BIOMARKERS AND RECEPTOR EXPRESSION IN NEUROENDOCRINE TUMOURS

by

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DECLARATION

I, Rajaventhan Srirajaskanthan, confirm that the work presented in this thesis is my own. Where work has been derived from other sources, I confirm that this has been indicated in the thesis.

.....

Dr. R. Srirajaskanthan

ABSTRACT

Neuroendocrine tumours (NETs) are uncommon tumours which have a diverse biology. The aims of this thesis were to identify potential new biomarkers and further develop understanding of tumour biology in NETs. The following were assessed i) somatostatin receptor (SSTR) and dopamine-2 receptor (D2R) expression in NETs, ii)HER expression and their associated prognosis, iii) Angiopoietin expression in NETs and their prognostic significance, iv) proteomic analysis of serum and NET cell lines to identify novel markers and finally v) the role of ^{68}Ga-DOTATATE PET in imaging of NETs.

Immunohistochemical studies were performed to determine whether SSTR and D2R are co-expressed in NETs. D2R was co-expressed with SSTR-2 and -5 in 93% of low grade tumours, with lower co-expression in higher grade tumours.

HER family of receptors are involved in oncogenesis; the expressions of these receptors were assessed. Immunohistochemical analysis of these receptors was performed in 82 cases. EGFR was expressed in 86%, HER-2 0%, HER-3 8.5% and HER-4 91.5%. The expression of EGFR was not associated with poor prognosis.

Angiopoietins (Ang) are involved in tumourogenesis. Serum Ang-1 and Ang-2 were measured in patients and healthy controls. Ang-2 was significantly higher in patients compared to controls. Patients with Ang-2 levels >4756pg/ml had a shorter time to progression.

Proteomic analysis using gel electrophoresis and LC/MS/MS of plasma from NET patients and established NET cell lines was performed to identify biomarkers. Proteomic

cell line analysis identified 17 proteins in all cell lines including Mac-2 binding protein. We validated Mac-2 binding protein and it appears to be a potential marker for NETs.

Finally, we performed a study to ascertain whether ^{68}Ga-DOTATATE PET identifies more lesions in NET patients in whom ^{111}In-DTPA-Octreotide showed faint/negative lesion uptake. ^{111}In-DTPA-Octreotide scintigraphy identified 27 lesions compared to 168 lesions identified with Ga-68-DOTATATE PET. ^{68}Ga-DOTATATE PET is a sensitive imaging modality for identifying NETs.

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Directly arising from research

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GLOSSARY

Abbreviations

AFP: alpha fetoprotein

Ang- Angiopoietin

AUC – area under the curve (relates to numerical value between 0.5-1 to assess accuracy of test)

Beta-HCG: Beta- Human chorionic gonadotrophin

CEA: carcinoembryonic antigen

CgA- Chromogranin A (a protein secreted from neuroendocrine cells. This acts as a biochemical marker of neuroendocrine tumour).

CT- computer tomography

CSV: comma separated value

CI: confidence interval

CM- culture media

DAB: diaminobenzidine tetrahydrochloride

DR- dopamine receptor

ENETS- European Neuroendocrine Tumour Society

ELISA: Enzyme linked immunoabsorbent assays

ECM- extracellular matrix

FPR: false positive rate

FDG: fluorodeoxyglucose

GEP: gastroenteropancreatic

HACE: hepatic artery chemoembolization

HER- human epidermal growth factor receptor

^{68}Ga-DOTATATE – ^{68}Gallium- [DOTA^{0},D-Phe^{1},Tyr^{3}]-octreotate

kDa- kilodaltons

^{111}In-DTPA-octreotide- ^{111}Indium- Diethylene triamine pentaacetic acid-Octreotide

IEF: isoelectric focussing

IGF: insulin like growth factor

LC- liquid chromatography

MIBG: meta-iodobenzylguanidine

MS- mass spectrometry

MEN: multiple endocrine neoplasia

mTOR: mammalian target of rapamycin

MRI- magnetic resonance imaging

NCBI: national centre for biotechnology information

NETs- Neuroendocrine tumour

PVDF: Polyvinylidene Fluoride

PET- positiron emission tomography, a nuclear medicine technique that enables visualization of tumours.

PCR: polymerase chain reaction

PSA: prostrate specific antigen

RRT: peptide radionuclide receptor therapy

RT-PCR: reverse transcriptase polymerase chain reaction

RIA: radio immunoassay

RECIST: Response Evaluation Criteria In Solid Tumors, radiological assessment tool for assessing tumour.

ROC curve- receiver operator characteristic curve

SELDI: Surface-enhanced laser desorption/ionization, method of mass spectromic proteomic analysis

Scintigraphy - A diagnostic test in which a two-dimensional picture of a body radiation source is obtained through the use of radioisotopes.

SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEER: surveillance, epidemiology and end results

SIRT: selective internal radiation therapy

SPECT: single photon emission computed tomography

SSTR- somatostatin receptor

STZ: Streptozotocin

TAE: transarterial embolization

TACE: transarterial chemoembolization

VEGF: Vascular endothelial growth factor

WHO- World health organisation

Chapter 1. Introduction

Abstract

Neuroendocrine tumours (NETs) are thought to arise from multipotent stem cells of the diffuse neuroendocrine system. The incidence is reported as 2.5-5 cases per 100 000 population and the incidence is rising. NETs are generally low grade indolent tumours however; the histological spectrum varies from low grade well differentiated tumours to high grade poorly differentiated tumours. A number of biochemical markers are available to diagnose these tumours, the most commonly used general marker is chromogranin A. Patients often present with advanced disease and treatment comprises of curative surgery or a number of palliative therapies. Biotherapy with somatostatin analogues and interferon-α, form the cornerstone of symptomatic therapy in patients with functional tumours. A number of other palliative therapies are available and advances are being made with new molecular targeted therapies.

1.1 Introduction

Neuroendocrine tumours (NETs) are derived from cells of the diffuse neuroendocrine system, which are present in organs throughout the body. Originally Pearse proposed that NETs developed from migration of cells from the neural crest, however, it is now thought that the tumour cells are derived from multipotent stem cells [1].

The term 'karzinoide' (meaning carcinoma like) was initially introduced by Siegfried Oberndorfer in 1907[2]. His initial observations describe small and multiple tumours which have the potential to become invasive but do not metastasize. Whilst some of these observations are correct these tumours do metastasize and should not be regarded as benign tumours. The term carcinoid tumour has historically been used, however, with advances in the understanding of the tumour biology and the recent WHO classification, the term NET or endocrine tumour is considered more appropriate.

1.1a Incidence

The reported incidence is 2.5 -5 cases per 100 000 population [3]. Approximately 0.5% of all malignant diseases are NETs of either bronchopulmonary or gastroenteropancreatic (GEP) origin [3]. There appears to be an increased incidence in small bowel NETs in blacks versus Caucasians in the USA [4]. The incidence of different NETs has risen over the last three decades; the greatest increase has been in bronchial and midgut NETs [5]. The increase in incidence of NETs will partly be due to improved diagnostic techniques both radiological and endoscopic.

1.1b Prevalence

Whilst the incidence of these tumours is relatively low, the long duration of disease means the prevalence is much higher than would be initially expected. Using the Surveillance, Epidemiology and End Results program registries (SEER 9), Yao et al demonstrated that prevalence of NETs was 35 per 100 000 population [6]. Recently studies have demonstrated an increased survival for patients leading an overall increase in prevalence [6;7].

1.1c Aetiology

Most cases are sporadic, however, some occur as part of genetic syndromes, these include multiple endocrine neoplasia -1 (MEN-1), MEN-2, Von Hippel Lindau syndrome and neurofibromatosis-1 [8]. Certain types of NETs are common in MEN-1, for example individuals with MEN-1 may develop gastric carcinoids. Non-functional pancreatic NETs and gastrinomas are also common with between 25-40% of MEN-1 patients developing gastrinomas. Bronchial NETs are relatively uncommon in MEN-1 with a reported incidence of 5% [9]. Midgut NETs are uncommon in MEN-1 patients.

1.2 Classification

Neuroendocrine cells were originally thought to be derived from neural crest cells as they expressed markers for neuronal differentiation and proteins involved in the biosynthesis of neurotransmitters. Pearse introduced the amine precursor uptake and decarboxylation (APUD) cell concept based on the ability of neuroendocrine cells to synthesize bioactive amines such as dopamine, adrenaline, noradrenaline, serotonin and histamine [1]. This theory however, is not thought to be correct since a number of studies have identified that gut endocrine cells share a common stem cell and are endodermally derived [10]. The currently accepted hypothesis proposes that the gut is derived from

endodermal stem cells. As it is developing the gut acquires an underlying tendency to differentiate into intestine. The endoderm has the potential to form a wide range of endocrine cell types. There is thought to be a pre-selected regional pattern of endocrine cells within the gut starts to develop, the factors responsible for directing this may be derived from the mesenchyme . Once these cells have started to develop their maturation is thought to be directed by the mesenchyme [11].

Neuroendocrine cells scattered within the gastrointestinal mucosa from stomach to colon represent the largest population of hormone-producing cells in the body. There are at least 14 different neuroendocrine cell types in the GI tract, all with a specific regional distribution [12]. The cells are specialized in the synthesis, storage and secretion of a wide range of polypeptide hormones and amines, all of which have an important role in bowel movement and fine tuning of hormonal secretion along the GI tract. Malignant change in specific neuroendocrine cells can lead to development of a number of different types of NETs [13].

In 1963, Williams and Sandler divided NETs according to their embryological origin into foregut (bronchial, stomach, pancreas, gall bladder and duodenum), midgut (jejunum, ileum, appendix and colon- up to ascending colon) and hindgut (transverse and remaining colon, rectum). This old type of classification is being replaced gradually; the WHO classification of endocrine tumours divides these tumours into well differentiated endocrine tumours, well differentiated endocrine carcinomas and poorly differentiated endocrine carcinomas. This classification is described in more detail in section 1.2a. More recently ENETS have developed a TNM classification system and grading system, this is further discussed in section 1.2. It is becoming apparent that tumours within each region can have markedly different clinical behaviour and

therefore, a shift towards tumours being categorized purely by anatomical location is being introduced [14]. For the purposes of the studies in this thesis the William and Sandler classification of NETs by embryological site has been used.

1.2a Pathology and Staging

NETs can exhibit a diverse spectrum of pathology, from benign tumours to highly aggressive poorly differentiated tumours [14]. The WHO classification is used for describing tumours of gut and pancreas [15]. Separate classifications systems are in use for bronchial, thymic and thyroid NETs. The WHO classification for tumours is based on degree of differentiation and clinical behaviour, there are three types see table 1.1.

1. well-differentiated endocrine tumours, with benign (1.1) or uncertain behaviour (1.2) at the time of diagnosis.
2. Well-differentiated endocrine carcinomas with low-grade malignant behaviour.
3. Poorly differentiated endocrine carcinomas, with high-grade malignant behaviour.

Table 1.1. WHO classification of NETs adapted from Koppel et al,[15].

Bronchial carcinoid tumours are classified into four groups dependent on histological parameters including mitotic activity and proliferation index. These groups are typical carcinoids, atypical carcinoids, large cell neuroendocrine carcinoma and small cell lung carcinoma [16]. Histological subtypes were diagnosed according to WHO Classification. Typical bronchial carcinoids are classified as carcinoid morphology with less than two mitotic figures per $2mm^2$ and no evidence of necrosis. Atypical carcinoids have carcinoid morphology with a mitotic count of 2-10 per $2mm^2$ and necrosis can be

present. LCNEC classification is based upon features of neuroendocrine morphology, mitotic rate up to 70 per 2 mm^2, necrosis, and cytologic features of large cell carcinoma. Small cell carcinoma has characteristic histological features such as small size, scant cytoplasm, high mitotic rate and large areas of necrosis.

The European Neuroendocrine Tumour Society (ENETS) proposed a TNM staging classification and this also includes a grading system of low, intermediate or high grade tumours dependent on their proliferation index, mitotic activity and histological phenotype [17-19]. Using this classification low grade tumour was regarded as mitotic count <2 per 10 high power fields (HPF) and Ki67 ≤2%, intermediate grade as having a mitotic count 2-20 per 10 HPF and Ki67 3-20% and high grade as mitotic count of >20 per 10 HPF and Ki67 >20.

ENETS have provided guidelines for staging tumours using a TMN classification (Table 1.2). The classification so far has been published for GEP NETs and not other NETs. Retrospective studies have shown prognostic value in this staging system.

1.3 Clinical features

NETs can be separated into non-functioning and functioning tumours. The majority (approximately 60%) are non-functional tumours i.e. with no symptoms attributable to secretion of metabolically active peptides. Functional tumours secrete substances that are metabolically active; which can lead to development of specific clinical syndromes. The most common functional syndrome is carcinoid syndrome which is thought to be due to secretion of amines, kallikrein and prostaglandins. Serotonin (5-hydroxytryptamine) is one of the main amines which is synthesized and secreted by these tumours.

