39%), diarrhoea (26%) and fatigue or tiredness (26%) as the most common first symptoms. Respondents with lung NENs (n=30) described cough (53%), wheeze (17%), pain (11%) and diarrhoea (11%) as the most common first symptoms.

Almost all respondents who reported symptoms prior to diagnosis (99%, 240/243) graded the severity of their symptoms on a 5-point scale (very mild, mild, moderate, severe, very severe) (Table 3-2). 63% of all those who responded reported their first symptom before diagnosis was severe or very severe (151/240). 70% of sbNEN respondents reported severe or very severe symptoms compared with 64% of pNEN and 58% of lung NEN respondents.

The median duration of the first symptom prior to diagnosis for all respondents was 30 months overall, 36 months for sbNENs, 24 months for pNENs and 48 months for lung NENs. The mean age at onset of first symptom was 51.6 years for all respondents and 55.2, 49.2 and 50.7 years for small bowel, pancreatic and lung NENs respectively. Over half of all respondents (56%) were aged over 50 years old at the time of developing their first NEN symptom. A greater percentage of small bowel NEN respondents (69%) were over 50 years old at the time of developing their first symptom than pancreatic (42%) and lung (54%) NEN respondents. This may reflect the differing epidemiology and tumour biology between small bowl and pancreatic NENs.
Figure 2-1. First symptom described by survey respondents categorised into seven symptom domains.

Respondents were asked to provide a free text response for their first symptom. These were searched by keywords in seven domains; diarrhoea, pain, flushing, cough, wheeze, fatigue/tired, and other. The responses are presented as percentages (n=303) and subgrouped by primary site of NEN, for example pancreatic (n=64) and small bowel (n=99) NENs.
Table 2-2. Severity of first NEN symptom reported by respondents

Respondents reported the severity of their first NEN symptom prior to diagnosis on a 5-point severity scale. Results are presented for respondents who provided an assessable response.

2.4.1.2 Other but more severe symptom
Over half of all symptomatic respondents (133/243, 55%) reported in free text that they had a different more severe symptom prior to diagnosis from their initial symptom. 59% of these respondents (78/133) described this additional more severe symptom in free text that was categorised into seven main areas; diarrhoea, pain, flushing, cough, wheeze, tiredness/ fatigue and other. 90% of respondents reported a single symptom. There was limited response data for NENs beyond those with pancreatic, small bowel and lung NENs. The three most common severe symptoms overall and for sbNEN, pNEN and lung NEN respondents were the same as those described as the first symptom. It is unclear if there is overlap in responses with those who reported two or more first symptoms as described earlier. 78% of all those who responded reported their severest symptom as severe or very severe (103/133). 80% of sbNEN respondents graded their severest symptom as severe or very severe compared with 90% of pNEN and 60% of lung NEN respondents.

2.4.1.3 Weight and appetite change
42% (126/303) of respondents reported no change to weight prior to their diagnosis, 31% reported weight loss (93/303) and 19% reported weight gain (58/303) (Table 2-3). There were similar proportions of sbNEN (37/99, 37%) and pNEN (19/64, 30%) respondents reporting weight loss, with a lower proportion for lung NENs respondents (10/51, 20%).
55% (168/303) of respondents reported no change to their appetite prior to diagnosis, 25% reported appetite loss (77/303) and 10% reported appetite gain (31/303) (Table 2-3). There were similar proportions of sbNEN (31/99, 31%) and pNEN (14/64, 22%) respondents reporting appetite loss, while there was a lower proportion for lung NENs respondents (9/51, 18%). However, the majority of sbNEN, pNEN and lung NEN respondents reported no change to their appetite prior to diagnosis; 61% (60/99), 53% (34/64) and 65% (33/51) respectively.

The mean weight change prior to diagnosis for respondents (142/303) was a loss of -1.6 kilograms (Figure 2-2). The mean weight change for sbNEN and pNEN respondents was a loss of -4.1 and -0.4 kilograms respectively. 52% of all respondents, 60% of sbNEN and 45% of pNENs reported greater than 4.5kg weight loss. Weight change was reported over a median duration of 12 months for respondents with a similar duration for pNENs (12 months) but longer duration for sbNENs (18 months). Cohorts of respondents who also reported a loss of appetite (number, %) had marked weight loss; -9.8kg overall (77/303, 25%), -11.7kg sbNENs (31/99, 31%), -6.6kg pNENs (14/64, 22%) and -10.8kg lung NENs (9/51, 18%).

The severity grading for weight change was similar for small bowel, pancreatic and lung NEN respondents with 30% reporting it as severe or very severe. 30% of sbNEN respondents reported that their appetite change was severe or very severe compared to 18% for pNENs and 14% for lung NENs. It is possible that the differences in appetite and weight are more pronounced for sbNEN respondents as they more directly relate to insidious mechanical and vascular changes in the enteric tract over a longer duration.
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Table 2-3. Responses to weight and appetite changes prior to diagnosis.

Respondents were asked if their weight or appetite had changed prior to diagnosis; decreased, increased, unchanged or unsure. Responses are reported as percentages and categorized by primary site.

<table>
<thead>
<tr>
<th>Site</th>
<th>No.</th>
<th>Loss</th>
<th>Gain</th>
<th>No change</th>
<th>Unsure</th>
<th>Decrease</th>
<th>Increase</th>
<th>No change</th>
<th>Unsure</th>
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<tr>
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<td>14%</td>
<td>36%</td>
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<td>21%</td>
<td>7%</td>
<td>50%</td>
<td>21%</td>
</tr>
<tr>
<td>Lung</td>
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<td>20%</td>
<td>33%</td>
<td>37%</td>
<td>10%</td>
<td>18%</td>
<td>10%</td>
<td>65%</td>
<td>8%</td>
</tr>
<tr>
<td>Not sure</td>
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<td>25%</td>
<td>15%</td>
<td>25%</td>
<td>25%</td>
<td>20%</td>
<td>15%</td>
<td>40%</td>
</tr>
<tr>
<td>Ovary</td>
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<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
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<td>5%</td>
<td>22%</td>
<td>20%</td>
<td>53%</td>
<td>5%</td>
</tr>
<tr>
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<td>0%</td>
<td>40%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>20%</td>
<td>80%</td>
<td>0%</td>
</tr>
<tr>
<td>Renal</td>
<td>1</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Small Bowel</td>
<td>99</td>
<td>37%</td>
<td>15%</td>
<td>42%</td>
<td>5%</td>
<td>31%</td>
<td>3%</td>
<td>61%</td>
<td>5%</td>
</tr>
<tr>
<td>Stomach</td>
<td>14</td>
<td>36%</td>
<td>7%</td>
<td>57%</td>
<td>0%</td>
<td>29%</td>
<td>0%</td>
<td>57%</td>
<td>14%</td>
</tr>
<tr>
<td>Unknown</td>
<td>33</td>
<td>30%</td>
<td>6%</td>
<td>52%</td>
<td>12%</td>
<td>27%</td>
<td>9%</td>
<td>58%</td>
<td>6%</td>
</tr>
<tr>
<td>Overall</td>
<td>303</td>
<td>31%</td>
<td>19%</td>
<td>42%</td>
<td>9%</td>
<td>25%</td>
<td>10%</td>
<td>55%</td>
<td>9%</td>
</tr>
<tr>
<td>Respondents</td>
<td>303</td>
<td>93</td>
<td>58</td>
<td>126</td>
<td>26</td>
<td>77</td>
<td>31</td>
<td>168</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 2-4. Weight change (kg) and duration of change.

Respondents were asked to quantify if their weight had changed prior to diagnosis. Mean weight change in kilograms and median duration of change in months were calculated and categorized by primary site. The percentage of respondents with weight loss of over 4.5 kilograms was also calculated.
Figure 2-2. Scatter plot of weight change for all respondents and lung, pancreas and small bowel NENs subgroups.

Respondents were asked to quantify if their weight had changed prior to diagnosis. The weight change in kilograms, either positive or negative (y-axis), is plotted for each respondent (x-axis) who reported a change. The change for lung, pancreas and small bowel NEN subgroups are also plotted.

2.4.1.4 Abdominal and back pain
69% of respondents (175/252, excluding those with lung NENs given pain locations) reported abdominal or back pain prior to diagnosis. A greater proportion of small bowel NEN (81%, 80/99) respondents reported pain than pNEN (55%, 35/64) respondents prior to the diagnosis. sbNEN respondents described the pain as starting in the periumbilical (26%) or pelvic area (24%) before becoming more generalized to the abdominal midline and lower abdomen (62%) prior to diagnosis (Figure 2-3). pNEN respondents described the pain as starting in the epigastric (39%) or upper right abdomen area (10%) before becoming more diffuse in it’s location prior to diagnosis. There was no differentiating characteristic to the pain experienced by sbNEN and pNEN respondents, or association with other intestinal symptoms (bowel frequency, consistency, ‘blockage’). The frequency of the pain was similar for both sbNENs and pNENs, occurring a few times a month or greater than once a week. sbNEN respondents described a more severe pain profile than pNEN respondents.
Chapter 2: Symptoms before diagnosis: a national survey of neuroendocrine neoplasm patients

Figure 2-3. Location of pain with percentages reported by small bowel and pancreatic NEN respondents at the start of the symptom and at the time of diagnosis.

The images above of abdominal and back sites of pain were displayed to respondents were able to indicate multiple sites of pain, both at the start of the symptom and at the time of NEN diagnosis. Percentage values are presented for each location and categorized by value; 0% - white, 1-9% - green, 11-19% - orange, 20-29% - red, and > 30% - black.
2.4.1.5 Bowel symptoms
Half of respondents (125/252, excluding lung NENs given the specific symptom) reported problems with their bowels prior to diagnosis. The proportion experiencing problems was greater for sbNENs (64%, 63/99) than for pNENs (36%, 23/64). The majority of pNEN and sbNEN respondents (67.5%) did not report problems with hard or lumpy stools (54/80). 80% of sbNEN and pNEN respondents (66/83) experienced problems with loose, mushy or watery stools prior to diagnosis. 46% of sbNEN respondents (21/46) described the problem with loose bowels as severe or very severe. 60% of small bowel NEN patients reported that three quarters of their stools were loose prior to diagnosis and 40% had this problem 75% of the time or more. 66% of sbNEN respondents (57/87) reported some degree of urgency to open their bowels and 78% (68/87) reported some degree of bloating. Less than 20% of sbNEN respondents reported black or bloody stools.

2.4.1.6 Other symptoms
67% of respondents (95/141, excluding those with lung NENs) reported skin flushing (defined in the questionnaire as redness and warm feeling) prior to diagnosis. Small bowel NEN (83%, 72/87) respondents reported skin flushing more than pNEN (43%, 23/54) respondents prior to the diagnosis. Almost a third of sbNEN respondents described the flushing as severe or very severe (31%, 22/72). Alcohol and large meals were reported by sbNEN respondents to make the flushing worse. 60% of respondents (84/141, excluding those with lung NENs) reported anxiety prior to diagnosis. Small bowel NEN (66%, 57/87) respondents reported anxiety more than pNEN (50%, 27/54) respondents prior to the diagnosis. A small proportion of sbNEN respondents described the anxiety as severe or very severe (16%, 9/57). Respondents reported nausea in 45% and vomiting in 36% of cases (65/146 and 52/146 respectively, excluding those with lung NENs) prior to diagnosis. 35% of respondents (49/139, excluding those with lung NENs) reported problems with breathing prior to diagnosis. The respondent data available limited further analysis of these symptoms.

2.4.2 Accessing healthcare services and diagnosis
The majority of respondents (80%) reported that they saw their GP with symptoms prior to their NEN diagnosis. Respondents reported they saw their GP over a median period of
18 months and a median of 5 interactions (Table 2-5). sbNEN respondents reported a longer duration of investigation in primary care with a median of 24 months.

<table>
<thead>
<tr>
<th>Site</th>
<th>No.</th>
<th>% seen by GP</th>
<th>Median No. times seen (IQR)</th>
<th>Median time investigated (months, IQR)</th>
<th>No.</th>
<th>% seen in clinic</th>
<th>Median No. times seen in clinic (IQR)</th>
<th>Median time investigated (months, IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas</td>
<td>52</td>
<td>73%</td>
<td>5 (2-9.8)</td>
<td>18 (3-48)</td>
<td>50</td>
<td>50%</td>
<td>3 (1-5)</td>
<td>3.0 (1.0-8.8)</td>
</tr>
<tr>
<td>Small Bowel</td>
<td>86</td>
<td>86%</td>
<td>5 (3-10)</td>
<td>24 (3.8-60)</td>
<td>85</td>
<td>62%</td>
<td>2 (1-4)</td>
<td>3.0 (1.0-12)</td>
</tr>
<tr>
<td>All sites</td>
<td>257</td>
<td>80%</td>
<td>5 (2.3-12)</td>
<td>18 (3-48)</td>
<td>246</td>
<td>58%</td>
<td>2 (0-4)</td>
<td>2.4 (1.0-12)</td>
</tr>
</tbody>
</table>

Table 2-5. Interactions and duration of investigation in primary and secondary care.

Respondents were asked if they attended primary care or secondary care clinics prior to their NEN diagnosis. The median number of attendances and duration of investigation prior to diagnosis was calculated, with subgroup analysis of pNENs and sbNENs.

Over half of respondents (58%, 122/210) reported that they first interacted with secondary care from a GP referral to a local hospital clinic (Table 2-6). Almost a third of respondents (31%, 66/210) reported that their first secondary care interaction was via an unplanned emergency admission from A&E. Again there was no marked difference in the routes that sbNEN and pNEN respondents first interacted with secondary care. 43% of pNEN and sbNEN respondents reported that they were investigated in gastroenterology clinics prior to diagnosis with others mainly investigated in surgical or oncology clinics. Respondents reported that they were investigated in clinic for a median of 2.4 months with a median of 2 attendances. The period managed and investigated in primary and secondary care management (median 18 and 2.4 months respectively) aligns with the period of respondents’ symptoms prior to diagnosis (median 30 months) and the referral route or potential delay in accessing secondary care. However, it was not possible to quantify if any overlap in primary and secondary care existed given the questionnaire design.
Table 2-6. Route of initial interaction with secondary care for investigation of symptoms.

Respondents were asked what their primary and secondary care healthcare practitioners initially thought was the cause of their symptoms from a list of the above symptoms. Key subgroups of pNENs and sbNEN were also analysed.

60% of sbNEN respondents (59/99) reported that they required surgery for their NEN compared to 65% of lung NEN (33/51) and 45% of pNEN respondents (26/64). 27% of sbNEN respondents (16/59) who had surgery reported that it took place as an emergency. 58% of sbNEN respondents (34/59) reported they had surgery at their local hospital. 78% of sbNEN respondents (77/99) reported spread or metastases at the time of diagnosis; 69% reported liver metastases (68/99), 39% lymph nodes disease (39/99) and 21% mesenteric disease (21/99). The extent of metastatic disease at diagnosis was greater than expected and potentially influenced by bias from self-selection of respondents with more advanced disease accessing the survey or recall bias in those with post-diagnosis disease progression. There was limited data for analysis on respondents’ previous cancer and family history profiles.

2.4.3 Irritable bowel syndrome versus NEN
Respondents reported that almost a quarter of GPs (24%, 39/163) thought the likely cause of their symptoms was IBS (Table 2-7). Respondents reported that over a third of healthcare practitioners in a secondary care setting thought the cause of their symptoms was either a NEN (21%) or cancer (15%). It is not clear if the index of suspicion for
malignancy in secondary care relates more to the setting per se, with a wider range of investigation modalities and cancer pathways, or from more clinically apparent tumour behaviour prior to diagnosis as well as potential recall bias favouring the eventual diagnosis setting. 30% of sb or pNEN respondents (47/155) reported that they were already diagnosed with IBS or were considered to have IBS from their NEN symptoms. The proportion was slightly greater for sbNEN respondents; just over a third (36%, 34/95) reported that IBS was considered or previously diagnosed.

Table 2-7. Initial cause or diagnosis for symptoms from primary care and secondary care.

<table>
<thead>
<tr>
<th>Cause / Site</th>
<th>Primary Care</th>
<th>Secondary Care</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pancreas</td>
<td>Small Bowel</td>
</tr>
<tr>
<td>Other</td>
<td>22%</td>
<td>25%</td>
</tr>
<tr>
<td>NET</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>Cancer</td>
<td>3%</td>
<td>4%</td>
</tr>
<tr>
<td>Not sure</td>
<td>9%</td>
<td>19%</td>
</tr>
<tr>
<td>IBS</td>
<td>16%</td>
<td>29%</td>
</tr>
<tr>
<td>Doctor not sure</td>
<td>8%</td>
<td>8%</td>
</tr>
<tr>
<td>Gall Stones</td>
<td>8%</td>
<td>12%</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>9%</td>
<td>16%</td>
</tr>
<tr>
<td>Menopause</td>
<td>2%</td>
<td>11%</td>
</tr>
<tr>
<td>Kidney stones</td>
<td>5%</td>
<td>3%</td>
</tr>
<tr>
<td>Depression</td>
<td>5%</td>
<td>3%</td>
</tr>
<tr>
<td>Constipation</td>
<td>3%</td>
<td>5%</td>
</tr>
<tr>
<td>Anaemia</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>Crohn’s</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>5%</td>
<td>4%</td>
</tr>
<tr>
<td>UTI</td>
<td>5%</td>
<td>3%</td>
</tr>
<tr>
<td>Chest infection</td>
<td>2%</td>
<td>2%</td>
</tr>
<tr>
<td>Haemorrhoids</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Respondents</td>
<td>64</td>
<td>99</td>
</tr>
</tbody>
</table>

Respondents were asked what their primary and secondary care healthcare practitioners initially thought was the cause of their symptoms from a list of the above symptoms. Key subgroups of pNENs and sbNEN were also analysed.

However, the proportion of respondents meeting all the criteria for functional diarrhoea was less than the proportion considered or previously diagnosed with IBS by healthcare practitioners. Approximately 5% of respondents’ (7/155) symptomatology reported in the questionnaire met the full criteria for functional diarrhoea (loose stool for at least 75% of stools occurring 75% of time without pain for over 3 months duration) in contrast to the 30% (47/155) who reported that IBS was considered or previously diagnosed by healthcare practitioners (Table 2-8). A fifth of sb or pNEN respondents (21%, 32/155)
met the part criteria for functional diarrhoea symptoms and duration if the pain criterion was not considered. Similarly, a quarter of sbNEN respondents (24%, 23/95) met the same part criteria for functional diarrhoea symptoms and duration, but only 4% (4/95) met the full criteria (including absence of pain). Other functional gastrointestinal disorder criteria were not incorporated into the questionnaire and this may represent part of the disparity between categorisation by reported symptomatology and consideration or diagnosis of IBS by healthcare practitioners. Respondents with red flag factors of age over 50 years or weight loss over 4.5kg reported that IBS was still considered. Over two thirds of sbNEN patients (68%, 23/34) who were considered or diagnosed with IBS by healthcare practitioners were over 50 years old at the start of their symptoms. Similarly, almost half those considered to have or diagnosed with IBS had weight loss of over 4.5kg (44%, 15/34) during the period of their symptoms. The survey did not ask respondents for the duration of their previous IBS diagnosis nor if the NEN related symptoms differed from these pre-existing IBS symptoms. However, there appears to be a disparity between achieving the IBS diagnostic criteria without red flag factors and healthcare practitioners’ consideration of the diagnosis.

<table>
<thead>
<tr>
<th>Site, subgroup by pain</th>
<th>Number</th>
<th>Functional diarrhoea criteria</th>
<th>IBS considered or diagnosed by healthcare practitioner</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>pNET</td>
<td>60</td>
<td>85%</td>
<td>15%</td>
</tr>
<tr>
<td>No Pain</td>
<td>25</td>
<td>37%</td>
<td>4%</td>
</tr>
<tr>
<td>Pain</td>
<td>35</td>
<td>48%</td>
<td>10%</td>
</tr>
<tr>
<td>sbNET</td>
<td>95</td>
<td>76%</td>
<td>24%</td>
</tr>
<tr>
<td>No Pain</td>
<td>15</td>
<td>12%</td>
<td>4%</td>
</tr>
<tr>
<td>Pain</td>
<td>80</td>
<td>64%</td>
<td>20%</td>
</tr>
<tr>
<td>All sbNET &amp; pNET</td>
<td>155</td>
<td>79%</td>
<td>21%</td>
</tr>
<tr>
<td>No Pain</td>
<td>40</td>
<td>21%</td>
<td>5%</td>
</tr>
<tr>
<td>Pain</td>
<td>115</td>
<td>58%</td>
<td>16%</td>
</tr>
</tbody>
</table>

Table 2-8. Symptoms of sbNEN and pNEN respondents achieving functional diarrhoea criteria and if IBS considered by healthcare practitioner.

The full criteria for functional diarrhoea is loose stool for at least 75% of stools occurring 75% of time without pain for over 3 months duration. SbNEN or pNEN respondents who achieved the full criteria are highlighted in yellow. Respondents also reported if their healthcare practitioner thought or diagnosed IBS from their symptoms.
2.5 Discussion

The survey is one of the largest of NEN patients exploring symptoms and healthcare access. The majority of respondents were from sbNEN, pNEN and lung NEN patients who reported free text symptoms that helped inform pre-diagnosis patterns. Small bowel NEN respondents frequently reported pain, flushing and diarrhoea while pancreatic NEN respondents reported pain, fatigue and diarrhoea. Lung NEN respondents frequently described cough and wheeze. The median duration in symptoms prior to diagnosis was 3 years, which may relate to the insidious tumour biology of the majority of NENs, delayed patient presentation to healthcare services as well as potential delays in both primary and secondary care.

Over half of all respondents were aged over 50 years old at the start of their symptoms with many reporting associated red flags of appetite and weight loss. The triad of age>50 years, weight loss >4.5kg and pain reported by NEN respondents is unlikely to be associated with functional diarrhoea and other benign conditions. Only a fraction of sbNEN and pNEN respondents met the symptom criteria for functional diarrhoea compared to a greater proportion labeled or continuing to be managed as IBS. A proportion of respondents may have had overlap in their IBS and NEN symptoms that masked an obvious change for earlier investigation or clinical assessment. The diagnostic rationale and pathways used by healthcare practitioners when assessing patients over the age of 50 with new symptoms, even if insidious in nature, should reflect the increased incidence of malignancy in this cohort.

Reduced health seeking behaviour in UK patients has been highlighted as a factor in later stage disease at presentation and consequent worse outcomes than other developed countries. The public health messaging around cancer symptomatology and the introduction of screening programmes can help improve outcomes but have no clear secondary benefit for other malignancies like NEN. Patients with symptoms like diarrhoea and pain may be investigated with simple and accessible diagnostics, like abdominal ultrasound, endoscopy and blood testing, that may be falsely reassuring given their low sensitivity for early NEN disease. Cross-sectional modalities like CT may reveal earlier stage disease, particularly when used in patients who are 50 years old with new symptoms.
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The survey was limited by its retrospective nature and inclusion of only historical NEN patients. There was a median of 4 years from diagnosis to completing the survey that could significantly bias a patient’s recall of symptoms. Indeed, events and symptomatology that occurred well after diagnosis, including post surgical symptoms, could easily be mixed with those occurring prior to diagnosis. Importantly, commonly experienced symptoms may have been reported by patients but may not relate to NEN symptoms; for example the flushing experienced with carcinoid syndrome compared with the menopause. There was no additional clinical questioning and validation of symptoms given the survey design. The survey was predominantly accessed via the NEN patient foundation’s digital promotional channels that could represent a self-selecting informed and proactive cohort of NEN patients. The responses may be not representative of the majority of NEN patients in the UK who may have different symptom, disease and healthcare experiences. The scope of the functional bowel criteria questions purely focused on functional diarrhoea (C4) due to survey design and respondents’ time constraints. Of note, pain is a feature of other functional bowel criteria and its absence may not be a clear differentiating symptom for a potential NEN. It is not clear from this survey if there are any differentiating NEN symptom characteristics from IBS as the questionnaire data was limited by design, both from acceptability and coverage, with no counterfactual cohort (functional diarrhoea) surveyed.

The survey also explored access to healthcare services and diagnostic work up without validation from actual healthcare records, particularly around the duration of primary and secondary management prior to diagnosis. The effect of information asymmetry and between patients and clinicians may limit the pre-diagnosis healthcare insights, including those around IBS, which respondents provided as part of this survey. However, the survey does highlight the potential value of prospectively surveying newly diagnosed patients using the broad functional bowel questions set with a counterfactual group of newly diagnosed IBS patients. Validation and additional information from healthcare records would add robustness and richness to the pre-diagnosis patient journey and touch points towards an earlier diagnosis. Analysis of data sources like the primary care Clinical Practice Research Datalink (CPRD) and secondary care Hospital Episode Statistics (HES) could help build the picture of the pre-diagnosis risk factors and signals of NENs in conjunction with a prospective patient survey (Pittayanon et al., 2014, Panitch et al.,
2002). Any interventions around clinical pathways, healthcare practices and patient beliefs would likely lead to an earlier diagnosis and possibly improved outcomes given the significant durations of symptoms that NEN respondents experience before diagnosis.
3 INVESTIGATION AND MANAGEMENT OF RECTAL NEUROENDOCRINE NEOPLASMS

3.1 Abstract

Background: Rectal neuroendocrine neoplasms (NENs) are increasingly identified at endoscopy the cause of which may be in part related to bowel cancer screening programmes.

Aims: A review of the literature is presented to aid clinicians in the diagnosis and management of rectal NENs.

Methods: A literature search was conducted through MEDLINE using search terms: rectal, rectum, carcinoid, neuroendocrine neoplasm, therapy, endoscopy, mucosal resection, submucosal dissection. Relevant articles were identified through manual review with reference lists reviewed for additional articles.

Results: The incidence of rectal NENs is approximately 1 per 100,000 population per year with the majority (80-90%) being <1cm and localised to the submucosa. Metastatic disease is infrequent (<20%) with risk factors including size, atypical appearance, grade, and depth of invasion. The primary resection modality influences complete resection rates and the need for secondary therapy. A thorough pre-resection diagnostic work up is required for lesions that are at higher risk of invasion and metastasis. Device-assisted endoscopic mucosal resection and endoscopic submucosal dissection are used to resect localised rectal NENs < 2cm. Transanal surgery is also used to resect localised 1-2cm
rectal NENs. Oncological surgical resection is used for rectal NENs that are >2cm or with invasion and regional disease. The treatment of advanced disease is multimodal.

Conclusions: The long-term tumour biology of small rectal NENs is unclear. There is uncertain impact from bowel cancer screening programmes on rectal NEN incidence, morbidity and mortality. Referral to NEN centres for patients with locally advanced disease or metastatic disease is recommended.

3.2 Introduction

Neuroendocrine neoplasms (NENs) of the rectum represent 34% of all diagnosed gastrointestinal NENs and are the most common behind small bowel NENs. Analysis of the Surveillance, Epidemiology and End Results (SEER) database from 1975 to 2008 reveals that all gastrointestinal NENs (apart from appendiceal) have increased in incidence (Tsikitis et al., 2012). The reported incidence of rectal NENs in the SEER database is approximately 1 per 100,000 population per year and accounting for 17.7% of all NENs (Modlin et al., 2003). A 10-fold increase in the age-adjusted incidence in rectal NENs has been observed over this period (Lawrence et al., 2011a). The rising incidence in rectal NENs is likely to be multifactorial with predominant contribution from improved diagnosis and coding rather than a significant increase in incidence in the population. Over the last 20 years there has been improved endoscopic lesion characterisation, wider access to routine colonoscopy and colorectal cancer screening programmes as well as more robust coding in cancer and histological registries. The majority of cases are diagnosed endoscopically with over 50% identified incidentally (Yoon et al., 2010). Most patients are asymptomatic with only a minority complaining of abdominal pain, rectal bleeding, weight loss or anorectal symptoms (Shebani et al., 1999). This chapter will focus on the endoscopic assessment and management of rectal NENs in addition to the histological assessment, staging, treatment and follow up of these tumours.

3.3 Methods

Chapter 3: Investigation and management of rectal neuroendocrine neoplasms


3.4 Results

3.4.1.1 Epidemiology
The mean age at diagnosis is typically younger (56 years) than other types of NENs. This age group (50-59 years) also exhibits the largest increase in incidence, most likely through their participation in screening colonoscopy programmes that were first introduced in the US in 2000 (Taghavi et al., 2013). Rectal NENs have been diagnosed in a greater proportion of patients than small bowel NENs in the overwhelming majority of years since screening colonoscopy started.

In the US, 27% of all NENs in Afro-American populations are diagnosed in the rectum, a 3-4 fold higher incidence over white Caucasian population, with a significant increase in race-by-year NEN incidence (Modlin et al., 2003). An increasing incidence of rectal NENs in US Hispanic and Asian ethnic groups has also been reported (Taghavi et al., 2013).

Analysis of the National Cancer Registry in England from 1971 to 2006, revealed a similar 10-fold increase in incidence of rectal NENs, which represent 8% of all GI NENs but are still lower than US and other Northern European countries (Ellis et al., 2010, Hauso et al., 2008). Rectal NENs are more commonly diagnosed in Asian populations representing 88-89% of all colorectal NENs in Japanese Asian populations (Konishi et al., 2006, Kotake et al., 2003). The reasons for the differences in distribution is unclear but may be related to regional colorectal endoscopy practice, variations in reporting as well as ethnicity-related genetic factors.

3.4.1.2 Impact of bowel cancer screening programmes
Rectal NENs diagnosed as a result of bowel cancer screening programmes are smaller and at an earlier stage than those diagnosed from non-screening endoscopy (Kaminski et
al., 2007a, Scherubl, 2009, Matsui et al., 1993). They are invariably 10mm or less and of grade 1. There are few epidemiological data on rectal NENs diagnosed in bowel cancer screening programmes. A Polish bowel cancer screening programme cohort of 50148 participants reported a prevalence of 0.05-0.07% or 50-70 per 100,000 colonoscopies performed (Kaminski et al., 2007a). The increased numbers of rectal NENs diagnosed in this cohort when compared to the SEER data is likely to reflect its underlying age-specific prevalence as this bowel cancer screening programme cohort were unselected and asymptomatic at endoscopy. A significant proportion of rectal NENs diagnosed in bowel cancer screening programmes are likely to be incidental findings but it is not clear whether there are more significant co-pathology identified and an increased synchronous malignancy rate with colorectal adenocarcinoma.

There are no clear data on differences in rectal NENs incidences with different bowel cancer screening programme strategies; direct to endoscopy (single screen) versus primary faecal occult blood test with secondary endoscopy (double screen). Future insights into the impact of different bowel cancer screening programme strategies on rectal NEN incidences may be explored in countries that have dual strategies. For instance, in the UK bowel cancer screening programme a single screen flexible sigmoidoscopy (single screen) at the age of 55 years old has been introduced in addition to an existing double screen (faecal occult blood test & endoscopy) at the age of 60-74 years old that is hypothesised to further prevent colorectal adenocarcinoma cases and deaths (Geurts et al., 2015). Analysis between two such screening cohorts may differentiate if the faecal occult blood test has additional diagnostic value above endoscopy per se for rectal NENs. It is also unclear whether bowel cancer screening programmes will have a significant positive impact on rectal NEN mortality in those screened as has been demonstrated with colorectal adenocarcinoma mortality as the natural disease history of small rectal NENs has not been characterised (Atkin et al., 2010, Scholefield et al., 2002). There are no long-term data on survival outcome differences between patients diagnosed within bowel cancer screening programmes and those diagnosed from non-screening endoscopy. A significant effect on prognosis and survival may be difficult to demonstrate given the total number of bowel cancer screening programme diagnosed rectal NENs is small. However, there has been a 20% improvement in the overall 5 year survival rate of rectal NENs since the era of bowel cancer screening programmes (Scherubl, 2009).
3.4.1.3 Histological assessment of rectal NENs

Rectal NENs are L cell type in origin with a trabecular growth pattern, sometimes with rosettes and tubular structures, granular chromatin, round-oval nuclei and indistinct nucleoli (Figure 3-1) (Caplin et al., 2012, Ramage et al., 2008). They express and stain for the general neuroendocrine markers chromogranin (although up to 50% can be negative) and synaptophysin as well as L cell specific markers such as glucagon-29, glucagon-37, glicentin, peptide YY, pancreatic polypeptide and their precursors (Federspiel et al., 1990, Kimura et al., 2000). Prostate-specific acid phosphatase is also expressed in the majority of rectal NENs (Federspiel et al., 1990).

The malignant potential of rectal NENs can be ascertained by the histological grade as classified by the mitotic index and from the expression of Ki-67, a tumour proliferation marker (Hotta et al., 2006, Shimizu et al., 2000). The North American Neuroendocrine Tumor Society (NANENS) Consensus Guidelines for staging and grading rectal NENs mirror those of the European Neuroendocrine Tumor Society (ENENS) guidelines, which have been retrospectively validated (Jann et al., 2011). Grade 1 rectal NENs have a mitotic index of less than 2 per 10 high-power fields and a Ki-67 less than≤2%. Grade 2 tumours have a mitotic index of 2-20 mitoses/ HPF and Ki-67 of 3-20% while grade 3 tumours have a mitotic index and Ki-67 greater than 20. Specific histological characteristics, such as grade, lymphovascular and perineural invasion, have prognostic implications beyond those described in the TNM staging and should be reported for all rectal NENs.
Figure 3-1. Rectal biopsy showing mucosal infiltration by a rectal NEN.

Left image is H&E staining and right image is immunohistochemistry showing tumour cells with weak staining for chromogranin.

3.4.1.4 Staging of rectal NENs

The staging of rectal NENs has two components; a pre-resection assessment based on diagnostic information and a post-resection assessment based on histological assessment of the resected specimen. The ENENS and Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) guidelines for TNM assessment of histological specimens are the same (Table 3-1) (Bosman, 2010b, Sobin et al., 2011, Edge et al., 2010). The staging is also similar with ENENS guidelines subclassifying Stage 1 into A and B depending if the tumour is 1cm or 1-2cm in diameter respectively (Table 3-2) (Rindi et al., 2007).
### Table 3-1. ENENS and UICC/AJCC TNM assessment of rectal NENs

<table>
<thead>
<tr>
<th>T Stage</th>
<th>T Description</th>
<th>N and M Stage</th>
<th>N and M Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumour cannot be assessed</td>
<td>Nx</td>
<td>Regional lymph node status not assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
<td>N0</td>
<td>Absence of lymph node metastasis</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour invades lamina propria or submucosa and size 2 cm or less</td>
<td>N1</td>
<td>Regional lymph node metastasis</td>
</tr>
<tr>
<td>T1a</td>
<td>Tumour size less than 1 cm in greatest dimension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1b</td>
<td>Tumour size 1-2 cm in greatest dimension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>Tumour invades muscularis propria or size more than 2 cm with invasion of lamina propria or submucosa</td>
<td>Mx</td>
<td>Distant metastasis not assessed</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour invades through the muscularis propria into the subserosa, or into non-peritonealised pericolic or perirectal tissues</td>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>T4</td>
<td>Tumour invades peritoneum or other organs</td>
<td>M1</td>
<td>Presence of distant metastasis</td>
</tr>
</tbody>
</table>

### Table 3-2. Staging of rectal NENs with ENENS sub-classifying Stage 1 into A and B by size

<table>
<thead>
<tr>
<th>Stage</th>
<th>T Stage</th>
<th>N Stage</th>
<th>M Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IA</td>
<td>T1a for ENETS</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IB</td>
<td>T1b for ENETS</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>T4</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>Any T</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
</tr>
</tbody>
</table>
3.4.2 Prognosis and survival of rectal NENs
Rectal NENs have the best overall survival of all gastroenteropancreatic NENs, this is largely due to the high incidence of small rectal NENs that have no evidence of invasion and carry an excellent long-term prognosis. The 5-year survival rates for rectal NENs from the SEER database and Norwegian Register of Cancer for the period 1993-2004 are high at 74%-88% (Hauso et al., 2008). The overwhelming majority of rectal NENs are small (<1cm) and confined to the submucosa (T1, 89%) with only a fraction that are greater than 2cm, invade the muscularis propria (T2, 5%) or beyond (T3 or T4, 6%) (Weinstock et al., 2013). Those that invade are more likely to be associated with progression and metastases (de Mestier et al., 2013). The majority of rectal NENs (80-88%) are localised at diagnosis with the remaining 12-20% diagnosed with regional lymph node spread and/or distant metastases (McDermott et al., 2014, Weinstock et al., 2013). Tumour stage at diagnosis is the most important prognostic factor; localised rectal NENs (T1, N0, M0) have a 5-year survival of 98-100% while those with regional (N1) and distant metastases (M1) have a survival of 54-74% and 15-37% respectively (Konishi et al., 2007, Shields et al., 2010, Jann et al., 2011). Favourable histological features include grade 1 and no lymphatic, vascular, perineural or muscularis propria invasion. Only a minority (2-6%) have lymphovascular or perineural invasion but this is associated with progression, metastases and worse survival (Shimizu et al., 2000, Koura et al., 1997). The reported incidence of nodal involvement for rectal NENs <1cm varies between 1-10%. For lesions between 1-2cm this increases to 30% and over 2cm this is 60%. The lesions with atypical endoscopic appearances also have a higher risk of nodal involvement. Endoscopists performing the index procedure should be aware that small rectal NENs can metastasize and hence a suspected lesion requires an adequate assessment of depth and stage.

3.4.3 Diagnostic appearances at endoscopy and endoscopic ultrasonography (EUS)
The endoscopic features of rectal NENs differ from those of adenomatous or hyperplastic polyps of the rectum. Typical features are of a small smooth sessile lesion, frequently less than 10mm in diameter, with a normal or yellow mucosal discolouration (reflecting the presence of chromogranin) (Figure 3-2) (Soga, 1997, Kim et al., 2008). These lesions arise in the deeper layers of the mucosa and raise the mucosal layer without clear surface distortion. Rectal NENs are commonly located proximal to 5cm from the anal margin.
The appearances of rectal NENs can be subtle, with only minimal mucosal elevation or just a fine overlying capillary network. Mucosal pit patterns, or their absence, can help differentiate a rectal NEN from hyperplastic and adenomatous lesions, particularly with endoscopic imaging techniques like narrow band imaging. Atypical features are more common in larger rectal NENs (greater than 5mm diameter) and include a semipedunculated or ulcerated orofungating shape, changes to the surface with depression, erosion or ulceration as well as a hyperaemic colour (Kim et al., 2008, Shim et al., 2004). Almost 80% of rectal NENs are less than 10mm in size with only 5% greater than 20mm (McDermott et al., 2014).

Figure 3-2. Endoscopic image of submucosal NEN

The lesion is approximately 10mm in size with a yellowish colouration, early central depression and normal overlying mucosa. EUS was performed to exclude invasion beyond the submucosa prior to the initial endoscopic resection.

3.4.3.1 Pre-therapeutic endoscopic assessment of rectal NENs
Endoscopists are well versed in the diagnostic tools for assessing mucosal lesions like adenomas, but they also need a high index of suspicion and a screening strategy for submucosal-appearing or atypical rectal lesions. A potential rectal NEN should be assessed for depth of invasion and the presence of metastatic disease as this affects prognosis and therapeutic strategy. This assessment is critical at the index endoscopy when the lesion should be carefully characterised for high-risk stigmata; suspicion of submucosal origin, size and concerning overlying mucosal change.

An attempt at index endoscopic resection should not be attempted if any high-risk stigmata are present that maybe associated with invasion or metastases. For example, an
ulcerated appearance or a lesions ≥2cm in size are likely to have deep submucosal invasion. A judicious biopsy may be taken for histological confirmation for suspected rectal NENs that are >0.5cm or with high-risk stigmata as histological grade can alter the choice of therapeutic modality. The site of an atypical or submucosal rectal lesion that is biopsied should be tattooed to allow the site to be easily identified for primary therapy and long-term surveillance.

Rectal EUS is helpful for assessing suitability for endoscopic resection by assessing depth of invasion and the presence of abnormal pathological nodes. MRI of the pelvis is also a useful modality for assessing depth of tumour involvement and nodal involvement. The therapeutic strategy for resecting rectal NENs should be guided by prognostic inferences and accurate staging.

Endoscopic ultrasonography (EUS) is useful for accurately determining size and depth of submucosal lesions like rectal NENs prior to endoscopic resection (Ishii et al., 2010, Kobayashi et al., 2005). They usually appear as a smooth homogeneously hypoechoic submucosal mass arising in the third layer with the second layer covering, but often becoming indistinct at its upper interface (Figure 3-3) (Matsumoto et al., 1991, Yoshikane et al., 1993). Correlation of EUS assessed size and depth with histological resection specimens is high with skilled operators.

Rectal NENs <0.5cm are unlikely to show evidence of invasion beyond the muscularis propria and consequently can be resected endoscopically. EUS should be performed prior to attempted resection of a rectal NEN that is more likely to have invasion of the muscularis propria or local lymph node disease, such as those that are >0.5cm in size or with atypical features like a semipedunculated or ulcerated orofungating shape, surface changes with depression, erosion, ulceration and hyperaemic colour. It is important to assess at EUS for local lymph nodes, especially if there is invasion beyond the muscularis propria, as additional diagnostic imaging may be required to assess for regional and distant disease. Hence, EUS is a core assessment modality for stratifying if a rectal NEN can be endoscopically resected or if surgical resection is required when invasion or metastases have been identified. The role of EUS in surveillance of resected NENs is discussed later in this chapter.
Figure 3-3. Endoscopic ultrasonography image of rectal NEN

The EUS demonstrates a smooth homogeneously hypoechoic submucosal masses (white arrows).

3.4.4 Radiological and functional imaging

3.4.4.1 Anatomical imaging

Magnetic resonance imaging (MRI) is a sensitive modality in rectal tumours for assessing stage and predicting circumferential resection margins (Beets-Tan et al., 2001). Rectal NENs appear as superficial, discrete, submucosal lesions with homogenous contrast enhancement (Kim et al., 2011, Rouse et al., 2008). The lesion usually has an isointense signal intensity on T1 weighted-images, and an isointense to hyperintense signal on T2 weighted images. A MRI of the pelvis is required for rectal NENs with size > 2cm, with invasion of the muscularis propria or beyond (T2-4) or with nodal disease (Caplin et al., 2012). A confident assessment of stage, prognosis and predicted resection margins can help guide the choice of therapeutic intervention (Taylor et al., 2011). A MRI of the pelvis is required when planning salvage therapy following an incomplete resection to assess for invasion, stage, and predicted resection margins (Figure 3-4).

MRI and EUS are complementary investigations for staging rectal NENs; MRI is more sensitive for assessing nodal disease but can miss T1 lesions, while EUS accurately differentiates T1 from T2 disease but under stages T4 disease (Fernandez-Esparrach et al., 2011, Li et al., 2015). CT complements MRI with staging by detecting perirectal fat and fascia infiltration as well as local lymph nodes. CT staging is required when nodal disease has been identified on EUS or MRI to assess for distant metastases. Therapy options in stage 4 rectal NEN disease can range from curative surgical resections through to debulking surgery and systemic medical therapy (Figure 3-5).
Figure 3-4. MRI pelvis image.

The MRI demonstrates regional lymph nodes (white arrows) from a grade 2 rectal NEN.

Figure 3-5. Surveillance MRI image.

The MRI demonstrates a new liver metastasis (white arrow) after a transanal endoscopic microsurgery (TEMS) resection of pT2, N0 rectal NEN.

3.4.4.2 Functional imaging
Functional somatostatin receptor imaging, either with scintigraphy or positron emission tomography CT (PET-CT), is useful for detecting metastatic lesions in G1 or G2 rectal
NENs (Teunissen et al., 2011). Radiolabelled somatostatin analogues bind to SSTR2 receptors expressed by NENs. Radiotracer accumulation in NEN deposits appears ‘avid’ on somatostatin receptor scintigraphy (discrete dark lesions) and PET-CT (discrete bright coloured lesions) when compared to physiological tissue uptake. PET-MRI is being evaluated as an alternative to PET-CT and offers the benefit of reduced ionizing radiation exposure (Figure 3-7).

An assessment should be made when lesions are greater than 2cm or if metastatic disease is suggested on CT. However, the sensitivity of Indium In-111 labeled Pentetreotide somatostatin receptor scintigraphy (OctreoScan, Mallinckrodt Medical B.V, Petten, The Netherlands) for detecting metastatic disease has not been established clearly establish in rectal NENs, with background activity also potentially obscuring the primary rectal lesion (Figure 3-6) (Kwekkeboom et al., 2000). Gallium-68 somatostatin receptor PET-CT offers higher sensitivity than somatostatin receptor scintigraphy for detecting NEN lesions (Figure 3-8) (Srirajaskanthan et al., 2010, Gabriel et al., 2007). It is superior to CT alone for staging rectal NENs and is more sensitive for detecting bone metastases (Prasad et al., 2011). Higher grade (grade 3) or poorly differentiated rectal NENs may not express somatostatin receptors and imaging with FDG PET-CT may be more beneficial in assessing the distribution of metastatic disease.
Figure 3-6. $^{111}$Indium-octreotide scintigraphy planar images.

The images demonstrate uptake in two small pelvic nodes (black arrows). This is best demonstrated on the Posterior planar image at 24 hours (furthest right image). The central uptake is accumulation of tracer in the bladder.

![Image of In-111 scintigraphy](image)

Figure 3-7. Gallium-68 somatostatin receptor PET-MRI image.

PET-MRI is being evaluated as an alternative functional imaging modality in NENs. The image demonstrates a rectal NEN demonstrating tracer uptake in an avid rectal mucosal lesion (white arrow highlighting a discrete bright coloured area).

![Image of Ga-68 PET-MRI](image)

Figure 3-8. Gallium-68 somatostatin receptor PET-CT image.

Investigation from the same patient as Figure 3-4 with tracer uptake in avid pelvic nodal disease (white arrows highlighting discrete bright coloured areas)
3.4.5 Therapeutic intervention for rectal NENs

The choice of therapeutic modality is based on a comprehensive diagnostic assessment as outlined and is aimed at achieving an optimal oncological resection with clear margins and no residual disease (Figure 3-9). There are no clear data from large multicenter studies and controlled trials to help guide the management of rectal NENs. The algorithms and management strategies outlined below are based on a review of the searched evidence, which is from predominantly single-center series and consensus guidelines, as well as the authors’ own expert practice in a regional referral center for NENs (Ramage et al., 2008, Pavel et al., 2012, Caplin et al., 2012, Ramage et al., 2016).

Endoscopic resection of a rectal NEN should ideally only take place after additional investigations have confirmed no invasion beyond the submucosa and the absence of regional disease. Oncological surgical resection is indicated if there is evidence of invasion involving the muscularis propria or regional disease. The grade of the NEN does influence therapy choice but may not be known until after the histological review of the resected specimen. An unresected rectal NEN <1cm can be empirically considered to be grade 1 given that the overwhelming majority are at histological assessment. Grade 1 NENs can be resected endoscopically while grades 2 or 3 NENs are more suitable for surgical resection. Importantly, the post-resection histological grade and stage may indicate that further secondary therapy is warranted.
Atypical rectal NEN features are a semipedunculated or ulcerated orofungating shape, surface changes with depression, erosion, ulceration and hyperaemic colour. LN – lymph nodes, APR - abdominoperineal resection, EMR – endoscopic mucosal resection, ESD – endoscopic submucosal dissection, TEMS - transanal endoscopic microsurgery, TAR – transanal resection.

3.4.5.1 Primary endoscopic resection
The choice of optimal endoscopic resection technique for rectal NENs depends on size, mucosal and submucosal appearance. Piecemeal biopsy removal of a rectal NEN is not recommended as it limits histological assessment, particularly of deep and lateral margins that affect complete resection rates. There is a greater chance of residual disease and recurrence following biopsy resection. Tattooing of rectal NENs is advocated to allow for easy identification of the site if secondary therapy is required and for long term surveillance. This is particularly important if piecemeal biopsy resection has been
attempted at the index endoscopy when only a small scar may be visible to indicate the site for salvage therapy.

Traditional endoscopic mucosal resection (EMR) using submucosal injection for lift prior to snare cautery resection is effective for lesions less than 0.5cm with low risk stigmata. Submucosal lifting helps ensure that the deep margin of the lesion is more likely to be resected with clear margins and no residual disease (Larghi and Waxman, 2007). An assessment with EUS is indicated if the lesion does not lift as there may be muscularis propria invasion, which is a contraindication for endoscopic resection.

Device-assisted EMR can help with ensuring clear deep resection margins for rectal NENs up to 1cm, especially those without mucosal changes. Cap-assisted EMR (EMR-C) suctions the lifted lesion into an attached cap prior to snare cautery resection (Inoue et al., 1992, Oshitani et al., 2000). The technique is useful for cutting the submucosal layer from the muscularis propria for a safe and clear deep margin. Similarly, ligation-assisted EMR (EMR-L) deploys bands around the base of tissue suctioned into an attached cap (Chaves et al., 1994, Fleischer et al., 1996). The band detaches the submucosa from the muscularis propria through contractile force prior to snare cautery resection. EMR-L compares favorably with endoscopic submucosal dissection (ESD) in small rectal NENs (0.5cm) in terms of ease of use, procedure duration and complete resection rates (Niimi et al., 2012, Choi et al., 2013). In particular, EMR-L is suited to NENs in the lower rectum as the complication of perforation is reduced at this site.

For rectal NENs from 0.5cm to 2cm in size, ESD has the advantage of wide en-bloc resection margins with clearance of the lateral and deep margins for a greater chance of complete resection when compared to device-assisted EMR (Figure 3-10 and 3-11) (Wang et al., 2015, Arezzo et al., 2015). This allows for the deep submucosal margin of a rectal NEN to be visualized and adequately resected. The recurrence rates of NENs resected by ESD are lower than that of EMR. However, ESD has a greater perforation risk than EMR and requires additional expertise to perform successfully. EMR using a dual-channel endoscope (EMR-D) has been shown to be comparable to ESD for complete resection rates in rectal NENs up to 16mm with the advantage of shorter procedure times and complications (Lee et al., 2013). The second channel allows forceps to be used to lift the lesion to help identify the deep submucosal lesion for safer deep resection. Surgical
options should be explored if device-assisted EMR or ESD is not available for 1-2cm rectal NENs.

**Figure 3-10. ESD of a submucosal lesion identified in the mid rectum.**

*The left image demonstrates the lesion with right image showing the appearances after endoscopic submucosal dissection (ESD), which was performed after EUS confirmed no nodal or invasive disease.*

Half of endoscopically resected small rectal NENs (0.5cm) may have histological evidence of lymphatic or vascular invasion but this is not associated with long-term recurrence or metastatic disease (Sekiguchi et al., 2015). Interestingly, curative resection rates are lower (65%), despite high EMR and ESD complete resection rates (88-92%), but are not associated with recurrent disease or a poorer prognosis in long-term series (Nakamura et al., 2015). Moreover, only a fraction of patients develop recurrence in instances of incomplete histological resection while under surveillance (Sung et al., 2012).
3.4.5.2 Secondary or salvage endoscopic resection
Small rectal NENs are frequently presumed to be of benign aetiology, such as a hyperplastic polyp or lipoma, with minimal clinical significance. Often an attempt at resection is performed at the index endoscopy using suboptimal techniques like cold biopsy or simple snare polypectomy. The deep margin can be involved if a rectal NEN is resected without submucosal lift or assisted devices. A pragmatic approach at the index endoscopy is to perform EMR with lift for any rectal lesion that is small (<0.5cm), solitary and without high-risk stigmata.

If the histological resection margins are involved, a further endoscopic assessment of the scar site is required. A post-resection EUS of the scar is useful, especially if the rectal NEN > 0.5cm, to assess for residual submucosal and nodal disease. A MRI pelvis can help delineate disease in cases of incomplete resection when salvage therapy is planned. Incomplete or failed primary resection of rectal NENs with residual disease does not
preclude salvage endoscopic resection. Secondary endoscopic therapy for failed primary resection, either from polypectomy or primary EMR, of small grade 1 or 2) rectal NENs has been shown to be effective using EMR-C (Jeon et al., 2011). ESD is also a safe and effective endoscopic salvage therapy and an attractive alternative to surgery (Hurlstone et al., 2008). However, ESD may be limited in availability when compared to EMR-C or surgery.

3.4.5.3 Surgical resection
A transanal surgical approach is advocated for rectal NENs up to 2cm in size without evidence of invasion of the muscularis propria or nodal disease (stage 1 disease). Lower rectal lesions (<5cm from anal verge) can be resected with conventional local transanal resection (TAR) techniques (Ishikawa et al., 2005). Middle and upper rectal lesions can be resected with transanal endoscopic microsurgery (TEMS) (Kinoshita et al., 2007, Nakagoe et al., 2003). The technique has advantages of accurate and magnified visualization of margins for wide and full-thickness excisions. TEMS can also be used for salvage therapy following an incomplete endoscopic resection with 30% of resection specimens having evidence of microscopic disease. Rectal NENs that are greater than 2cm in size, grade 3 or invasive (stage 2-4) should have oncological surgical resection (anterior resection or abdominoperineal resection) with total mesorectal excision. This is based on the increased risk of metastatic disease, particular with higher-grade tumours.

3.4.6 Surveillance of rectal NENs
The surveillance protocol for rectal NENs is guided by the risk of recurrence based on factors like size, grade and stage (Figure 3-12) (Caplin et al., 2012). Additional poor prognostic markers like lymphovascular or perineural invasion should be taken into consideration when establishing the surveillance protocol. The guidance differs between those with complete resection margins and those with incomplete margins but no residual disease. This again emphasises the need for a definitive initial therapy aimed at complete resection.
3.4.6.1 Completely resected stage 1 rectal NENs

Grade 1 or 2 rectal NENs < 1cm in size can be discharged from surveillance given the low risk of recurrence. However, grade 3 NENs of the same small size have a greater chance of recurrence and should have annual surveillance with colonoscopy as a minimum. The surveillance should continue for 5 years and then default to those for an adenomatous polyp. For all rectal NENs 1-2cm in size, irrespective of grade, surveillance at 12 months is advocated with colonoscopy, EUS and MRI given the greater chance of recurrence. The surveillance should again then default to those for an adenomatous polyp.

All completely resected rectal NENs >2cm in size should have surveillance (colonoscopy, EUS and MRI) given the risk of recurrence with the interval dependent on grade; annually for grade 1 or 2 rectal NENS and at 4-6 months for grade 3 rectal NENs during the first year and then annually. Surveillance using a biomarker of NEN disease, such as chromogranin A, may provide additional information if it were elevated at diagnosis and normalised following resection as an increase may indicate recurrence.

3.4.6.2 Incompletely resected stage 1 rectal NENs

For incompletely resected grade 1 rectal NEN <1cm without overt evidence of residual disease 6 monthly surveillance with EUS is advocated for 2 years. EMR-C or ESD of the scar site should also be considered for further wider clearance and repeat histological
assessment. This is a similar strategy to that advocated for secondary or salvage endoscopic resection if residual NEN disease was present.

3.4.6.3 Completely resected stage 2-4 rectal NENs
Stage 2 or 3 rectal NENs that have complete resections with curative intention should have 6-monthly surveillance for 5 years with CT of the chest, abdomen, and pelvis given the risk of metastatic disease. Often a MRI pelvis can be helpful to discriminate between disease and post surgical changes like fibrosis or inflammation. Stage 4 rectal NENs should similarly have a 6-monthly surveillance protocol with CT with a minimum of 5 years with some centres advocating up to 10 years. Biomarkers paired with radiological imaging may be useful in the surveillance period if they were abnormal and responded after curative resection.

3.4.7 Management of recurrent and metastatic disease
The management of metastatic disease to the liver or other distant site is beyond the scope of this chapter but is outlined in consensus guidelines (Pavel et al., 2012, Anthony et al., 2010, Caplin et al., 2012). The overriding treatment paradigm is that surgical resection offers the only chance for cure and that a multidisciplinary team should guide an individual patient’s treatment. The primary rectal NEN should be treated to palliate symptoms such as rectal bleeding or to reduce the risk of incipient bowel obstruction. However, unlike NENs from other primary sites like the small bowel, there is no survival benefit from resection of the primary rectal NEN in advanced metastatic disease. Liver directed and systemic therapy options exist for patients with advanced disease.

3.5 Discussion
Rectal NENs are increasing in incidence and are often incidentally diagnosed at colonoscopy. The impact of different bowel cancer screening strategies on the natural history of rectal NENs is uncertain with unproven effect on morbidity and mortality. Endoscopists need to be aware how specific characteristics, such as size and atypical features, influence the likelihood of invasion, regional and metastatic disease. A thorough pre-resection endoscopic assessment, with additional diagnostics like EUS and MRI, should directly affect the choice of resection modality to maximise complete resection rates and long-term outcomes. Patients with locally advanced and metastatic rectal NEN disease should be referred to expert NEN centres as the choice of therapy can be
multimodal over the patient’s disease history. The development of international NEN disease registries would further improve our understanding of rectal NEN tumour biology and the choice of therapies.
4 ILEO-COLONIC NEUROENDOCRINE NEOPLASMS IDENTIFIED IN THE ENGLISH BOWEL CANCER SCREENING PROGRAMME (BCSP)

4.1 Abstract

Background: Ileo-colonic NENs are diagnosed as part of bowel cancer screening programmes (BCSP) but the incidence and characteristics are not known, particularly with faecal occult blood (FOBT) screening.

Aims: To identify and characterise NENs diagnosed from the English BCSP, a double screen programme using guaiac FOBT and colonoscopy, by analysing the national colorectal screening database and validating the findings with individual BCSP centers.

Methods: The Exeter database was interrogated by running queries to identify participants with coded NENs (2006-2014). A written proforma was sent to the responsible BCSP clinician for validation and characterisation.

Results: 13,061,716 participants were adequately screened with gFOBT with 259,765 participants with definitively abnormal results. There were 146 unique participants with NEN-related codes from 216,707 BCSP colonoscopies. The incidence rates per 100,000 colonoscopies were 29 rectal, 18 colonic and 11 for ileal NENs. The incidence of all NENs was 28 times greater than the published population incidence. The majority of
rectal NENs had grade 1 (80%) and stage T1 (85%) disease. Over half of ileal NENs (54%) in this study had T3/4 invasive disease, with 85% having nodal and 36% having metastastic disease.

Conclusions: The characteristics and incidence of ileal and colonic NENs suggest that gFOBT may play a screening role in addition to the role of colonoscopy seen with rectal NENs. Additional data from BCSPs, including from those with different screening strategies, will help our understanding if colorectal cancer screening helps identify occult NENs at an earlier stage for better outcomes.

4.2 Introduction

Neuroendocrine neoplasms, particularly rectal NENs, can be identified through routine colonoscopy but there are limited data on those identified through bowel cancer screening programmes of age specific populations (Basuroy et al., 2016). Rectal NENs diagnosed as a result of bowel cancer screening programmes are smaller and at an earlier stage than those diagnosed from non-screening endoscopy (Kaminski et al., 2007b, Scherubl, 2009, Matsui et al., 1993). They are invariably 10mm or less and grade 1. There are few epidemiological data on rectal NENs diagnosed in bowel cancer screening programmes. The Polish bowel cancer screening programme adopts a single screen strategy with colonoscopy alone and reported a cohort of 50148 participants with a rectal NEN prevalence of 0.05-0.07% or 50-70 per 100,000 colonoscopies performed (Kaminski et al., 2007b). The increased numbers of rectal NENs diagnosed in this cohort when compared to the SEER data (approximately 1 per 100,000 population per year) are likely to reflect its underlying age-specific prevalence from screening an asymptomatic population rather than from diagnoses from colonoscopies performed for other indications (Modlin et al., 2003, Kaminski et al., 2007b). A significant proportion of rectal NENs diagnosed in bowel cancer screening programmes are likely to be incidental findings but it is not clear whether there are more significant co-pathology identified and an increased synchronous malignancy rate with colorectal adenocarcinoma. There are no clear data on differences in rectal NENs incidences with different bowel cancer screening programme strategies; direct to endoscopy (single screen) versus primary faecal occult blood test with secondary endoscopy (double screen). There are no data on small bowel NENs identified from terminal ileal intubation at BCSP colonoscopy.
4.2.1.1 The English bowel cancer screening programme

The incidence in the UK of colorectal cancer is 184 per 100,000 population per year in 60-75 year olds (CancerResearchUK, 2015). This is the population targeted in the English bowel cancer screening programme, which uses a double screening strategy of primary guaiac faecal occult blood testing (gFOBT) and secondary colonoscopy (Hardcastle et al., 1996). Three postal kits for gFOBT are sent to invited participants and returned for analysis. The iron-containing component of haemoglobin (haeme) has peroxidase activity that turns guaiac blue when hydrogen peroxide is added. False positives can occur as peroxidases are found in common foodstuffs (Young et al., 2002). Participants are invited to attend for the secondary screening test with colonoscopy if the primary screening gFOBTs are abnormal. BCSP colonoscopy requires completion and visualization of the whole colon to the caecum, the most proximal part of the colon, but does not necessarily mandate terminal ileal intubation.

In the English BCSP roll out period (July 2006 – October 2008) 2.1 million participants were invited to complete gFOBT testing with 1.08 million (51%) returning the required number of test kits (Logan et al., 2012). 21,106 participants (2%) who completed gFOBT had abnormal results and were offered to attend for colonoscopy. 17,518 participants attended for colonoscopy with 10% (1772 participants) being diagnosed with colorectal cancer. There are no published data on the numbers or characteristics of neuroendocrine neoplasms identified from the English BCSP.

This study aims to identify the number of NENs diagnosed in the English BCSP through a two-step process; a primary query of the BCSP database to identify participants with NENs and secondary data validation with reporting centres to corroborate database coding and to further characterise identified NENs.

4.3 Methods

A service evaluation application was submitted to English BCSP Research Committee, Public Health England (PHE) and NHS Health Research Authority (HRA) Confidential Advisory Group (CAG). A CAG proportionate review approved the project’s information governance structure. A PHE Office of Data Release (ODR) contract (ODR1516_038) was issued to Hampshire Hospitals NHS Foundation Trust to allow time limited
Chapter 4: Ileo-colonic neuroendocrine neoplasms identified in the English bowel cancer screening programme (BCSP)

pseudonymised data access for record linkage and validation. The project was completed within the time period stipulated (March 2016) by the ODR contract. Mortality data was not available as part of the ODR contract. All data was pseudonymised using a study identifier with linkage known only to an independent study coordinator.