Stage	T†	N‡	M§
0	Tis	N0	M0
I	T1	N0	M0
IIA	T2	N0	M0
IIB	T3	N0	M0
IIIA	T4	N0	M0
IIIB	Ay T	N1	M0
IV	Any T	Any N	M1

Table 1.2. TNM staging system for foregut NETs proposed by ENETs [20;21]

T indicates tumour classification; N, lymph node status; M, metastatic status.
† T0 indicates no evidence of primary tumour, Tis tumour in situ/dysplasia (size <5mm); T1, gastric or duodenal tumour invading lamina propria or submucosa and size <10mm or pancreatic tumour limited to pancreas and size <20mm; T2, gastric or duodenal tumour invading the muscularis propria or submucosa, or size >10mm, or pancreatic tumour limited to pancreas and size between 20-40mm; T3, gastric or duodenal tumour penetrating the serosa, or duodenal tumour infiltrating the pancreas, or pancreatic tumour limited to the pancreas and size >40mm, or pancreatic tumour invading the duodenum or common bile duct; T4, gastric, duodenal or pancreatic tumour invading adjacent structures.
‡ N0 indicates absence of regional lymph node metastasis; N1, invasion of regional lymph nodes. § M0 indicates absence of distant metastasis, M1, presence of distant metastasis.

1.3a Carcinoid syndrome

Carcinoid syndrome occurs in 20-30% of patients with mid-gut carcinoid tumours and approximately 5% of bronchial carcinoids [22]. Other foregut tumours (e.g. pancreatic neuroendocrine tumours) can cause carcinoid syndrome although this is uncommon (1%). Hind gut tumours are generally non-functional and occasionally cause carcinoid syndrome.

Carcinoid syndrome is usually seen in patients with liver metastases (in 95% patients), but excess tachykinins and serotonin production from retroperitoneal metastases or ovarian tumours can bypass the liver to cause carcinoid syndrome.

Normally serotonin is synthesized from tryptophan, and is subsequently metabolized by monoamine oxidase to 5-hydroxyindoleacetic acid (5-HIAA), which is subsequently secreted in the urine in healthy individuals. Approximately 99% of tryptophan is used for the synthesis of nicotinic acid and less than 1% converted to 5-HT. However, in patients with carcinoid tumours there is a shift towards the production of 5-HT. The increased production of 5-HT and other products and their direct release into the systemic circulation, due to liver metastases, leads to the development of carcinoid syndrome [23].

Patients often describe having symptoms for many months prior to presentation. The two most common symptoms are diarrhea and flushing, whilst wheeze occurs less commonly. Often diarrhoea is associated with crampy abdominal pain and urgency, and can occur during both day and night. Flushing is characteristically described as a sudden onset of pink to red discoloration involving the face and upper trunk. This usually lasts a few minutes and can occur intermittently throughout the day. Triggers leading to flushing and diarrhoea include stress, tyramine-containing foods (chocolate, bananas, and walnuts) and alcohol. Classic facies of carcinoid syndrome consists of pink-to-red discoloration around the face and upper trunk. In patients with atypical flushing, which may last for several hours, telangiectasia and hypertrophy of the face may be seen. Wheeze is caused by bronchial constriction mediated via tachykinins and bradykinins. This is more common in those with bronchial carcinoid tumors.

A raised jugular venous pressure and features of right heart failure may be present in patients with carcinoid heart disease related to carcinoid syndrome. Right-sided cardiac

murmurs of tricuspid regurgitation and pulmonary stenosis may be heard on cardiovascular examination [24].

Other hormone-related manifestations include morphoea (subcutaneous thickening of the lower limbs) and a pellagra-type rash if nicotinic acid deficiency has been induced. With severe long-standing hepatomegaly or local infiltration, IVC obstruction or even lymphangiectasia leading to ascites may occur.

A number of other clinical syndromes occur in NETs dependent on the hormones secreted. Table 1.3 demonstrates the clinical features seen in different types of NETs.

1.4 Diagnostic investigations

Diagnosis of NETs requires biochemical, topographical imaging and importantly histological diagnosis. Efforts should be made to identify primary site of tumour, which can be difficult, since some primary lesions are small and not detected by conventional cross sectional imaging.

1.4a Biochemical tests

Patients with suspected NETs should undergo biochemical testing, including fasting gut hormones (glucagon, vasoactive intestinal peptide, somatostatin, gastrin), Chromogranin A (CgA) and pancreatic polypeptide [17]. Neuroendocrine cells contain typical secretory granules, called large dense-core vesicles based on their characteristic appearance on electron microscopy. In addition to the specific peptide hormones or neuropeptides, these granules also contain one or more secretogranin proteins. The first member of this family and the most widely studied is CgA.

Site	Clinical features	Cell type	MEN-1
Pancreatic			
Insulinoma	Hypoglycaemia, whipples triad, clammy, sweating, weight gain	Beta islet cell	5-10%
VIPoma	Werner- Morrison syndrome, watery diarrhoea	VIP	10%
Glucagonoma	Diabetes Mellitus, Necrolytic migratory erythema		5-10%
Somatostatinoma	Gallstones, Diabetes Mellitus, Steatorrhoea	D cells	5-10%
Gastrinoma	Zollinger Ellison syndrome	G cells	25%
Non-functional	Symptoms related to mass effect		15%
Bronchial	Majority non-functional, 8% carcinoid syndrome, atypical flushing	Pulmonary neuroendocrine cells,	5%
Midgut	Majority non functional, 40% develop carcinoid syndrome	K, L , Motilin	
Hindgut	Usually non-functional, however tumours may secrete somatostatin other peptide and occasionally carcinoid syndrome may occur	Enterochromaffin cell	

Table 1.3. The clinical features of NETs. Midgut tumours arise from the jejunum to caecum and hindgut encompasses tumours from the ascending colon to rectum.

Since CgA is stored in a majority of different NETs, its release to the circulation can be used as a 'general' marker for various NETs. This is of particular interest in 'nonfunctioning' tumours, which may lack other suitable markers. Currently CgA is the best general biochemical marker for NETs currently available [25;26]. The plasma concentrations of CgA are elevated in various peptide-producing NETs and the highest concentrations are noted in patients with metastatic carcinoid tumours, particularly midgut carcinoids. Tumour burden and plasma CgA concentrations has been studied and it shown that in patients with midgut carcinoids those with multiple liver metastases had significantly higher concentrations of CgA than those with only a few liver metastases or

lymph node metastases only [27]. A study by Arnold et al [28] demonstrated increased plasma chromogranin A in patients with metastatic neuroendocrine tumours is predictive for shorter survival. There was a modest correlation between chromogranin A concentrations and hepatic tumour burden.

There are some other pitfalls in the interpretation of CgA concentrations. 'False-positive' elevation of CgA can be seen in patients with renal impairment, liver failure, atrophic gastritis and inflammatory bowel disease [29]. Also patients receiving proton pump inhibitors have a slight increase in CgA due to gastrin-induced ECL cell hyperplasia. Furthermore, physical stress induced by exercise or trauma can produce a slight elevation in CgA (about twofold) [30].

The introduction of radioimmunoassay for various peptide hormones led to increased diagnosis of NETs. The peptides and amines secreted by NETs may serve as tumour markers not only for diagnosis but also for follow-up of treatment. Biochemical tumour markers can be divided into specific and general markers, see Table 1.4. Specific markers include urinary 5-HIAA, neuropeptide K for carcinoid syndrome. For specific pancreatic tumours other markers can be measured for example glucagon in glucagonoma, vasointestinal peptide for VIPoma. General tumour markers for NETs include Chromogranin A and B and pancreatic polypeptide, see table 1.4.

Routine lab tests including Full Blood Count, Urea and Electrolytes, Liver Function Tests, CEA, AFP, HCG-beta, Ca-19.9, ESR should be performed. Urinary 5-HIAA should also be performed in all patients with suspected carcinoid syndrome [31]. AFP and HCG-beta if elevated are poor prognostic indicators in NETs [31]. Table 1.4, shows the different biochemical tests that used for diagnosis of NETs.

A good biomarker needs to be sensitive and specific, whilst CgA provides good sensitivity and specificity it is not 100% for the reasons described above. CgA has been shown in some studies to provide prognostic information though this has not been demonstrated in all studies [28].

There is a need for other markers which provide better prognostic information and also markers that may be used in conjunction with CgA to identify NETs. One of the main focuses in this thesis is to identify new plasma markers which can be used for diagnosis of NETs or aid prognosis.

Type of tumour	Plasma marker	Urinary marker
Carcinoid	Chromogranin A Chromogranin B Neuron Specific Elonase HCG-beta Substance P Gherlin Neuropeptide K Alpha fetoprotein	5-Hydroxyindoloacetic acid
Phaeochromocytoma	Chromogranin A Chromogranin B Neuron Specific Elonase HCG-beta Neuropeptide Y Metanephrins Alpha fetoprotein	Catecholamines Vanillylmandelic acid Dopamine Homovallinic acid
Pancreatic NETs	Chromogranin A Chromogranin B Pancreatic polypeptide Neuron Specific Elonase HCG-beta Alpha fetoprotein Glucagon Gastrin Insulin Somatostatin Calcitonin Vasoactive intestinal peptide	

Table 1.4. General and specific biochemical plasma markers raised in NET dependent on anatomical site.

1.4b Histology

Histology remains the gold standard for diagnosing NETs. Immunohistochemistry should be performed using a panel of antibodies to general neuroendocrine markers. These include chromogranin A, synaptophysin, PGP9.5. In addition the tumour should be stained with an antibody to the Ki67 protein, since the Ki67 proliferation index is of benefit in grading tumours [17].

The histological characteristics of NETs vary according to the degree of differentiation. Low grade NETs originating from the gut were previously termed ‘typical’ carcinoids; these tumours had classic histological architecture of trabecular, or ribbon like cell clusters, with little of no cellular pleomorphism and occasional mitoses. The higher grade and poorly differentiated tumours had increased mitotic activity and evidence of necrosis. The WHO classification gives clear parameters for categorizing NETs into the three main categories described earlier, see Table 1.1 [15].

1.5 Imaging

Cross sectional imaging is principally with contrast computer tomography (CT) including arterial phase enhancement abdomen, chest and pelvis. Magnetic resonance imaging (MRI) is the most sensitive modality for liver metastases [32]. Studies of CT in carcinoid tumours show an overall sensitivity of 80% in detecting lesions [33;34]. Both MRI and CT appear to have similar sensitivities for localization of pancreatic NETs; however most studies involve small numbers and heterogeneous cases [35-37]. Both modalities are important in identifying extent of metastatic disease, and assessing response to treatment. In cases where the primary is not identified, both modalities can be useful in identification of primary lesions. As technology improves there will be advances in the role of cross sectional imaging [34]. The sensitivity and specificity of CT and MRI alone are lower than in combination of ^{111}In-Octreotide scan with CT or MRI [38].

1.5a Nuclear Medicine

Nuclear medicine (NM) imaging is important in staging of disease and suitability for therapy with somatostatin analogues and peptide receptor therapy. The two main NM scans used in staging NETs are ^{111}In-Octreotide, ^{123}I-MIBG, with the advent of newer modalities including PET scanning being introduced.

There are 5 different somatostatin receptor (SSTR) subtypes all of which have strong affinity to somatostatin [39]. ^{111}In-DTPA-Octreotide (Octreotide™) is a somatostatin analogue which has a strong affinity to SSTR-2 and to lesser extent SSTR-5 receptors. Most NETs predominantly express SSTR-2. Synthetic radiolabelled SSTR analogues such as ^{111}In-DTPA-Octreotide enable somatostatin receptor scintigraphy (SRS) to be performed [40]. Scintigraphy is a technique which leads to the production of two-

dimensional images of the distribution of radioactivity in tissues, following the internal administration of a radiopharmaceutical imaging agent, the images being obtained by a scintillation camera. Spatial localization using 2-D planar images can be inaccurate especially when imaging the abdomen. Development of single photon emission computed tomography (SPECT) has enabled much better spatial resolution within the abdomen also increased lesion detection using this technique. SPECT imaging is a technique whereby the gamma photon–emitting radionuclides are detected by one or more gamma cameras rotated around the patient, using the series of two-dimensional images to recreate a three-dimensional view.

SRS is now established in localizing NET [41]. Prospective studies have shown that inclusion of SRS in the diagnostic work-up of patients alters management in up to 47% of cases [42]. The sensitivity of SRS for detection of GEP NETS has been well studied. The sensitivity has been reported between 67-100%, with no significant difference in carcinoid tumours from foregut, midgut or hindgut origin [43-45]. With pancreatic NETs sensitivity of SRS is dependent on the type of functional tumour. Gastrinomas have reported sensitivity between 56-80%, VIPoma is 60-70% and insulinoma is lower at 50% due to a lower expression of SSTR-2 [40]. With phaeochromocytomas SRS is often negative and other imaging modalities such as Meta-iodobenzylguanidine (MIBG) should be used. Medullary thyroid cancer express SSTR-1 therefore may be negative on SRS. False positive scans can be seen in patients with chronic inflammation, granulomatous disease. SRS detection is also affected by the size of NET and will often not detect lesions <1cm [46]. The current gold standard nuclear medicine imaging modality for NETs is regarded as being ^{111}In-DTPA-Octreotide; however other techniques such as PET scanning are producing promising results.

Meta-iodobenzylguanidine (MIBG) has been used for two decades to visualize carcinoid tumours. The method was initially developed to detect phaeochromocytomas. MIBG shares the same method of uptake as norepinephrine and is not dependent on SSTR receptor expression. In phaeochromocytomas ^{123}I-MIBG has sensitivity of 87% and specificity of 99%, however for carcinoid tumours only has 50% sensitivity and specificity; whilst in pancreatic NETs uptake may be seen in <10% cases [47]. In general ^{123}I-MIBG scintigraphy was shown to be less sensitive than ^{111}In-DTPA-Octreotide scintigraphy in identifying carcinoid tumours [47].