4.3.1 BCSP (Exeter) database search
English bowel cancer colonoscopy data is stored on the Exeter database managed by PHE. Queries were jointly developed with PHE to capture potential NEN-related search terms across relevant data tables in the Exeter database. The ‘polyp architecture’, ‘SNOMED’ and ‘lesion type’ data tables were identified as relevant to NEN related colonoscopy findings (IHTSDO). Queries using the NEN search terms outlined in the Table 4-3 were run for each data table to identify BCSP participants attending for colonoscopy with NEN-related coding from 2006 to December 2014. In addition participation data at primary screening and secondary screening was requested to support the calculation of NEN diagnosis rates in the BCSP. The anatomical site for the NEN related findings were broadly categorized into colorectal, ileal, appendiceal and unknown sites. Colorectal NENs were subcategorized into colonic and rectal anatomical site. Incidences per 100,000 colonoscopies performed were calculated and reported by anatomical site as well. There was no coding in the Exeter database for size, histological features and staging for non-colorectal pathology such as NENs.

<table>
<thead>
<tr>
<th>POLYP ARCHITECTURE</th>
<th>SNOMED</th>
<th>LESION TYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocrine Tumour</td>
<td>Endocrine carcinoma</td>
<td>Endocrine carcinoma</td>
</tr>
<tr>
<td>(Carcinoid)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant carcinoma</td>
<td>Goblet cell endocrine tumour</td>
<td></td>
</tr>
<tr>
<td>Malignant carcinoma</td>
<td></td>
<td>Mixed endocrine tumour/Adenocarcinoma</td>
</tr>
</tbody>
</table>

Table 4-1. NEN related database search terms across tables in the English BCSP database.
Chapter 4: Ileo-colonic neuroendocrine neoplasms identified in the English bowel cancer screening programme (BCSP)

Three Exeter database tables were identified with coding relating to NEN; polyp architecture, SNOMED and lesion type. The investigators and PHE jointly authored the NEN search terms.

4.3.2 Data validation proforma
A written proforma was sent to the responsible BCSP clinician for all participants identified with NEN related search terms. The proforma contained validation questions for NEN diagnosis as well as additional details on tumour characterization, diagnostic and therapeutic modalities as well as referrals, follow-up and patient support (Appendix 1). Non-identifiable clinical reports were reviewed if provided as supplementary data. Histological grading was classified by the mitotic index and from the expression of Ki-67, a tumour proliferation marker (Hotta et al., 2006, Shimizu et al., 2000). There was no independent histopathological review of tissue specimens performed as per the study’s application, review and approval. TNM staging of NENs was reported by European Neuroendocrine Tumor Society (ENENS) guidelines, with sub-classification of T1 stage rectal NENs by size (T1a <1cm, T1b 1-2cm), if provided on the written proforma or sufficiently described on the supplementary reports (Jann et al., 2011). Metastatic disease was judged if present on radiological imaging, functional imaging or on EUS in line with ENENS guidance. Follow-up communication on a maximum of three occasions was made to encourage completion of proformas and for missing data queries.

4.3.2.1 CHARACTERISTICS OF NEUROENDOCRINE NEOPLASMS
- Confirmation of the type of tumour and site if NEN
- Maximum tumour dimension of NEN
- Depth of invasion
- Presence of vascular invasion
- Grade of tumour and actual Ki-67 index
- Presence of metastatic disease present at diagnosis
- TNM staging of tumour (TNM 7) and type of staging (radiological versus pathological)
- Pathology reporting Standards (ENENS TNM, WHO 2004, WHO 2010)
• Results of staging CT scan, CT colonography, pelvic MRI, rectal EUS (if performed)
• Presence of pathological lymph nodes on MRI or EUS for rectal NENs
• Results of chromogranin A or B
• Presence of other significant pathology identified, like cancer or inflammatory bowel disease.

4.3.2.2 INITIAL PATIENT MANAGEMENT AND REFERRALS
• Type of Multi-Disciplinary Meeting (MDM) and any recommendations or guidance
• Details of specialty referrals made for management (i.e. surgery, oncology, gastroenterology) and if NEN center referral was made.
• Details of additional management recommendations made and if altered planned care

4.3.2.3 TREATMENT AT DIAGNOSIS
• Details of colonoscopy report including details of management of NEN lesion with EMR or standard polypectomy.
• Details of further therapeutic procedure including endoscopic resection and surgery.
• Histological resection data including grade, resection margins and lymph nodes status
• Other therapies including on metastases and additional histological grade data.

4.3.2.4 PATIENT FOLLOW UP
• Duration and frequency of follow up
• Surveillance or follow-up modalities, including imaging, chromogranins, and endoscopy
• Disease recurrence with details on sites and therapies.
• Status of patient in follow-up (active, exited surveillance or death)

4.3.2.5 PATIENT INFORMATION AND SUPPORT
• Patients informed about the presence of a NEN
• Sources of information given to patient
4.3.3 Data analysis
Data coding and analysis was performed with Microsoft® Excel® for Mac (2011). Missing data was coded as unknown. Coding rates per 100,000 colonoscopies performed were calculated and reported by anatomical site as well. Statistical testing was not performed given the lack of relevant comparative data for NENs identified in BCSPs. Data were presented as medians with interquartile ranges and percentages with absolute numbers (% identified number / total number) for groupings identified during the analyses.

4.4 Results

4.4.1 BCSP (Exeter) database search

4.4.1.1 Participation data during study period
23,405,057 invitations to participate in the English BCSP were sent from 2006 until December 2014. Of these invitations 13,061,716 participants were adequately screened with three gFOBT equivalent to a 55.8% uptake for the primary screening test. 259,765 participants had definitively abnormal gFOBT results equivalent to 1.99% of those adequately screened. 216,707 participants had colonoscopies equivalent to 83.42% uptake for the secondary screening test in those with abnormal gFOBT and 1.66% of total of participants who were adequately screened. The screening data for each year of the BCSP categorised by participant sex are outlined in the appendices.

4.4.1.2 NEN search terms results
There were 146 unique BCSP participants with NEN related codes across the three database tables in this time period (Table 4-2). All of the 64 participants coded in the ‘polyp architecture’ table with the term ‘Endocrine Tumour (Carcinoid)’ were unique and were not additionally coded in either the ‘SNOMED’ or ‘lesion type’ tables. There were 82 unique participants coded in either the ‘SNOMED’ or ‘lesion type’ tables with a proportion coded in both tables (28 out of 82 participants).
Table 4-2. Result from NEN related database search of the English BCSP database.

Three Exeter database tables were searched with coding relating to NEN. Participants can be coded across multiple tables. The numbers of participants returned per table and per NEN search term are outlined. 146 unique participants were identified through the searches.

4.4.1.3 NEN coding rate by year

On average 16 participants are coded each year with NEN related terms since the inception of BCSP, or 21 participants per year if the first two years of the BCSP roll out are excluded (Table 4-3). The BCSP becomes fully established from 2010 onwards (colonoscopy volumes greater than 30,000 per year) when approximately 26 participants are coded per year. Approximately 60% of the 146 participants with NEN codes were male (n=87).
Table 4-3. Participants coded with a NEN each year of the BCSP with gender, colonoscopy volume and average numbers.

146 unique participants were identified through the searches of the English BCSP database across the study period (2006 to December 2014). BCSP colonoscopy volume data and participants (total, female, male) coded are presented for each year. Average numbers of participants coded per year for the whole BCSP period and excluding the initial roll out period (2006/07) are outlined and rounded to the nearest whole number.

4.4.1.4 NENs by site and incidence

As expected with colonoscopy screening, the majority of the 146 participants with NENs coded had lesions in the colorectal region (n=102, 70%) (Table 4-4a). Other sites include ileal (n=24, 16%), unknown (n=18, 12%) and appendiceal (n=2, 1%, coded as ‘goblet cell’) NENs. There were differences in percentages between male (M) and female (F) participants for colorectal (F – n=39, 66%; M – n=63, 72%) and ileal (F – n=13, 22%; M – n=11, 13%) sites but not with appendiceal sites (2% F vs. 1% M). The absolute numbers of ileal NEN sites were small and there was no data on terminal ileal intubation rates to explain the greater percentage of ileum NENs in female participants.
Subcategorisation of colorectal NENs (n=102, 70%) by anatomical site revealed that rectal NENs (n=62, 42%) were the most common. The rectum as an anatomical site was the most common across both female (n=28, 47%) and male (n=34, 39%) participants (Table 4-4b and 4-4c). However, the second most common site was different between genders; ileal NENs in female (n=13, 22%) and colonic NENs in male participants (n=28, 33%). Coding for colonic NENs in female participants was much lower (n=11, 19%) than in male participants (n=28, 33%), which cannot be attributed to differences in visualization of the whole colon during BCSP colonoscopy.

The coding rate for all NENs was 67 per 100,000 colonoscopies per year (146 NENs coded in 216,707 BCSP colonoscopies during study period) with coding rates for rectal, colonic and ileum NENs of 29, 18 and 11 per 100,000 colonoscopies per year respectively. The gender differences in absolute numbers outlined above for ileal and rectal NENs are reflected in differences in coding rates by anatomical site. In female participants, the incidence of NENs is 66 per 100,000 colonoscopies per year with rates for rectal, ileal and colonic of 31, 14 and 12 per 100,000 colonoscopies per year respectively. In male participants, the incidence of NENs is 69 per 100,000 colonoscopies per year with rates for rectal, colonic and ileum of 27, 23 and 9 per 100,000 colonoscopies per year respectively.
Table 4-4a. All participants coded with a NEN each year of the BCSP categorised by anatomical site with corresponding coding rates.

Table 4-4 (a-c). Participants coded with a NEN each year of the BCSP categorised by anatomical site with corresponding coding rates.

Table 4-4a outlines the data for all participants while 4-4b and 4-4c presents data for female and male participants respectively. 146 unique participants were identified through the searches of the English BCSP database across the study period (2006 to December 2014). Sites of coded NENs are presented with colorectal subcategorized into colonic and rectal sites. Average numbers of participants coded per anatomical site per year for the whole BCSP period and excluding the initial roll out period (2006/07) are outlined and rounded to the nearest whole number (excluding appendiceal NENs where numbers are too few). Coding rates for all NENs and by anatomical site are calculated using the volume of BCSP colonoscopy and presented as per 100,000 colonoscopies per year.
Table 4-4b. Female participants coded with a NEN each year of the BCSP categorised by anatomical site with corresponding coding rates.

<table>
<thead>
<tr>
<th>Year</th>
<th>Total NET</th>
<th>Colorectal NET</th>
<th>Colonic NET</th>
<th>Rectal NET</th>
<th>Ileum NET</th>
<th>Appendix NET</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2007</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2008</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2009</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2010</td>
<td>8</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2011</td>
<td>13</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2012</td>
<td>11</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2013</td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2014</td>
<td>9</td>
<td>7</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>39</td>
<td>11</td>
<td>28</td>
<td>13</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>% of total</td>
<td>100%</td>
<td>66%</td>
<td>19%</td>
<td>47%</td>
<td>22%</td>
<td>2%</td>
<td>10%</td>
</tr>
<tr>
<td>Average</td>
<td>6.6</td>
<td>4.3</td>
<td>1.2</td>
<td>3.1</td>
<td>1.4</td>
<td>0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Exclude 2006/2007</td>
<td>8.43</td>
<td>5.57</td>
<td>1.6</td>
<td>4</td>
<td>1.9</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Incidence</td>
<td>66</td>
<td>43</td>
<td>12</td>
<td>31</td>
<td>14</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 4-4c. Male participants coded with a NEN each year of the BCSP categorised by anatomical site with corresponding coding rates.

<table>
<thead>
<tr>
<th>Year</th>
<th>Total NET</th>
<th>Colorectal NET</th>
<th>Colonic NET</th>
<th>Rectal NET</th>
<th>Ileal NET</th>
<th>Appendix NET</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2007</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2008</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2009</td>
<td>8</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2010</td>
<td>15</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2011</td>
<td>17</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>2012</td>
<td>15</td>
<td>10</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2013</td>
<td>14</td>
<td>9</td>
<td>2</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2014</td>
<td>12</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>87</td>
<td>63</td>
<td>29</td>
<td>34</td>
<td>11</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>% of total</td>
<td>100%</td>
<td>72%</td>
<td>33%</td>
<td>39%</td>
<td>13%</td>
<td>1%</td>
<td>14%</td>
</tr>
<tr>
<td>Average / year</td>
<td>9.7</td>
<td>7</td>
<td>3.2</td>
<td>3.8</td>
<td>1.2</td>
<td>0.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Exclude 2006/2007</td>
<td>12.43</td>
<td>9</td>
<td>4.1</td>
<td>4.9</td>
<td>1.6</td>
<td>0.1</td>
<td>1.7</td>
</tr>
<tr>
<td>Coding rate</td>
<td>69</td>
<td>50</td>
<td>23</td>
<td>27</td>
<td>9</td>
<td>1</td>
<td>9</td>
</tr>
</tbody>
</table>
4.4.1.5 BCSP to population ratios

Colorectal cancer and NEN populations rates from SEER and Cancer Research UK data (per 100,000 per year) and corresponding BCSP coding rates (per 100,000 colonoscopies per year) are presented in Table 4-5 with BCSP to population rate ratios calculated (Logan et al., 2012, Banck et al., 2013, Modlin et al., 2008). The BCSP incidence of CRC is 55 times greater than the same age specific population incidence. This reflects the purpose of the BCSP double screening strategy with participants with abnormal gFOBT being offered colonoscopy to help identify colorectal cancer. The BCSP incidence of NENs is 28 times greater than the population incidence (not age specific) from the SEER database. This reflects the positive effect, though attenuated when compared to colorectal cancer, of the double screening strategy in identifying NENs. The greatest effect in ratios was for coded colorectal NENs (ratio 36), particularly for colonic NENs (ratio 45) over rectal NENs (ratio 32). The BCSP to population ratios for coded ileal and appendiceal NENs are lower at 12 and 5 respectively.

<table>
<thead>
<tr>
<th>Type of cancer and anatomical site</th>
<th>Population rate per 100,000 per year</th>
<th>BCSP rate per 100,000 colonoscopies per year</th>
<th>Ratio (BCSP/Population)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal cancer</td>
<td>184</td>
<td>10,100</td>
<td>55</td>
</tr>
<tr>
<td>All NET</td>
<td>2.4</td>
<td>67</td>
<td>28</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>0.9</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Appendixal</td>
<td>0.2</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Colorectal</td>
<td>1.3</td>
<td>47</td>
<td>36</td>
</tr>
<tr>
<td>Other colonic</td>
<td>0.4</td>
<td>18</td>
<td>45</td>
</tr>
<tr>
<td>Rectal</td>
<td>0.9</td>
<td>29</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 4-5. Population and BCSP rates for colorectal cancer and coded NENs categorised by anatomical site with BCSP to population ratios.

Population incidence rates for colorectal cancer and neuroendocrine neoplasms are presented per 100,000 per year (Logan et al., 2012, Banck et al., 2013, Modlin et al., 2008). Published diagnosis rates for colorectal cancer from the English BCSP and NEN coding rates from this study are presented as BCSP rates per 100,000 colonoscopies per year. The ratios of BCSP to population rates are rounded to the nearest whole number.
4.4.2 Data validation proforma
Proformas were distributed via email and post to the responsible BCSP clinician and site coordinator for each of the 146 participants identified as part of the database search. The return rate for proformas was high (82%, 119/146) as a result of follow-up communication for missing proformas. Nine of the participants were validated as adenocarcinomas by the BCSP site and excluded from further analysis; they were incorrectly coded in the database tables as ‘endocrine carcinoma’ (n=7), ‘mixed endocrine tumour/adenocarcinoma’ (n=1) and ‘endocrine tumour (carcinoid)’ (n=1). Five of these incorrectly coded cases were from a single BCSP site, which may represent problems with local coding as this represented all the potential NEN participants from that site. The characteristics of the remaining 110 validated NEN lesions in BCSP participants are discussed in more depth in this section.

4.4.2.1 Characteristics of neuroendocrine neoplasms
The overwhelming majority of participants with validated NEN lesions were well differentiated (85%, 94/110) with only a minority poorly differentiated (8%, 9/110) or goblet cell carcinoid (appendiceal site) and MANEC (both 2%, both 2/110) (Table 4-6).

<table>
<thead>
<tr>
<th>Differentiation of NET</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated NET</td>
<td>94</td>
</tr>
<tr>
<td>Poorly differentiated NEC (small cell)</td>
<td>4</td>
</tr>
<tr>
<td>Poorly differentiated endocrine carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Poorly differentiated NEC (large cell)</td>
<td>2</td>
</tr>
<tr>
<td>Goblet Cell Carcinoid</td>
<td>2</td>
</tr>
<tr>
<td>Mixed adenoneuroendocrine carcinoma (MANEC)</td>
<td>2</td>
</tr>
<tr>
<td>Other / -</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>110</strong></td>
</tr>
</tbody>
</table>

Table 4-6. Differentiation or histological appearance of validated NENs.

Validation proformas were sent to BCSP sites to complete for the 146 coded NENs as part of secondary validation and to characterise in more detail. 119 proformas were returned with 9 participants validated as incorrectly coded. The histological appearance
and/or differentiation as reported on the proforma or supporting anonymised reports were analysed.

4.4.2.1.1 Anatomical Site
Anatomical site was recorded for almost all of the returned validated NEN proformas (109/110) (Table 4-7). Almost half of the NENs identified in participants were rectal (49%, 54/110) with the remaining being ileal (27%, 30/110), colonic (16%, 17/110) and appendiceal (7%, 8/110). The percentage and absolute numbers of rectal NENs are in keeping with the database coding for participants. The numbers of ileal NENs (30/110) were greater in number than those coded on the BCSP database (24/146). In contrast, validated colonic NENs were less in number (17/110) than expected from the database (40/146). There were a small number of appendiceal NENs (7%, 8/110) reported to be diagnosed from participation in the BCSP. One case of appendiceal NEN was identified at endoscopy with an abnormal appendiceal orifice appearance in the caecum, while the others were identified incidentally at surgery or on histology following a right hemicolecotomy for colorectal cancer.

There was good concordance between the database coded and clinician validated anatomical site where both were available (101/110). An ‘unknown’ database coded anatomical site was clarified in nine instances by returned validation proformas, predominantly affecting validated appendiceal NENs (6/9) and ileal NENs (3/9). The discordance in numbers for colonic, ileal and appendiceal NENs was likely related to the return rate of proformas (82%) as well asClarifying a proportion of the ‘unknown’ database anatomical sites (9/110). Additionally, ileal and appendiceal NENs may have been incorrectly coded as colonic NENs on the BCSP database. Importantly, these changes in validated numbers for all anatomical sites from the proforma would impact on the incidence and ratio calculations outlined earlier. In particular, the incidences and ratios for appendiceal and ileal NENs would increase, as the numbers in the validated cohort are greater than those in the total identified coded; 30 validated ileal NENs versus 24 database, and 8 validated appendiceal NENs versus 2 database.
Table 4-7. Anatomical site of validated BCSP NENs with lesion size and histological grade.

Validation proformas confirmed 110 NENs with supporting details on anatomical site, lesion size and histological grade as reported by BCSP sites or from supporting anonymised reports. Size data is reported in millimetres for median and interquartile ranges (IQR) in brackets (excluding IQR for all NENs). Histological grading was classified by the mitotic index and from the expression of Ki-67, a tumour proliferation marker (Hotta et al., 2006, Shimizu et al., 2000, Jann et al., 2011).

4.4.2.1.2 Histological Grade
The grade of was reported on the proforma or in available from anonymised reports for 98/110 participants (Table 4-7). 85% of participants with this data available were reported as grade 1 (83/98) with 8% as grade 2 (8/98) and 7% as grade 3 (7/98). Grade data was available for 87% rectal NENs (47/54) with 91% reported as grade 1 (43/47), 2% grade 2 (1/47) and 6% grade 3 (3/47). Grade data was available for 86% of ileal NENs (26/30) with 96% reported as grade 1 (25/26) and 4% grade 2 (1/26). Grade data was available for 94% of 16/17 colonic NENs (16/17) with 56% reported as grade 1 (9/16), 19% grade 2 (3/16) and 25% grade 3 (4/16).

4.4.2.2 Rectal NENs
The validation proformas and supporting reports described 54 rectal NENs (Table 4-7). They were small with a median size of 5mm and a narrow interquartile range of 3-6.75mm. The largest lesion was reported as 40mm and the smallest lesion as 1mm. 85% of rectal NENs were reported as being less than 10mm in size (46/54) with 11% between 10-20mm (6/54) and 4% greater than 20mm (2/54).
Data on T stage was available for 87% of rectal NEN cases (47/54) (Table 4-8). Irrespective of size, rectal NENs were early stage (T1 – 85%, 40/47) with only a small proportion that invaded the muscularis propria or beyond (T2 – 4%, 2/47; T3 -11%, 5/47). The overwhelming majority of rectal NENs less than 10mm were staged at T1a (38/39) where data was available, with only one case staged at T2 invading beyond the submucosa into the muscularis propria. Rectal NENs between 10-20mm were staged as 33% T1b (2/6) with the remainder invading into the muscularis propria (T2, 17%) and through into the subserosa, non-peritonealised pericolic or perirectal tissues (T3, 50%). Both rectal NENs greater than 20mm invaded into the subserosa, non-peritonealised pericolic or perirectal tissues (T3, 100%).

Data on N stage was available for 34% of rectal NEN cases (18/54) with a significant number not assessed (denoted ‘x’, 29/54) or unknown (denoted ‘-‘, 7/54). This may be related to endoscopy being the predominant resection modality for T1 rectal NENs when nodal status may not be assessed by other modalities. All rectal NENs less than 10mm did not have nodal disease (12/12) where this data was available and assessed. Half of 10-20mm rectal NENs had nodal disease (2/4) where available and assessed. Both rectal NENs greater than 20mm had nodal disease.

Data on M stage was available for 26% of rectal NEN cases (14/54). Again this may be related to endoscopy being the predominant resection modality for the majority of rectal NENs when metastatic disease may not be assessed by other modalities. All rectal NENs less than 10mm did not have metastatic disease (11/11) where this data was available or assessed. Half of 10-20mm rectal NENs had metastatic disease (2/4) where available and assessed. Metastases were present in the one rectal NEN greater than 20mm where assessed or reported. Free text data for the specific sites of metastatic disease was limited and not suitable for analysis.
Table 4-8. TNM staging data for validated rectal NENs categorised by size.

Validation proformas confirmed 54 rectal NENs with supporting details on TNM staging reported by BCSP sites or from supporting anonymised reports (Jann et al., 2011). TNM data that were not assessed are reported as ‘x’ and when not available as ‘-’. Percentages are rounded to nearest whole %.
4.4.2.3 Ileal NENs
Table 4-7 outlines the size data for the 30 validated ileal NENs. Lesions had a median size of 15mm with an interquartile range of 11.75-25.25mm. T stage data were available for 93% of ileal NEN cases (28/30) reflecting the role of cross sectional imaging for ileo-colonic lesions prior to consideration for surgical resection and histological staging. Nearly half of ileal NENs (46%, 13/28) with T stage data did not invade beyond the muscularis propria and were stage as T1 (21%, 6/28) or T2 (25%, 7/28). The remainder (54%, 15/28) were reported to invade into the subserosa or beyond into the visceral peritoneum and were stage as T3 (32%, 9/28) or T4 (21%, 6/28).

Data on N stage were available for 90% of ileal NEN cases (27/30). 85% of ileal NENs (23/27) with N stage data were reported to have nodal disease (N1). Data on M stage were available for 37% of ileal NEN cases (11/30). 36% of ileal NENs (4/11) with M stage data were reported to have metastases. 44% of participants with nodal disease (4/9) had metastatic disease as well. Free text data for the sites for metastatic disease was limited and not suitable for analysis.

4.4.2.4 Role of diagnostics
Staging CT was performed in 71% (78/110) of participants with a validated NEN lesion. 54% of participants with rectal NENs (29/54) underwent staging CT. This may be influenced by the fact that most rectal NENs had limited disease that did not invade the muscularis propria (T1). The majority of ileal NENs (93%, 28/30) had staging CT likely due to more advanced disease and planned management with surgical resection. Free text or anonymised copies of reports were rarely completed and not suitable for analysis. CT colonography was rarely performed (5/110) given colonoscopy was the screening test. Chromogranin blood testing was performed in 34% of participants (37/110) with the greatest coverage in ileal NENs (63%, 19/30).

Pelvic MRI was performed in 35% (19/54) of participants with rectal NENs. Data on the status of pathological lymph nodes on pelvic MRI were limited with few completed proformas and reports describing abnormal nodes (2/11). EUS was performed in a limited number of rectal NENs (7%, 4/54) potentially related to the early stage of disease as well as access to the diagnostic modality. There were no data on pathological local lymph nodes status at EUS.
4.4.2.5 Associated pathology
Non-NEN pathology was identified in 60% (53/88) of participants with NENs with assessable data that could range from colorectal cancer to adenomas or other unreported pathology. Colorectal cancer was a co-malignancy in 9% participants (8/88). The NEN was an incidental finding in 75% (6/8) of these cases. Appendiceal NENs were associated with colorectal cancer as a co-malignancy in 5/6 instances (83%). Invariably the appendiceal NEN was an incidental post-resection histological finding following hemicolectomy for a colorectal cancer identified during the colonoscopy. Adenomas were identified at BCSP colonoscopy in 55% (48/87) of participants with assessable data; 74% of rectal (32/43), 43% of appendiceal (3/7) and 35% of ileal (8/23) NENs. Reporting of the numbers of adenomas identified in participants was limited and not suitable for analysis.

4.4.2.6 Initial patient management and referrals
The overwhelming majority of participants (94%, 102/108) with a validated NEN were discussed in a multidisciplinary meeting (MDM), mostly in colorectal MDMs (82%, 89/108) rather than a specific NEN MDM (12%, 13/108). Additional recommendations were made in 82% of instances (63/77).

BCSP participants with a NEN were referred to a number of specialties, sometimes multiple specialties. Surgery (65/110), NEN centers (35/110) and oncology (24/110) were the main referral specialties. The data on the type of review offered by the NEN center and its recommendations were limited and not being suitable for analysis.

4.4.2.7 Treatment at diagnosis
Therapy was attempted at the index BCSP colonoscopy in 83% of rectal NENs (45/54) with polypectomy in 73% (33/45) and EMR in 27% (12/45) instances. Data on subsequent endoscopic or surgical procedures following the index BCSP endoscopy were available for 85% participants (94/110). All participants with ileal and appendiceal NENs had a subsequent procedure following the index BCSP endoscopy. 37% of participants with rectal NENs (16/43) had a subsequent therapy where this data was available. This is on the background of a high proportion (83%) having primary therapy at the index BCSP endoscopy. Data on the type of secondary therapy performed was complete for 63/110 participants and are outlined in Table 4-9.
Table 4.9. Surgical or secondary therapy for validated BCSP NENs by anatomical site.

<table>
<thead>
<tr>
<th>Anatomical Site</th>
<th>Number</th>
<th>EMR</th>
<th>ESD</th>
<th>Lap. surgery</th>
<th>Open surgery</th>
<th>Other</th>
<th>TEMS</th>
<th>-</th>
</tr>
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<tr>
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<td>1</td>
<td>8</td>
<td>7</td>
<td>2</td>
<td>7</td>
<td>44</td>
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<tr>
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<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>38</td>
</tr>
<tr>
<td>Colonic</td>
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<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Ileal</td>
<td>30</td>
<td>12</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Appendiceal</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td>2</td>
<td>1</td>
<td>24</td>
<td>27</td>
<td>2</td>
<td>7</td>
<td>47</td>
</tr>
</tbody>
</table>

Abbreviations: EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection; TEMS, transanal endoscopic microsurgery; Lap. Surgery, laparoscopic surgery; ‘-‘, therapy data not available.

The number of lymph nodes removed during surgery was available for the 55% of participants undergoing surgery (60/110). The median number of lymph nodes involved was 2 with an interquartile range of 1-6. Data on histological resection margins, as assessed from the proforma or from histology reports, were available for 65% of validated NENs (72/110); 77% with a R0 resection (56/72), 18% for R1 resection (13/72) and 4% R2 resection (3/72). The majority of R1 resections were for rectal NENs (76%, 10/13) and all R2 resections were for proximal colonic or ileal NENs. Therapies on metastases were performed on seven participants, ranging from the use of somatostatin analogues through to hepatic resections for metastases and systemic chemotherapy.

4.4.2.8 Patient follow up
Arrangements for planned follow up were reported on the proforma for 66 participants with a median planned follow-up of 36 months (range 0-120 months) with an interquartile range of 6-60 months. Follow up was on an annual basis for 31 participants and on a six monthly or more frequent basis for a further 45 participants. Data on adherence to a follow up schedule was completed for 45% of participants (49/110) with full adherence reported in 84% of this cohort (41/49). CT was reported as the main surveillance modality during the follow-up period, mostly for participants with ileal NENs. In particular, endoscopy
(25/28) was recommended for following up rectal NENs. Surveillance was often reported as multimodal using a combination of diagnostic tests (i.e. MRI, chromogranins, EUS, octreoscan).

Data on the recurrence of NEN disease was available for 60% of the participants (66/110). Recurrence was reported in 14% (9/66) of all participants with 14% for ileal NENs (3/22) and 3% for rectal NENs (1/31). Data on the site of recurrence, whether local, regional or metastatic, and the modality of therapy used to manage recurrence was incomplete and not suitable for analysis. Data on the participant’s status in follow up was available for 44% participants (48/110) with 65% under active follow-up, 21% exiting surveillance and 15% reporting to have died. However, there was no validated external mortality and cause of death data available for this study.

4.4.2.9 Patient information and support
Participants were informed of the NEN diagnosis in 97% of occasions (73/75) where data were assessable. Proforma reporting of information distribution was limited, with NEN related literature or details of the NEN patient foundation (NPF) confirmed to be given to only 10% of validated NEN participants (11/110). Specialist nurse support was offered to 80% of participants (45/56) through predominantly non-NEN clinical nurse specialists (77%, 35/45). The data on participants with advanced disease referred to NEN centers or offered clinical trials was incomplete and not suitable for analysis.

4.5 Discussion
This study adds to the published data to date on neuroendocrine neoplasms identified through BCSPs, reporting on both the characteristics of rectal and ileal NENs, as well being the first study of double screening (FOBT and colonoscopy) BCSPs.

However, there were study limitations resulting from the two-stage methodology of primarily interrogating the BCSP database to identify participants with NEN codes and secondarily validating those results via written proformas sent to BCSP sites. There was no specific code for ‘neuroendocrine neoplasm’ in the database. Some potential NENs lesions may have been missed if not captured by the NEN-related surrogate codes based on ‘endocrine’ and ‘carcinoid’. Additionally, under-reporting of NENs may have
impacted on the study results as coding of non-CRC and non-adenomatous lesions in the English BCSP was not mandatory during the study period. The calculation of NEN coding rates per 100,000 colonoscopies per year performed and population to BCSP ratios were solely based on the primary stage data from querying the BCSP database, without secondary validation of diagnosis. Discordance in numbers of coded on the BCSP database and validated NEN numbers would influence these calculations. Importantly, there were no independent histopathological assessments to confirm NEN diagnoses, grade and differentiation, nor central review of radiology and other diagnostics to confirm staging assessments.