1.5b Positron Emission Tomography

Positron emission tomography (PET) is a technique that produces a three-dimensional image or picture of functional processes in the body. The system detects gamma rays emitted indirectly from a radionuclide tracer usually bound to a biologically active molecule or receptor ligand/ analogue. The radionuclide tracer then accumulates in areas of interest and computer reconstruction allows development of 3-D images. Newer scanners combine PET with either CT or MRI thereby enabling better topographical localization. The radio-isotope usually has a short half-live and undergoes positron emission decay, whereby it emits a positron and after travelling a few millimetres the positron encounters and annihilates with an electron, producing a pair of photons. These often move in opposite directions, i.e. 180° apart. These are detected when they reach a scintillator in the scanning device creating a burst of light. If a biologically active tracer, such as fluorodeoxyglucose (FDG) is used then metabolic activity in terms of regional glucose uptake can be determined; which can be useful in determining the response to treatment for example following chemotherapy. ^{18}FDG-PET is suitable for high grade

tumours; however its role in low grade tumours due to their slow glucose turnover is unclear [48]. However, it has been used in imaging gastrointestinal stromal tumours which can be well differentiated and have a low mitotic activity [49;50]. PET scanning in other malignancies is well established; however its role in NETs is still evolving.

New agents of interest include ^{68} Ga-DOTA-Octreotide and ^{68}Ga-DOTA-Octreotate, 5-hydroxytryptophan (5-HTP) and 3,4-dihydroxyphenylalanine (DOPA). Studies with ^{68}Ga-DOTA-Octreotide showed it had a greater sensitivity than conventional SRS [51;52]. Preliminary studies using these different agents have been published, most generally showing improved sensitivity over ^{111}In-DTPA-Octreotide scintigraphy. Some agents such as 5-HTP and DOPA which may have good sensitivities are not able to demonstrate SSTR-2 status, which would be important if considering these patients for radiotargeted therapy. In this thesis the role of ^{68}Ga-DOTATATE PET in patients with negative or weak uptake on ^{111}In-DTPA-Octreotide scintigraphy is assessed.

1.5c Endoscopy

If the primary site has not been identified by conventional imaging, it is worthwhile performing endoscopy of the upper and lower gastrointestinal tract (GIT). In addition if patients are known to have a primary lesion in the GIT, endoscopy will allow visualization of the lesion and the option of histological diagnosis. For detection of gastric, pancreatic and duodenal lesions, endoscopic ultrasound (EUS) is a sensitive method for staging disease and providing information regarding depth of invasion and potential resectability [53]. In addition biopsies can be performed to provide histological diagnosis. EUS has an accuracy of 90% in staging of rectal carcinoids [54].

Capsule endoscopy can be used to diagnose small bowel carcinoid tumours, and appears to be at least as sensitive as enteroscopy at identifying lesions. Obviously the drawback is the inability to obtain a histological diagnosis. In small case series there appears to be advantage of capsule endoscopy over conventional small bowel investigations using CT and barium follow-through [55]. To exclude possibility of obstruction barium follow through or patency capsule study should be performed prior to capsule endoscopy.

For bronchial NETs which commonly arise in large to midsize airways bronchoscopy is of use in assessing the lesion and obtaining histological diagnosis [5].

1.6 Management

Therapies for NETs incorporate those required for symptom control, due to hormonal secretion from tumours and also anti-proliferative therapies. The management of NETs requires the use of a number of different therapies including surgery, biotherapy, chemotherapy, peptide receptor targeted therapy and tumour embolization. The best way to provide the most appropriate management plan for patients is through a multidisciplinary approach. Different therapies may be required at different clinical stages, and in patients with indolent disease and mild symptoms merely symptomatic relief may be all that is required for some years.

1.6a Surgery

Primary resection of NETs is recommended for all patients with localized disease, unless contraindicated or patient's decline surgical therapy. A proportion of primary small bowel ileal tumours present with bowel obstruction, which requires urgent surgical

intervention. Survival of patients, who have curative resections, is better than those with unresectable disease[17].

Patients with advanced functional tumours, in whom maximal medical therapy is unable to control symptoms, surgical resection of tumour enable better symptom control. However, in patients with non-syndromic tumours, the value of surgery cannot be assessed in the same way; in these patients alterations in survival time would be the best method to assess benefit of surgery.

The role of surgery for hepatic metastases is more difficult. Only 10-15% of patients with liver metastases has unilobar diseae and is suitable for surgical resection. The majority of patients have bilobar disease. In studies looking at surgical resection vs. TACE and/or TAE, there is a significant survival benefit for patients undergoing surgery[22]. However, most of these studies are biased since patients not suitable for surgery usually have bilobar and larger volume disease.

There is a general consensus that if the primary can be resected or has been resected and that >95% of the tumour load can be surgically resected there is a role for debulking surgery. Patients with liver metastases that have curative resections have a 5 year survival of 75-81%[17]. There is currently no randomised control data showing whether this aggressive surgical approach offers any survival benefit. These studies are required to help determine whether this approach is valid.

1.6b Somatostatin analogues

Somatostatin is a small polypeptide hormone which occurs naturally in the human body and binds with a high affinity to the five recognized SST receptors. Activation of SST receptors leads to activation of common signalling pathways such as inhibition of adenyl cyclase and modulation of mitogen activated protein kinase through G-protein dependent mechanisms [56]. The effect of somatostatin on tumour growth may be through the suppression of synthesis and secretion of growth factors and growth promoting hormones (see Figure 1.1). Somatostatin also appears to inhibit angiogenesis and cell proliferation in *in vitro* models. Its anti-angiogenic effect appears to be through inhibition of angiogenic factors like VEGF, IGF-1 and PDGF [57;58].

In view of the short half life of SST, synthetic analogues were developed, initially Octreotide™ and subsequently Lanreotide. Short acting Octreotide which needs to be administered three times a day and long acting Octreotide-LAR and Lanreotide Autogel with 28 day duration of action [59]. Both are equally effective at controlling symptoms related to carcinoid syndrome. Improvement is seen in approximately 85% of cases [60]. Biochemical markers such as CgA and urinary 5-HIAA are found to decrease by at least 50% in 60-80% of cases following therapy [61]. In a study performed by Garland et al, of 27 patients with positive SRS and commenced on Octreotide LAR all had good symptom control initially. However, the majority of patients developed progressive disease and required further therapies for symptom control [62]. Side-effects with somatostatin analogues include gastro-intestinal disturbances, including pancreatic insufficiency that may require enzyme replacement therapy, gallstones and glucose intolerance.

Anti-proliferative effects of SST analogues have been demonstrated in vitro in a number of studies and a number of clinical studies have demonstrated slower rate of progression in patients receiving SST analogues. Recently the results from the PROMID study were published, this was a placebo-controlled, double blind, prospective randomized study to examine the effect octreotide on tumour growth in patients with NETs. It demonstrated median time to tumour progression in the octreotide LAR and placebo groups was 14.3 and 6 months, respectively (hazard ratio [HR] = 0.34; 95% CI, 0.20 to 0.59; P<0.001) [63].

Tolerance to SST analogues is a recognized phenomenon and there is a need for new biotherapy agents. Parsireotide a new multi-ligand SST analogue is currently being trialled. Recent studies have demonstrated that the majority of NETs co-express dopamine and somatostatin receptors, which have led to development of chimeric agents which have shown promising results in NET cell lines [64;65].

1.6c Interferon α

Interferon therapy has been used for symptomatic control in patients with NETs since 1982. It has been found to be beneficial in reducing symptoms of flushing and diarrohea in patients with carcinoid syndrome in 50-60% of cases. Significant biochemical responses are reported in 40-50% of cases [66]. Its mechanism is action is unclear though is thought to act through antisecretory and immunomodulatory functions. Its anti-tumour effect is generally reported to be <20% partial response rate in most studies. In a study of 111 patients treated with interferon-α 15% demonstrated a greater than 50% reduction in tumour size [67].

Studies have shown disease stabilization occurs in 40% of patients with combined therapy with somatostatin analogues and interferon-α, which is similar to that of SST analogues alone [68]. A randomised study with over 100 patients showed there was no significant survival benefit of SST analogues with interferon-α compared to SST analogues alone [69].

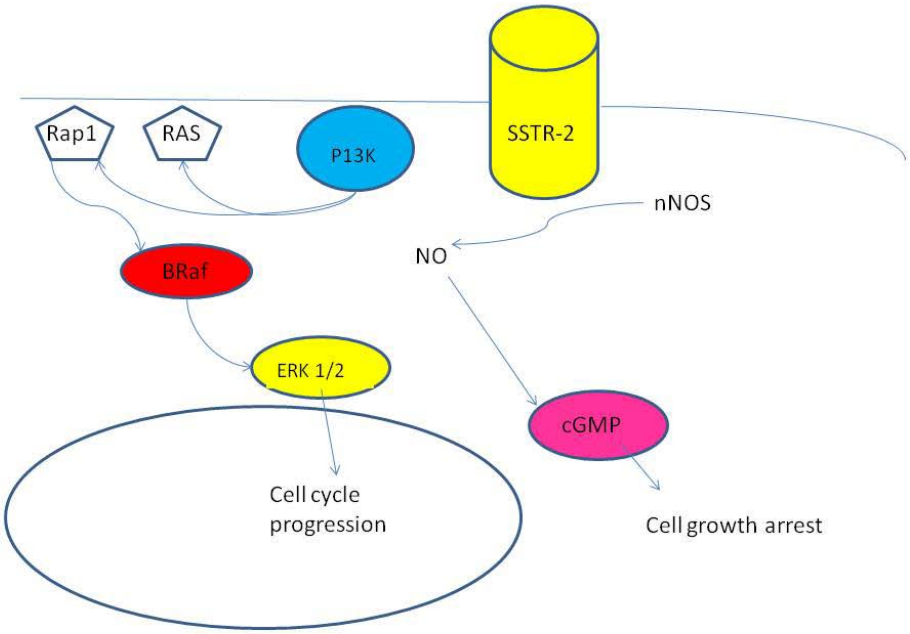


Figure 1.1. The downstream signalling pathways activated following somatostatin binding to the SSTR-2 receptor.

1.6d Chemotherapy

Chemotherapy has been widely used in the treatment of NETs for over three decades. Its precise role is not clearly defined; it is however often used as first line therapy for unresectable poorly-differentiated NETs and pancreatic well differentiated NETS, which are often chemosensitive. Studies have demonstrated wide variation in response rates

with chemotherapy; this may in part be due to inclusion of different types and grades of NETs (see Table 1.5). Overall response rate for intestinal carcinoid is <30% [70-77].

1.6e Hepatic artery embolization

Metastases from NETs are often predominantly in the liver and therefore embolization of the liver can result in necrosis of tumour tissue and consequent decrease in hormonal secretion. Embolization is commonly performed radiologically and can be performed with particles or chemoembolization. Contraindications to performing hepatic artery embolization include; portal vein thrombosis, liver failure, biliary reconstruction and poor performance status of patient. Symptomatic response is seen in 40-80% of cases [78], with a biochemical response of 50-75% for hepatic embolization and 12-75% for hepatic chemoembolization [79] (see table 1.6). A study by Gupta et al, demonstrated no additional benefit of TACE (with doxorubicin) to TAE in metastatic midgut tumours. Complications post procedure includes ileus, portal vein thrombosis, hepatic abscess, hepatic fistula, encephalopathy, and renal insufficiency.

Hepatic embolization of liver metastases with ^{90}Yttrium selective internal radiation therapy (SIRT) has been in use for over a decade to treat patients with primary and secondary liver cancers [80]. There have been a number of small non-randomised trials to assess the role of ^{90}Yttrium microspheres in NETs liver metastases [81-83]. A study by Kennedy et al in 148 patients with unresectable NET liver metastases demonstrated stable disease in 22.7%, partial response in 60.5% and complete response in 2.7% [81]. In all three small studies Yttrium-90 microspheres has been well tolerated.

Study	Regimen	No of pts	Objective response (%)	Response duration (months)	Median survival (months)
Well differentiated GEP NETs					
Moertel [75]	STZ +cyclophosphamide STZ + 5-FU	42 47	33 26	17 17	28.4 small bowel 28.4 pancreas
Moertel [77]	Dox+ STZ STZ+ 5-FU	36 33	69 45	20 6.9	26 18
Eriksson [70]	Dox + STZ STZ +5-FU	25 19	24 11	22	
Sun [84]0)	Dox + 5-FU STZ+ 5-FU	85 78	15.9 16	4.5 5.3	
Kulke [85]	Irinotecan+ cisplatin	18	78 (only s.d)	4.5	11.4
Kulke [72]	Gemcitabine	18	65 (only s.d)	8.3	11.5
Rivera [86]	Dox+ STZ+ 5-FU	12	55	15	21
Kulke [73]	Thalidomide + temozolomide	29	25	13.5	
Poorly differentiated GEP NETs					
Moertel [76]	Etoposide + cisplatin	18	67	8	19
Mitry [87]	Etoposide + cisplatin	41	42	9	15
Mani [88]	Irinotecan + cisplatin	20	58	4	

Table 1.5. Chemotherapy studies performed to date in NETs separated into well differentiated and poorly differentiated tumours. s.d= stable disease. Objective response is a reduction in tumour size, either partial >25% of tumour volume or complete response. STZ, streptozoscin; 5-FU, 5-flurouracil; dox, doxorubicin.

Study	Type of NET	HACE or HAE	No. of patients	Clinical response	Biochemical response	Radiological response
Rusniewski [89]	Carcinoid	HACE	24	73%	57%	33%
Therasse [90]	Carcinoid	HACE	23	100%	91%	35%
Clouse [91]	Carcinoid	HACE	14	90%	69%	78%
Diaco [92]	Carcinoid	HACE	10	100%		60%
Roche [93]	Carcinoid	HACE	14	70%	75%	86%
Kim [94]	Pancreatic Carcinoid	HACE	14 16	- -	90% 75%	50% 25%
Drougas [95]	Carcinoid	HACE	14	66%	100%	
Gupta [79]	Carcinoid Pancreatic	HAE or HACE	69 54			67% 35%
Marrache [96]	Carcinoid Pancreatic	HACE	48 19	91%	65%	37%

Table 1.6. Response rates to hepatic embolization and chemoembolization.

HACE; hepatic artery chemoembolization, HAE; hepatic artery embolization. Biochemical response is >50% reduction in tumour markers CgA. Radiological response is reduction in tumour volume >50% using cross sectional imaging.

1.6f Radionuclide peptide receptor therapy

The first reports of ^{131}I-MIBG therapy in NETs were reported in 1994 [97]. Since then a number of studies have been published showing variable radiological response rates, with symptom response between 60-80%, with median duration of response between 6-24 months [98-102]. Treatment is well tolerated and toxicity is often limited usually to temporary myleosupression following therapy.

The over expression of SSTR-2 has facilitated the development of targeted peptide receptor therapy. The mechanism of action appears to be that the radiopeptide binds to SSTR-2 receptor and is internalized to the cell therefore delivering radioactivity. The beta emitting radionuclides then irradiate neighbouring tumour cells. Contraindications include bone marrow suppression, renal impairment, liver failure, very poor performance

status and inability to self care. A number of studies have been published using peptide receptor radionuclide therapy, however, the criteria for objective response has varied in studies, see table 1.7. Two radiopeptides that are currently in use these are ^{90}Yttrium and ^{177}Lutetium. Unfortunately there are no randomised studies of peptide-receptor radionuclide therapy, which makes evaluating their true benefit and optimal radionuclide difficult.

Kwekkeboom et al recently published the largest series to date of over 500 patients treated with ^{177}Lu-DOTATATE [103]. Of these patients response data was available in 310; of which 2% had complete response, 28% partial response, 16% had minor response. The median time to progression was 40 months and median overall survival from start of treatment was 46 months, median survival from diagnosis was 128 months. The overall survival for these patients seems much higher than historic controls were survival was usually around 60 months.

The main adverse effect with radiopeptide receptor therapy is related to bone marrow suppression and this is usually a cumulative effect, often seen after 3 doses of therapy. Other side effects include fatigue, tiredness, nausea and occasionally liver failure. Long term impairment of renal function can occur, and therapy is contraindicated in patients with poor renal function. Bodei et al, reported a decrease in creatinine clearance of between 5-10% in 20 of 23 patients treated with Y^{90} at one year post therapy [104].

1.7 Future therapies

Understanding the biology of cancer has improved over the last few decades and this has led to the development of new potential targets for treatment. There is a new focus for 'targeted therapies' which have had success in other cancers for example Trastuzumab in

breast cancer and Imatinib in management of chronic myelogenous leukaemia. With new agents being trialled the radiological objective response rates are usually low, however disease stabilization and time to progression seem to more useful markers of effectiveness of these therapies especially in low grade GEP NETs.

Authors	**No.**	**Response (%)**				
		CR	PR	MR	SD	PD
Y^{90}-DOTATOC						
Otte [105]	29	0	2 (7%)	4 (14%)	20 (69%)	3 (10%)
Waldherr [106]	39	2 (55%)	7 (18%)	n/a	27 (69%)	3 (8%)
Bodei [107]	29	1 (3%)	7 (24%)	n/a	14 (48%)	7 (24%)
Valkema [108]	52	0	5 (10%)	7 (13%)	29 (56%)	14 (26%)
Y^{90}-Lanreotide						
Virgolini [109]	39	0	0	8 (20%)	17 (44%)	14 (36%)
Y^{90}-DOTATATE						
Baum [110]	75	0	28 (37%)	n/a	39 (52%)	8 (11%)
Lu^{177}- DOTATATE						
Kwekkeboom [103]	310	5 (2%)	96 (28%)	51 (16%)	107 (35%)	61 (20%)

Table 1.7. Peptide receptor studies looking at different radiopeptides and response rates seen in NETs. No., number of cases; CR, complete response; PR, partial response; MR, minor response; SD, stable disease; PD, progressive disease. Criteria for response used WHO criteria.

1.7a Tyrosine kinase inhibitors

The tyrosine kinase receptor family comprise approximately 20 different classes including platelet derived growth factor receptors (PDGFR), c-kit and epidermal growth factor receptor (EGFR); they have become increasingly important as targets for cancer treatment. Inhibition of different receptor tyrosine kinases has shown promising results in a number of cancers.Currently a number of trials are underway assessing different receptor tyrosine kinase inhibitors in NETs.

Immunohistochemical studies have demonstrated that PDGF and c-kit receptors are expressed in the majority pancreatic NET cells as well as in the stroma [111]. Expression of c-kit and PDGF receptors in midgut tumours is less clear with one study showing no expression for c-kit and only 38% expressing PDGFR-alpha [112]; other groups have shown c-kit expression in midgut carcinoid tumours [113]. There has been one phase II study assessing the response rate of Imatinib, a specific tyrosine kinase inhibitor directed at the *TK* domain in *abl* (the Abelson proto-oncogene), c-kit and PDGF-R (platelet-derived growth factor receptor). Twenty seven patients with advanced carcinoid tumours received Imatinib of which 1 had a partial response, 17 had stable diseases and 9 progressive with disease. Median progression free survival was 24 weeks and median overall survival was 36months [114]. A single agent therapy shows only a modest effect, possibly combination with other therapies could improve the effect.

EGFR is a member of the HER family of transmembrane tyrosine kinase receptor. It is involved in the pathophysiology of a number of malignancies. EGFR and pEGFR has been shown to be over expressed in NETs [115], pEGFR expression has been associated with worse survival in patients with pancreatic endocrine tumours [116]. A phase II study of gefitinib (EGFR inhibitor) in patients with progressive metastatic NETs (57 carcinoid tumour and 39 islet cell carcinomas) showed a modest treatment response with 3 cases having a partial response and 2 minor response by radiological criteria and progression free survival at 6 months was 61% in carcinoid tumour group and 31% in islet cell carcinoma [117]. As a single agent although well tolerated this therapy has limited effect.

Sunitinib malate, an oral tyrosine kinase inhibitor with action against all VEGFRs, PDGFR, stem-cell factor receptor and FMS-like tyrosine kinase-3 was studied in a phase

II trial. One hundred and seven patients received Sunitinib with overall response rate of 16.7% and 68% stable disease, median time to progression was 7.7 months for pancreatic NET and 10.2 for carcinoid patients [118]. In view of this response a further randomised study to assess Sunitinib have been undertaken with good responses identified in the patients with well differentiated pancreatic NETs [119].

Sorafenib, a small molecule inhibitor of Raf kinase, VEGFR-2 and PDRGFR-β tyrosine kinase domains is being explored in patients with metastatic NETs. Preliminary results available in 41 patients showed partial responses in 10% and minor responses in 29% of cases. However, 43% of patients developed grade 3-4 toxicity. Further studies using Sorafenib in conjunction with cyclophosphamide are currently underway.

Other trials are currently in progress assessing other VEGF inhibitors [120;121]; this includes PTK787/ ZK222584 (PTK/ZK), an oral angiogenesis inhibitor which selectively blocks VEGF receptor tyrosine kinase signalling from all known VEGFRs. Twenty patients have been enrolled in a phase II, open label study. All had progressive disease prior to enrolment and following therapy 50% had achieved stable disease at 6 months and 27% at 1 year, median time to progression was 7 months. There were no complete or partial responses by radiological criteria. However, seven patients stopped therapy due to adverse effects [121]. It appears to have some antiproliferative activity inducing disease stabilization.

1.7b Tyrosine kinase antibody inhibitors

Angiogenesis is essential for the growth of tumours; the process of new blood vessel development is complex with a number of pro- and anti-angiogenic factors involved.

Angiogenic inhibitors are able to block pathways and factors and thereby directly or indirectly inhibit tumour growth. Vascular endothelial growth factor (VEGF) is a potent promoter of angiogenesis and is expressed in NETs. Studies looking at VEGF expression in NETs found its expression to be present in low grade well differentiated NETs, however, loss of VEGF expression was noted in poorly differentiated tumours. A phase II study of Octreotide plus bevacizumab or octreotide plus PEG interferon, found that 18% (n=4) in the bevacizumab arm had partial response and 95% no progression at 18 weeks compared to one partial response with PEG interferon and 68% progression free survival at 18 weeks. So it appears progression free survival was better in patients on bevacizumab [122]. Currently studies are underway using bevacizumab in combination with other therapies. A phase II study using bevacizumab and temozolomide has shown promising early results [123]. Another study looking at capecitabine, oxaliplatin, and bevacizumab is still recruiting but shows this combination appears to be generally well tolerated; of the 13 patients enrolled 4 have achieved partial response and 6 stable disease [74].

1.7c mTOR inhibitors

Mammalian target of rapamycin (mTOR) is a threonine kinase that regulates cell growth and also mediates signalling to key downstream receptors including IGF receptor and EGFR. mTOR is thought to play a role in sporadic NETs as well as pancreatic islet cell tumours in patients with tuberous sclerosis [124]. Studies in pancreatic neuroendocrine cell lines (BON-1) have shown that rapamycin inhibits mTOR leading to decreased IGF-1 and neuroendocrine tumour cell growth [125].

Temsirolimus was trialled as a single agent in a phase II study where it had modest effects. Thirty seven patients with advanced NETs and evidence of progressive disease were treated on an intention to treat basis. Partial radiological response occurred in 2 cases (5.6%) and disease stabilization in 21 (58.3%); median time to progression of 6 months [126]. A number of patients suffered from fatigue, hyperglycaemia and rash.

Yao et al, recently published the results of a phase II study using everolimus and Sandostatin LAR in patients with advanced NET. They observed a response rate of 20% in the 60 patients enrolled, 70% achieved stable disease, the overall median progression free survival was 60 weeks, and survival at 3 years is 78% [127]. These promising results have led to two everolimus trials, RADIANT-1 and RADIANT-2. RAD001 In Advanced Neuroendocrine Tumors-1 is an open label, stratified, single-arm phase II study of everolimus in patients with advanced pancreatic NETs having failed previous cytotoxic chemotherapy. RADIANT-2 is a randomised double-blind, placebo controlled, multi-centre phase III study of octreotide combined with everolimus or placebo in patients with advanced NETs.

1.7d Immunotherapy

The aim of T-cell immunotherapy is to enhance the immune response against tumour-associated antigens; several different approaches are currently being trialled. The ideal response will be to create a therapy that targets tumour cell with minimal effects on normal tissue. Dendritic cells are regarded as ‘professional’ antigen presenting cells and dendritic cell based immunization can lead to immune memory [128]. Dendritic cells play a central role in the initiation and regulation of immune responses. This action has been utilized to present tumour material to cytotoxic T cells.

Only small case series using immunotherapy have been published in NETs. A study by Scholt in three patients with pancreatic NETs showed evidence of biochemical response and no tumour progression at 20 months post therapy [129]. In a study of 20 patients with a variety of different cancers including 4 patients with NETs, patients were administered dendritic cell therapy with adjuvant interleukin-2 for 12 days post therapy [130]. Survival for the patients with NETs ranged from 4-32 months. Other potential immunotherapy techniques include genetically engineered T-cells with altered functions to enable them to target tumour tissue [131].

1.8 Tumour angiogenesis

Angiogenesis is essential for tumour development and is thought to arise secondary to an increase in proangiogenic factors within tumours that leads to development of a vascular network which is essential for tumours to grow beyond 2-3mm [132]. A number of different angiogenic factors have been described which seem to interact in a number of complex pathways. It is now assumed that the critical event in the regulation of angiogenesis is the signalling cascade involving vascular endothelial growth factor (VEGF). This conclusion is based first of all on the biological properties of this growth factor; as VEGF has been shown to induce angiogenesis in both *in vivo* and *in vitro* models [133;134]. In addition to the VEGF signalling system the angiopoietins/ Tie2 receptor system is necessary for vascular system development in embryogenesis [135](see Figure 1.2).

VEGF and angiopoietins are factors that have been extensively studied in certain cancers. VEGF expression has been demonstrated in NETs using immunohistochemistry. However, to date there are no reports regarding the expression

or role of angiopoietins in NETs. The angiopoietin family is comprised of four members Angiopoietin-1 to Angiopoietin-4.