The written validation proforma that was distributed to BCSP sites had a high return rate of 82% but a number of data fields were incomplete, which particularly limited the insights on managing and supporting BCSP participants diagnosed with NENs. Notwithstanding issues around BCSP coding and study data coverage, factors like endoscopic recognition, particularly of small incidental rectal NENs, and histological reporting practices for non-CRC non-adenomatous ‘incidental’ lesions may lead to underreporting and an underestimation of NENs diagnosed in the English BCSP.

NENs were identified with a higher ratio (28 times greater) in the English double screen BCSP than in the SEER database. The ratios were lower than that seen with colorectal cancer between an age-specific population and BCSP incidences (55 times greater). The incidence of colorectal cancer and NENs are likely to increase with age and the SEER data used in this study to calculate incidence ratios was not age-specific, with the true age-specific incidence of NENs in the BCSP population of 60-74 year olds likely to be higher. This would likely reduce the described ratios of BCSP NEN rates to age-specific NEN incidence, limiting the effect further of the double screen BCSP strategy in identifying new NEN cases. Therefore, double screening with colonoscopy and gFOBT may not be as effective a population screening methodology for identifying asymptomatic participants with ileo-colonic NENs as it is for colorectal cancer. The Polish BCSP uses single screen endoscopy and reported 24 rectal NEN in 50,148 colonoscopies, an incidence of approximately 48 per 100,000 colonoscopies performed (Kaminski et al., 2007b). This is higher than the incidence of rectal NEN of 29 per
100,000 colonoscopies per year reported in this double screen English BCSP study. It was not possible to differentiate the effect of gFOBT primary screening from secondary colonoscopy for identifying NENs in this English BCSP. However, the assumption is that endoscopy itself, rather than gFOBT, may be the most critical component for screening for rectal NENs. They are invariably small smooth lesions with minimal mucosal disruption that only rarely ulceration. It would seem unlikely that a rectal NEN would result in occult bleeding and a positive FOBT during screening. Importantly, English BCSP colonoscopy is only offered to the 2% of participants who have abnormal gFOBTs, suggesting that there maybe a large undiagnosed cohort of rectal NENs in the remaining 98% of participants who have normal gFOBTs.

There appears to be no differences in rectal NEN characteristics in this study of the double screen English BCSP and the Polish single screen cohort. Rectal NENs were almost half of all NENs identified in this study and were small (85%<10mm, median size 5mm), which compares favourably to the Polish BCSP that reported a median size of 6mm and typical endoscopic features in 84% of cases. The majority of rectal NENs in this study were grade 1 (80%) and early T stage (T1 – 85%, 40/47). Only a small proportion invaded the muscularis propria (T2 – 4%, 2/47) or beyond (T3 -11%, 5/47). Nodal disease was present in 22% (4/18) and metastases in 14% (2/14) of participants with complete data. However, a large proportion of clinical proformas and data returned for validation did not assess for nodal (Nx, 54%) or metastatic disease (Mx, 61%), or this data was unknown (‘-‘, 13%). The majority of rectal NENs managed with endoscopy alone were T1 stage, which implies the risk of nodal and metastatic disease is low and does not necessarily require EUS or MRI investigation (Basuroy et al., 2016). Thus, the true proportion of rectal NEN participants with advanced disease in the English BCSP will be less than calculated in this study if the numbers for Nx and Mx staging were also included. Interestingly, the staging of rectal NENs in the English BCSP is similar to that described in published non BCSP data where the majority are limited to the submucosa (T1, 89%) with only a fraction with invasive disease (T2, 5%; T3 or T4, 6%) and regional disease affecting lymph nodes or distant metastases (12-20%) (McDermott et al., 2014, Weinstock et al., 2013).
Future insights into the role of FOBT and endoscopy in identifying NENs through screening of asymptomatic populations may come through recent changes in the English BCSP. The recent introduction of a single screen flexible sigmoidoscopy (‘bowel scope’) BCSP at the age of 55 years, in addition to the existing double screen BCSP for 60-75 years old, is hypothesized to further prevent colorectal adenocarcinoma cases and deaths (Geurts et al., 2015). This may help quantify the effect of endoscopy itself in identifying rectal NENs and the true underlying incidence in unselected participants. The incidence of rectal NENs in an age-specific population attending the direct-to-test flexible sigmoidoscopy BCSP should mirror the prevalence of all 55 year olds in England.

There are no published data on the role of FOBT for aiding the diagnosis of gastrointestinal NENs. Ileal NENs identified in this study were advanced and not associated with CRC suggesting that FOBT may play a role in aiding the diagnosis of asymptomatic ileal NENs. 54% of participants with ileal NENs in this study had invasive disease (T3/4) with 85% having nodal disease (N1) and 36% having metastases (M1). These luminal lesions can often have an ulcerated appearance that may lead to occult blood loss and FOBT positivity. The current gFOBT assay will be replaced in England by the quantitative faecal immunochemical test (FIT), which is specific for human blood, easier to process and more acceptable for participants to use (PHE, 2016). FIT requires only one small faecal sample from a single bowel motion compared to two samples from three different motions for gFOBT. A recent trial of FIT against gFOBT in the English BCSP demonstrated an increase in uptake of 7% to 66.4%, particularly from previous non-responders, men and more deprived populations (Moss et al., 2016). This change in primary screening test led to a twofold increase for colorectal cancer and fivefold increase for advanced adenomas. The quantitative FIT result can be used to determine an acceptable positivity rate for screening with concentrations up to 40 μg Hb/g faeces achieving increased colorectal cancer detection rates and up to 180 μg Hb/g faeces for increased advanced adenomas detection.

Increased numbers of incidental NENs diagnosed through population screening for colorectal cancer appears to have limited impact on services with approximately 26 participants diagnosed per year through BCSP in England. The majority of cases were discussed in colorectal MDMs, managed by surgeons and with limited clinical nurse
support for patients. Referral pathways to NEN specialists for opinions or management appear to be less mature and maybe geographically dependent. It is not clear if the volume of bowel scope flexible sigmoidoscopy will result in far greater numbers of participants diagnosed with rectal NENs who may require specialist input from non-BCSP related clinical services, like advanced endoscopy services (for EUS and ESD) and NEN centers. The endoscopist’s index of suspicion for atypical rectal lesions is an important factor that affects pick up rates and successful management of rectal NENs (Basuroy et al., 2016).

From a cancer outcomes perspective, there were no survival outcome data available for this study and there are no other published data demonstrating survival differences between NEN patients diagnosed within BCSPs and those diagnosed otherwise. It is unclear if there will be a significant positive impact on rectal NEN morbidity and mortality as has been demonstrated with CRC mortality (Atkin et al., 2010, Scholefield et al., 2002). The natural disease history and tumour biology of small incidental colorectal NENs, like rectal NENs, has not been characterised. However, ileal NENs identified in the English BCSP were more locally advanced with nodal and metastatic disease. It is likely that this represents significant clinical disease at the time of the BCSP colonoscopy, which invariably would have led to symptoms with possible increased morbidity and mortality if diagnosed later. While there has been a 20% improvement in the overall 5 year survival rate of rectal NENs since the era of BCSPs, demonstrating a true mortality effect from BCSP on NENs may be difficult given the total numbers diagnosed are small (Scherubl, 2009). The 5-year survival rates for rectal NENs from the SEER database and Norwegian Register of Cancer for the period 1993-2004 are high anyway at 74%-88% (Hauso et al., 2008).
5 MARKERS OF CARCINOID

HEART DISEASE IN SMALL BOWEL NENs

5.1 Abstract

Introduction: NT-proBNP (NTP) is suggested as the best current biomarker to screen for CHD and monitor for heart failure (HF) (suggested cut off of >260 pg/ml). A number of other markers have been explored to diagnose and prognosticate in heart failure but have not been explored in sbNEN patients with CS and CHD. Galectin-3 (GAL3) promotes fibroblast proliferation and collagen synthesis and correlates with worse outcomes in HF. ST2 (or IL-1 R4) is an interleukin-1 receptor that signals the severity of cardiac remodelling and tissue fibrosis in response to cardiac events and HF. Adrenomedullin (ADM) is elevated in heart failure and is reported to have prognostic value in sbNENs. Calprotectin is an inflammatory protein increased in heart failure and correlating with severity.

Methods: Three groups of sbNEN patients (n=37) were identified with blood released from the King’s College Hospital Institute of Liver Studies biobank; CHD (Group A, n=10), non-functional (Groups B, n=12, normal CgA, 5HIAA, BNP), functional (Group C, n=15, ↑chromogranin A (CgA) & urine 5HIAA, normal BNP). Analysis was performed using NTP, GAL3, ST2, Calprotectin and adrenomedullin commercial assays. Statistical analysis was performed with SPSS.
Results: The median values for NTP in the CHD cohort was above the 260 pg/ml cut off. Median values for calprotectin were elevated across all three groups. ST2, GAL3 and adrenomedullin were not elevated. The Kruskal–Wallis test across the 3 patient groups was significant for NTP (p=<0.001) but not for ST2, GAL3, adrenomedullin and calprotectin. The Mann-Whitney U-test was significant (p< 0.05) between the CHD and both other groups but was not significant (p=0.12) between the functional and non-functional groups. There was significant correlation between GAL3 and calprotectin.

Discussion: The results corroborate the role of NTP in CHD for NEN patients. ST2 may play a role in combination with NTP for risk stratification in CHD and heart failure. GAL3 requires further evaluation given its possible role in the development of cardiac fibrosis. Its value may be for screening at an earlier stage of CHD. Larger studies of putative markers at various stages of sbNEN disease and in CHD, such as early, advanced and post valve surgery, may be of benefit. A panel of fibrosis and inflammatory markers in combination with natriuretic peptides may be clinical value in sbNEN and CHD patients.

5.2 Introduction

Guidance recommends screening for carcinoid heart disease (CHD) with the biomarker N-terminal pro-brain natriuretic peptide (NT-proBNP) (Ramage et al., 2012, Bhattacharyya et al., 2008, Korse et al., 2009, Davar et al., 2017). Other biomarkers have been investigated in CHD or more broadly in heart failure. Activin A has high sensitivity (87%) but low specificity (57%) for detecting CHD and is an independent predictor of mortality in sbNENs (Bergestuen et al., 2010, Zahid et al., 2015). The American College of Cardiology Foundation (ACCF) and American Heart Association (AHA) joint guidelines for managing heart failure recommend a combination of the natriuretic peptides with fibrosis markers, particularly for prognostication (Writing Committee et al., 2013). A number of potential biochemical markers are associated with inflammation and fibrosis in heart failure, rather than purely with volume overload and natriuresis like NT-proBNP, that may also be of value in screening for CHD. Such markers may be of value both in advanced CHD with volume overload as well as in early CHD with active fibrosis.
but without volume overload. Four potential markers were evaluated in this study; adrenomedullin, calprotectin, galectin-3, and soluble ST2.

5.2.1.1 Adrenomedullin
Adrenomedullin (ADM) has been suggested as a putative biomarker for NEN disease itself as well as being associated with cardiac failure. It is a potent vasodilator and natriuretic peptide that is secreted from the ventricular myocardium in heart failure (Jougasaki et al., 1995). The profiles of plasma and pericardial ADM in heart failure are dissimilar suggesting differences in regulation (Nishikimi et al., 2004). The activity of ADM in the vasculature is exerted via calcitonin receptor-like receptor, increasing cAMP and nitric oxide synthesis via intracellular signal transduction pathways (Cheung and Tang, 2012, Wong et al., 2012). ADM in the heart inhibits hypertrophy and, importantly, fibrosis suggesting a compensatory role in heart failure and myocardial infarction. This is in contrast to other markers explored in this study.

ADM is reported to be involved in tumour progression, being secreted by tumour cells and associated stromal cells, acting as a growth factor, preventing apoptosis, inducing angiogenesis, increasing metastatic potential and blocking immune-surveillance (Larrayoz et al., 2014). The production of ADM in tumours is related to pro-inflammatory substances and oxidative stress. Plasma ADM is increased in malignancy and tissue ADM is overexpressed in a range of tumours, including NENs. It is significantly elevated, particularly with small bowel NENs and in those with progressive disease (Pavel et al., 2006). Over half of tumour samples had greater than 5% cytoplasmic staining for ADM. There were no published data on plasma ADM levels in CHD prior to this study.

5.2.1.2 Calprotectin
Low-grade inflammation is thought to be important in the pathogenesis of cardiac disease including heart failure (Yndestad et al., 2006, Kruzliak et al., 2014). Calprotectin is an inflammatory cascade protein that is released from the cytosol of neutrophils and monocytes (Hessian et al., 1993). It promotes extravasation of leukocytes at sites of inflammation by facilitating attachment and weakening of tight junctions. Consequently, calprotectin is released in inflammation into circulating plasma (Frosch et al., 2000). Plasma calprotectin is increased in heart failure and may be associated with severity
(Jensen et al., 2012). There were no published data on plasma calprotectin levels in CHD or NENs prior to this study.

5.2.1.3 Soluble ST-2
Suppression of tumorigenicity 2 (ST2, also known as IL-1 R4 and T1) is a member of the interleukin-1 receptor family and a marker of cardiac fibrosis and ventricular remodeling (Bayes-Genis et al., 2015, Bayes-Genis et al., 2014). ST2 has a transmembrane receptor form (ST2L) and a soluble decoy form (sST2). IL-33 reduces fibrosis and hypertrophy through binding to ST2L on cardiac tissue (Januzzi, 2013). Elevated levels of sST2 are associated with the severity of symptomatic heart failure and cardiovascular mortality as well as helping with the optimisation of therapy through serial measurement (Wettersten and Maisel, 2016, Januzzi et al., 2015). There were no published data on ST2 in CHD or NENs prior to this study.

5.2.1.4 Galectin-3
Galectin-3 (Gal-3) is a soluble beta-galactoside-binding lectin that has been implicated in the pathophysiology of heart failure (de Boer et al., 2009). Gal-3 is released from activated cardiac macrophages and stimulates myofibroblasts that leads to fibrosis and cardiac remodeling (Sharma et al., 2004). Gal-3 has prognostic value in both acute and chronic heart failure with an association with all-cause mortality but not with cardiovascular mortality (Filipe et al., 2015). Gal-3 does not add to risk stratification in heart failure, in contrast to ST2, in a head to head study (Bayes-Genis et al., 2014). There were no published data on Gal-3 in CHD or NENs prior to this study.

CHD is characterised by the presence of metastatic functional small bowel NENs that secrete vasoactive substances that cause inflammation and fibrosis. In contrast to NT-proBNP (NTP), which is associated with volume overload and natriuresis, these four circulating markers are either associated with cardiac fibrosis, inflammation or NEN disease itself. Therefore, they may have value as potential biomarkers of CHD, particularly in patients with early cardiac disease without volume overload or cardiac strain. The putative markers were explored in a comparative study with the recommended NTP in three clinically distinct subpopulations of sbNEN patients, who were biochemically non-functional, functional, or with diagnosed carcinoid heart disease. The non-functional cohort was included as a control group for the biomarkers.
5.3 Methods

An application was made to the King’s College Hospital (KCH) Institute of Liver Studies biobank (HTA reference 12378) for approval to use archived samples from consented sbNEN patients.

5.3.1 Cohorts of small bowel NEN patients

The tumour board clinical database (LiverWare) was interrogated to identify subgroups of sbNENs as part of a wider approved service evaluation. All patients identified had a histologically confirmed low-grade small bowel NEN. The presence of residual disease was not evaluated or stratified as part of this study. The following keyword searches of the ‘problem list’ table in the LiverWare database were used to identify sbNEN patients with CHD; carcinoid heart disease, valve replacement, tricuspid, pulmonary, aortic, mitral, regurgitation, stenosis, heart failure. Validation of CHD was performed in conjunction with the electronic healthcare records, including with echocardiography results.

Plasma radioimmunoassay chromogranin A (CgA) and urinary 5HIAA measurements (24 hour or random levels) were used to differentiate functional and non-functional subgroups. Brain-type natriuretic peptide (BNP) was the assay routinely used in KCH for CHD heart failure assessment during the period of sample archiving in the biobank.

King’s College Hospital (KCH) Institute of Liver Studies biobank staff confirmed that subgrouping was appropriate and selected the final patient samples in each cohort with valid consent. Frozen samples were released anonymously (0.5ml EDTA blood and 0.5ml clotted serum) to the investigators and immediately transferred without freeze-thaw to KCH Viapath freezers. KCH Viapath laboratory technicians performed the assays.

5.3.1.1 Group A – Carcinoid heart disease (CHD)

The presence of CHD was confirmed by auditing the electronic patients records. The urine 5HIAA was elevated above the normal range (5HIAA >4 mg/g creatinine) with radioimmunoassay CgA above the normal range (two assays over period of collection >6 nmol/L and >60 pmol/L). A total of 10 patient samples were released for analysis.
5.3.1.2 Group B – Non-functional (NF)
The urine 5HIAA was within the normal range (5HIAA <4 mg/g creatinine) with radioimmunoassay CgA within normal range (two assays over period of collection <6 nmol/L and <60 pmol/L). Heart failure was excluded in this cohort with a normal range brain-type natriuretic peptide (BNP <100pg/ml). A total of 12 patient samples were released for analysis.

5.3.1.3 Group C – Functional (F)
The urine 5HIAA was elevated above the normal range (5HIAA >4 mg/g creatinine) with radioimmunoassay CgA above the normal range (two assays over period of collection >6 nmol/L and >60 pmol/L). Heart failure was excluded in this cohort with a normal range brain-type natriuretic peptide (BNP <100 pg/ml). A total of 15 patient samples were released for analysis.

5.3.2 Assay methodology
5.3.2.1 NT-proBNP
NT-proBNP reagent kit (Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey GU16 8QD) is a two-site chemiluminescence immunoassay. The assay consists of a bead-bound polyclonal sheep antibody and a sheep polyclonal anti-NT-proBNP antibody conjugated to alkaline phosphatase from bovine calf intestine in a buffer as the reagent. A soluble sandwich complex only develops in the presence of insulin molecules that bridge the two antibodies. Therefore, only peptides that also bridge the antibodies are quantitated. A chemiluminescence substrate captures the amount of alkaline phosphatase when measured by light emission read with a high sensitivity photon counter. This is directly proportional to the concentration of NT-proBNP in the samples. The samples were analysed on the Siemens Immulite 2000 analyser.

Individual aliquots of clotted serum samples (50 μL) for each patient were thawed and incubated at 37°C simultaneously with both antibodies in a reaction tube. The tube was intermittently agitated to develop the soluble sandwich complex between the bead bound and buffer antibodies. The reaction tube was then spun at high speed to force reaction fluid up and into the coaxial sump chamber of the Siemens Immulite 2000 analyser. The reaction tube was serially washed to remove unbound material from the bead and the
inner tube. The chemiluminescent substrate was added to the reaction tube and light emission read with a high sensitivity photon counter to ascertain the amount of NT-proBNP present.

The details of precision, linearity, sensitivity, and reference ranges for the NTP assay are outlined below:

5.3.2.1.1 Precision

<table>
<thead>
<tr>
<th></th>
<th>Intra-assay precision</th>
<th>Inter-assay precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level 1</td>
<td>Level 2</td>
</tr>
<tr>
<td>Mean (ng/L)</td>
<td>35.6</td>
<td>1430</td>
</tr>
<tr>
<td>SD</td>
<td>1.92</td>
<td>42.4</td>
</tr>
<tr>
<td>CV (%)</td>
<td>5.4</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Coefficient of variation (CV)

5.3.2.1.2 Linearity

Up to 35,000 ng/L, any results above this level were reported as >35,000 ng/L. Samples were not diluted if above this range.

5.3.2.1.3 Sensitivity

The minimum detectable concentration of NT-proBNP assay is 10 ng/L.

5.3.2.1.4 Reference range

The current quoted reference range for NT-proBNP is:

<table>
<thead>
<tr>
<th>Age</th>
<th>NT-proBNP ng/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;75 yrs</td>
<td>125</td>
</tr>
<tr>
<td>&gt;75 yrs</td>
<td>450</td>
</tr>
</tbody>
</table>

5.3.2.2 ST2

The ST2 assay (R&D Systems, 9 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB) is a quantitative sandwich enzyme immunoassay technique containing NS0-expressed recombinant human ST2. A monoclonal antibody specific for ST2 is pre-coated onto a microplate with any ST2 present in samples binding to the immobilised
antibody. A second sandwiching HRP-linked polyclonal antibody for ST2 then is able to provide colour intensity that can be measured and is proportional to the sample ST2.

Individual aliquots of EDTA plasma samples (50 µL) for each patient were thawed. Standards and samples were pipetted into the antibody pre-coated microplate wells. Unbound substances were washed away and an HRP-linked polyclonal antibody specific for ST2 was added to the wells. Excess antibody-enzyme reagent was washed away and substrate solution of TMB was added to the wells for colour development. Sulphuric acid was then added to stop colour development to allow intensity measurement.

The details of precision, linearity, sensitivity, and reference ranges for the ST2 assay are outlined below:

5.3.2.2.1 Precision

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-Assay Precision</th>
<th>Inter-Assay Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>N</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mean (ng/L)</td>
<td>273</td>
<td>628</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>15.2</td>
<td>27.5</td>
</tr>
<tr>
<td>CV (%)</td>
<td>5.6</td>
<td>4.4</td>
</tr>
</tbody>
</table>

5.3.2.2.2 Linearity

Up to 40.0 µg/L, any results above this concentration were reported as >40.0 µg/L. Samples were not diluted if above this range.

5.3.2.2.3 Sensitivity

Patient results less than 5.05 ng/L are reported as <5.05 ng/L.

5.3.2.2.4 Reference range

The current quoted reference range for ST2 is:

<table>
<thead>
<tr>
<th></th>
<th>Mean (µg/L)</th>
<th>Range µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>13.0</td>
<td>6.74 – 20.4</td>
</tr>
<tr>
<td>EDTA plasma</td>
<td>12.2</td>
<td>4.90 – 19.9</td>
</tr>
<tr>
<td>Heparin plasma</td>
<td>12.3</td>
<td>5.72 – 19.8</td>
</tr>
</tbody>
</table>
5.3.2.3 Galectin-3

The Galectin-3 assay (Quantikine ELISA kit distributed by R & D Systems Europe, 19 Barton Lane, Abingdon Science Park, Abingdon, Oxon, OX14 3NB) is a quantitative sandwich enzyme immunoassay technique containing an E-coli expressed recombinant human galectin-3. A monoclonal antibody specific for galectin-3 is pre-coated onto a microplate with any galectin-3 present in samples binding to the immobilised antibody. A second sandwiching horseradish peroxidase linked (HRP) polyclonal antibody for galectin-3 then is able to provide colour intensity that can be measured and is proportional to the sample galectin-3.

Individual aliquots of EDTA plasma samples (100 µL) for each patient were thawed. Standards and samples were pipetted into the antibody pre-coated microplate wells. Unbound substances were washed away and an HRP-linked polyclonal antibody specific for galectin-3 was added to the wells. Excess antibody-enzyme reagent was washed away and substrate solution of TMB was added to the wells for colour development. Sulphuric acid was then added to stop colour development to allow intensity measurement.

The details of precision, linearity, sensitivity, and reference ranges for the galectin-3 assay are outlined below:

5.3.2.3.1 Precision

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Mean (µg/L)</td>
<td>0.79</td>
<td>2.46</td>
<td>5.11</td>
<td>0.80</td>
<td>2.59</td>
<td>5.50</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.03</td>
<td>0.09</td>
<td>0.18</td>
<td>0.05</td>
<td>0.16</td>
<td>0.32</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.3</td>
<td>3.8</td>
<td>3.5</td>
<td>5.8</td>
<td>6.0</td>
<td>5.8</td>
</tr>
</tbody>
</table>

5.3.2.3.2 Linearity

Up to 10.0 µg/L, any results above this concentration were reported as >10.0 µg/L. Samples were not diluted if above this range.
5.3.2.3.3 Sensitivity
Patient results less than 0.016 µg/L are reported as <0.016 µg/L.

5.3.2.3.4 Reference range
The current quoted reference range for Galectin-3 is 2.40 – 15.7 µg/L.

5.3.2.3.5 Standardisation
This immunoassay is calibrated against highly purified E-coli-expressed recombinant human Galectin-3 produced by R & D Systems

5.3.2.4 Calprotectin
The Calprotectin assay (CALPROLAB™ Calprotectin ELISA (ALP) distributed by CALPRO Labs, Arnstein Arnebergsvei 30, 1366 Lysaker, Norway) is a quantitative sandwich enzyme immunoassay (ELISA) technique. A monoclonal antibody specific for Calprotectin is pre-coated onto a microplate with any Calprotectin present in samples binding to the immobilised antibody. The addition of enzyme-labelled, immunoaffinity-purified Calprotectin-specific antibodies provides colour intensity that can be measured that is proportional to the sample Calprotectin. The assay is calibrated using Calprotectin purified from leukocyte extract. The CALPROLAB™ Calprotectin ELISA (ALP) assay has been developed and validated mainly for faecal samples but is used for plasma/serum samples.

Individual aliquots of EDTA plasma samples (200 µL) for each patient were thawed and diluted 1:20 as per the protocol. Standards and samples were pipetted into the antibody pre-coated microplate wells. Unbound substances were washed away and an enzyme-linked polyclonal antibody specific for Calprotectin was added to the wells. Excess antibody-enzyme reagent was washed away and substrate solution was added to the wells for colour development. Colour intensity was measured on the ELISA plate reader set at 405nm.

The details of precision, linearity, sensitivity, and reference ranges for the Calprotectin assay are outlined below:

5.3.2.4.1 Intra-assay Precision
Tested using one kit lot; six samples were diluted 1:20 and tested with 10 replicates in one run:
5.3.2.4.2 Inter-assay Precision
Precision between plasma samples was not available for this kit.

5.3.2.4.3 Linearity
The assay is linear up to 500 µg/L, any results above this concentration are reported as >500 µg/L. Samples were not diluted if above this range.

5.3.2.4.4 Sensitivity
The assay can detect calprotectin as low as 5 µg/L.

5.3.2.4.5 Reference range
Calprotectin reference ranges are 0.15 to 0.9 mg/L (Kristinsson et al., 1998).

5.3.2.5 Adrenomedullin
The adrenomedullin assay (Uscn Life sciences Inc 108 Zhuanyang Avenue, Economic and Technological Development Zone, Wuhan 430056 China) uses a competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific for human ADM is pre-coated onto a microplate. A competitive inhibition reaction occurs between unlabelled human ADM (standards or samples) and biotin labelled human ADM for the pre-coated antibody specific for human ADM on the microplate. The addition of avidin conjugated to Horseradish Peroxidase (HRP) provides colour intensity that is inversely proportional to the concentration of ADM in the sample.

Individual aliquots of EDTA plasma samples (100 µL) for each patient were thawed. Standards and samples were pipetted into the antibody pre-coated microplate wells. Unbound substances were washed away and an avidin conjugated to HRP was added to
the wells and incubated. A substrate solution was added to the wells and the colour intensity measured that was inversely proportional to the sample ADM concentration.

The details of precision, linearity, sensitivity, and reference ranges for the adrenomedullin assay are outlined below:

5.3.2.5.1 *Intra assay precision*
Three samples with high, medium and low concentrations were measured 20 times in the same batch. CV = <10%

5.3.2.5.2 *Inter assay precision*
Three samples with high, medium and low concentrations were measured 20 times between batches. CV = <12%

5.3.2.5.3 *Linearity*
Up to 1000 ng/L, any results above this concentration were reported as >1000 ng/L. Samples were not diluted if above this range.

5.3.2.5.4 *Sensitivity*
Patient results less than 1.43 ng/L are reported as <1.43 ng/L.

5.3.2.5.5 *Reference range*
The current quoted reference range for ADM using this method is 9.4 – 12.0 ng/L.

5.3.3 Statistical Methods
The Shapiro Wilk test for normality was used for continuous variables, with non-parametric data reported as median values with interquartile ranges. Logarithmic transformation of continuous data was not performed for additional normality testing given the small sample size and lack of dilution if out of concentration range. Comparison of non-parametric data between cohorts was performed using the Kruskal-Wallis test with the Mann-Whitney Test used for inter-group comparisons. *P* values less than 0.05 were considered significant. Analysis was performed using IBM SPSS Statistics Version 23.00 (Armonk, NY: IBM Corp.).
5.4 Results

The background data for the three anonymised cohorts of sbNEN patients (A=CHD, B=Non-Functional (NF), C=Functional (F)) are outlined in Table 5-1.

<table>
<thead>
<tr>
<th>Group</th>
<th>ALL</th>
<th>A - CHD</th>
<th>B – Non Functional (NF)</th>
<th>C – Functional (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>37</td>
<td>10</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Female (%)</td>
<td>11 (30%)</td>
<td>5 (50%)</td>
<td>3 (25%)</td>
<td>3 (20%)</td>
</tr>
<tr>
<td>Mean age</td>
<td>62</td>
<td>61.8</td>
<td>61.2</td>
<td>62.8</td>
</tr>
<tr>
<td>(Range)</td>
<td>(38.3-79.9)</td>
<td>(38.3-75.2)</td>
<td>(40.1-79.9)</td>
<td>(44.1-79.5)</td>
</tr>
<tr>
<td>Median age</td>
<td>65.1</td>
<td>64.7</td>
<td>61.8</td>
<td>65.1</td>
</tr>
</tbody>
</table>

**Table 5-1. Baseline patient characteristics of the studied sbNEN cohorts**

5.4.1 Statistical analysis by assay

5.4.1.1 Distributions plots and parametric assessment

The distribution plots and statistics for each assay, including ranges, are outlined in Figure 5-1 and Table 5-2 respectively. Two results in the ST2 assay group were >40 ug/L and were analysed as 40 ug/L. Tests for parametric distribution for each assay using the Shapiro Wilk statistic (Table 5-3) highlight that the distributions are non-parametric. The medians for NTP, ST2, Galectin3 and ADM were within the normal reported range for each assay. The medians for Calprotectin were above the upper limit of the normal range (Table 5-2).
Chapter 5: Markers of carcinoid heart disease in small bowel NENs

<table>
<thead>
<tr>
<th>Statistic</th>
<th>ST2</th>
<th>Galectin3</th>
<th>Calprotectin</th>
<th>AdrenoM</th>
<th>NTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Median</td>
<td>13.58</td>
<td>5.43</td>
<td>1.2</td>
<td>3.9</td>
<td>121</td>
</tr>
<tr>
<td>Minimum</td>
<td>4.67</td>
<td>3.56</td>
<td>0.43</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Maximum</td>
<td>40</td>
<td>9.8</td>
<td>6.06</td>
<td>18.77</td>
<td>16983</td>
</tr>
<tr>
<td>Range</td>
<td>35.33</td>
<td>6.24</td>
<td>5.63</td>
<td>18.77</td>
<td>16959</td>
</tr>
<tr>
<td>Interquartile Range</td>
<td>6.9</td>
<td>2.64</td>
<td>1.03</td>
<td>4.68</td>
<td>315</td>
</tr>
<tr>
<td>Percentiles 25</td>
<td>10.595</td>
<td>4.53</td>
<td>0.79</td>
<td>2.525</td>
<td>65</td>
</tr>
<tr>
<td>Percentiles 50</td>
<td>13.58</td>
<td>5.43</td>
<td>1.2</td>
<td>3.9</td>
<td>121</td>
</tr>
<tr>
<td>Percentiles 75</td>
<td>17.495</td>
<td>7.165</td>
<td>1.82</td>
<td>7.205</td>
<td>380</td>
</tr>
<tr>
<td>Skewness</td>
<td>1.557</td>
<td>0.685</td>
<td>2.246</td>
<td>1.638</td>
<td>6.002</td>
</tr>
<tr>
<td>Kurtosis</td>
<td>2.443</td>
<td>-0.542</td>
<td>5.817</td>
<td>2.691</td>
<td>36.313</td>
</tr>
</tbody>
</table>

Table 5-2. Assay statistics for exploratory biomarkers and NT-proBNP.