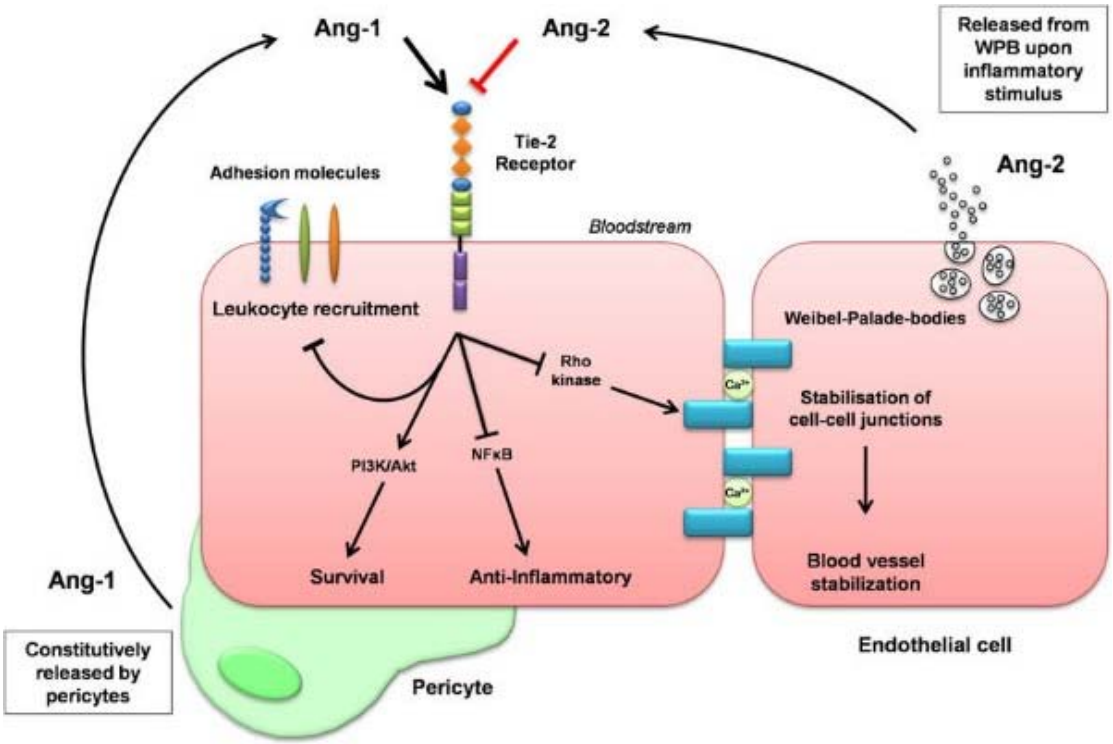


Figure 1.2. Postulated pathways of action of Ang-1 and Ang-2. Ang-2 blocks the binding of Ang-1 to the Tie-2 receptor. This leads to inhibition of the survival and anti-inflammatory actions which are normally triggered by Ang-1 ligand binding.

Most research to date has focussed on Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2), which have both been demonstrated to bind to Tie-2 receptor. It appears that Ang-2 blocks the binding of Ang-1 to Tie-2, which consequently promotes angiogenesis. However, the true interaction of these factors and consequent downstream effects are thought to be much more complex. Studies have demonstrated induction and up-regulation of Tie-2 and Ang-2 expression in endothelial cells are regulated by hypoxia and proinflammatory cytokines, such as TNF-α [136;137]. These same stimuli down-regulate

the expression of Ang-1, this suggests an inverse relationship between Ang-1 and Ang-2 [138].

In the era of targeted therapies a number of studies in NETs have demonstrated good response in terms of disease stabilization using angiogenic inhibitors, such as Bevacizumab. As part of this thesis serum Ang-1 and Ang-2 have been measured and compared in patients with NETs to the circulating concentrations in control subjects.

1.9 Cellular receptors and targeted therapy in NETs

Chemotherapy and radiotherapy show limited efficacy in most patients with midgut NETs. Pancreatic tumours are relatively chemo-responsive with a 40-65% reported response rate; whilst mid-gut NETs have a response rate of 20-30%. The low response to traditional therapies has led to development of targeted therapies in NETs. Following the success of Imanitinib in chronic myeloid leukaemia and gastrointestinal tumours, targeted therapies offer a new avenue for potential therapy.

NETs are known to express SST receptors 1-5 in differing concentrations and this has led to development of somatostatin analogues to control symptoms and tumour progression in patients. Recently studies have demonstrated that other G-protein coupled receptors specifically dopamine receptors can dimerize with each other as well as with SST receptors leading to augmentation of their effect. This has led to development of chimeric molecules to target both the dopamine-2 receptor and the SST-2 receptor. The expression of dopamine-2 receptors in NETs using immunohistochemistry has not been reported previously. A study was performed to determine whether dopamine and somatostatin receptors are co-expressed in NETs.

The Epidermal growth factor receptor (EGFR) family of receptors comprise of 4 members, EGFR, HER-2, HER-3 and HER-4. Of these the most extensively studied are EGFR and HER-2, both of which have been shown to have important roles in oncogenesis. Over expression of HER family receptors is associated with reduced survival in patients with breast, colon and ovarian cancer [139-143].

The four HER receptors share structural homology which includes two cysteine-rich regions in their extracellular domain, and a kinase domain flanked by the carboxy-terminal tail which possesses tyrosine autophosphorylation sites. HER-3 is devoid of intrinsic kinase activity, whilst HER-2 seems to have no direct ligand [144-146]. Hetero- or homo- dimerism is required for initiation of downstream signalling pathways; since HER-2 has no direct ligand it often heterodimerizes with EGFR or HER-3. To date, ten genes have been identified to encode ligands to this group of receptors. Epidermal growth factor, amphiregulin and Transforming growth factor α bind EGFR specifically, whilst neuregulins bind HER-3 and HER-4. Betacellulin, epiregulin bind to both EGFR and HER-4. Ten possible homo- and hetero- dimers can be formed from HER receptors [146].

Inhibition of HER-2 receptor in breast cancer using Trastuzumab a humanised monoclonal antibody specific to HER-2 has demonstrated improved prognosis in patients with HER-2 positive breast cancer- HER-2 staining identified using immunohistochemistry. EGFR inhibition with cetuximab in conjunction with radiotherapy in head and neck cancers demonstrated improved prognosis compared to radiotherapy alone.

It is well established that growth factor binding to the extracellular region of EGFR promotes dimerization of the monomeric receptor and increases the tyrosine kinase activity of its intracellular domain [147] (Figure 1.3). Receptor molecules in the ligand-induced EGFR dimer become tyrosine autophosphorylated. The resulting phosphotyrosines recruit the SH2 domains of multiple downstream signalling molecules, thus initiating an array of intracellular signalling pathways (Figure 1.4).

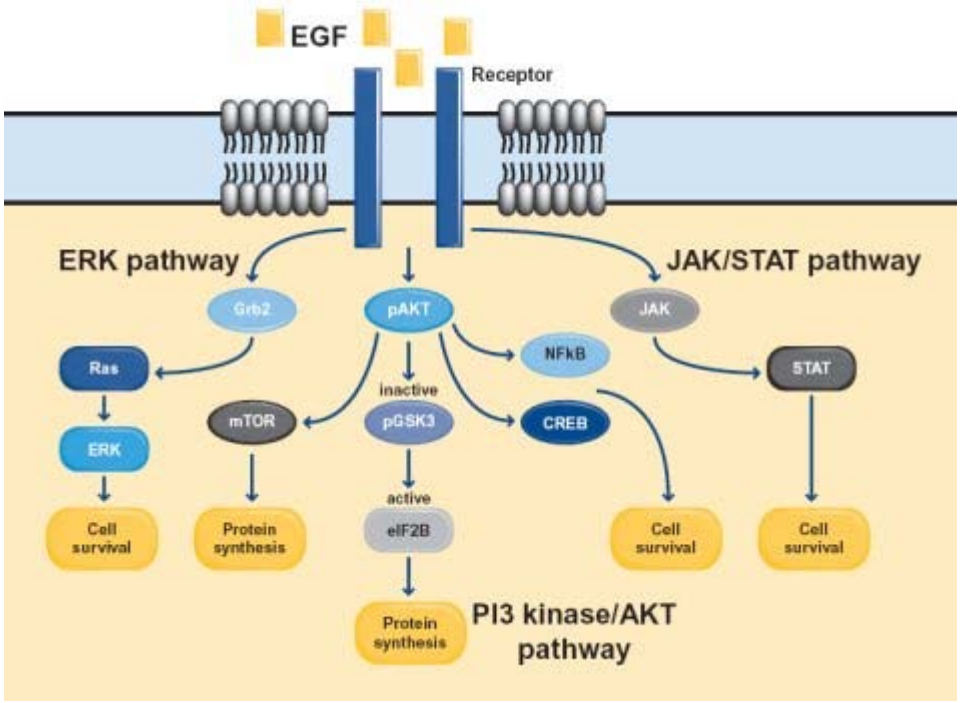


Figure 1.3. EGFR receptor and downstream pathways

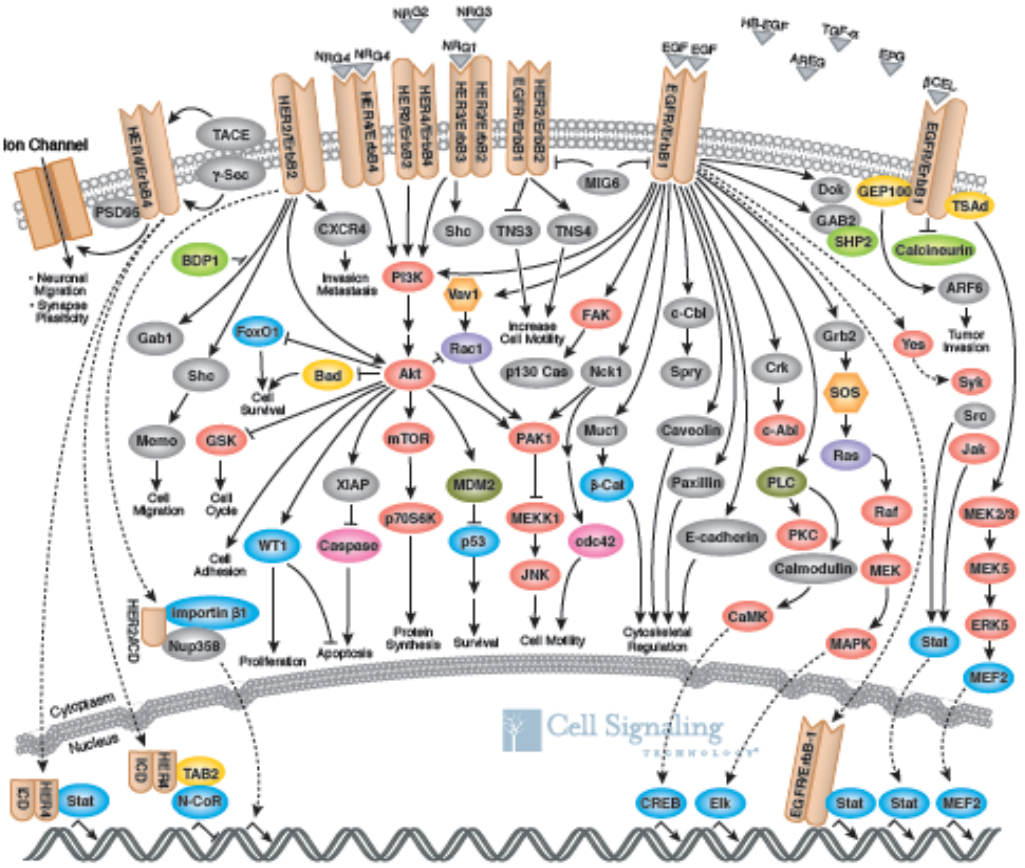


Figure 1.4. Schematic diagram showing EGFR family receptor pathways (from Cell signalling technology).

1.10 Rationale of Thesis

The first part of this thesis was focussed on identifying new therapeutic targets to help in the management of NETs. Two experiments were performed the first studied expression of the somatostatin and dopamine family of receptors in NETs using immunohistochemistry. Somatostatin analogues have been used for symptomatic control for over two decades, and there is evidence to suggest they have anti-proliferative actions. The mechanisms underlying this are complex and a number of different pathways have been postulated. Recently studies have shown dimerization of SSTR-5 and D2R as well as SSTR-2 and D2R following activation by ligands to both dopamine and SSTR-2 and SSTR-5 receptors. Development of chimeric molecules which bind both SSTR and D2R receptors have shown promising results in cell lines and also

patients with pituitary adenomas. Co-expression of SSTR and D2R receptors in NETs has not previously been demonstrated. Therefore, an immunohistochemical study was undertaken looking at SSTR-2, SSTR-5 and D2R expression in NETs.

The second study was to examine the HER family of receptors. HER family comprises four receptors (EGFR, HER-2, HER-3, HER-4). EGFR and HER-2 are both known to be essential in oncogenesis and targeted therapies are now available to inhibit these receptors. As mentioned earlier studies have examined EGFR and HER-2 expression in NETs but no studies have examined all members of the HER family. There is no evidence regarding whether expression of the HER family of receptor is associated with poorer prognosis

In addition to identifying novel markers, serum analysis of angiopoietins was performed in patients with NETs and healthy controls to determine whether angiopoietins were raised in patients. Furthermore, analysis was performed to determine whether angiopoietins provide any prognostic information

Identification of new biomarkers in NETs is required due to the lack of prognostic and diagnostic markers currently available. Whilst CgA is widely used marker, its role is limited in providing prognostic information and dependent on the specific assay used. Therefore the second part of this thesis is focussed on aiming to identify new diagnostic tools for the management of NETs; specifically aimed at developing new potential biomarkers for identification and prognostication of disease, evaluating new radiological techniques and finally to demonstrate dual expression of somatostatin and dopamine-2 receptors in NET, since this may provide a potential new therapeutic options.

Determination of new biomarkers was achieved using proteomic techniques focussed on plasma and NET cell lines. A number of different proteomic approaches are available for analysis of different media; this study used 2-D gel electrophoresis with MS/MS for analysis of plasma. For analysis of conditioned media from NET cell lines, 1-D gel electrophoresis and MS/MS was used. The use of plasma and cell lines for identifying potential markers of cancer has been well validated in previous studies.

Currently a combination of nuclear medicine and traditional cross sectional imaging techniques are employed in aiding in the diagnosis of NETs. Two nuclear medicine scans currently used are ^{111}In-DTPA-octreotide scintigraphy and ^{123}I-MIBG. However, as described earlier neither of these techniques are a 100% sensitive nor specific. Furthermore, positive uptake with ^{111}In-DTPA-octreotide scintigraphy is used to determine the treatment with peptide receptor targeted therapy. The advent of ^{68}Ga-DOTATATE PET offers a new imaging modality for identifying SSTR-2 positive tumours. The clinical benefit of this technique over ^{111}In-DTPA-octreotide scintigraphy has yet to be elucidated. A study was performed to compare the total number of lesions identified using ^{68}Ga-DOTATATE PET in patient with negative or equivocal uptake on ^{111}In-DTPA-octreotide scintigraphy and whether clinical management is altered.

Chapter 2: Expression of somatostatin and dopamine 2 receptors in neuroendocrine tumours.

Abstract

Introduction: Somatostatin and dopamine receptors are both G-protein coupled receptors. Somatostatin receptor (SSTR) expression in neuroendocrine tumours has been well characterised. There is evidence of dopamine receptor expression in neuroendocrine tumours. This study examined expression of D2R and SSTR-2 and SSTR-5 receptors using immunohistochemistry in tumour tissue from patients with neuroendocrine tumours.

Methods: Consecutive samples of formalin-fixed paraffin-embedded tumour tissue were available from 56 patients with a histologically confirmed diagnosis of neuroendocrine tumour (NET). The study population was divided into low grade (n=29), intermediate grade (n=18) and high grade (n=9). Immunohistochemical evaluation was performed for the expression of SSTR-2a, SSTR-5 and D2 receptors (D2R).

Results: Both SSTR-2 and SSTR-5 were expressed in 100% of low grade, 94.4% of intermediate grade and 66.7% of high grade NETs. D2R was expressed in 93.1% low grade, 77.8% intermediate and 44.4% of high grade tumours. Co-expression of all three receptors was present in 93.1% of low grade tumours. There was an inverse correlation of SSTR-2 ($r=-0.380$, $p<0.005$) and SSTR-5 ($r=-0.472$, $p<0.0001$) with tumour grade. D2R was positively correlated with SSTR-2 ($r=0.269$, $p=0.041$) and SSTR-5 ($r=0.267$,

p=0.045). Also D2R expression was inversely correlated with grade of tumour (r=0.395, p=0.006). Octreoscan correlated with SSTR-2, SSTR-5 and D2R receptor expression.

Conclusion: Dopamine-2 receptor is expressed in the majority of low and intermediate grade tumours. It is co-expressed with SSTR-2 and SSTR-5 in the majority of cases. The advent of new chimeric molecules that bind both somatostatin and dopamine receptors may provide a new therapeutic option in the management of neuroendocrine patients.

2.1 Introduction

The somatostatin and dopamine systems constitute two major receptor networks that share similar structural features [148]. Both families of receptors are G-protein coupled membrane receptors. There are five subtypes of somatostatin receptor (SSTR) which have been characterised to date [149;150]. Neuroendocrine tumours (NETs) are known to over express SSTR most commonly SSTR-2 and SSTR-5. SSTR-1 and SSTR-3 are expressed less frequently, whilst SSTR-4 is only occasionally expressed in NETs [151-153]. There is some heterogeneity with regards to SSTR receptor expression, dependent in part on the type of tumour. Somatostatin plays an important role in controlling cell function including inhibition of hormone release, neurotransmission and angiogenesis [154;155]. Over time there is evidence of tolerance to somatostatin analogues and some loss in their efficacy [156].

Currently five dopamine receptors have been cloned and characterised. They modulate activity of adenylate cyclase via G-proteins [157;158]. Generally dopamine receptors (DR) are sub-divided to two subfamilies, the DR1-like (DR1 and DR5) and the D2R-like (D2R, DR3 and DR4). Co-expression of dopamine and somatostatin receptors in endocrine tumours including pituitary tumours has been demonstrated [159]. Expression of dopamine receptor-2 (D2R) and SSTRs has been demonstrated in BON-1 a pancreatic neuroendocrine cell line [160]. A study of SSTR and D2R co-expression in 35 gastroenteropancreatic tumours using RT-PCR demonstrated SSTR-1, SSTR-2 and D2R receptor expression in all cases [65]. Pivonello et al, have demonstrated D2R receptor expression in 10 of 10 cases of NETs, however, in two of these cases staining was very weak [161]. New chimeric somatostatin-dopamine compounds such as BIM-23A387 have shown promising results *in vitro* studies in pituitary adenomas and lung carcinoma cell lines.

The aim of this study was to demonstrate, using immunohistochemistry, whether somatostatin and dopamine receptors are co-expressed in NETs and whether there is any difference in expression between different grades of NETs [159;162].

2.2 Materials and Methods

Consecutive samples of formalin-fixed paraffin-embedded tumour tissue were available from 56 patients with a histologically confirmed diagnosis of neuroendocrine tumour. All cases had been assessed for grade of tumour; including Ki67 proliferation index and/or number of mitoses per 10 high power fields were available in all cases. Of the tumour samples 39 were surgical specimens from patients who had undergone tumour resection. The remainder were from tumour biopsies. The study population included all major NET subtypes including: foregut (n=20), midgut (n=25), hindgut (n=3), ovarian (n=1) and NETs of unknown primary (n=7). Tumours were graded using the system proposed by ENETS consensus group [18;21;163]. Using this classification low grade tumour was regarded as mitotic count $\leq$ 2 per 10 high power fields (HPF) and Ki67 $\leq$ 2%, intermediate grade as having a mitotic count 2-20 per 10 HPF and Ki67 2-20% and high grade as mitotic count of >20 per 10 HPF, Ki67 >20 and appropriate poorly differentiated tumour morphology. Twenty nine patients had a low grade tumour, 18 were intermediate grade and 9 high grade tumours. The proposed criteria currently only encompasses GEP NETs, however, for the purpose of this study tumours of unknown primary, ovarian and bronchial NETs were graded using the same criteria. The study was performed under the auspices of the Royal Free Hospital Pathology Department ethics recommendation for the studies of archive histology samples. Clinical details

including sex, age at diagnosis and previous nuclear medicine imaging was collected where available.

3 micrometer sections of tumour tissue were dewaxed three times in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation in 1% hydrogen peroxide and diluted in acetone, for 10 minutes. For SSTR-2a and dopamine 2 receptor staining, slides were submersed in 10mM citric acid (pH=6.0) for 10 minutes in microwave at 600w, then cooled immediately in water. Slides for somatostatin 5 were submersed in 10mM citric acid (pH=6.0) for 20 minutes in microwave at 600w; then left to cool at room temperature for 20 minutes. Following which all specimens were washed in TBS-Tween. All slides were blocked with normal serum for 30 minutes.

Primary antibodies comprised: anti-SSTR2a antibody (SS-800) a polyclonal rabbit antibody raised against the c-terminus of human SSTR-2a receptor (Gramsch Laboratories, Germany). Anti-SSTR-5 antibody (SS-890) a polyclonal rabbit antibody raised against the c-terminus of the human SSTR-5 receptor (Gramsch Laboratories, Germany). Anti-D2R antibody (NLS-1403) a polyclonal rabbit antibody (Novus Biologicals, USA). Sections were incubated with anti-SSTR2a 1:800 for 1 hour, anti-SSTR5 1: 800 with 1% bovine serum albumin as antibody diluent and incubated overnight. Anti-D2R 1:600 with 0.2% bovine serum albumin as antibody diluents and incubated overnight.

For D2R and SSTR-5 slides biotinylated secondary antibody was used with slides incubated for 30mins. The antibody binding was visualized using a DAB peroxidase substrate kit. The sections were counterstained with Mayer's haematoxylin for 3.5

minutes. For SSTR-2 slides envision polymer kit was used and antibody binding was visualised using a DAB peroxidise substrate kit.

Positive controls for anti-SSTR-2a and anti-SSTR-5 were pancreatic tissue; negative controls included substitution of the primary antibody with normal sera. Positive controls for D2R included bowel tissue; negative controls included substitution of the primary antibody with normal sera.

2.2a Histological interpretation

Tumours were classified according to their site of origin, degree of differentiation and their initial mitotic index. Two examiners (R.S and J.W) performed the interpretation of immuno-histological staining for the antibodies studied, independently of each other. Any discordant results were then reviewed together to reach agreement or determine an average value for disputed sections. At time of interpretation neither examiner was aware of the histological grade of the tumour or the results from staining of other receptors. Scoring was based on intensity of staining whereby 0= negative, 1= weakly positive, 2= moderate, 3= strongly positive. The extent of tumour staining was also scored, whereby 10 random high power fields were assessed and the average percentage of positive staining cells in which: 1= <25%, 2= 25-75% and 3=>75%. The product of the density of staining and the percentage of tumour cells staining positive was used as the immunohistochemical score, giving final values of 0,1,2,3,4,6,9 [164]. Scores of 0 were counted as negative, 1-2 weak staining, 3-4 moderate staining and 6-9 strong staining.

2.2b Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Spearman's correlation test for nonparametric data was performed to reveal any relationships between receptors studied: SSTR-2, SSTR-5 and DR-2, tumour grade (low, intermediate and high), anatomical site of primary tumour and Octreoscan result.

2.3 Results

Fifty six tumour samples were examined, 25(44.6%) from males and 31 (55.4%) from females (Table 2.1). The median age of patients at diagnosis was 56.5 years (18-80). All samples had a histologically confirmed diagnosis of NET. Overall, 52 of 56 (92.9%) patients expressed SSTR-2; the staining was predominantly membranous with some cytoplasmic staining of the tumour cells. Fifty two of 56 (92.9%) cases were positive for SSTR-5 again staining was predominantly membranous and slight cytoplasmic staining. Forty five of 56 (80.3%) cases stained positive for DA2R receptor, tumour cells displayed both membranous and cytoplasmic staining (Figure 2.1).

	Low grade	Intermediate grade	High grade	Total
Male	16	5	4	25
Female	13	13	5	31
Total	29	18	9	56
Median age (range)	60 (18-78)	62 (22-75)	44.5 (34-80)	56.5 (18-80)
Location of primary				
Foregut	9	6	5	20
Midgut	17	7	1	25
Hindgut + ovarian	1	2	1	4
Unknown	2	3	2	7

Table 2 .1. Characteristics of the patients, grade and origin of tumours.

In low grade tumours: SSTR-2 staining was positive in 29/29 (100%), of which 22 (75.9%) had strong staining (IHC score≥6). SSTR-5 was positive in 29/29 (100%), of which 22 (75.9%) exhibited strong staining. D2R was positive in 27/29 (93.1%) cases, of which 19 (65.5%) had strong staining, see Figure 2.2a.

In intermediate grade tumours: SSTR-2 was expressed in 17/18 (94.4%), of which 9 cases (50%) has strong staining. SSTR-5 was expressed in 17/18 (94.4%) samples, of which 11 (61.1%) exhibited strong staining. D2R was expressed in 14/18 (77.8%) samples, of which 7 cases (38.9%) had strong staining, Figure 2.2b.

In high grade tumours: SSTR-2 was expressed in 6/9 (66.7%), of which 3 (33.3%) exhibited strong staining, SSTR-5 was expressed in 6/9 (66.7%) of which 2 cases (22.2%) showed strong staining. D2R receptor was expressed in 4/9 (44.4%) strong staining seen in 1 (11.1%) of cases, Figure 2.2c.

Co-expression of all three receptors was seen in 93.1% of low grade tumours and 72.2% of intermediate and only 33.3% high grade tumours. Only in two cases was staining for all three receptors negative, one was a large cell neuroendocrine carcinoma of the lung and the other a high grade colonic NET. There was no difference in pattern of receptor expression dependent on primary site of tumour, see Table 2.2. The specific staining patterns seen in tumours of different primary sites and tumour grades are shown in Table 2.2.