ST2: 6.74 - 20.4 ug/L, Galectin-3: 2.4 – 15.7 ug/L, Calprotectin: 0.15 to 0.9 mg/L, AdrenoM: 9.4 – 12.0 ng/L, NTP: <75yrs: 125 ng/L >75yrs: 450 ng/L

<table>
<thead>
<tr>
<th>Statistic</th>
<th>df</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST2</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>Galectin3</td>
<td>37</td>
<td>0.016</td>
</tr>
<tr>
<td>Calprotectin</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>NTP</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>AdrenoM</td>
<td>37</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5-3. Shapiro Wilk statistics for parametric distribution of assay results.
Figure 5-1. Distribution plot for exploratory biomarkers and NT-proBNP assays.

5.4.1.2 Normal Q-Q plots
Normal Q-Q plots for each assay (Figure 5-2) confirm a distribution of observed results away from the expected straight-line result for a parametric distribution.
Figure 5-2. Normal Q-Q plots for each assay with an expected straight-line.

5.4.1.3 Tukey box plots by assay

The Tukey box plots for each of assay are outlined in Figure 5-3. The median, first and third quartiles are described as well as the whiskers describing the last datum within a 1.5x IQR range (i.e., the upper whisker is highest case within 1.5 times IQR and the lower whisker is the lowest case within 1.5 times IQR). Outlier results are displayed with corresponding sample ID (i.e. from 1-37) for each assay’s dataset. The most extreme
outlier across all assays was for the NTP (16,983 ng/L) leading to a flattened Tukey box plot. This sample was from the CHD cohort and there is no clinical data available on the severity of heart failure at sample time point.

Figure 5-3. Tukey box plots for each assay.

*Upper whisker is highest case within 1.5 times IQR and the lower whisker is the lowest case within 1.5 times IQR. Outlier results are displayed with corresponding sample ID.*
5.4.2 Statistical analysis by groups

5.4.2.1 Distributions statistics and parametric assessment

The distribution statistics for each assay by group (Group A (CHD) =10, Group B (non functional) =12, Group C (functional) =15) are described in Table 5-4 (a-e). The two results in the ST2 assay >40 ug/L (analysed as = 40 ug/L) were both in the CHD (Group A) and neither were from the patient with the largest NTP result. The median values for ST2, Galectin-3 and ADM were within the normal range across all three sbNEN cohorts. The median value for calprotectin was elevated across all three sbNEN groups. The median value of NTP in the CHD cohort was elevated above the normal range while those in the NF and F cohorts were within the range. Only 1 out of 10 in the CHD had a NTP value less than the cut off of 260 pg/ml (equivalent to 260 ng/L used in this study) reported as useful in CHD.

<table>
<thead>
<tr>
<th>ST2</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>17.205</td>
<td>12.765</td>
<td>12.63</td>
</tr>
<tr>
<td>Variance</td>
<td>143.392</td>
<td>40.509</td>
<td>14.973</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>11.97466</td>
<td>6.36469</td>
<td>3.86955</td>
</tr>
<tr>
<td>Minimum</td>
<td>10.28</td>
<td>4.67</td>
<td>6.71</td>
</tr>
<tr>
<td>Maximum</td>
<td>40</td>
<td>22.38</td>
<td>22.03</td>
</tr>
<tr>
<td>Range</td>
<td>29.72</td>
<td>17.71</td>
<td>15.32</td>
</tr>
<tr>
<td>Interquartile Range</td>
<td>22.66</td>
<td>13.36</td>
<td>4.4</td>
</tr>
<tr>
<td>Skewness</td>
<td>0.583</td>
<td>0.116</td>
<td>0.622</td>
</tr>
<tr>
<td>Kurtosis</td>
<td>-1.5</td>
<td>-1.45</td>
<td>1.35</td>
</tr>
</tbody>
</table>

ST2: 6.74 - 20.4 ug/L

Table 5-4a. Distribution statistics for ST2 by group.
Galectin-3: 2.4 – 15.7 ug/L

Table 5-4b. Distribution statistics for Galectin-3 by group.

<table>
<thead>
<tr>
<th>Galectin-3</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>5.065</td>
<td>6.57</td>
<td>5.43</td>
</tr>
<tr>
<td>Variance</td>
<td>2.969</td>
<td>3.758</td>
<td>3.126</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>1.72313</td>
<td>1.93844</td>
<td>1.76809</td>
</tr>
<tr>
<td>Minimum</td>
<td>3.56</td>
<td>3.68</td>
<td>3.56</td>
</tr>
<tr>
<td>Maximum</td>
<td>8.7</td>
<td>9.8</td>
<td>9.57</td>
</tr>
<tr>
<td>Range</td>
<td>5.14</td>
<td>6.12</td>
<td>6.01</td>
</tr>
<tr>
<td>Interquartile Range</td>
<td>3.08</td>
<td>3.52</td>
<td>1.99</td>
</tr>
<tr>
<td>Skewness</td>
<td>0.676</td>
<td>0.231</td>
<td>1.227</td>
</tr>
<tr>
<td>Kurtosis</td>
<td>-0.789</td>
<td>-1.113</td>
<td>1.084</td>
</tr>
</tbody>
</table>

Calprotectin: 0.15 to 0.9 mg/L

Table 5-4c. Distribution statistics for Calprotectin by group.

<table>
<thead>
<tr>
<th>Calprotectin</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>0.995</td>
<td>1.5</td>
<td>1.19</td>
</tr>
<tr>
<td>Variance</td>
<td>3.831</td>
<td>0.657</td>
<td>0.577</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>1.95722</td>
<td>0.81042</td>
<td>0.75976</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.43</td>
<td>0.43</td>
<td>0.48</td>
</tr>
<tr>
<td>Maximum</td>
<td>6.06</td>
<td>3.47</td>
<td>3.35</td>
</tr>
<tr>
<td>Range</td>
<td>5.63</td>
<td>3.04</td>
<td>2.87</td>
</tr>
<tr>
<td>Interquartile Range</td>
<td>2.08</td>
<td>1.03</td>
<td>0.99</td>
</tr>
<tr>
<td>Skewness</td>
<td>1.614</td>
<td>1.205</td>
<td>1.19</td>
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<tr>
<td>Kurtosis</td>
<td>1.378</td>
<td>2.592</td>
<td>1.791</td>
</tr>
</tbody>
</table>
Chapter 5: Markers of carcinoid heart disease in small bowel NENs

NTP: <75yrs: 125 ng/L >75yrs: 450 ng/L

Table 5-4d. Distribution statistics for NTP by group.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>537.5</td>
<td>65</td>
<td>117</td>
</tr>
<tr>
<td>Variance</td>
<td>26950776</td>
<td>6726.992</td>
<td>11117.21</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>5191.4136</td>
<td>82.01824</td>
<td>105.4382</td>
</tr>
<tr>
<td>Minimum</td>
<td>202</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td>Maximum</td>
<td>16983</td>
<td>317</td>
<td>430</td>
</tr>
<tr>
<td>Range</td>
<td>16781</td>
<td>293</td>
<td>401</td>
</tr>
<tr>
<td>Interquartile Range</td>
<td>570.25</td>
<td>74.25</td>
<td>117</td>
</tr>
<tr>
<td>Skewness</td>
<td>3.147</td>
<td>2.083</td>
<td>1.699</td>
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<tr>
<td>Kurtosis</td>
<td>9.928</td>
<td>4.929</td>
<td>3.234</td>
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</tbody>
</table>

ADM: 9.4 – 12.0 ng/L

Table 5-4e. Distribution statistics for Adrenomedullin by group.

<table>
<thead>
<tr>
<th>AdrenoM</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>2.75</td>
<td>3.885</td>
<td>4.9</td>
</tr>
<tr>
<td>Variance</td>
<td>20.263</td>
<td>21.324</td>
<td>13.514</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>4.50144</td>
<td>4.6178</td>
<td>3.67614</td>
</tr>
<tr>
<td>Minimum</td>
<td>1.75</td>
<td>1.98</td>
<td>0</td>
</tr>
<tr>
<td>Maximum</td>
<td>15.86</td>
<td>18.77</td>
<td>12.24</td>
</tr>
<tr>
<td>Range</td>
<td>14.11</td>
<td>16.79</td>
<td>12.24</td>
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<tr>
<td>Interquartile Range</td>
<td>5.72</td>
<td>4.18</td>
<td>4.69</td>
</tr>
<tr>
<td>Skewness</td>
<td>1.852</td>
<td>2.37</td>
<td>0.8</td>
</tr>
<tr>
<td>Kurtosis</td>
<td>3.329</td>
<td>6.272</td>
<td>-0.215</td>
</tr>
</tbody>
</table>

Table 5-4(a-e). Distribution statistics for assays for each group.

Group A – CHD, B – non functional (NF), C – functional (F). The distributions for each group are presented in Tables 5-4a for ST2, 5-4b for galectin-3, 5-4c for calprotectin, 5-4d for NTP and 5-4e for adrenomedullin.
5.4.2.2 Tukey box plots by groups

The Tukey box plots for each assay by group are outlined in Figure 5-4. The most extreme outlier was for NTP (16,983 ng/L) in the CHD cohort (A) leading to a flattened Tukey box plot across all three cohorts on the data output.

![Tukey box plots for each assay by group](image)

**Figure 5-4. Tukey box plots for each assay by group.**

_A – CHD, B – non-functional, C – functional. Upper whisker is highest case within 1.5 times IQR and the lower whisker is the lowest case within 1.5 times IQR. Outlier results are displayed with corresponding sample ID._
5.4.2.3 Kruskal-Wallis test

Statistical analysis was performed by rank with the Kruskal-Wallis test between groups A (CHD), B (NF) and C (F) given the non-parametric distribution of the assays (Table 5-5). The Kruskal-Wallis test showed no statistical difference between the groups for ST2, Galectin-3, Calprotectin and Adrenomedullin assays. There was a statistical difference with Kruskal-Wallis test between the sbNEN groups for NTP (Chi Squared -21.179, df -2, p=.000).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Group</th>
<th>N</th>
<th>Mean Rank</th>
<th>Chi-Square</th>
<th>df</th>
<th>Asymp. Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>10</td>
<td>25</td>
<td></td>
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</tr>
<tr>
<td>ST2</td>
<td>B</td>
<td>12</td>
<td>17.08</td>
<td>4.228</td>
<td>2</td>
<td>0.121</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>15</td>
<td>16.53</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>A</td>
<td>10</td>
<td>17.55</td>
<td></td>
<td></td>
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<tr>
<td>Galectin3</td>
<td>B</td>
<td>12</td>
<td>21.08</td>
<td>0.687</td>
<td>2</td>
<td>0.709</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>15</td>
<td>18.3</td>
<td></td>
<td></td>
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<td>A</td>
<td>10</td>
<td>18.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calprotectin</td>
<td>B</td>
<td>12</td>
<td>19.75</td>
<td>0.102</td>
<td>2</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>15</td>
<td>18.87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>10</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTP</td>
<td>B</td>
<td>12</td>
<td>11.42</td>
<td>21.179</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>15</td>
<td>16.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>10</td>
<td>16.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdrenoM</td>
<td>B</td>
<td>12</td>
<td>20.83</td>
<td>1.034</td>
<td>2</td>
<td>0.596</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>15</td>
<td>19.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5-5. Non-parametric statistical testing (Kruskal-Wallis) of assays between groups.**

*Total of 37 samples tested with each assay across three groups; A-CHD, B-non-functional NENs, C-functional NENs.*
5.4.2.4 Mann-Whitney Test

The statistical difference with Kruskal-Wallis test across the groups for the NTP assay was explored using the Mann Whitney U test (Table 5-6). The Mann Whitney U test was statistically different for NTP between group A (CHD) and both groups B (NF) and C (F). There was no significant difference for NTP between group B and C with the Mann Whitney test. This suggests that NTP is significantly elevated in the CHD when compared to both the functional and non-functional sbNEN cohorts.

### Table 5-6: Mann-Whitney Test Statistics

<table>
<thead>
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<th>NTP</th>
<th>Mean Rank</th>
<th>Sum of Ranks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GroupNo</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>20.1</td>
<td>201</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>8.27</td>
<td>124</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td></td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Test Statistics°</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTP</td>
</tr>
<tr>
<td>Mann-Whitney U</td>
</tr>
<tr>
<td>Wilcoxon W</td>
</tr>
<tr>
<td>Z</td>
</tr>
<tr>
<td>Asymp. Sig. (2-tailed)</td>
</tr>
<tr>
<td>Exact Sig. [2*(1-tailed Sig.)]</td>
</tr>
</tbody>
</table>

### Table 5-6: Wilcoxon Test Statistics

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<th>Sum of Ranks</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>GroupNo</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>17.4</td>
<td>174</td>
</tr>
<tr>
<td>B</td>
<td>12</td>
<td>6.58</td>
<td>79</td>
</tr>
<tr>
<td>Total</td>
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<td></td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test Statistics°</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTP</td>
</tr>
<tr>
<td>Mann-Whitney U</td>
</tr>
<tr>
<td>Wilcoxon W</td>
</tr>
<tr>
<td>Z</td>
</tr>
<tr>
<td>Asymp. Sig. (2-tailed)</td>
</tr>
<tr>
<td>Exact Sig. [2*(1-tailed Sig.)]</td>
</tr>
</tbody>
</table>
Table 5-6. Non-parametric statistical testing (Mann Whitney U test) of NTP between groups.

*Group A- CHD, B- non-functional, C-functional.*

5.4.2.5 Correlation analysis

Correlation plots across the five assays studied (ST2, galectin-3, calprotectin, NTP and adrenomedullin) are outlined below in Figure 5-5(a-d). Spearman’s rank order correlation was performed between assays with the results outlined in the Table 5-7. There was a strong positive correlation between Calprotectin and Galectin-3 which was statistically significant at the 0.01 level (two tailed) (Correlation Coefficient $r = 0.461$, $p = 0.004$).
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**Table 5-7. Spearman’s rank order correlation between assays.**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Test Statistics</th>
<th>ST2</th>
<th>Galectin3</th>
<th>Calprotectin</th>
<th>NTP</th>
<th>AdrenoM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST2</td>
<td>Correlation Coefficient</td>
<td>1</td>
<td>0.171</td>
<td>0.029</td>
<td>0.219</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td></td>
<td>0.312</td>
<td>0.866</td>
<td>0.194</td>
<td>0.523</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Galectin3</td>
<td>Correlation Coefficient</td>
<td>0.171</td>
<td>1.461**</td>
<td>0.206</td>
<td>0.033</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.312</td>
<td></td>
<td>0.004</td>
<td>0.221</td>
<td>0.848</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Calprotectin</td>
<td>Correlation Coefficient</td>
<td>0.029</td>
<td>.461**</td>
<td>1</td>
<td>0.179</td>
<td>-0.131</td>
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<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.866</td>
<td>0.004</td>
<td></td>
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<td>0.441</td>
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<td>37</td>
<td>37</td>
</tr>
<tr>
<td>NTP</td>
<td>Correlation Coefficient</td>
<td>0.219</td>
<td>0.206</td>
<td>0.179</td>
<td>1</td>
<td>-0.126</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.194</td>
<td>0.221</td>
<td>0.29</td>
<td></td>
<td>0.459</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>AdrenoM</td>
<td>Correlation Coefficient</td>
<td>0.108</td>
<td>0.033</td>
<td>-0.131</td>
<td>-0.126</td>
<td>1</td>
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<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>0.523</td>
<td>0.848</td>
<td>0.441</td>
<td>0.459</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
</tbody>
</table>

**Correlation is significant at the 0.01 level (2-tailed).**
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Figure 5-5a. Correlation plot for ST2.

Figure 5-5b. Correlation plot for Galectin-3.
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Figure 5-5c. Correlation plot for Calprotectin.

Figure 5-5d. Correlation plot for NTP

Figure 5-5. Correlation plots between the assays.

The correlation plots for each assay are grouped in Figure 5-5a for ST2, 5-5b for galectin-3, 5-5c for calprotectin, and 5-5d for NTP (y-axis) against other assays (x-axis)

5.5 Discussion

The results corroborate published literature reporting the value of the natriuretic heart failure marker NTP in patients with carcinoid heart disease. NTP was only elevated above the suggested cut off 260 pg/ml in the cohort of patients with an existing diagnosis of CHD (median 578 pg/ml) (Bhattacharyya et al., 2008). This may also indicate an element of volume overload and clinical heart failure at the time of sampling in these patients.

However, there are limitations with this study given the nature of the archived samples with relatively few relevant clinical data points relating to CHD. The KCH ILS dataset did not include scores for severity of heart failure, either clinical or echocardiographic
features, nor any details of management of CHD and CS, for instance with valve replacements and medical therapy respectively.

Although, the median NTP value was within the normal range across both the functional and non-functional sbNEN cohorts, it was elevated above the cut off 260 pg/ml in 2 out of 15 of the functional sbNEN cohort. This is despite the cohort being screened to have a temporally matched brain-type natriuretic peptide (BNP) result within the normal range (<100 pg/ml). This may be related to the stability of BNP and NTP after sampling as well as differences in clearance from the circulation. BNP is synthesised in the ventricular myocardium as proBNP and released into the circulation when it is cleaved into two fragments; the biologically active C-terminal fragment BNP and the biologically inactive N-terminal fragment NT-proBNP (Weber and Hamm, 2006, de Lemos et al., 2003). BNP is cleared by binding to natriuretic peptide receptors with subsequent inactivation by proteolysis. In contrast, NTP is mainly cleared by renal excretion. NTP has a half-life of 120 minutes in contrast to BNP with a half-life of 20 minutes. The differences in clearance and half-lives mean that NTP is the more stable in circulation with levels six fold greater than that of BNP. Both NTP and BNP levels are increased with worsening renal function, although both are similarly affected in mild to moderate renal impairment (Luchner et al., 2005). The corresponding renal function (calculated glomerular filtration rate) for the anonymised patient samples in this study was not available for correlation with NTP or the other assays.

Low values of natriuretic BNP or NTP effectively exclude heart failure. However, in instances of minimal myocardial stretch such as in heart failure with preserved ejection fraction, the BNP or NTP can be falsely low. The American College of Cardiology Foundation (ACCF) and American Heart Association (AHA) joint guidelines for managing heart failure recommend a combination of the natriuretic peptides with fibrosis markers like ST2 or galectin-3 as being advantageous, particularly for prognostication (Writing Committee et al., 2013). ST2 is suggested to be superior to Galectin-3 when combined with NTP in improving risk stratification in chronic heart failure (Bayes-Genis et al., 2014). A cut off of 35 ng/mL (=35 µg/L) for ST2 has been suggested associated with increased risk of adverse outcomes (Ky et al., 2011). Two patients in the CHD cohort had ST2 values above this cut off with no correlation between ST2 and NTP results in
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this study. There are no outcome data available to suggest if ST2 is associated with worse outcomes when compared to other CHD patients in the cohort.

The fibrosis marker Galectin-3 appears to be of limited value in CHD from this study with normal range results across all three cohorts. It has been hypothesised that Galectin-3 may have a role in earlier stages of fibrosis development and ventricular remodeling acting as an “upstream” signal of the state of myocardial fibrosis in heart failure (Bayes-Genis et al., 2014). Whether Galectin-3 has a role in assessing early fibrosis and risk of progressive CHD will require further evaluation with larger cohorts of patients with a wide set of clinical and cardiac data points.

Interestingly, there was correlation between Galectin-3 and Calprotectin in this study. The reason for this is not clear apart from the fact that both are released from inflammation associated cells. Calprotectin is an inflammatory cascade protein released from neutrophils and monocytes while galectin-3 is released from activated macrophages. The median calprotectin was elevated above the normal range across all three cohorts of sbNEN patients suggesting that it may be related to the presence of disease itself or as a non-specific inflammatory marker. Elevated levels of plasma calprotectin have been reported in colorectal, breast, ovarian, endometrial and cervical cancers (Ni Bhriain et al., 2009, Kostakis et al., 2010, Kristinsson et al., 1998). Calprotectin-laden neutrophil migration is a feature of intestinal inflammation with leakage in the lumen at sites of mucosal breaks allowing for faecal measurement (Roseth et al., 1997, Walsham and Sherwood, 2016). Faecal calprotectin has clinical utility in excluding inflammatory bowel disease (IBD) in patients likely to have irritable bowel syndrome. The assay also has value for assessing disease activity, response and relapse in IBD. Faecal calprotectin is elevated in patients with colorectal cancer and pre-malignant adenomatous polyps (Tibble et al., 2001). The role of faecal calprotectin as a diagnostic tool in sbNENs has not been explored but may be of clinical utility given that these tumours are often associated with luminal mucosal disruption, sometimes resulting in ulceration.

Circulating adrenomedullin was not elevated across any of the three cohorts in this small study. This is in contrast to earlier published data that reported elevated tissue levels in sbNENs suggesting it as a putative prognostic marker (Pavel et al., 2006). There appeared to be no superimposed ventricular secretion of ADM in the CHD cohort, although the
severity of heart failure was not known at the time of sampling. Other natriuretic peptides, like NTP, were elevated in the CHD cohort in this study. This suggests that ADM has limited value as a natriuretic and vasodilator cardiac marker in CHD.

The role of medical therapy in managing sbNEN patients may limit the value of fibrosis and inflammatory cardiac markers. Small bowel NEN patients with CS, especially in the context of CHD, receive medical therapy aimed at reducing the secretion of serotonin from tumour deposits (Grozinsky-Glasberg et al., 2015). Somatostatin analogues (SSAs) and radiopeptide nuclide therapy reduce levels of serotonin leading to a reduction in CS symptoms. Reductions in peak circulating serotonin, vasoactive peptides and cytokines with therapy may also attenuate the secretion of measurable fibrosis and inflammatory cardiac markers. The patients in the functional and CHD cohorts in this study were established on medical therapy so any damping effect on the secretion of markers could not be evaluated without pre-therapy or washout sampling. Further studies with longitudinal testing of fibrosis and inflammatory markers in sbNEN patients may be able to ascertain if there are any effects from initiating therapy across early and late disease. The availability of outcome data will provide some clarity as to their clinical utility, especially prognostic value. However, there has been no published data to date to suggest that therapy, either with medical management or tumour debulking, affects the progression of CHD (Moller et al., 2003).

The role of fibrosis markers in CS does require further exploration given that fibrosis plays a key role in the development in CHD. The value of current fibrosis markers in a disease that predominantly causes fibrosis of the cardiac valves, rather than myocardium, is not clear. The various stages of CHD, from minimal valvular fibrosis and carcinoid plaques in early disease through to functional valve effects and heart failure in late disease, have not been well characterised. Current modalities for diagnosis CHD focus at valvular changes like tricuspid regurgitation and pulmonary stenosis that are associated with late disease. There are no diagnostic tools for identifying early CHD prior to the development of overt valvular changes. It may well be that these markers are only of value in combination with NTP for chronic heart failure risk stratification purposes rather than individually to identify active fibrosis of uncertain and potentially limited clinical significance. Larger studies at various stages of CHD, such as early, advanced and post valve surgery, in patients with CS may be of benefit.
Chapter 6: Proteomic signatures of pancreatic NENs

6 PROTEOMIC SIGNATURES OF PANCREATIC NENs

6.1 Abstract

Background: Proteomic studies with isobaric labelling with tandem mass tags (TMT) allows for the quantification of proteins between tumour and normal tissue. There has been no published proteomic study of non-functional pancreatic NENs to date.

Aims: To identify proteins that are of interest that are significantly different in abundance in G1 non-functional pancreatic NENs.

Methods: Experiments were performed on fresh frozen paired tumour and adjacent normal pancreatic tissue from seven patients. TMT 6plex intact protein labeling was separately performed on both the supernatant (100µg per sample) and pellet samples (50µg per sample) from three pNENs. TMT 10plex labelling was performed on four other paired pNEN samples (10µg per sample). Labeled samples were subject to gel electrophoresis and in-gel tryptic digestion with peptide extracts quantified by liquid chromatography–mass spectrometry (LC–MS/MS). Statistical testing was used to identify labelled proteins that were significantly different with fold changes between tumour and control supporting relative changes in abundance. These proteins were reviewed in open-source bioinformatics platforms, such as GO Slim, KEGG and Reactome pathways, to identify a list of proteins of interest.

Results: Complete sets of labelled peptides and associated proteins differed between the experiments; 4972 sets of peptides and 790 proteins for the supernatant, 3424 peptides and 729 proteins for the pellet, 1918 peptides and 364 proteins for the four pNENs. Significant proteins were identified; 156 from the supernatant, 19 from the pellet and 28 proteins from the four pNENs. There was mapping of 187 unique proteins to cancer pathways, in particular RAS and PI3K-Akt signalling pathways, with ten proteins of
interest identified; Neudesin, Tenascin-X, Actin-related protein 3, Fibulin-1, Moesin, Secretogranin-2, CD63 antigen, Tropomyosin 3, 14-3-3 protein beta/alpha and Calnexin.

Conclusions: This exploratory quantitative proteomic study using fresh frozen tissue samples yielded a number of proteins that may be involved in cancer development through extracellular matrix interactions, cytoskeletal changes and intracellular signalling. Further studies to validate the findings and to correlate with clinical data are required.

6.2 Introduction

Quantitative proteomics provides a list of identified proteins as well as quantifies the relative changes between disease and normal samples to enable statistical analysis for differences in protein expression and as well as pathway mapping. A number of quantitative methodologies exist ranging from historical gel-based proteomics through to ‘shot gun’ or ‘bottom up’ strategies using mass spectrometry of peptides with stable isotope labeling, label-free and targeted techniques (Cheung and Juan, 2017, Maes et al., 2015, Pan et al., 2013). Extracted proteins in gel-based proteomics, such as 1D gel and 2D polyacrylamide gel electrophoresis (PAGE), undergo separation by isoelectric focusing (IEF), based on their isoelectric point (pI), as well as by molecular weight via sodium dodecyl sulphate (SDS)-PAGE respectively. Mass spectrometry consists of an ionization source that generates ions of target molecules such as peptides and an analyzer that sorts molecules by mass-to-charge ratio (m/z). One ‘shot gun’ proteomics approach uses stable isotope labelling to modify the differential masses of peptides from different sources. For tissue samples, isobaric labeling methods using tandem mass tags (TMT) generates a ratio in signal intensities during tandem mass spectra (MS/MS) that reflects the relative expression levels of the peptides in the different samples from fragmentation of the unique reporter label ions from the peptide precursor (Thompson et al., 2003). Label-free quantification of proteins, such as used with the FFPE neuroendocrine studies outlined earlier, quantify either via peptide spectral count or peptide ion current intensity. The approach requires more computational effort than labeled approaches and, for peptide spectral count quantification, lower sensitivity for low abundance proteins. Label-free quantification also requires multiple injections of the sample to reduce error in data acquisition from signal differences in the mass spectrometer. In contrast, TMT labelling
of paired samples supports a stable run with statistical power for signal normalization without the need for multiple sample injections.

This proteomic study aims to identify proteins of interest, differential protein expression and signalling pathways in G1 pancreatic NENs.

6.3 Methods

An application was submitted and granted by the Research Ethics Committee, National Research Ethics Service, NHS Health Research Authority for the study of proteomic and immunohistochemical signatures of neuroendocrine neoplasms (REC reference: 14/LO/1867, IRAS project ID: 155954, 13 October 2014). The study was granted local approval (KCH14-187) by King’s College Hospital NHS Foundation Trust and a material transfer agreement established with Centre of Excellence for Mass Spectrometry (CEMS), The Institute of Psychiatry, Psychology & Neuroscience (IoPPN), Kings College London. An additional application was made to the King’s College Hospital (KCH) Institute of Liver Studies biobank (HTA reference 12378) for approval to use fresh frozen (FF) tissue samples previously collected from consented pNEN patients.

The tumour board clinical database (LiverWare) was interrogated to identify pNENs as part of a wider approved service evaluation. All patients identified had a histologically confirmed non-functional low-grade pNEN on resection specimens. King’s College Hospital (KCH) Institute of Liver Studies biobank or histopathology staff confirmed and selected the final patient samples with valid consent. Frozen tissue resection samples were released anonymously (paired normal and tumour samples) to the investigators and immediately transferred without freeze-thaw to Centre of Excellence for Mass Spectrometry (CEMS), The Institute of Psychiatry, Psychology & Neuroscience (IoPPN), Kings College London freezers prior to processing within 24 hours.

6.3.1 Three pairs pNEN: supernatant

Three pairs of normal pancreas and pNEN tumour samples were released for the initial experiment as outlined below.
6.3.1.1 Cell Lysis stage
Frozen tissue was thawed to room temperature and 500 μL of ice-cold phosphate-buffered saline (PBS) was added to each sample. The samples were vortexed for 5 seconds, left for a further 5 seconds each and then aspirated. The addition of PBS wash was repeated five times with a final aspiration to remove any residual blood that may contaminate the protein result. 500 μL of lysis buffer (8M Urea, 75mM NaCl, 50mM Tris, phosphatase inhibitor and protease inhibitor) was added to each tissue sample and left for 1 hour at room temperature. An additional 200 μL of lysis buffer was added to sample 5 & 6 (pair 3) given the volume of tissue sample. Each specimen was homogenized with a cleaned glass homogenizer and sonicated to break up fibrous tissue. The samples were spun down for 15 minutes at 40000 rpm at 4 °C and the supernatant aspirated into new correspondingly labelled microfuge tubes. Any remaining fibrous tissue pellets were frozen for use at the later stage.

6.3.1.2 Quantification of Total Protein concentration – Nanodrop
2 μL of supernatant from each sample was combined with 98 μL lysis solution to produce 1:50 dilution of each. Three Nanodrop spectrophotometer calculations (mg/ml) were attempted for each sample and an average calculated to quantify the protein concentration of the 1:50 supernatant dilution.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Origin</th>
<th>Type</th>
<th>Specimen ID</th>
<th>Biobank ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pair 1</td>
<td>Normal</td>
<td>S00017973</td>
<td>201102690</td>
</tr>
<tr>
<td>2</td>
<td>Pair 1</td>
<td>Tumour</td>
<td>S00017974</td>
<td>201102690</td>
</tr>
<tr>
<td>3</td>
<td>Pair 2</td>
<td>Normal</td>
<td>S00017415</td>
<td>201102530</td>
</tr>
<tr>
<td>4</td>
<td>Pair 2</td>
<td>Tumour</td>
<td>S00017416</td>
<td>201102530</td>
</tr>
<tr>
<td>5</td>
<td>Pair 3</td>
<td>Normal</td>
<td>S00014795</td>
<td>201101813</td>
</tr>
<tr>
<td>6</td>
<td>Pair 3</td>
<td>Tumour</td>
<td>S00014796</td>
<td>201101813</td>
</tr>
</tbody>
</table>
The protein concentration of the undiluted supernatant for each sample was calculated from the 1:50 supernatant concentration. The appropriate volume and dilution for each sample was calculated to give an estimated 100 µg of protein. The samples were dried down in the speed vacuum prior to labelling.