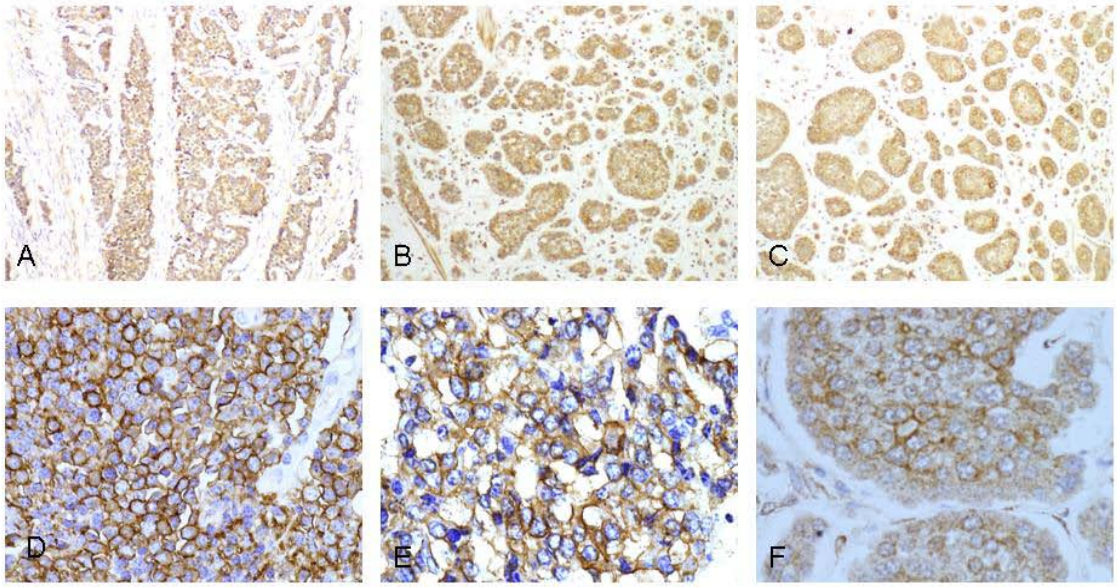
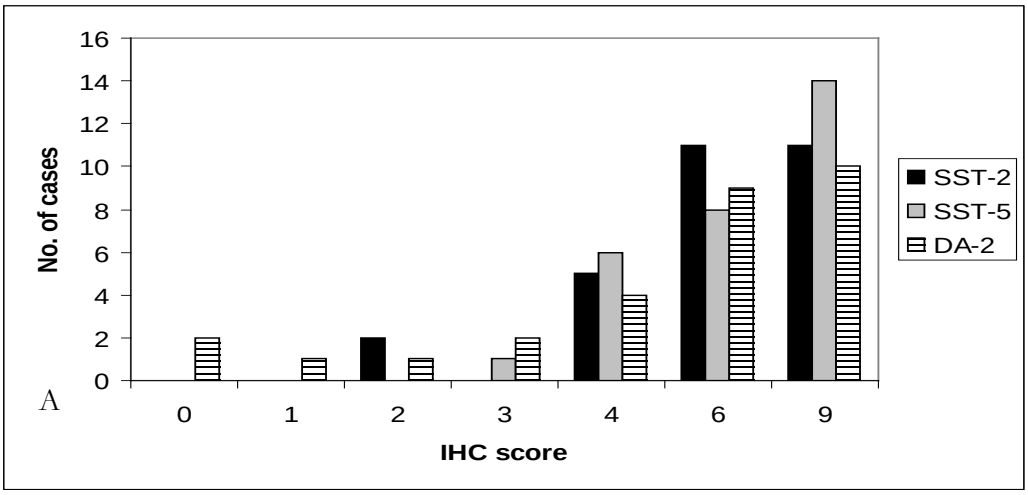
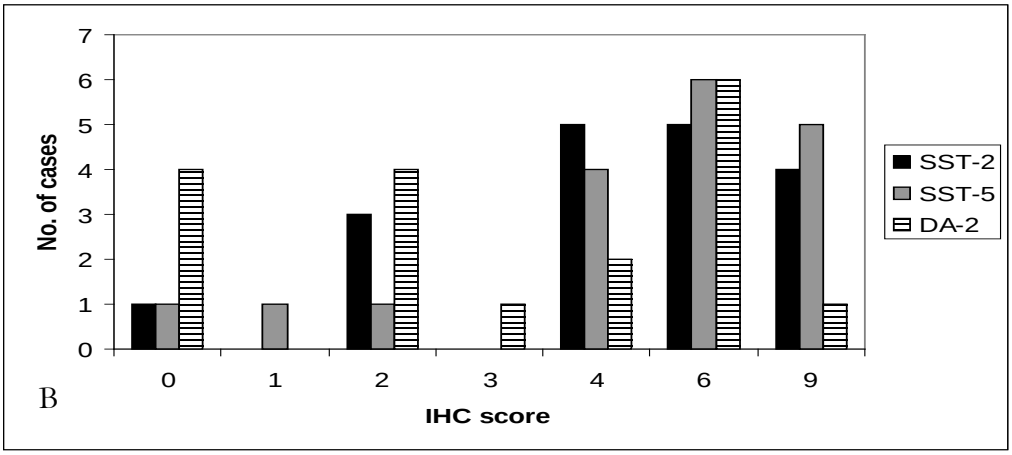


Figure 2. 1. Immunohistochemical staining of SSTR-2,SSTR-5 and D2R in NETs. A) SSTR- 2 staining in an ileal NET, predominantly membranous with some cytoplasmic staining of tumour cells, x 200 magnification. B) SSTR-5 staining shows predominantly membranous with some cytoplasmic staining, x 200 magnification. C) Dopamine-2 receptor staining shows predominantly membranous staining, x200 magnification. Images A to C are consecutive slides from same patient. D) SSTR-2 staining in low grade NET, shows strong membranous staining, x 400 magnification. E) SSTR-5 staining in NET, strong membranous staining, x 400 magnification. F) D2R staining in NET, marked membranous staining and some cytoplasmic staining, x 400 magnification.





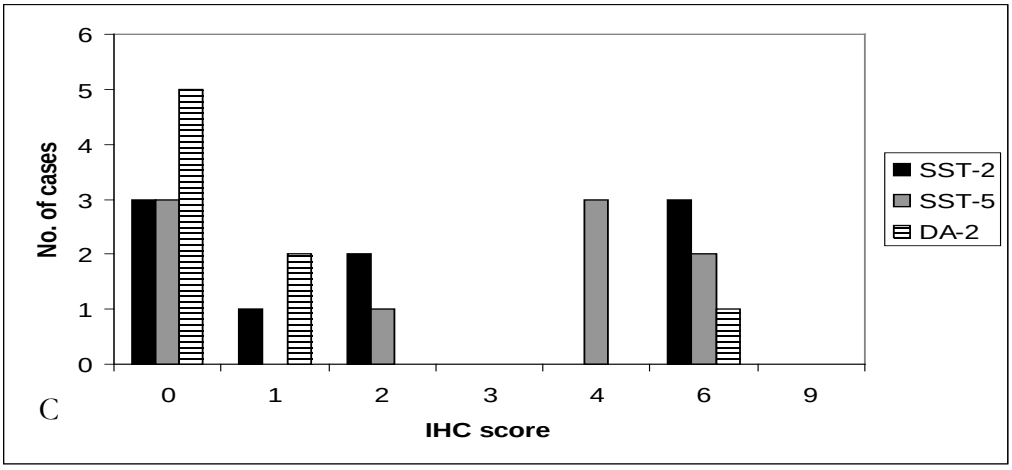


Figure 2.2. Bar charts demonstrating strength of staining for SSTR and D2R. A) Breakdown of tumour samples according to the overall score for immunohistochemical staining in low grade tumours. The method for creating a immunohistochemical score is described in the methods. B) Immunohistochemical scores for intermediate grade tumours. C) Immunohistochemical score for high grade tumours, it can be seen a large number of these cases are negative or weak staining for all three antibodies.

There was strong positive correlation between SSTR-2 and SSTR-5 receptors (r, 0.625 $p<0.001$) and also SSTR-2 and SSTR-5 expression correlated with positive Octreoscan, see Table 2.3. In addition grade of tumour was inversely correlated (i.e. low grade tumours had high SSTR expression and high grade had lower SSTR expression) with SSTR-2 ($r =– 0.38$, $p<0.005$) and SSTR-5 ($r = -0.0472$, $p< 0.005$). D2R was positively correlated with SSTR-2 ($r = 0.269$, $p< 0.05$) and SSTR-5 expression ($r = 0.267$, $p <0.05$). D2R expression was also inversely correlated with tumour grade ($r= -0.357$, $p <0.005$). Octreoscan reports were available in 29 cases, there was a positive correlation between D2R expression and positive Octreoscan ($r=0.395$, $p<0.05$).

2.4 Discussion

This study has demonstrated co-expression of SSTR-2, SSTR-5 and D2R in a wide variety of NETs using immunohistochemistry. Overall expression of D2R expression was in 80.3% of cases, with significantly higher expression in low grade compared to intermediate and high grade NETs. Both SSTR-2 and SSTR-5 receptor expression was identified in 92.9% of cases, with higher expression seen in low and intermediate grade tumours compared to high grade tumours. Immunohistochemical and RT-PCR studies have shown similar levels of SSTR expression in NETs [165]. Furthermore, lower SSTR-2 and SSTR-5 expression has been demonstrated in higher grade tumours [166]. This correlates with negative somatostatin receptor scintigraphy seen in these patients. Papotti illustrated there was >90% correlation between IHC and RT-PCR in identification of SST 2,3 and 5 receptors [165].

Contrary to a previous study [65] indicating 100% D2R expression in 36 NETs using RT-PCR this study found lower levels of receptor expression and also a difference in

expression between high and low grade tumours a finding not previously described. This could be due to a number of reasons; possibly related to low concentrations of receptor expression in these specimens which were below the threshold of detection by immunohistochemistry or that D2R is not expressed in all NETs.

Location	Grade/ Type	Total Cases	SSTR-2		SSTR-5		D2R		Octreotide Scan	+ive scan
			+ive	Score	+ive	Score	+ive	Score		
Bronchial	A.C/ INTER	1	1	4	1	6	1	3	1	1
	LCNEC/HIGH	2	0		1	4	0		1	0
Gastric	LOW	1	1	9	1	9	1	6	0	n/a
	INTER	1	1	2	1	6	1	2	0	n/a
	HIGH	1	1	6	1	6	0	0	1	0
MTC	LOW	2	2	4-9	2	4-9	2	4	1	1
Pancreatic	LOW	6	6	4-9	6	6-9	5	4-9	4	4
	INTER	2	2	4	2	6-9	2	4-6	2	2
	HIGH	3	2	2-6	2	4	1	2	2	2
Duodenal	LOW	1	1	3	1	6	1	9	0	n/a
Ileal	LOW	14	14	1-9	14	4-9	14	1-9	11	11
	INTER	7	7	3-9	7	4-9	7	4-6	3	3
	HIGH	1	1	6	0		0		0	n/a
Colon	LOW	3	3	6-9	3	4-9	3	3-9	1	1
	INTER	2	2	2	2	4	1	9	1	1
	HIGH	1	0		0		0		0	n/a
Ovarian	INTER	1	1	9	1	9	1	6	1	1
Unknown	LOW	2	2	4-6	2	6	1	2	0	n/a
	INTER	3	3	6-9	3	4-6	1	4	0	n/a
	HIGH	2	2	1-3	2	2-6	2	3-6	0	n/a

Table 2.2. SSTR-2, SSTR-5 and D2R receptor expression in NETs. Grade of tumour was based on proposed ENETS classification (see methods). +ive, is number of positive cases for which staining was seen with specific antibody. Score, is based on the histological score calculated from intensity of staining and area of tumour stained (see methods). Octreotide scan was performed in some patients total number is listed in column (Octreotide scan) and the number positive is listed in +ive scan column.

		SSTR-2	SSTR-5	D2R
SSTR-2	Correlation Coefficient		**0.625**	**0.269**
	Sig. (2-tailed)		**<0.0001**	**0.041**
SSTR-5	Correlation Coefficient	**0.625**		**0.267**
	Sig. (2-tailed)	**<0.0001**		**0.045**
Grade	Correlation Coefficient	**-0.380**	**-0.472**	**-0.357**
	Sig. (2-tailed)	**0.003**	**<0.0001**	**0.006**
Octreoscan	Correlation Coefficient	**0.803**	**0.360**	**0.395**
	Sig. (2-tailed)	**<0.0001**	**0.043**	**0.025**
Location	Correlation Coefficient	0.803	0.360	0.395
	Sig. (2-tailed)	1.00	0.108	0.958

Table 2.3. Correlation Table for SSTR-2, SSTR-5 and D2R receptor expression and a number of other parameters. Correlations were performed using a Spearman non-parametric correlation test. Significant results are in bold text, with p value <0.05. Tumour grade is inversely correlated with receptor expression.

In this study there was a significant difference in SSTR-2, SSTR-5 and D2R expression between low and high grade tumours. This may be in part because high grade tumours are poorly differentiated and loose SSTR expression. In this study we did not identify any significant difference between receptor expression in pancreatic and midgut NETs.

Strong correlation between SSTR2 receptor staining using immunohistochemistry and somatostatin scintigraphy has been reported [149]. In this study positive immunohistochemical staining of D2R was also correlated with positive Octreoscan findings, which is unsurprising since the high concentration of co-expression of SSTR-2 and D2R receptors. Since the majority of low and intermediate grade tumours co-express somatostatin and D2R, patients with positive somatostatin scintigraphy could potentially find benefit from therapy with new chimeric agents with affinity for SSTR and D2R receptors perhaps without the need for additional testing for D2R expression.

A number of different chimeric molecules combining somatostatin and dopamine receptor agonists have been developed. Promising results with *in vitro* studies have been observed with BIM-23A387 which has affinity for SSTR2 and D2R and BIM-23A760 with affinity for SSTR-2, SSTR-5 and D2R [167]. These chimeric compounds that bind both dopamine and SSTRs have also shown encouraging results *in vivo* and *in vitro* with pituitary adenomas [168;169]. The lower expression of D2R in intermediate/high grade NETs could be an important factor when determining possible sensitivity of these tumours to treatment with new chimeric somatostatin-dopamine compounds.