### 6.3.1.3 TMT intact protein labelling protocol

The Thermo Scientific TMT Isobaric Mass Tagging Kit quantifies labelled proteins extracted from tissue. The isobaric tagging reagent has the same nominal parent (precursor) mass and is composed of an amine-reactive NHS-ester group, a spacer arm and an MS/MS reporter (Figure 6-1). A unique isobaric mass tag is used for each sample with a corresponding unique reporter mass result in the MS/MS spectrum (126-131Da for TMT6 Isobaric Label Reagents). These reporter ions are in the low mass region of the MS/MS spectrum and are used to report relative protein expression levels during peptide fragmentation.
Figure 6-1 (a, b). TMT isobaric mass tag and TMT6plex.

a - the isobaric tagging reagent is composed of an amine-reactive NHS-ester group, a spacer arm and an MS/MS reporter. b - unique isobaric mass tags are used for each sample with a corresponding unique reporter mass result in the MS/MS spectrum (126-131Da for TMT6 Isobaric Label Reagents).

Intact proteins were labelled with TMT reagents that are amine-reactive and modify lysine residues as well as the protein N-termini. This allows for combining labelled samples earlier in the sample process to reduce sample variability and to enable single processing for fractionation (separation) in the 1D-PAGE and digestion. Protein from tissue samples have amine-based buffers and thiol reagents removed before reduction and alkylation. Samples are labelled with the TMT Reagents and then are mixed at the 6plex level. 1D-PAGE resolves complex samples into sections prior to tryptic protein digestion and peptide extraction for LC-MS/MS analysis. The gel stage resolves the proteins by molecular weight with the gel cut into sections to reduce the complexity of the whole sample being analysed by mass spectrometry.

2.5μL of 2% sodium dodecyl sulfate (SDS) was added to denature each of the dried protein samples followed by 22.5μL of ultrapure H20. 25μL of 200mmol tetraethylammonium bromide (TEAB), a dissolution buffer, was added to each sample.
followed by 2.6\(\mu\)L of tris(2-carboxyethyl)phosphine (TCEP), a reducing agent of disulfides, and heated to 55 °C for 1 hour. 2.8\(\mu\)L of indole-3-acetic acid (IAA), an alkylating reagent, was added and left at room temperature for 1 hour. 18.2\(\mu\)L of each individual TMT 6plex isobaric label reagent was added to each corresponding sample and left at room temperature for 1 hour. 3.9\(\mu\)L of hydroxylamine (1:20), a quenching reagent, was added to each sample and left at room temperature for 15 minutes. All six labelled samples were combined in one tube and a further 49.2 \(\mu\)L (6x8.2\(\mu\)L) of hydroxylamine added. The samples were frozen at -80 °C for 30 minutes and dried in speed vacuum.

**Figure 6-2.** Proteomic workflow for TMT 6plex labelling and LC-MS/MS.

Tumour and normal tissue samples were individually processed to lyse cells and to assess protein concentration. TMT labelling of calculated protein amounts was performed for each sample before being combined and run through gel electrophoresis. The stained gel was sectioned and in-gel tryptic digestion performed prior to peptide extraction. The extracts were analysed by LC-MS/MS to quantify individual tags and associated peptide fragments.
6.3.1.4 Gel Electrophoresis, Colloidal Staining and Destaining

The combined TMT labelled sample was boiled in 50μL Laemmli buffer for 10 minutes. 5μL of SeeBlue® Plus2 Pre-stained Protein Standard was slowly expelled into cassette well 1 of the NuPAGE® 10% BIS-Tris polyacrylamide gel and 25μL of the labelled sample was loaded into both well 3 and well 5. NuPAGE® MES SDS Running Buffer was added to chamber and electrophoresis started with 50 volts for 30 minutes to ensure samples enter the stacking gel from each well. The gel was then resolved with 150 volts for 60 minutes until the dye front travelled to the bottom. The gel was fixed with 40mls of 7% Acetic Acid / 40% methanol solution for 30 minutes on an orbital shaker at 60 RPM. The gel was stained with dilute colloidal concentrate (Coomassie Brilliant Blue (CBB)) for 48 hours on orbital shaker at 60 RPM until bands are clear. Destaining was performed with 40mls - 7% Acetic Acid / 40% methanol solution for 15 minutes with a further destain with 40mls - 2% Acetic Acid / 25% methanol solution for 30 minutes. The gel was stored in ultrapure water at 4 °C.

![Image of gel electrophoresis](image)

**Figure 6-3. Gel electrophoresis of three pairs pNEN supernatant.**

*The combined sample of TMT 6plex labelled samples (three pairs of normal and tumour pNENs) was introduced into lane 3 and 5 with a protein standard into lane 1. The gel*
was resolved until the dye front reached the bottom and then stained, destained. The standard weights (kDa) are displayed to the left of the gel.

6.3.1.5 In-gel tryptic digestion and peptide extraction
The gel bands were divided into 5 equal sections, cut into 2mm³ pieces and transferred into correspondingly labelled microfuge tubes. The gel pieces in each microfuge tube were repeatedly washed with 100mM ammonium bicarbonate (Ambic) for 5 minutes and dehydrated with acetonitrile (ACN), prior to being dried in the speed vacuum for 5 minutes. The gel pieces were rehydrated in 10mM dithiothreitol (DTT) for 45 minutes at 56 °C to disrupt disulfide bonds for protein unfolding. Any excess liquid was decanted and the gel pieces were dehydrated again with ACN before being dried in the speed vacuum for 5 minutes. The gel pieces were rehydrated in 55mM iodoacetamide (IAA) for 20 minutes in the dark at room temperature to irreversibly alkylate sulphhydryl groups. Excess liquid was removed and any remaining stain completely removed with the addition of 50% ACN/50% 100mM Ambic at 1000rpm at 37°C. The excess liquid was removed and the gel pieces dehydrated with 100% ACN before being dried in the speed vacuum for 5 minutes. A 30µL aliquot of trypsin (13ng/µL) with 200µL of 50mM Ambic was added to rehydrate the gel pieces at 4 °C for 20 mins. Unabsorbed trypsin was removed and the gel pieces covered with a minimal volume of 50mM Ambic (200µL) before being incubated at 37 °C for 2 hours and then overnight at room temperature. The supernatant was then decanted from the gel pieces and collected in a new microfuge tube. The gels were washed with a minimal volume of 50mM Ambic to immerse the pieces for 5 minutes at 37 °C. The gel pieces were dehydrated with ACN for 10 minutes at 37 °C and the supernatant collected in the tube. The Ambic and ACN step was repeated to ensure as much extracted peptide was collected. The pooled peptide extract was dried and stored at -80 °C.

6.3.1.6 Liquid chromatography–mass spectrometry (LC–MS/MS)
The dried down pooled peptide extract was thawed and made up to 50 µL with 50mM Ambic. 10 µL of the pooled sample was injected onto a Thermo pre-column (EASY-Column, 2 cm, ID 100 µm, 5 µmC18-A1), using the Proxeon EASY-nLC II system (Thermo Fisher Scientific). Peptides were then resolved using an increasing gradient of 0.1% formic acid in ACN (5–50% over 115 min) through a Thermo analytical column (EASY-Column, 10 cm, ID75 µm, 3 µm C18-A2) at a flow rate of 300 nL/min. Mass
spectra were acquired on an LTQ Orbitrap Velos (Thermo Fisher Scientific) throughout the chromatographic run (115 min), using Top10 HCD method combining microscans following each FTMS scan (2× μScans at 30,000 resolving power @ 400 m/z). Higher energy collision induced dissociation (HCD) was carried out on 10 of the most intense ions from each FTMS scan then put on a dynamic exclusion list for 30 s (20 ppm m/z window). AGC ion injection target for each FTMS scan were 1,000,000 (500 ms max injection time). AGC ion injection target for each HCD scan were 10,000 (50 ms max ion injection time).

6.3.2 Three pairs pNEN: pellet

The pellet samples stored following lysis of the paired samples were analysed with modifications to the above methodology outlined below; different lysis buffer and protein quantification using Bradford assay.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Origin</th>
<th>Type</th>
<th>Specimen ID</th>
<th>Biobank ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - Pellet</td>
<td>Pair 1</td>
<td>Normal</td>
<td>S00017973</td>
<td>201102690</td>
</tr>
<tr>
<td>2 - Pellet</td>
<td>Pair 1</td>
<td>Tumour</td>
<td>S00017974</td>
<td>201102690</td>
</tr>
<tr>
<td>3 - Pellet</td>
<td>Pair 2</td>
<td>Normal</td>
<td>S00017415</td>
<td>201102530</td>
</tr>
<tr>
<td>4 - Pellet</td>
<td>Pair 2</td>
<td>Tumour</td>
<td>S00017416</td>
<td>201102530</td>
</tr>
<tr>
<td>5 - Pellet</td>
<td>Pair 3</td>
<td>Normal</td>
<td>S00014795</td>
<td>201101813</td>
</tr>
<tr>
<td>6 - Pellet</td>
<td>Pair 3</td>
<td>Tumour</td>
<td>S00014796</td>
<td>201101813</td>
</tr>
</tbody>
</table>

6.3.2.1 Cell lysis stage

The lysis buffer was altered to a urea:thiourea with the addition of the detergent SDS to aid lysis and solubilisation of proteins from the fibrous pellets (Xavier et al., 2010). 100μL of lysis buffer (7.5M Urea, 2M Thiourea, 0.1% SDS, phosphatase inhibitor and protease inhibitor) was added to the thawed pellets after the supernatant was aspirated. The samples were left overnight at room temperature and then homogenized with a clean glass homogenizer and left in a cold room overnight. Additional 600μL of lysis buffer added to Sample 2 (S00017974) and all samples were spun down for 45 minutes at 14000rpm at 4°C. The supernatant from each sample was aspirated into a new correspondingly labelled microfuge tube.
6.3.2.2 Quantification of Total Protein concentration – Bradford Assay
A stock of Bovine Serum Albumin (BSA - LOT number – A9647-50G) was made to 3mg/ml concentration with the lysis buffer. The Bradford standards (ID 1-7) of concentration zero to 0.5µg/µL were prepared with the corresponding volume of BSA and lysis buffer as outlined below. 10µL from standard (ID 1-7) and 10µL from undiluted samples (1-6) were each separately added to three individual plate wells (for mean absorbance calculations) in a 96 well microtiter plate assay (Figure 6-4). 200µL of diluted Bio-Rad dye reagent (10ml dye with 40ml ultrapure H2O) was added to each well of either standard or samples. The dye reagent and standard or samples were mixed with a microplate mixer and the absorbance of plate read at 595 nm for 1 second. The absorbance data was imported in Excel to produce standard curve and sample protein measurements. The absorbances of the standard 1-7 are outlined below with samples 3 and 5 absorbance reading higher than the standard range. Samples 3 and 5 were diluted with lysis buffer to concentrations 1:2 (20:20 lysis µL) and then again 1:10 (10:90 lysis µL) to assess the absorbance of each 10µL samples across three new wells when mixed with dye reagent as outlined above. The mean absorbance of the 1:10 dilution of samples 3 and 5 were within the standard range (see below) and sample protein concentrations were calculated in Excel (see below) against the standard curve (Absorbance= 1.063x (Concentration (µg/µL)) + 0.5428)). A volume of each sample equivalent to 50µg of protein was dried down for labelling. A smaller amount of protein was used to manage overall volume for labelling, as the protein concentration in the pellet samples was lower when compared to the initial supernatant (100µg of protein for each sample with smaller required sample volume).

<table>
<thead>
<tr>
<th>Standard ID</th>
<th>BSA standard concentration (mg/mL)</th>
<th>Volume of BSA stock (mL)</th>
<th>Volume of lysis buffer (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>2</td>
<td>0.025</td>
<td>4.22</td>
<td>495.78</td>
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<td>0.05</td>
<td>8.45</td>
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</tr>
<tr>
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<td>0.1</td>
<td>16.89</td>
<td>483.11</td>
</tr>
<tr>
<td>5</td>
<td>0.2</td>
<td>33.78</td>
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</tr>
<tr>
<td>6</td>
<td>0.4</td>
<td>67.57</td>
<td>432.43</td>
</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>84.46</td>
<td>415.54</td>
</tr>
</tbody>
</table>
Slope (1.063) and intercept (0.5428) calculated from BSA concentration and absorbance ($R^2=0.977$).

Absorbance $= 1.063 \times \text{(Concentration (µg/µL))} + 0.5428$

<table>
<thead>
<tr>
<th>Standard ID</th>
<th>BSA conc. (mg/mL)</th>
<th>Absorbance Well 1</th>
<th>Absorbance Well 2</th>
<th>Absorbance Well 3</th>
<th>Mean Absorbance</th>
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<tbody>
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<td>0.597</td>
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<tr>
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<td>0.59</td>
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<td>0.922</td>
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<table>
<thead>
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<th>Absorbance Well 3</th>
<th>Mean Absorbance</th>
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<td>0.642</td>
<td>0.646</td>
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<tr>
<td>(1:10) 3</td>
<td>1.053</td>
<td>1.098</td>
<td>1.132</td>
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<tr>
<td>(1:10) 5</td>
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<td>1.016</td>
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<td>6</td>
<td>1.22</td>
<td>1.176</td>
<td>1.1</td>
<td>1.165</td>
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</table>
Figure 6-4. Bradford assay for protein quantification for three pairs pNEN pellets.

The Bradford standard (1-7) was added to three wells per row (labelled 1-7). The six undiluted samples (N) were added to three wells adjacent to the standard. Samples 3 and 5 read above the standard so were diluted (1:2 and 1:10) and added to the adjacent wells.

Calculation of undiluted µg/µl & volume required for 10, 50 and 100 µg protein

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Calculated µg/µl</th>
<th>Undiluted µg/µl</th>
<th>µl for 10µg total</th>
<th>µl for 50µg total</th>
<th>µl for 100µg total</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.088</td>
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<td>(1:10) 3</td>
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</tr>
<tr>
<td>(1:10) 5</td>
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<td>21.895</td>
</tr>
<tr>
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<td>0.586</td>
<td>17.076</td>
<td>85.382</td>
<td>170.764</td>
</tr>
</tbody>
</table>

6.3.2.3 TMT intact protein labelling, Gel Electrophoresis, and remaining steps
The pellet protein samples were labelled (126-131Da for TMT6 Isobaric Label Reagents) using the same methodology as outlined above with all samples combined in one tube (RB TMT 6-1). The samples were prepared and loaded (40µL) into well lanes 2-8 of the NuPAGE® 10% BIS-Tris Gel (LOT 14020771) with 5µL of SeeBlue® Plus2 Pre-stained
Protein Standard in well lane 1. The labelled sample was loaded across more well lanes given the spread of protein beyond the initial loaded lanes in the first gel. The gel was run as outlined above with fixation, colloidal staining and destaining performed with the same methodology. The In-Gel digestion and peptide extraction was performed as outlined earlier. The peptide extract was similarly loaded for LC–MS/MS with the same methodology for data processing and analysis.

6.3.3 Four pairs pNEN
A further four pairs of normal pancreas and pNEN tumour samples were released for a follow on experiments. Paired tissue from a pancreatic adenocarcinoma resection specimen was used to complete the TMT 10plex labelling. This sample was included to assess the extent of labelling across but was not analysed at a bioinformatics level.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Source</th>
<th>Origin</th>
<th>Type</th>
<th>Specimen ID</th>
<th>Biobank ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – N1</td>
<td>Biobank</td>
<td>Pair 1 - NET</td>
<td>Normal</td>
<td>S00016202</td>
<td>201102212</td>
</tr>
<tr>
<td>2 – T1</td>
<td>Biobank</td>
<td>Pair 1 - NET</td>
<td>Tumour</td>
<td>S00016203</td>
<td>201102212</td>
</tr>
<tr>
<td>3 – N2</td>
<td>Biobank</td>
<td>Pair 2 – NET</td>
<td>Normal</td>
<td>S00019832</td>
<td>201103181</td>
</tr>
<tr>
<td>4 - T2</td>
<td>Biobank</td>
<td>Pair 2 – NET</td>
<td>Tumour</td>
<td>S00019833</td>
<td>201103181</td>
</tr>
<tr>
<td>5 – N3</td>
<td>ILS histo</td>
<td>Pair 3 - NET</td>
<td>Normal</td>
<td>299/15 pancreas</td>
<td>-</td>
</tr>
<tr>
<td>6 – T3</td>
<td>ILS histo</td>
<td>Pair 3 - NET</td>
<td>Tumour</td>
<td>299/15 tumour</td>
<td>-</td>
</tr>
<tr>
<td>7 – N4</td>
<td>ILS histo</td>
<td>Pair 4 - NET</td>
<td>Normal</td>
<td>3766/14 pancreas</td>
<td>-</td>
</tr>
<tr>
<td>8 – T4</td>
<td>ILS histo</td>
<td>Pair 4 - NET</td>
<td>Tumour</td>
<td>3766/14 cyst</td>
<td>-</td>
</tr>
<tr>
<td>9 – N5</td>
<td>ILS histo</td>
<td>Pair 5 - Adeno</td>
<td>Normal</td>
<td>2468/11 pancreas</td>
<td>-</td>
</tr>
<tr>
<td>10 – T5</td>
<td>ILS histo</td>
<td>Pair 5 - Adeno</td>
<td>Tumour</td>
<td>2468/11 tumour</td>
<td>-</td>
</tr>
</tbody>
</table>

6.3.3.1 Adaptations from pellet lysis methodology
The lysis preparation used with the pellet was used for the five pairs of tissue as a harsher lysis mix. The thawed samples were washed with PBS and cut into smaller pieces prior to 600 μL lysis buffer being added. The samples were glass homogenized and stored overnight at room temperature. The samples were homogenized and sonicated on two
Further occasion with an interim spin for 60 minutes at 40000rpm at 4 °C to ensure disruption of fibrous tissue to aid lysis. 1ml acetone was added to sample N1 and N2 to disrupt the lipid layer to aid aspiration of the supernatant. The supernatants and pellets samples were spun for 20 minutes at 14000rpm at 4 °C prior to Bradford assay.

The bovine serum assay (BSA) standards (see below and Figure 6-5) and microplate with samples were prepared as per the methodology outlined above. The undiluted samples mean absorbance was beyond the standard and a 1:50 dilution (2μL sample :98μL lysis) was prepared for all samples (N1-N5, T1-T5). The Bradford assay was repeated with new standards and the diluted samples. The absorbances were within the standard and the protein concentrations of the undiluted samples were calculated. A volume of either undiluted (30μl) or 1:10 dilutions (10μl :90μl lysis) from each sample was taken to provide enough for 10 μg protein and volume considerations for TMT 10-plex labelling.

<table>
<thead>
<tr>
<th>Standard ID</th>
<th>BSA conc. (mg/mL)</th>
<th>Absorbance Well 1</th>
<th>Absorbance Well 2</th>
<th>Absorbance Well 3</th>
<th>Mean Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.34</td>
<td>0.345</td>
<td>0.348</td>
<td>0.344</td>
</tr>
<tr>
<td>2</td>
<td>0.025</td>
<td>0.374</td>
<td>0.385</td>
<td>0.383</td>
<td>0.381</td>
</tr>
<tr>
<td>3</td>
<td>0.05</td>
<td>0.388</td>
<td>0.391</td>
<td>0.402</td>
<td>0.394</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>0.431</td>
<td>0.428</td>
<td>0.439</td>
<td>0.433</td>
</tr>
<tr>
<td>5</td>
<td>0.2</td>
<td>0.528</td>
<td>0.526</td>
<td>0.536</td>
<td>0.53</td>
</tr>
<tr>
<td>6</td>
<td>0.4</td>
<td>0.599</td>
<td>0.636</td>
<td>0.667</td>
<td>0.634</td>
</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>0.646</td>
<td>0.68</td>
<td>0.705</td>
<td>0.677</td>
</tr>
</tbody>
</table>

Slope (0.6612) and intercept (0.3642) calculated from BSA conc and absorbance (R²=0.9817).

\[ \text{Absorbance} = 0.6612 \times (\text{Concentration (μg/μL)}) + 0.3642 \]
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Samples

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Absorbance Well 1</th>
<th>Absorbance Well 2</th>
<th>Absorbance Well 3</th>
<th>Mean Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>0.386</td>
<td>0.389</td>
<td>0.381</td>
<td>0.385</td>
</tr>
<tr>
<td>N2</td>
<td>0.398</td>
<td>0.399</td>
<td>0.406</td>
<td>0.401</td>
</tr>
<tr>
<td>N3</td>
<td>0.707</td>
<td>0.709</td>
<td>0.713</td>
<td>0.71</td>
</tr>
<tr>
<td>N4</td>
<td>0.593</td>
<td>0.592</td>
<td>0.59</td>
<td>0.592</td>
</tr>
<tr>
<td>N5</td>
<td>0.413</td>
<td>0.418</td>
<td>0.418</td>
<td>0.416</td>
</tr>
<tr>
<td>T1</td>
<td>0.472</td>
<td>0.464</td>
<td>0.465</td>
<td>0.467</td>
</tr>
<tr>
<td>T2</td>
<td>0.543</td>
<td>0.542</td>
<td>0.502</td>
<td>0.529</td>
</tr>
<tr>
<td>T3</td>
<td>0.576</td>
<td>0.567</td>
<td>0.571</td>
<td>0.571</td>
</tr>
<tr>
<td>T4</td>
<td>0.66</td>
<td>0.654</td>
<td>0.663</td>
<td>0.659</td>
</tr>
<tr>
<td>T5</td>
<td>0.44</td>
<td>0.448</td>
<td>0.455</td>
<td>0.448</td>
</tr>
</tbody>
</table>

**Figure 6-5. Bradford assay for protein quantification in four pairs pNEN pellets.**

The Bradford standard (1-7) was added to three wells per row (labelled 1-7). Diluted normal samples (N1-N5) were added to three wells adjacent to the standard with diluted tumour samples (T1-T5) added adjacent to the normal samples.
Calculation of undiluted μg/μl & volume required for 10, 50 and 100 μg protein

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Undiluted μg/μl</th>
<th>μl for 10μg total</th>
<th>μl for 50μg total</th>
<th>μl for 100μg total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>1.6</td>
<td>6.251</td>
<td>31.256</td>
<td>62.512</td>
</tr>
<tr>
<td>N2</td>
<td>2.784</td>
<td>3.592</td>
<td>17.958</td>
<td>35.915</td>
</tr>
<tr>
<td>N3</td>
<td>26.124</td>
<td>0.383</td>
<td>1.914</td>
<td>3.828</td>
</tr>
<tr>
<td>N4</td>
<td>17.202</td>
<td>0.581</td>
<td>2.907</td>
<td>5.813</td>
</tr>
<tr>
<td>N5</td>
<td>3.944</td>
<td>2.536</td>
<td>12.678</td>
<td>25.357</td>
</tr>
<tr>
<td>T1</td>
<td>7.775</td>
<td>1.286</td>
<td>6.431</td>
<td>12.862</td>
</tr>
<tr>
<td>T2</td>
<td>12.463</td>
<td>0.802</td>
<td>4.012</td>
<td>8.024</td>
</tr>
<tr>
<td>T3</td>
<td>15.664</td>
<td>0.638</td>
<td>3.192</td>
<td>6.384</td>
</tr>
<tr>
<td>T4</td>
<td>22.293</td>
<td>0.449</td>
<td>2.243</td>
<td>4.486</td>
</tr>
<tr>
<td>T5</td>
<td>6.313</td>
<td>1.584</td>
<td>7.92</td>
<td>15.84</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Label</th>
<th>Sample / volume / named</th>
<th>Vol. required μl</th>
<th>H20 required for TMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>126</td>
<td>Undiluted/ 30 μl / N1S 30μl</td>
<td>6.251</td>
<td>16.2</td>
</tr>
<tr>
<td>N2</td>
<td>127n</td>
<td>Undiluted/ 30 μl / N2S 30μl</td>
<td>3.592</td>
<td>18.9</td>
</tr>
<tr>
<td>N3</td>
<td>127c</td>
<td>1:10 dilution / N3S 1:10</td>
<td>3.828</td>
<td>18.7</td>
</tr>
<tr>
<td>N4</td>
<td>128n</td>
<td>1:10 dilution / N4S 1:10</td>
<td>5.813</td>
<td>16.7</td>
</tr>
<tr>
<td>N5</td>
<td>128e</td>
<td>Undiluted/ 30 μl / N5S 30μl</td>
<td>2.536</td>
<td>20</td>
</tr>
<tr>
<td>T1</td>
<td>129n</td>
<td>Undiluted/ 30 μl / T1S 30μl</td>
<td>1.286</td>
<td>21.2</td>
</tr>
<tr>
<td>T2</td>
<td>129c</td>
<td>1:10 dilution / T2S 1:10</td>
<td>8.024</td>
<td>14.5</td>
</tr>
<tr>
<td>T3</td>
<td>130n</td>
<td>1:10 dilution / T3S 1:10</td>
<td>6.384</td>
<td>16.1</td>
</tr>
<tr>
<td>T4</td>
<td>130c</td>
<td>1:10 dilution / T4S 1:10</td>
<td>4.486</td>
<td>18</td>
</tr>
<tr>
<td>T5</td>
<td>131</td>
<td>Undiluted/ 30 μl / N1S 30μl</td>
<td>1.584</td>
<td>20.9</td>
</tr>
</tbody>
</table>

The TMT labelling methodology was the same as outlined above, apart from the volume of ultrapure H20 being adjusted for each sample to make up the volume to 25μL after the
initial addition of 2.5\(\mu\)L of 2% SDS. A TMT 10plex isobaric label was used to label the ten samples. The labelled samples were combined (RB TMT 10-1) and quenching 10x8.2\(\mu\)L hydroxylamine added prior to being frozen at -80°C for 30 minutes and dried in speed vac for 4 hours.

The combined sample was re-suspended in 25\(\mu\)L 50mM Ambic and 25\(\mu\)L 2x Laemmli buffer for gel electrophoresis. SeeBlue® Plus2 Pre-stained Protein Standard (5\(\mu\)L) was added into gel well lanes 1 and 6, with 25 \(\mu\)L of sample added to well lanes 3 and 4. The electrophoresis was run until the dye front reach the bottom of the resolving gel. Fixation, colloidal staining and destaining, in-gel digestion and peptide extraction was performed with the same methodology (Figure 6-6). Apart from a change to TMT 10plex modification, the peptide extract was loaded for LC–MS/MS with the same methodology for data processing and analysis.

![Figure 6-6. Gel electrophoresis of four pairs pNEN supernatant.](image)

The combined sample of TMT 10plex labelled samples (four pairs of normal and tumour pNENs, one pair of pancreatic adenocarcinoma) was introduced into lane 3 and 4 with a protein standard into lane 1 and 6. The gel was resolved until the dye front reached the
bottom and then stained, destained. The standard weights (kDa) are displayed to the left of the gel.

6.3.4 Data processing and analysis

6.3.4.1 Peptide identification

Peak lists were extracted from Xcalibur Raw data files using Proteome Discoverer 1.4 and datasets combined for Mudpit analysis prior to being searched using Mascot 2.2 search engine. Figure 6-7 illustrates the overall workflow used for peptide identification and quantification. The spectrum files node was used to select the raw data files of interest. Spectrum selector node was set to its default values, therefore the data was not smoothed, no signal to noise threshold was set, and no charge state filtering or de-isotoping took place. The node was programmed to search for tryptic peptides with up to 3 missed cleavages (C-Term K/R restrict P), with dynamic modifications set as carbamidomethyl (C). TMT 6plex or 10plex labels at the N-terminus and lysine residues, in combination with methionine oxidation, were dynamic modifications. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance 0.5 Da for both database search engines. Once peptides were identified using each of the search method, the Percolator node filtered identifications of basis of q-values, which were estimated on the basis of the target-decoy search approach. To filter out target peptide spectrum matches (target-PSMs) over the decoy-PSMs a fixed false discovery rate (FDR) of 1% was set at the peptide level. The peptide confidence level was set to low (P=0.05) with filter ‘Peptides per Protein’ and ‘Count only rank 1 peptides’ options activated. ‘Count peptide only in top scored proteins’ were deactivated. Proteome Discover 1.4 was used to export the list of identified peptides to MS Excel.
6.3.4.2 Peptide and protein quantification, statistical analysis and bioinformatics resources

Proteome Discover 1.4 was used to export the list of identified peptides to Excel. Peptides with TMT 6plex or 10plex (N-terminal, K) modifications were searched. The pancreatic adenocarcinoma sample pair (labels 128c, 131) was not included for bioinformatics analysis as only a single paired sample used to assess completeness of labelling with the 10plex. Peptides with missing labels in either normal or tumour samples in any of the pairs were removed to ensure only quantified labelled peptides remained for statistical analysis across the plexes. The most common associated UniProt protein group accession for each peptide was used for descriptors and for protein-level summation.

6.3.4.2.1 Peptide level - Fold Chang

Ratios of peptides labelled across all specimens were calculated for paired samples, and all summed tumour versus control samples, to describe relative abundance. A summed fold change of less than 0.5 or greater than 2 was considered as reduced or increased.
respectively in abundance in the tumour compared to the normal tissue. Peptides were ordered by size of summed fold change.

6.3.4.2.2 Peptide level - Statistical testing
Student's paired t-Test, with a two-tailed distribution and equal variance, was performed for paired samples with significance at $p \leq 0.05$ (5%) level to identify peptide quantification differences between paired control (6plex labels - 126, 128, 130; 10plex labels 126, 127n, 127c, 128n, 128c) and tumour (6plex - 127, 129, 131; 10plex - 129n, 129c, 130n, 130c, 131) samples. UniProt protein accession numbers of significant peptides were used to identify proteins of interest with corresponding ratios of tumour to control. Logarithmic transformation of calculated ratios (log2) and p values (log10) was performed to generate a tornado plot of transformed peptide ratios versus significance levels.

6.3.4.2.3 Protein level - Fold Change
Quantification at a protein level was calculated from median values for each label for first rank peptides associated with each protein’s UniProt accession number. A summed scaled ratio for tumour versus control at a protein level was calculated from the ratio of tumour and control summed median values per label per UniProt accession number. Proteins were ordered by size of summed scaled fold change for each UniProt accession number.

6.3.4.2.4 Protein level - Statistical testing
Similarly to peptide analysis, Student's paired t-Test, with a two-tailed distribution and equal variance, was performed for paired samples with significance at $p \leq 0.05$ (5%) level to identify protein group quantification differences between paired median control (6plex labels - 126, 128, 130; 10plex labels 126, 127n, 127c, 128n, 128c) and tumour (6plex - 127, 129, 131; 10plex - 129n, 129c, 130n, 130c, 131) samples. Logarithmic transformation of calculated ratios (log2) and p values (log10) was performed to generate a tornado plot of transformed protein ratios and significance levels.

6.3.4.2.5 Bioinformatics
Qualitative analysis of biological processes was performed using a single list, across all three experiments (6plex supernatant & pellet, 10plex), of UniProt protein accession numbers identified through protein-level significance testing (5% level) of median control versus tumour samples where a minimum of three peptides contributed. A number of bioinformatics resources, such as WEB-based GEne SeT AnaLysis Toolkit
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6.4 Results

The following results section outlines both peptide level and protein level quantitative analysis of labelled control and tumour NEN samples. The TMT labelling was performed at a protein level prior to in-gel tryptic digestion for peptide extraction. The TMT labels at the N-terminus and lysines with the mass spectrometer measuring the cleaved mass reporter label and the corresponding peptide. The extent of labelling of all peptides is assessed below with drop off potentially due to the mass spectrometer not measuring corresponding peptides when a label was detected or vice versa.

6.4.1 Three pairs supernatant – TMT 6plex

A total of 11966 peptide sequences were identified with medium or high confidence level from LC-MS/MS across the three sets of paired samples. Almost three quarters (72%) of these peptides had at least one TMT 6plex (N-terminal, K) modifications (8677 peptides), which were overwhelmingly identified with high confidence level (94%). Over 57% of these peptides (4972/8677) had quantifiable N-terminal, K modifications across all three paired samples. These completed sets of modified peptides represented 41% of the total identified (4972/11966) from LC-MS/MS. There were 790 distinct proteins when summing complete sets of modified peptides.

6.4.1.1 Peptide level results

There were statistically significant (p<0.05) differences in the quantity of modified peptides between normal and tumour pairs for 1830 peptide sequences. These peptide sequences were associated with 564 protein group accessions. The overwhelming majority (96%, 1759/1830) of these peptides had a greater than twofold increase in abundance in tumour versus normal samples (ratio > 2.0). Less than 3% (52/1830) of these significant peptides had a less than twofold decrease in abundance in tumour versus normal samples (ratio <0.5).
Significant peptides of note that are confirmatory of pancreatic neuroendocrine neoplasms samples include the neuroendocrine secretory granules chromogranin A (p<0.05, 2 separate peptide sequences), chromogranin B (secretogranin 1, p<0.05, 4 separate peptide sequences) and chromogranin C (secretogranin 2, p<0.0.5, 7 separate peptide sequences). Figure 6-8(a) plots the logarithmic transformed relative abundance tumour: normal (log2ratio) against p-value (log10) for all peptides.

6.4.1.2 Protein level results
There were statistically significant (p<0.05) differences in the quantity of proteins between normal and tumour pairs for 156 distinct proteins (filtered for at least three contributory peptides). The overwhelming majority of proteins (146/156) had a greater than twofold increase in the abundance in tumour versus normal samples. Of the remaining proteins, nine had a less than twofold increase in abundance and one had a greater than twofold decrease (40S ribosomal protein S3a). Figure 6-8(a) plots the logarithmic transformed relative abundance tumour: normal (log2ratio) against p-value (log10) for all 790 distinct proteins with complete sets of peptide modification data (unfiltered by number of contributory peptides).

6.4.2 Three pairs pellet – TMT 6plex
A total of 8915 peptide sequences were identified with medium or high confidence level from LC-MS/MS across the three sets of paired samples. Over half (52%) of these peptides had at least one TMT 6plex (N-terminal, K) modifications (4692 peptides), which were overwhelmingly identified with high confidence level (97%). Almost three quarters (73%) of these peptides (3424/4692) had quantifiable N-terminal, K modifications across all three paired samples. These completed sets of modified peptides represented 38% of the total identified (3424/8915) from LC-MS/MS. There were 729 distinct proteins when summing complete sets of modified peptides.

6.4.2.1 Peptide level results
There were statistically significant (p<0.05) differences in the quantity of modified peptides between normal and tumour pairs for 234 peptide sequences. These peptide sequences were associated with 101 protein group accessions. The overwhelming majority (87%, 204/234) of these peptides had a greater than twofold increase in abundance in tumour versus normal samples. 12% (29/234) of these significant peptides
had a less than twofold decrease in abundance in tumour versus normal samples. Significant pNEN confirmatory peptides include neuroendocrine secretory granule chromogranin B (secretogranin 1, p<0.05, 2 separate peptide sequences). Figure 6-8(b) plots the logarithmic transformed relative abundance tumour: normal (log2ratio) against p-value (log10) for all peptides.

6.4.2.2 Protein level results
There were statistically significant (p<0.05) differences in the quantity of proteins between normal and tumour pairs for 19 distinct proteins (filtered for at least three contributory peptides). The majority of proteins (15/19) had a greater than twofold increase in the abundance in tumour versus normal samples. The remaining proteins had a greater than twofold decrease (4/19). Figure 6-8(b) plots the logarithmic transformed relative abundance tumour: normal (log2ratio) against p-value (log10) for all 729 distinct proteins with complete sets of peptide modification data (unfiltered by number of contributory peptides).

6.4.3 Four pairs – TMT 10plex
A total of 14077 peptide sequences were identified with medium or high confidence level from LC-MS/MS across the three sets of paired samples. Almost three quarters (67%) of these peptides had at least one TMT 10plex (N-terminal, K) modifications (9458 peptides), which were overwhelmingly identified with high confidence level (96%). In contrast to the earlier results using the 6plex, 20% of these peptides (1918/9458) had quantifiable 10plex N-terminal, K modifications across all paired samples. The variable TMT 10plex labelling of the pair samples probably relates to the different amounts of protein used; 10 μg protein for each 10plex sample compared to 100μg and 50μg protein for each supernatant and pellet 6plex sample respectively. These completed sets of modified peptides represented 13% of the total identified (1918/14077) from LC-MS/MS. There were 364 distinct proteins when summing complete sets of modified peptides.

6.4.3.1 Peptide level results
There were statistically significant (p<0.05) differences in the quantity of modified peptides between normal and tumour pairs for 353 peptide sequences. These peptide sequences were associated with 121 protein group accessions. The overwhelming majority (94%, 333/353) of these peptides had a greater than twofold increase in
abundance in tumour versus normal samples. Significant pNEN confirmatory peptides include neuroendocrine secretory granule chromogranin A (p<0.05, 1 separate peptide sequences). Figure 6-8(c) plots the logarithmic transformed relative abundance tumour: normal (log2ratio) against p-value (log10) for all peptides.

6.4.3.2 Protein level results
There were statistically significant (p<0.05) differences in the quantity of proteins between normal and tumour pairs for 28 distinct proteins (filtered for at least three contributory peptides). All proteins had a greater than twofold increase in the abundance in tumour versus normal samples. Figure 6-8(c) plots the logarithmic transformed relative abundance tumour: normal (log2ratio) against p-value (log10) for all 364 distinct proteins with complete sets of peptide modification data (unfiltered by number of contributory peptides).
Figure 6-8(a). Three pairs supernatant pNEN volcano plots – peptide (left) and protein (right)
Figure 6-8(b). Three pairs pellet pNEN volcano plots – peptide (left) and protein (right)
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Figure 6-8(c). 4 paired pNEN volcano plots – peptide (left) and protein (right)

**Figure 6-8 (a-c). Volcano plots of fold change and p-value of labelled peptide and proteins**

*a – three pairs supernatant, b – three pairs pellet, c - 4paired pNEN. The x-axis represents fold-changes of tumour versus normal pancreatic tissue (log2 of fold change), and the y-axis represents the statistical significance p-value (−log10 of p-value).*
6.4.4 Combined proteins

Combining the significantly different protein-level analysis between tumour and normal tissue from the three experiments (6plex supernatant – 156 proteins, pellet – 19 proteins, 10plex 4pNEN – 28 proteins) resulted in 187 unique protein accession numbers. The combined list of significant proteins are outlined in the appendices with UniProt accession numbers, Entrez Gene code, number of peptides, fold change and p-value. Figure 6-9 illustrates the distribution and overlap of unique accession numbers across the three experiments. There was no identical significant protein that was identified across the three experiments. Three quarters of the combined UniProt accession numbers were unique to the supernatant experiment (76%, 142/187) that had the greatest amount of labelled protein (600µg total labelled).

![Figure 6-9. Venn diagram of overlapping significant proteins from the three experiments](image)

The three experiments yielded different and differing numbers of significant protein; supernatant -156 proteins, pellet – 19 proteins, 4pNEN – 28 proteins). 187 unique proteins were identified across all three experiments.

6.4.4.1 Geneontology (GO) Slim classification

The 187 protein accession numbers were mapped to 174 unique Entrez Gene ID with 13 accession numbers not mapped as associated with multiple or no Entrez Gene IDs. The majority of proteins were associated with biological processes like metabolic function (121/174), biological regulation (115/174) and cell component organisation (100/174)
(Figure 6-10). Biological processes of interest include those associated with response to stimuli (97/174), cell communication (67/174) and cell proliferation (22/174) that may relate to tumour development. The majority of proteins were vesicle (145/174) and membrane (105/174) cellular components that may reflect secretory and signalling nature of neuroendocrine neoplasms. The overwhelming majority of proteins were involved in protein binding (151/174) as a molecular function that again may reflect signalling within neuroendocrine neoplasms.
Figure 6-10. GO Slim summary of combined proteins by biological process, cellular component or molecular function.

The height of the bar represents the number of user list genes observed in the category. Charts from WebGestalt (WEB-based Gene SeT AnaLysis Toolkit).
6.4.5 Reactome and KEGG pathway analysis
The combined list of 187 significant proteins were analysed for mapping to biological pathways via Reactome (https://reactome.org/) (Fabregat et al., 2018). The proteins were mapped to 772 pathways (Figure 6-11) of which 57 pathways had 10 or more proteins mapped (see appendices for list of these pathways). The top 10 pathways ranked by number of proteins mapped are outlined in Table 6-1. The coverage of proteins mapped support involvement in signalling (signal transduction, post-translational protein modification) and secretion (vesicle-mediated transport, neutrophil degranulation) in neuroendocrine neoplasms.

<table>
<thead>
<tr>
<th>Pathway identifier</th>
<th>Pathway name</th>
<th>No. Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-HSA-168256</td>
<td>Immune System</td>
<td>63</td>
</tr>
<tr>
<td>R-HSA-392499</td>
<td>Metabolism of proteins</td>
<td>58</td>
</tr>
<tr>
<td>R-HSA-1430728</td>
<td>Metabolism</td>
<td>54</td>
</tr>
<tr>
<td>R-HSA-162582</td>
<td>Signal Transduction</td>
<td>51</td>
</tr>
<tr>
<td>R-HSA-168249</td>
<td>Innate Immune System</td>
<td>44</td>
</tr>
<tr>
<td>R-HSA-1266738</td>
<td>Developmental Biology</td>
<td>36</td>
</tr>
<tr>
<td>R-HSA-597592</td>
<td>Post-translational protein modification</td>
<td>36</td>
</tr>
<tr>
<td>R-HSA-1643685</td>
<td>Disease</td>
<td>34</td>
</tr>
<tr>
<td>R-HSA-5653656</td>
<td>Vesicle-mediated transport</td>
<td>29</td>
</tr>
<tr>
<td>R-HSA-6798695</td>
<td>Neutrophil degranulation</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 6-1. Top 10 Reactome pathways with number of mapped proteins

The 187 proteins were uploaded to Reactome (https://reactome.org/) and mapped to all pathways. No. Proteins - number of proteins mapped to pathway (Fabregat et al., 2018).
The combined list of 187 significant proteins were analysed for mapping to specific KEGG pathways related to cancer (Luo and Brouwer, 2013, Luo et al., 2017) (Figure 6-12 a, b, c).

The membrane located tropomyosin 3 (P06753, TPM3) interacts upstream of the Ras signalling pathway. Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 (P62873, GNB1) is involved in the transduction of trans-membrane chemokine signalling and interacts with PI3K and Ras activity. The cytoplasmic protein Ras homolog gene family, member A (P61586, RHOA) is downstream of both Ras and mTORC2, and is involved in cytoskeleton organization, cell motility, tissue invasion and metastases. Other downstream proteins include Ras-related protein Ral-A (P11233, RALA), involved in gene expression and endocytosis, and Ras-related protein Rap-1A (P62834, RAP1A), involved in the Rap1 signalling pathway.

Collagen, type IV, alpha (UniProt ID- P02462, Entrez Gene ID - COL4A1) interacts with extracellular membrane (ECM) receptors involved with focal adhesion upstream of the PI3K-Akt signalling pathway. Heat shock protein HSP 90-beta (P08238, HSP90AB1) is a cytoplasmic molecular chaperone upstream of AKT that promotes the maturation, structural maintenance and regulation of specific target proteins involved in cell cycle control and signal transduction. 14-3-3 protein beta/alpha (P31946, YWHAB) is downstream of AKT, involved in the regulation of signalling pathways, like FoxO, that affect cell survival and cell cycle progression.

A number of proteins mapped to proteoglycans involved in cancer. As well as the pathway mentioned above, Ras homolog gene family, member A (P61586, RHOA) is a downstream of the proteoglycan hyaluronan and transmembrane protein relating to breast cancer (CD44). CD63 antigen (P08962, CD63) plays a role in the activation of a number of cellular signalling cascades and is downstream of the chondroitin and dermatin sulfate proteoglycans. In heparin sulfate proteoglycans cancer pathways, basement membrane-specific heparan sulfate proteoglycan core protein (P98160, HSPG2) is involved in angiogenesis with Moesin (P26038, MSN) involved with tumour cell migration and invasion. Finally, Glutathione S-transferase alpha 1 (P08263, GSTA1) is involved in the conjugation of glutathione that has a role in evading apoptosis.
Chapter 6: Proteomic signatures of pancreatic NENs

Figure 6-11. Reactome overview of biological pathways

Yellow lines represent proteins mapped to pathways. Image from Reactome (https://reactome.org/, accessed 2018) (Fabregat et al., 2018)
6-12(a). Proteins involved in KEGG pathways in cancer (mapped proteins highlighted in red).
6-12 (b). Proteins involved in RAS signalling pathways (mapped proteins highlighted in red)
Chapter 6: Proteomic signatures of pancreatic NENs

6-12 (c). Proteins involved in the PI3K-Akt signalling pathways (mapped proteins highlighted in red)

Figure 6-12 (a, b, c). Mapping of proteins to specific KEGG pathways related to cancer.
6.4.6 Proteins of interest

A number of proteins were identified that were thought to be of interest for future studies.

Neudesin (Q9UMX5, NENF) acts as an extracellular neurotrophic factor that promotes cell proliferation via MAPK1/ERK2, MAPK3/ERK1 and AKT1/AKT pathways. It is thought to play a role in breast tumourigenesis and is over expressed in other carcinomas of the uterine cervix, lymphoma, colon, lung, skin and leukaemia (Han et al., 2012).

Tenascin-X (P22105, TNXB) mediates interactions between cells and the extracellular matrix. It is thought to accelerate collagen fibril formation and may play a role in the growth of epithelial tumors. It potentially is a secretory and immunohistochemical marker of ovarian cancer (Kramer et al., 2015).

Actin-related protein 3 (P61158, ACTR3) functions as a component of the Actin-related protein 2/3 (Arp 2/3) complex, which is involved in regulation of actin polymerization that mediates the formation of branched actin networks. Dysregulation of Arp2/3 is thought to play a role in tumour progression through invasion and metastases (Molinie and Gautreau, 2018, Zhang et al., 2017).

Fibulin-1 (P23142, FBLN1) plays a role in cell adhesion and migration along protein fibers within the extracellular matrix (ECM). It has been implicated as a tumour suppressor by inhibiting cell motility and reducing tumour growth (Qing et al., 1997, Hayashido et al., 1998).

Moesin (P26038, MSN) is involved in major cytoskeletal structures and plays a role in regulating the proliferation, migration, and adhesion of human cells. The ezrin-radixin-moesin (ERM) family of proteins is expressed in cancer, particularly epithelial origin, and has been implicated in progression (Clucas and Valderrama, 2014). Moesin’s expression in gastric adenocarcinoma was related to poor prognosis (Jung et al., 2013).

Secretogranin-2 or Chromogranin C (P13521, SCG2) is a neuroendocrine secretory granule protein, which is the precursor for biologically active peptides secretoneurin, which exerts chemotaxic effects on specific cell types, and manserin. It is highly
expressed in advanced prostate cancer and may contribute to the neuroendocrine differentiation by promoting the formation of secretory granules and the proliferation of cancer cells (Courel et al., 2014). Another neuroendocrine secretory granule, chromogranin A, is a general circulating biomarkers in NENs and is routinely used biomarker in with diagnostic, prognostic and predictive value (Lawrence et al., 2011b, Vinik et al., 2009).

Tropomyosin 3 (P06753, TPM3) is implicated in stabilizing cytoskeleton actin filaments in non-muscle cells as well as being an upstream protein in the Ras signaling pathway. Elevated TPM3 levels and cytoplasmic protein expression have been described in hepatocellular cancer (Lam et al., 2012). The Tropomyosin isoforms, including TPM3, have been detected at higher levels in ovarian cancer patients when compared to controls and are suggested as putative markers (Tang et al., 2013).

14-3-3 protein beta/alpha (P31946, YWHAB) is an adapter protein implicated in the regulation of a general and specialized signaling pathways. It has been described as a putative tissue biomarker of response to neo-adjuvant chemotherapy in ER-positive breast cancer (Hodgkinson et al., 2012). The urinary levels of 14-3-3 protein beta/alpha were associated with advanced stage and poor survival in patients with clear cell renal cell carcinoma (Kaneko et al., 2016).

Calnexin (P27824, CANX3) is a calcium-binding protein that interacts with newly synthesized glycoproteins in the endoplasmic reticulum and may it may play a role in receptor-mediated endocytosis at the synapse. Calnexin has been reported as a potential novel sero-diagnostic marker for lung cancer and of prognostic significance in colorectal cancer (Kobayashi et al., 2015) (Ryan et al., 2016). Both 14-3-3 beta/alpha and calnexin may potentially be involved in tumor recurrence and the malignant properties of lung adenocarcinoma that contribute to poor prognosis (Okayama et al., 2014).

CD63 antigen (P08962, CD63) plays a role in cellular signaling cascades and is thought to contribute to malignancy, including invasion and metastases (Tominaga et al., 2014, Seubert et al., 2015). Positive staining for CD63 has been described in lung cancer, melanoma and pancreatic cancer (Kim and Kwon, 2010, Lupia et al., 2014, Khushman et
al., 2017). Tumour-derived exosomes with surface CD63 are thought to be closely related to tumour development and metastases (Yu et al., 2015).

6.5 Discussion

This proteomic study of pancreatic neuroendocrine neoplasm tissue demonstrated an abundance of significant proteins across seven paired fresh frozen samples. The data are limited by the small pNEN sample size used, the relative distribution of significant proteins across the three experiments and the lack of validation that require additional evaluation.

Historically, proteomic studies have used formalin-fixed samples from histopathology archives as a source of larger numbers of samples and often with accompanying clinical data for correlation with outcomes (Gustafsson et al., 2015). The same approach to quantification with tandem mass tag labels can be applied (Maes et al., 2013). However, FFPE proteomic workflow is open to variation with a number of pre-analytical factors that affect analysis as well as additional processing for protein extraction (Mason, 2016).

The relative distribution in numbers of proteins across the three experiments relates to the different focus of the experiments (supernatant vs. pellet) and different protein amounts used for labeling (10, 50, 100µg per sample). The initial supernatant TMT 6plex yielded an abundance of unique proteins from a total of 600µg protein labeled. As a consequence, the amount chosen in subsequent experiments (300µg for pellet and 100µg for 4pNEN) was reduced. Time and cost limited the ability to re-run the pellet and 4pNEN experiments with increased amounts of protein for labeling.

Confirmatory studies are required to validate the expression of the proteins in pancreatic NENs that were identified in this study. The Western blot technique is able to identify specific proteins from a complex mixture of proteins extracted from cells through the use of specific antibodies (Hnasko and Hnasko, 2015, Mahmood and Yang, 2012). Immunohistochemistry performed on tissue, similarly with antibodies to the specific proteins of interest, allows for validation in larger cohorts of neuroendocrine neoplasms patients with the potential for correlation of expression with clinical outcomes, like recurrence, progression and survival (Matos et al., 2010).
However, a number of proteins of significance from this study were associated with cancer pathways, including RAS, PI3K-Akt and proteoglycan signaling. Proteins of interest were identified that are thought to contribute to the tumourigenesis as well as the development of invasion and metastases in other cancers. These proteins of interest have not previously been described for pancreatic neuroendocrine neoplasms and require further studies for validation as potential biomarkers or therapeutic targets.

Secretory proteins of interest included chromogranin C / secretogranin-2, part of the granin family that are associated with neuroendocrine neoplasms (Gut et al., 2016). Chromogranin A is a recommended as a marker for non-functional pancreatic NENs (Falconi et al., 2016). Differences in serum chromogranin A and B may be associated with NENs of different origin, with an isolated abnormal chromogranin B associated with pancreato-duodenal NENs (Basuroy et al., 2015). Similarly, circulatory chromogranin C may be of value as a circulating biomarker for pancreatic NENs.

A number of proteins of interest have a putative role in cancer progression via interactions with the extracellular matrix (ECM); Neudesin, Tenascin-X and Fibulin-1. Pancreatic NENs and pancreatic adenocarcinoma are associated with fibrovascular stromal changes and there is increasing focus on targeting the tumour stroma through the relationship between pancreatic cancer cells and the ECM (Kasajima et al., 2015, Vennin et al., 2018). Other proteins of interest are involved in the cytoskeleton and may have roles in adhesion and migration; actin-related protein 3, moesin, and tropomyosin 3. The cytoskeleton is thought to play a role in the development of tumour cell migration, invasion and metastasis (Fife et al., 2014). Other proteins identified in this study, like 14-3-3 protein beta/alpha, CD63 antigen, mapped to specific cancer pathways, particularly RAS and PI3K-Akt signaling pathways, which may be potential targets for therapy.
7 CONCLUSION

7.1.1.1 Chapter 2
Patients with NENs experience symptoms prior to diagnosis that are of an unclear pattern and duration, often misdiagnosed through overlap with benign conditions such as functional bowel disorders. The novel data reported from a patient survey established pre-diagnosis symptom patterns, duration of symptoms, and differentiation from functional conditions that are reported for the first time. The most frequent incorrect initial diagnosis was Irritable Bowel Syndrome in patients with GEP NENs with respondents accessing healthcare services repeatedly with a broad range of persistent or evolving symptomatology. A significant number of these patients are over 50 years of age and, therefore, investigation of other pathology should be considered prior to making a diagnosis of IBS. Only 5% of respondents met the criteria for functional diarrhoea Rome Criteria (C4). Better awareness in primary care of alarm symptoms and the Rome criteria for functional bowel disorders may lead to earlier referral to secondary care for investigation to exclude malignancy. An increased level of suspicion in primary care for malignancy in patients with persistent symptoms or red flags may lead an earlier referral to secondary care cancer services that may lead to an earlier diagnosis, improved quality of life and clinical outcomes.

7.1.1.2 Chapter 3
While the long-term tumour biology of rectal NENs may not be clear, they are increasing in incidence and endoscopists need to be aware of the importance of performing a thorough pre-resection assessment. Risk factors for metastatic disease include size, atypical appearance, grade, and depth of invasion. Pre-resection diagnostic work ups may include EUS, anatomical and functional imaging. The choice of primary resection modality influences complete resection rates and the need for secondary therapy. Device-assisted endoscopic mucosal resection and endoscopic submucosal dissection are used to resect localised rectal NENs < 2cm. Transanal surgery is also used to resect localised 1-2cm rectal NENs. Oncological surgical resection is used for rectal NENs that are >2cm
Chapter 7: Conclusion

or with invasion and regional disease. The treatment of advanced disease is multimodal and referral to NEN centres for patients with locally advanced disease or metastatic disease is recommended.

7.1.1.3 Chapter 4
Screening programmes for other malignancies, like colorectal cancer, may incidentally diagnose asymptomatic NENs and represent an opportunity for earlier identification of patients. This is the first systematic analysis of NENs diagnosed through a double screening BCSP of gFOBT and colonoscopy. There were low numbers and rates of NENs each year of the BCSP in England. The characteristics of rectal NENs were similar to that published for a single screening BCSP but the diagnosis rate per 100,000 colonoscopies was lower. This suggests that double screening with a primary gFOBT screen is not as helpful as screening with colonoscopy itself for rectal NENs. However, the role of gFOBT in screening for colonic or ileal NENs is not clear as these lesions could be associated with occult bleeding and were reported for the first time in this study. The impact on morbidity and mortality of being diagnosed through a screening programme, rather than later once symptomatic, requires further investigation with outcome and comparative data. This will help with our understanding if colorectal cancer screening helps identify NENs, particularly ileal and colonic origin, at an earlier stage leading to better outcomes.

7.1.1.4 Chapter 5
Carcinoid heart disease (CHD) develops in patients with carcinoid syndrome through fibrosis of cardiac valve disease. The recommended marker for CHD is NT pro BNP (NTP) that is elevated in volume overload and natriuresis that occurs in advanced CHD. This study of fibrosis, inflammation and disease markers did not identify alternative or complementary biomarkers to NTP for screening of CHD. This confirms the consensus guidance for screening and assessing CHD with NTP. The mechanism or causation of the increased circulating calprotecin was uncertain and its role as a faecal and circulatory biomarker in small bowel neuroendocrine disease should be further evaluated. The dampening effect of medical therapy on such markers is unclear. Additional studies would require a larger sample number with robust cardiac and clinical baseline characterization of the stages of both NEN and CHD disease.
7.1.1.5 Chapter 6
This study reported for the first time proteomic analysis of fresh frozen low-grade pNENs and yielded information on cancer pathways and proteins of interest. The labeling of samples with isobaric tandem mass tags allows for quantification of proteins between tumour and normal samples. 187 proteins were significantly different in their abundance in low-grade pancreatic NEN across three experiments from seven patient samples. Proteins mapped to well described cancer pathways, like PI3-Akt and Ras signaling, as well as a number supported by evidence from other malignancies. Proteins of interest were identified that spanned potential mechanism for NEN development from extracellular matrix (ECM), cytoskeleton and cellular signaling interactions. Further study of these proteins is required for reproducibility and validation, with Western blot and immunochemistry, as well as assessing clinical utility with linkage to clinical and outcome data.

Strategies aimed at individualizing or personalizing healthcare for patients with neuroendocrine neoplasms can span from symptom origination through to diagnosis, either from screening or a heightened index of suspicion, towards managing complex disease, such as sequelea like carcinoid heart disease, and the development of biomarkers. The thesis explored themes in the first part around symptoms patterns prior to diagnosis, individualizing care for rectal NEN patients, diagnoses through colorectal cancer screening programmes, and in the second part around biomarkers in carcinoid heart disease and in pancreatic neuroendocrine neoplasms.
8 REFERENCES


References


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SURVEY MONKEY: NEUROENDOCRINE NEOPLASMS- SYMPTOMS BEFORE DIAGNOSIS

https://www.surveymonkey.com/r/diagnosisNET

Final version: 15 September 2014
Survey opened: 20 September 2014
Survey closed: 5 May 2015
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4. First Symptom

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Neuroendocrine Tumours - Symptoms Before Diagnosis

5. First Symptom
- Question: How long did you have [X] for BEFORE you were diagnosed?
  - Please type a response using a number. For example “60” rather than “6yrs”.
  - Hand
  - Date

6. How severe was the [X] that you experienced BEFORE you were diagnosed?
  - None
  - Mild
  - Moderate
  - Severe
  - Very severe

7. Did anything make the [X] better?
  - If you are not sure, please leave question blank.

8. Did anything make the [X] worse?
  - If you are not sure, please leave question blank.

9. Apart from the [X], did you experience a DIFFERENT MORE SEVERE symptom BEFORE you were diagnosed?
  - Yes
  - No
  - Move

Neuroendocrine Tumours - Symptoms Before Diagnosis

6. Worst Symptom
- Question: What was the MOST SEVERE symptom that you experienced BEFORE you were diagnosed with a NET?
  - The worst symptom that you experienced.

7. Worst Symptom
- Question: How severe was the [X] that you experienced BEFORE you were diagnosed with a NET?
  - Very mild
  - Mild
  - Moderate
  - Severe
  - Very severe

8. How long did you have [X] for BEFORE you were diagnosed?
  - Please type a response using a number. For example “60” rather than “6 yrs”.
  - Hand
  - Date

9. Did anything make the [X] better?
  - If you are not sure, please leave question blank.

10. Did anything make the [X] worse?
    - If you are not sure, please leave question blank.

11. Apart from the [X], did you experience a DIFFERENT MORE SEVERE symptom BEFORE you were diagnosed?
    - Yes
    - No
    - Move

12. How long did you have [X] for BEFORE you were diagnosed with a NET?
    - Please type a response using a number. For example “60” rather than “6 yrs”.
    - Hand
    - Date

13. Did your weight change BEFORE you were diagnosed with the NET?
    - Weight gain
    - Weight loss
    - No change in weight
    - Move

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Neuroendocrine Tumours - Symptoms Before Diagnosis

9. Weight Change

16. How much [KG] did you notice BEFORE you were diagnosed with a NET?
Answer in KG of kilograms, please or stone.
Please type a response using a number. For example "50" rather than "50 KG".

Weight

17. How long did you have [MTH] for BEFORE you were diagnosed?
Please type a response using a number. For example "9" rather than "9 MTH".

MTH

18. How severe was the [SIG] that you experienced BEFORE you were diagnosed?

- Very mild
- Mild
- Moderate
- Severe
- Very severe

Neuroendocrine Tumours - Symptoms Before Diagnosis

10. Appetite

21. Did your appetite change BEFORE you were diagnosed with the NET?

- Increased appetite
- Decreased appetite
- No change in appetite
- None

Neuroendocrine Tumours - Symptoms Before Diagnosis

11. Appetite

22. How long did you have the [SIG] BEFORE you were diagnosed with a NET?
Please type a response using a number. For example "6" rather than "6 MTH".

MTH

23. How severe was the [SIG] that you experienced BEFORE you were diagnosed?

- Very mild
- Mild
- Moderate
- Severe
- Very severe

Neuroendocrine Tumours - Symptoms Before Diagnosis

10. Type of NET

25. What type of neuroendocrine tumour have you been diagnosed with?
This is where the NET started or the Primary site.

- Small bowel
- Pancreas
- Lung
- Liver
- Other
- None

(please specify)
Chapter 9: Appendices

Neuroendocrine Tumours - Symptoms Before Diagnosis

25. How would you describe the pain or discomfort?

If you are not sure, please leave question blank.

- Sharp
- Burning
- Sharp and burning
- Tenderness
- Swelling
- Other (please specify): ____________

26. Did this pain or discomfort build up to a steady, severe level?

- No
- Yes
- Same
- Less
- More

27. How long did the pain or discomfort last before it disappeared?

Please type a response using a number. For example '8' rather than 'Yes'.

- Minutes
- Hours

28. Did this pain or discomfort occur and then completely disappear during the same day?

- Yes
- No

29. Did you experience the pain or discomfort at night?

- Yes
- No
- Some nights
- Every night
- Other

30. Did anything make the pain BETTER?

If you are not sure, please leave question blank.

- Yes
- No

31. Did anything make the pain WORSE?

If you are not sure, please leave question blank.

- Yes
- No

40. How often did this pain or discomfort get better or stop AFTER you had a bowel movement?

- Always
- Sometimes
- Other
- Never

41. Before you were diagnosed, did this pain or discomfort occur ONLY DURING your menstrual bleeding and not at other times?

This question is aimed at WOMEN. Please answer 'Yes', 'No', or 'Not applicable' if had
the menopause change in life.

For MEN please check 'Not applicable'.

- Yes
- No
- Not applicable

42. When this pain or discomfort STARTED, did you have any of these problems?

Please select how frequently you experienced for each symptom.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Very often</th>
<th>Often</th>
<th>Sometimes</th>
<th>Rarely</th>
<th>Never</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache or facial discomfort</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach pain or bloating</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscular stiffness or discomfort</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other discomfort (please specify)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Neuroendocrine Tumours - Symptoms Before Diagnosis

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### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 17. Fullness

**Q.44. How long did you feel uncomfortably full after a regular meal BEFORE you were diagnosed?**

<table>
<thead>
<tr>
<th>Time</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than one week</td>
<td></td>
</tr>
<tr>
<td>One to three weeks</td>
<td></td>
</tr>
<tr>
<td>More than three weeks</td>
<td></td>
</tr>
<tr>
<td>One day</td>
<td></td>
</tr>
<tr>
<td>Less than one week</td>
<td></td>
</tr>
<tr>
<td>One day or less</td>
<td></td>
</tr>
</tbody>
</table>

**Q.45. How often did you have trouble finishing a regular sized meal BEFORE you were diagnosed?**

<table>
<thead>
<tr>
<th>How often</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Never</td>
<td></td>
</tr>
<tr>
<td>Less than once a week</td>
<td></td>
</tr>
<tr>
<td>One to three times a week</td>
<td></td>
</tr>
<tr>
<td>More than three times a week</td>
<td></td>
</tr>
<tr>
<td>One day or less</td>
<td></td>
</tr>
<tr>
<td>More than one day</td>
<td></td>
</tr>
</tbody>
</table>

#### 18. Nausea

**Q.46. How often did you have bothersome nausea BEFORE you were diagnosed?**

<table>
<thead>
<tr>
<th>How often</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Never</td>
<td></td>
</tr>
<tr>
<td>Less than once a week</td>
<td></td>
</tr>
<tr>
<td>One to three times a week</td>
<td></td>
</tr>
<tr>
<td>More than three times a week</td>
<td></td>
</tr>
<tr>
<td>One day or less</td>
<td></td>
</tr>
<tr>
<td>More than one day</td>
<td></td>
</tr>
</tbody>
</table>

**Q.47. How long did you have bothersome nausea for BEFORE you were diagnosed?**

<table>
<thead>
<tr>
<th>Time</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than one week</td>
<td></td>
</tr>
<tr>
<td>One to three weeks</td>
<td></td>
</tr>
<tr>
<td>More than three weeks</td>
<td></td>
</tr>
<tr>
<td>One day</td>
<td></td>
</tr>
<tr>
<td>Less than one week</td>
<td></td>
</tr>
<tr>
<td>One day or less</td>
<td></td>
</tr>
</tbody>
</table>

#### 19. Vomiting

**Q.48. How often did you vomit BEFORE you were diagnosed?**

<table>
<thead>
<tr>
<th>How often</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Never</td>
<td></td>
</tr>
<tr>
<td>Less than once a week</td>
<td></td>
</tr>
<tr>
<td>One to three times a week</td>
<td></td>
</tr>
<tr>
<td>More than three times a week</td>
<td></td>
</tr>
<tr>
<td>One day or less</td>
<td></td>
</tr>
<tr>
<td>More than one day</td>
<td></td>
</tr>
</tbody>
</table>
## Chapter 9: Appendices

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 21. Yelling

**Q21. How long did you have symptoms before you were diagnosed?**

Please type a response using a number. For example “10” rather than “ten”.

- **Years:**
- **Months:**
- **Days:**

**Q22. How long did you have problems with your bowel habit before you were diagnosed?**

Please type a response using a number. For example “10” rather than “ten”.

- **Years:**
- **Months:**
- **Days:**

**Q23. How severe were the bowel problems that you experienced before you were diagnosed?**

- **Very severe**
- **severe**
- **Moderate**
- **mild**
- **None**

**Q24. Did any of these make your bowel worsen before you were diagnosed?**

- **Hard**
- **Loose**
- **Diarrhea**
- **Blood**
- **Other**

**Q25. Did you have any other symptoms before you were diagnosed?**

- **No**
- **Yes**

---

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 22. Bowel

**Q26. How often were you passing your stools each day or each week before you were diagnosed?**

Please type a response using a number. For example “10” rather than “ten”.

- **Days:**
- **Weeks:**

**Q27. Did you have any problems with your bowel before you were diagnosed?**

For example, developing diarrhea or constipation as a problem.

- **No**
- **Yes**

---

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 23. Bowel

**Q28. How did your bowel habit change?**

- **Hard**
- **Loose**
- **Diarrhea**
- **Blood**
- **Other**

**Q29. How long did you have problems with your bowel habit before you were diagnosed?**

Please type a response using a number. For example “10” rather than “ten”.

- **Years:**
- **Months:**
- **Days:**

**Q30. How severe were the bowel problems that you experienced before you were diagnosed?**

- **Very severe**
- **Severe**
- **Moderate**
- **Mild**
- **None**

---

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 24. Bowel

**Q31. How often did you have problems with hard or easy stool before you were diagnosed?**

- **None**
- **Mild**
- **Moderate**
- **Severe**
- **Other**

**Q32. Did you have any other symptoms before you were diagnosed?**

- **No**
- **Yes**

---

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 25. Bowel

**Q33. How did your bowel habit change?**

- **Hard**
- **Loose**
- **Diarrhea**
- **Blood**
- **Other**

**Q34. How long did you have problems with your bowel habit before you were diagnosed?**

Please type a response using a number. For example “10” rather than “ten”.

- **Years:**
- **Months:**
- **Days:**

**Q35. How severe were the bowel problems that you experienced before you were diagnosed?**

- **Very severe**
- **Severe**
- **Moderate**
- **Mild**
- **None**

**Q36. Did any of these make your bowel worse before you were diagnosed?**

- **Hard**
- **Loose**
- **Diarrhea**
- **Blood**
- **Other**

**Q37. Did you have any other symptoms before you were diagnosed?**

- **No**
- **Yes**

---

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 26. Bowel

**Q38. How often were you passing your stools each day or each week before you were diagnosed?**

Please type a response using a number. For example “10” rather than “ten”.

- **Days:**
- **Weeks:**

**Q39. Did you have any problems with your bowel before you were diagnosed?**

For example, developing diarrhea or constipation as a problem.

- **No**
- **Yes**

---

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 27. Bowel

**Q40. How did your bowel habit change?**

- **Hard**
- **Loose**
- **Diarrhea**
- **Blood**
- **Other**

**Q41. How long did you have problems with your bowel habit before you were diagnosed?**

Please type a response using a number. For example “10” rather than “ten”.

- **Years:**
- **Months:**
- **Days:**

**Q42. How severe were the bowel problems that you experienced before you were diagnosed?**

- **Very severe**
- **Severe**
- **Moderate**
- **Mild**
- **None**

---

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 28. Bowel

**Q43. How often did you have problems with hard or easy stool before you were diagnosed?**

- **None**
- **Mild**
- **Moderate**
- **Severe**
- **Other**

**Q44. Did you have any other symptoms before you were diagnosed?**

- **No**
- **Yes**

---

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 29. Bowel

**Q45. How did your bowel habit change?**

- **Hard**
- **Loose**
- **Diarrhea**
- **Blood**
- **Other**

**Q46. How long did you have problems with your bowel habit before you were diagnosed?**

Please type a response using a number. For example “10” rather than “ten”.

- **Years:**
- **Months:**
- **Days:**

**Q47. How severe were the bowel problems that you experienced before you were diagnosed?**

- **Very severe**
- **Severe**
- **Moderate**
- **Mild**
- **None**

**Q48. Did any of these make your bowel worse before you were diagnosed?**

- **Hard**
- **Loose**
- **Diarrhea**
- **Blood**
- **Other**

**Q49. Did you have any other symptoms before you were diagnosed?**

- **No**
- **Yes**
Chapter 9: Appendices

Neuroendocrine Tumours - Symptoms Before Diagnosis

24. Bowels

91. How long did you have hard or lumpy stools BEFORE you were diagnosed with a NET?

Please type a response using a number. For example '6' rather than 'six'.

Name: ____________________________

Date: ____________________________

92. How severe was the problem with hard lumpy stools that you experienced BEFORE you were diagnosed?

• never
• a little
• a lot
• uncomfortable
• very severe

93. How often did you have the sensation that the stool could not be passed (it was blocked) BEFORE you were diagnosed?

• never
• a little
• a lot
• uncomfortable
• very uncomfortable

94. How long did you have the sensation that the stool could not be passed (it was blocked) BEFORE you were diagnosed?

Please type a response using a number. For example '6' rather than 'six'.

Name: ____________________________

Date: ____________________________

Neuroendocrine Tumours - Symptoms Before Diagnosis

25. Bowels

95. How severe was the problem with loose, mushy stools that you experienced BEFORE you were diagnosed?

• very mild
• a little
• a lot
• uncomfortable
• very uncomfortable

96. Were at least three-quarters (75%) of your usual bowel, mushy or watery BEFORE you were diagnosed?

• yes
• no

97. How long did you have loose or mushy stools BEFORE you were diagnosed with a NET?

Please type a response using a number. For example '6' rather than 'six'.

Name: ____________________________

Date: ____________________________

Neuroendocrine Tumours - Symptoms Before Diagnosis

26. Bowels

98. How often did you have problems with loose, mushy or watery stools BEFORE you were diagnosed?

• never
• a little
• a lot
• uncomfortable
• very uncomfortable

99. Did you have a bout of diarrhoea before you were diagnosed?

• yes
• no

100. Did your stools become more frequent

• yes
• no
Chapter 9: Appendices

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 28. Urgency

- **Q28:** How long did you have problems with making to the toilet to open your bowel BEFORE you were diagnosed?
- **Please type a response using a number. For example “0” rather than “Yes”:**
  - Years: [ ]
  - Months: [ ]
  - Days: [ ]

#### 29. Blushing

- **Q29:** How often did you suffer from blushing symptoms BEFORE you were diagnosed with the NET?
  - Never
  - Less than once a month
  - Once a month
  - Twice a week
  - Three or more times a week
  - Three or more times a week
  - Twice a day
  - More than once a day
  - Never

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 30. Blushing

- **Q30:** How long did you suffer from blushing BEFORE you were diagnosed with the NET?
- **Please type a response using a number. For example “0” rather than “Yes”:**
  - Years: [ ]
  - Months: [ ]
  - Days: [ ]

#### 31. Hunger

- **Q31:** How often did you notice you were hungry BEFORE you were diagnosed?
  - Never
  - Less than once a month
  - Once a month
  - Twice a week
  - Three or more times a week
  - Three or more times a week
  - Twice a day
  - More than once a day
  - Never

- **Q32:** How often did you notice black stools BEFORE you were diagnosed?
  - Never
  - Less than once a month
  - Once a month
  - Twice a week
  - Three or more times a week
  - Three or more times a week
  - Twice a day
  - More than once a day
  - Never

- **Q33:** How often did you want to eat before you were diagnosed?
  - Never
  - Less than once a month
  - Once a month
  - Twice a week
  - Three or more times a week
  - Three or more times a week
  - Twice a day
  - More than once a day
  - Never

- **Q34:** How often did you have problems with skin feeling (numbness or warm feeling) BEFORE you were diagnosed with the NET?
  - Never
  - Less than once a month
  - Once a month
  - Twice a week
  - Three or more times a week
  - Three or more times a week
  - Twice a day
  - More than once a day
  - Never
Chapter 9: Appendices
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36. Breathing

How long did you have problems with breathing BEFORE you were diagnosed?

Please type a response using a number. For example '10' rather than 'Yes'.

None

One

Two

More

How severe was the breathing problem that you experienced BEFORE you were diagnosed?

Very mild

Mild

Severe

Very severe

How far were you able to walk with the breathing problem BEFORE you were diagnosed?


37. Other symptoms

What other symptoms did you experience BEFORE you were diagnosed?

Other symptoms that you believe were related to the NET, if there were no other symptoms, leave this answer blank.


38. GP

Did you see a GP about any symptoms or problems related to the neuroendocrine tumor BEFORE you were diagnosed?

Yes

No

Which symptoms did you discuss with your GP BEFORE you were diagnosed?


How long was the GP investigating any symptoms BEFORE you were diagnosed?


How many times did you see a GP about your symptoms BEFORE you were diagnosed?


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<table>
<thead>
<tr>
<th>Question</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>48. GP 3: What was the problem that caused the patient to seek medical advice when they first noticed symptoms?</td>
<td></td>
</tr>
<tr>
<td>49. Did your GP perform any investigations to investigate your symptoms before you were diagnosed?</td>
<td>Yes, No</td>
</tr>
<tr>
<td>50. What investigations did your GP arrange for you before you were diagnosed?</td>
<td></td>
</tr>
<tr>
<td>51. Were the results of these tests normal?</td>
<td>Yes, No</td>
</tr>
<tr>
<td>52. GP 4: What was the problem that caused the patient to seek medical advice when they first noticed symptoms?</td>
<td></td>
</tr>
<tr>
<td>53. Did your GP perform any investigations to investigate your symptoms before you were diagnosed?</td>
<td>Yes, No</td>
</tr>
<tr>
<td>54. What investigations did your GP arrange for you before you were diagnosed?</td>
<td></td>
</tr>
<tr>
<td>55. Were the results of these tests normal?</td>
<td>Yes, No</td>
</tr>
</tbody>
</table>

*Note: The questions and options are screenshot from a survey or questionnaire form.*
Neuroendocrine Tumours - Symptoms Before Diagnosis

Chapter 9: Appendices

46. Did you receive any medication or buy any from a pharmacy to help with the symptoms BEFORE you were diagnosed?
- Antidepressants
- Analgesics
- Painkillers
- Tranquilizers
- Sedatives
- Antiemetics
- Steroids
- Hormones
- Hormone replacement therapy
- Anticoagulants
- Anti-epileptics
- Anticonvulsants
- Antihistamines
- Antihypertensives
- Diuretics
- Cardiovascular drugs
- Diabetics
- Insulin
- Glucose
- Other medications

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Neuroendocrine Tumours - Symptoms Before Diagnosis

47. Hospital 1

71. How many times did you attend the hospital BEFORE you were diagnosed?
- Yes
- No

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Neuroendocrine Tumours - Symptoms Before Diagnosis

48. Hospital 2

72. What specialist team did you attend BEFORE you were diagnosed with the NET?
- Yes
- No

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Neuroendocrine Tumours - Symptoms Before Diagnosis

49. Hospital 3

73. What did your specialist team think the problem was initially?
- Yes
- No

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Neuroendocrine Tumours - Symptoms Before Diagnosis

**102. At this time, did you think there was another problem causing your symptoms?**

☐ Yes

☐ No

☐ Not sure

Neuroendocrine Tumours - Symptoms Before Diagnosis

**47. Hospital 3**

Q10. What problem did you think was causing your symptoms when you were being investigated by the hospital?

If you are not sure, please leave question blank.

Neuroendocrine Tumours - Symptoms Before Diagnosis

**48. Hospital 4**

Q106. Are there any other medical problems that you think were related to the NET?

If you are not sure, please leave question blank.

**111. Did you need to have surgery for the NET around the time you were diagnosed?**

☐ Yes

☐ No

☐ Not sure

Neuroendocrine Tumours - Symptoms Before Diagnosis

**49. Surgery**

Q112. Have you diagnosed with the NET only after surgery?

☐ Yes

☐ No

☐ Not sure

Q113. Did you need to have emergency surgery for the NET?

☐ Yes

☐ No

☐ Not sure

Q114. Did you have surgery for the NET at the local hospital?

☐ Yes

☐ No

☐ Not sure
Chapter 9: Appendices

### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 9. Diagnosis

**9.1. At the time of diagnosis, had the neuroendocrine tumour spread to other sites?**

- [ ] Yes
- [ ] No

For instance to lymph nodes or the liver.

**9.2. Which sites had the tumour spread to at the time of diagnosis?**

- [ ] Liver
- [ ] Lungs
- [ ] Bone
- [ ] Pancreas
- [ ] Other

*Note the primary site of metastasis.*

#### 10. Other

**10.1. Did you suffer from any of these conditions before you were diagnosed with the NET?**

- [ ] Carpal tunnel syndrome
- [ ] Hypertension
- [ ] Gastric ulcer
- [ ] Carcinoid syndrome
- [ ] Pancreatic cancer
- [ ] Adenocarcinoma
- [ ] Breast cancer
- [ ] Colorectal cancer
- [ ] Nerve tumours
- [ ] Ovarian cancer
- [ ] Kidney cancer
- [ ] Osteosarcoma
- [ ] Soft tissue sarcoma

#### 10.2. What operations did you have BEFORE you were diagnosed with the NET?**

- [ ] None

#### 10.3. Were you diagnosed with cardiac heart disease at the time of diagnosis?**

- [ ] Yes
- [ ] No
- [ ] Don’t know

#### 10.4. Have you ever been diagnosed with another type of cancer?**

- [ ] Yes
- [ ] No
- [ ] Don’t know

---

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### Neuroendocrine Tumours - Symptoms Before Diagnosis

#### 54. Other 2

<table>
<thead>
<tr>
<th>Question</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>123. Do you drink alcohol?</td>
<td>Yes, No, Less than usual</td>
</tr>
<tr>
<td>123. Do you smoke cigarettes?</td>
<td>No, Less than usual, Less than usual smoking</td>
</tr>
</tbody>
</table>

#### 55. Other 3

<table>
<thead>
<tr>
<th>Question</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>124. Is there a family history of any of these conditions?</td>
<td>Yes, No, Less than usual</td>
</tr>
<tr>
<td>124. Is there a family history of any of these cancers?</td>
<td>Yes, No, Less than usual</td>
</tr>
<tr>
<td>125. Is there a family history of any of these intraabdominal organs?</td>
<td>Yes, No, Less than usual</td>
</tr>
</tbody>
</table>

#### 56. Other family history

<table>
<thead>
<tr>
<th>Question</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>126. Which relative(s) were diagnosed and which type of NET did they have?</td>
<td>Yes, No</td>
</tr>
</tbody>
</table>

#### 57. Open response

<table>
<thead>
<tr>
<th>Question</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>128. Is there anything else that you would like to mention or share?</td>
<td>Yes, No</td>
</tr>
</tbody>
</table>
# Neuroendocrine Tumours - Symptoms Before Diagnosis

## 9. Demographic data

**What is your ethnic group?**

- Asian
- Black
- Chinese
- Indian
- Other Asian background
- Other Black background
- Other Chinese background
- Other Indian background
- Other Other background
- Other
- Mixed
- Not stated

## 9. Final Page

*Notes for completing the survey: Your responses will help us understand the needs of those affected by NET tumors once diagnosed. To ensure confidentiality, no personal data will be used in any analysis.*

Please refer to the next pages for more information. You can also visit [the NET website](#) or [contact](#).
DATA VALIDATION FORM SENT TO BCSP SITES FOR COMPLETION

Towards an earlier diagnosis of Neuroendocrine Tumours (NETs): Does Faecal Occult Blood testing identify ileo-colonic NET in the NHS Bowel Cancer Screening Programme?

Service Evaluation approved by the BCSP Research Committee, NHS HRA Confidentiality Advisory Group & PHE Office of Data Release

BCSP diagnosed Colorectal/Terminal Ileum NETs – Patient Data Collection Forms

PATIENT Details:

<table>
<thead>
<tr>
<th>NHS Number</th>
<th>DOB (MM/YY)</th>
<th>Postcode (1st part)</th>
<th>BCSP Site Name</th>
<th>Consultant</th>
<th>Procedure Type</th>
<th>Procedure Date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NEUROENDOCRINE NEOPLASM:

1. **Type of Tumour?** Please attach anonymised Pathology reports
   - Well differentiated NET (incl ‘carcinoid’): 1
   - Poorly differentiated NEC (small cell ca): 2
   - Poorly differentiated NEC (large cell): 3
   - Poorly differentiated endocrine carcinoma: 4
   - Goblet Cell Carcinoma: 5
   - Mixed Adeno-Neuroendocrine carcinoma (MANEC): 6
   - Other: 7; please specify...

2. **Site of NET**
   - Rectum = 1
   - Recto/sigmoid = 2
   - Sigmoid colon = 3
   - Descending colon = 4
   - Transverse colon = 5
   - Ascending colon = 6
   - Caecum = 7
   - Appendix = 8
   - Ileum = 9
   - Other = 10; please specify...

3. **Maximum tumour dimension of NET?** (mm)

4. **Depth of invasion?**
   - Mucosa only = 1
   - Submucosa = 2
   - Muscle invasion = 3
   - Peritoneal invasion = 4

5. **Presence of vascular invasion?**
   - No = 0
   - Yes = 1
   - Not Known = 2

6. **Grade of tumour?**
   - G1 (Ki67 < 2% or less): 1
   - G2 (Ki67 = 3-20%): 2
   - G3 (Ki67 > 20%): 3

7. **Actual Ki-67 index?** (% can be referred to as MIB-1)

8. **Was metastatic disease present at diagnosis?**
   - No = 0
   - Yes = 1
   - Not Known = 2

9. **Stage of tumour (TNM)?**
    - T=  
    - N=  
    - M=  

10. **Type of staging?**
    - Radiological = 1
    - Pathological = 2

11. **Pathology reporting Standards?**
    - ENETS TNM = 1
    - WHO 2004 = 2
    - WHO 2010 = 3

12. **Was a staging CT scan performed?**
    - No = 0
    - Yes = 1
    - Not Known = 2

  Results and please attach anonymised report: ..........................................................

13. **Were bloods sent for chromogranin A or B?**
    - No = 0
    - Yes = 1
    - Not Known = 2

  Results and please attach anonymised report: ..........................................................

BCSP CR/TI NETs Data Collection Forms v2.0 28.05.15

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14. Was a CT Colonography performed? [ ] No = 0  Yes = 1  Not Known = 2
   Results and please attach anonymised report: .................................................................

15. Was a staging Pelvic MRI performed? [ ] No = 0  Yes = 1  Not Known = 2
   Results and please attach anonymised report: .................................................................

16. Were Pathological Lymph nodes present on pelvic MRI? [ ] No = 0  Yes = 1  Not done = 2
   If Yes: Site/s? ...................................................................................................................

17. Was a Rectal EUS performed? [ ] No = 0  Yes = 1  Not Known = 2
   Results and please attach anonymised report: .................................................................

18. Were Pathological Lymph nodes present on rectal EUS? [ ] No = 0  Yes = 1  Not Known = 2

19. Was other significant pathology identified? (eg cancer, IBD) [ ] No = 0  Yes = 1  Not Known = 2
   If Yes – please state other pathologies: .............................................................................

INITIAL PATIENT MANAGEMENT

1. Multi-Disciplinary Meeting (MDM) type? [ ] None = 0  Colorectal = 1  Specialist NET = 2

2. Were clear follow-up recommendations proposed for the NET? [ ] No = 0  Yes = 1  Not Known = 2

3. What guidance/recommendations were given for NET management? (Please attach anonymised MDM outcome) ......................................................................................................................

INITIAL PATIENT REFERRAL

1. Were any referrals made to: (please tick all referrals made) Surgery? [ ]
   Specialist NET centre? [ ] Oncology? [ ] Endocrinology? [ ] Gastroenterology? [ ]
   Consultant / Location: ....................................................................................................
   Consultant / Location: ....................................................................................................

2. If referred to a NET centre - was the patient seen at the NET centre (= 1) or written communication only with the NET Centre (= 2)?  Not Known = 3
   NET Centre Location: ....................................................................................................
   NET Specialist: ..............................................................................................................

3. Were additional recommendations made? [ ] No = 0  Yes = 1  Not Known = 2
   If yes - recommendation details: ....................................................................................

4. Did these significantly alter patient care? [ ] No = 0  Yes = 1  Not Known = 2
   If yes – details: ..............................................................................................................
TREATMENT AT DIAGNOSIS

1. **Initial Colonoscopy**: please supply anonymised report
   - EMR: □ No = 0  Yes = 1  Not Known = 2
   - Standard Polypectomy: □ No = 0  Yes = 1  Not Known = 2

2. **Subsequent Surgical procedures**?
   - No = 0  Yes = 1  Not Known = 2
   - Date: □ □ □ □ □ □ □

3. **Type of Surgical procedure on primary tumour**?
   - Please supply anonymised operation note
   - EMR = 1  ESD = 2  TEMS = 3  Laparoscopic surgery = 4  Open Surgery = 5
   - Other = 6 - please specify:

4. **Number of LN’s removed**?
   - None = 0  1 – 5 nodes = 1  6 - 10 = 2  >10 = 3

5. **Ki-67 index from primary site**?
   - □ % (can be referred to as MIB-1)

6. **Histological resection margins**?
   - R0 = 0  R1 = 1  R2 = 2  Not Known = 3

7. **Therapy on metastases performed**?
   - □ No = 0  Yes = 1  Not Known = 2
   - Date: □ □ □ □ □ □ □

   **Site/s of metastases receiving therapy**?
   - Type of intervention on metastases?

8. **Ki-67 index from metastasis site**?
   - □ % (can be referred to as MIB-1)

9. **Other therapy performed**?
   - □ No = 0  Yes = 1  Not Known = 2
   - Date: □ □ □ □ □ □ □ Therapy type
   - Date: □ □ □ □ □ □ □ Therapy Type
   - Date: □ □ □ □ □ □ □ Therapy Type

PATIENT FOLLOW UP

1. **How long was follow-up for the patient planned for**?
   - □ Years or □ Months

2. **How frequently was follow-up planned**?
   - □ Annually (tick) or □ Monthly (number i.e. 6)

3. **Was length and frequency of follow-up adhered to**?
   - □ No = 0  Yes = 1  Not Known = 2
   - If not – please give reason:

4. **Protocol for follow up**: please tick methods used:
   - CT Scan □  MR □  Octreoscan □  PET □  EUS □  Chromogranin A / B □
5. Was any **new disease** found at follow up?  
   - No = 0  
   - Yes = 1  
   - Not Known = 2
   
   **If Yes** - please tick all site(s):  
   - Local recurrence at primary site  
   - Regional nodal disease  
   - Metastatic disease  
   - Not Known

6. **Date of progression/recurrence** post diagnosis?

7. **Therapy** performed on recurrent or progressive disease?  
   - No = 0  
   - Yes = 1  
   - Not Known = 2
   
   **Date:**  
   - Therapy Type
   
   **Date:**  
   - Therapy Type
   
   **Date:**  
   - Therapy Type

8. **Patient status?**  
   - Active follow-up = 1  
   - Exit surveillance = 2  
   - Died = 3  
   - Not Known = 4

**PATIENT INFORMATION and SUPPORT**

1. Were patients **informed** about the presence of a NET?  
   - No = 0  
   - Yes = 1  
   - Not Known = 2

2. **Information** given to patient? **please tick all applicable**:
   - **NET patient support websites** suggested
   - **NET literature provided**
   - Not Known
   - Other:  

3. Were patients given **specialist nurse support?** (NET or otherwise?)  
   - No = 0  
   - NET CNS = 1  
   - Other CNS = 2  
   - Not Known = 3

**PATIENTS with ADVANCED DISEASE**

1. Were patients subsequently seen or discussed in a **NET Centre?**  
   - No = 0  
   - Yes = 1  
   - Not Known = 2

   **If Yes** – please specify centre:

   **NET Specialist:**

   Were patients offered entry into **clinical trials?**  
   - No = 0  
   - Yes = 1  
   - Not Known = 2

---

**Form completed by:**  
**Signed:**  
**Role:**

**THANK YOU FOR PROVIDING INFORMATION TO ASSIST DEVELOPMENT OF CLINICAL GUIDELINES FOR THIS PATIENT GROUP**

If you have any queries, please email: katy.o'donnell@nhs.net or Tel: 01256 360427/6

Please return this form, with anonymised copies of reports, by POST marked ‘Private and Confidential’ to:

Katy O'Donnell, Study Coordinator, HPB Office, Pelican Centre, The Ark, Basingstoke & North Hampshire Hospital, Basingstoke, RG24 9NN
ENGLISH BCSP SCREENING DATA DURING STUDY

<table>
<thead>
<tr>
<th>Year</th>
<th>Invited</th>
<th>FOBT adequately screened</th>
<th>Definitive FOBT abnormal</th>
<th>Definitive FOBT normal</th>
<th>Uptake</th>
<th>Coloscopy</th>
<th>Abnormal % of adequately screened</th>
<th>Definitive FOBT abnormal w/o coloscopy</th>
<th>Population adequately completed screening</th>
<th>Colonoscopy rate of total FOBT adequately screened</th>
<th>Colonoscopy uptake rate of FOBT abnormal</th>
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<tr>
<td>2006</td>
<td>54,189</td>
<td>27,815</td>
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<td>341</td>
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<td>312</td>
<td>27,503</td>
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<td>370,903</td>
<td>7,811</td>
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<td>5,340</td>
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<td>368,432</td>
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<td>15,419</td>
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<td>997,652</td>
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<td>2.17%</td>
<td>4,844</td>
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<td>35,556</td>
<td>1.88%</td>
<td>6,337</td>
<td>2,223,614</td>
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<td>34,196</td>
<td>1.83%</td>
<td>5,499</td>
<td>2,165,315</td>
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<td>86.15%</td>
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<td>32,249</td>
<td>1.80%</td>
<td>7,021</td>
<td>2,173,777</td>
<td>1.48%</td>
<td>82.12%</td>
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Total 23,405,057 13,061,716 259,765 12,801,951 55.81% 216,707 1.99% 43,058 13,018,658 1.66% 83.42%

Average/year 2,600,362 1,451,302 28,863 1,422,439 55.81% 24,079 1.99% 4,784 1,446,518 1.60% 83.42%

Exclude 2006/20 3,232,050 1,809,000 35,000 1,773,100 55.96% 30,147 1.98% 5,754 1,803,246 1.68% 83.97%
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<thead>
<tr>
<th>Year</th>
<th>Invited</th>
<th>FOBT adequately screened</th>
<th>Definitive FOBT abnormal</th>
<th>Definitive FOBT normal</th>
<th>Uptake</th>
<th>Colonoscopy</th>
<th>Abnormal % of adequately screened</th>
<th>Definitive FOBT abnormal w/o colonoscopy</th>
<th>Population adequately completed screening</th>
<th>Colonoscopy rate of total FOBT adequately screened</th>
<th>Colonoscopy uptake rate of FOBT abnormal</th>
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<tr>
<td>2006</td>
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<td>119</td>
<td>14,239</td>
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<td>966</td>
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<td>78.32%</td>
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<td>83.05%</td>
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<td>Definitive FOBTs abnormal</td>
<td>Definitive FOBT normal</td>
<td>Uptake</td>
<td>Colonoscopy</td>
<td>Abnormal % of adequately screened</td>
<td>Definitive FOBT abnormal w/o colonoscopy</td>
<td>Rectoscopy rate of total FOBT adequately screened</td>
<td>Colonoscopy uptake rate of FOBT abnormal</td>
<td></td>
</tr>
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<td>2006</td>
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<td>2,367</td>
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<td>2,785</td>
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<td>1,605,030</td>
<td>861,921</td>
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<td>857,695</td>
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<td>24,043</td>
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Average/year: 1,279,670 680,145 16,748 663,397 52.24% 14076 2.46% 2671 677,473 2.08% 84.05%
Excluded 2005/20: 1,590,412 847,346 20,791 826,456 52.02% 17508 2.45% 2192 844,054 2.08% 84.65%
# COMBINED LIST OF 187 UNIQUE SIGNIFICANT PROTEINS

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<th>Accession</th>
<th>Gene</th>
<th>Peptides</th>
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<td>PSMD7</td>
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<td>ARPC3</td>
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<td>MYH11</td>
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