In conclusion this study has demonstrated co-expression of D2R and SSTR-2 and SSTR-5 in NETs, with higher expression of D2R in low grade rather than high grade NETs.

Chapter 3: Expression of the HER 1-4 family of receptor tyrosine kinases in Neuroendocrine Tumours

Abstract

Introduction: The type I receptor tyrosine kinase family comprises four homologous members: Epidermal growth factor receptor (EGFR), HER-2, HER-3 and HER-4. Studies have shown that EGFR and HER-2 play a critical role in oncogenesis. This study sought to determine the pattern of expression and the prognostic significance of EGFR, HER-2, HER-3 and HER-4 in variety of neuroendocrine tumours using immunohistochemistry.

Methods: Receptor expression in 82 paraffin embedded specimens of neuroendocrine tumours was studied using immunohistochemistry. The pattern and protein expression concentrations for each receptor were correlated with clinical and pathological parameters.

Results: EGFR expression was identified in 86.6% samples, HER-2 was not expressed in any samples, HER-3 was expressed in 8.5% samples and HER-4 was expressed 91.5%. EGFR and HER-4 were co-expressed in 79.3% of cases. HER-3 was correlated with better survival. EGFR was not associated with poor prognosis.

Discussion: EGFR, HER-2 and HER-4 expression is not associated with poorer survival. HER-3 expression is correlated with better prognosis. Over expression of EGFR and HER-4 may offer potential new therapeutic targets.

3.1 Introduction

The HER family is comprised of four distinct receptors: EGFR, HER-2, HER-3 and HER-4. These are transmembrane receptors composed of an extracellular ligand-binding domain and a cytoplasmic region with enzymatic activity [146;170]. To transduce signals the receptors need to either hetero- or homo-dimerize following ligand binding [170]. HER receptor phosphorylation activates a cascade of signalling pathways that include controlling apoptosis via PKB/Akt and mitogenic pathways via Ras/ MAP kinase [171]. These routes are thought to regulate cellular growth differentiation, proliferation, angiogenesis and apoptosis.

Development of humanized antibodies against EGFR and HER-2, have enabled inhibition of the downstream signalling pathways, consequently leading to improved survival in these patients [115;172]. EGFR inhibition by humanised anti-EGFR antibodies (e.g. Cetuximab) has shown positive results in head and neck cancers in combination with radiotherapy. Trastuzumab (Herceptin) is a fully humanized monoclonal antibody that binds to the extracellular domain of HER-2 and has antiproliferative activity against breast cancers over expressing HER-2 [173].

Recent studies have shown that HER family of receptors play a critical role in progression of various cancers [174-176]. Previous studies have demonstrated the expression of EGFR in NETs [115;116]. Previous studies demonstrated high EGFR expression in NETs [115]. A number of studies have been performed to assess HER-2 expression in a number of different types of NETs, demonstrating different concentrations of expression in various NETs [115;177-180]. Expression of all four members of the HER family has not been studied in NETs.

The aim of this study was to evaluate the expression of HER-2, HER-3 and HER-4 in NETs by immunohistochemistry and its association with EGFR and b) to correlate the extent of expression with clinico-pathological parameters.

3.2 Materials and Methods

Consecutive samples of formalin-fixed paraffin-embedded tumour tissue were available from 82 patients with a histologically confirmed diagnosis of NET. Of these 58 were from surgical resection from patients who had undergone an operation and tumour resection. A further 24 samples were from tumour biopsies. The study population included all major NET subtypes including: foregut, mid-gut, hindgut, bronchial, paraganglioma and NETs of unknown primary (see Table 3.1). Demographic details, including tumour stage and survival data. Tumours were graded where possible using the TNM system proposed by ENETS consensus group [19;21]. Using this classification low grade tumour was regarded as mitotic count <2 per 10 high power fields (HPF) and Ki67 ≤2%, intermediate grade as having a mitotic count 2-20 per 10 HPF and Ki67 3-20% and high grade as mitotic count of >20 per 10 HPF and Ki67 >20. This classification currently only encompasses gastroenteropancreatic NETs, for the purposes of this study this classification was expanded to include other types of NETs. The study was performed under the auspices of the Royal Free Hospital Pathology Department ethics recommendation for the studies of archive histology samples.

Three micrometer sections of tumour tissue were dewaxed three times in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation in 1% hydrogen peroxide, diluted in acetone, for 10 minutes. For HER-3 antibody studies the samples were submersed in 10mM citric acid (pH=6.0) and microwaved at 600 watts for 20 minutes; then allowed to cool at room temperature. Slides for HER-2 and HER-4

studies were immersed in 10mM citric acid (pH=6.0) and placed water bath at 98°C for 45 minutes, following which they were removed and cooled at room temperature for 20 minutes. Specimens were washed in TBS-Tween and pre-incubated with avidin and biotin diluted in 3% normal serum for 20 minutes each.

Primary antibodies comprised: anti-HER-2 polyclonal rabbit (DAKO Ltd), anti-HER-3 rabbit monoclonal antibody (DAKO Ltd), anti-HER-4 polyclonal rabbit antibody (Labvision Ltd). Sections were then incubated with anti-HER 2 antibody (1:250), anti-HER-4 antibody (1:50) and anti-HER-3 antibody (1: 50) were incubated for 1 hour. Biotinlyated 2 antibody was used with slides incubated for 30minutes. The antibody binding was visualized by using a DAB peroxidase substrate kit. The sections were counterstained with Mayer's haematoxylin for 3.5 minutes.

Negative controls included substitution of the primary antibody with normal sera. Breast cancer tissue was used for positive controls and determining optimal pre-treatment conditions for all antibodies.

The EGFR studies [181] were reviewed in this study in order to correlate EGFR immunohistochemical findings with those of HER-2, HER-3 and HER-4. This study initially included 98 NET specimens, however due to limited availability of tissue immunohistochemical analysis of HER-2, HER-3 and HER-4 was performed in 82 of these cases. The remaining 16 cases which had been stained for EGFR were excluded from the analysis.

3.2a Histological interpretation

Tumours were classified according to their site of origin, degree of differentiation and their initial mitotic index. Two examiners (R.S and J.W) performed the interpretation of immuno-histological staining for the antibodies studied independently of each other. Any discordant results were then reviewed together to reach agreement or determine an average value for disputed sections. The same score was achieved independently in 94% (77/82) of cases. Scoring was based on intensity of staining of tumour cells whereby 0= negative, 1= weakly positive, 2= moderate, 3= strongly positive. Then extent of tumour staining was also scored, whereby 10 random high power fields were assessed and the average percentage of positive staining cells in which: 1= <25%, 2= 25-75% and 3=>75%. The product of the density of staining and the percentage of tumour cells staining positive was used as the histological score, giving final values of 0,1,2,3,4,6,9. Scores of ≤2 were counted as negative and scores >2 were classed as positive [164;181].

3.3 Results

Tumour tissue was available from 82 patients with a histologically confirmed diagnosis of NET. All 82 cases were negative for HER-2 (see Figure 3.1). Seven (8.5%) cases were positive for HER-3 staining; the staining in these cases was predominantly cytoplasmic with some membranous staining.

The surrounding stroma showed weak or negative staining in the majority of cases. Of the seven cases that were positive, 3 were paragangliomas, 3 foregut and one mid-gut tumour (see Table 3.2). Seventy five (91.5%) cases were positive for HER-4 antibody, with staining predominantly membranous and cytoplasmic (see Figure 3.2). Seventy one

of the 82 (86.5%) cases reviewed for EGFR staining were positive for EGFR expression, the staining of which was predominantly cytoplasmic and perinuclear.

Four cases over-expressed EGFR only, 10 cases expressed only HER-4 receptor and none expressed HER-3 receptor alone. EGFR, HER-3 and HER-4 were all expressed in 6 cases. EGFR and HER-4 were co-expressed in 65 (79.3%) cases. There minimal weak staining of the surrounding stroma in cases with EGFR, HER-3 and HER-4. Tumour grade could be assessed in 66 of the 82 cases, who had tissue available for MIB-1 or Ki67 proliferation index staining.

	Number	%
Patients	82	
Age		
Median	59	
Range	32-88	
Gender		
Male	33	40.2
Female	49	59.8
Primary site		
Thymic	1	1.2
Thyroid	4	4.9
Bronchial	4	4.9
Gastric	3	3.7
Pancreatic	18	23.2
Duodenal	1	1.2
Jejunal-Ileal	21	25.6
Appendiceal	8	9.8
Colon	4	6.1
Ovarian/ Cervical	4	4.9
Paraganglioma	6	6.1
Unknown	8	8.5

Table 3.1. Patient group characteristics

Of these 44 were low grade, 6 intermediate grade and 16 high grade. Multivariant statistical analysis did not show any correlation between tumour grade and expression of EGFR, HER-2, HER-3 or HER-4. There was no correlation of expression of EGFR with HER-3 or HER-4. No correlation\n between HER-3 and HER-4 expression. There was no significant difference in expression of EGFR, HER-3 or HER-4 between fore-, mid- or hind- gut tumours; with EGFR and HER-4 being co-expressed in all different types of NETS.

HER-2 and HER-4 had a negative correlation. HER-3 was positively correlated with survival using Spearman correlation (r= 0.272, p= 0.05). EGFR, HER-2 and HER-4 had no significant correlation with survival (see Table 3.3).

Site	**No**.		**EGFR**			**HER-3**			**HER-4**	
		+ case	intensity	area	+case	intensity	area	+ case	intensity	area
Thymic	1	1	2	3	0	0	0	1	2	3
Thyroid	4	4	1-3	1-3	0	0	0	3	1-3	2-3
Bronchial	4	4	2	2	1	3	1	4	2-3	2-3
Gastric	3	3	2	1-3	0	0	0	3	3	2-3
Pancreatic	18	14	1-3	1-3	2	2	1-2	14	1-3	1-3
Duodenal	1	1	2	3	0	0	0	1	3	3
Jejunal-Ileal	21	19	1-3	1-3	1	2	3	19	2-3	1-3
Appendiceal	8	8	2-3	1-3	0	0	0	8	2-3	2-3
Colon	4	2	2	2	0	0	0	4	2-3	2-3
Ovarian	3	3	2	2	0	0	0	3	2-3	2
Cervical	1	1	2	2	0	0	0	1	2-3	2
Paraganglioma	6	5	1-3	1-3	3	1-3	2-3	6	2-3	2-3
Unknown	8	6	1-3	1-3	0	0	0	8	1-3	2-3
TOTAL	82	71			7			75		

Table 3.2. Immunohistochemistry for EGFR, HER-3 and HER-4 in 82 neuroendocrine tumours. (No) is number of cases in total, (+) case is the number of cases with positive uptake (i.e. score >2). Intensity scored 1-3, where: 1- weak, 2- moderate and 3- intense. Area scored 1-3, where 1- <25%, 2- 25-75% and 3- >75%.

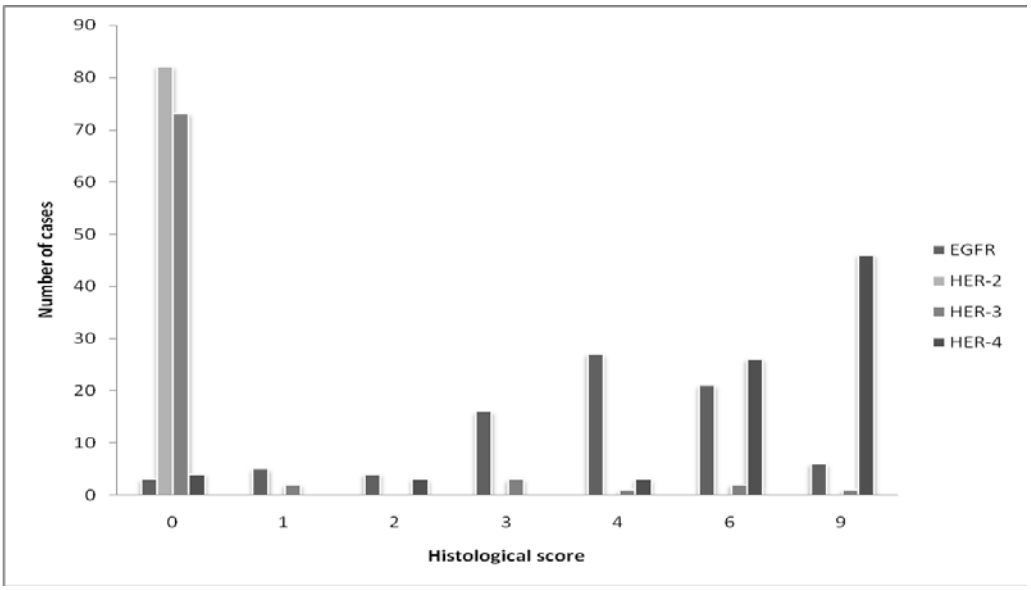


Figure 3.1. Scoring of tumour samples according to immunohistochemical staining. The method of creating a histological score for immunohistochemical staining is described in the methods.

		HER1	HER3	HER4
HER2	Correlation Coefficient	.086	-.103	-.505(**)
	Sig. (2-tailed)	.447	.364	.000
HER3	Correlation Coefficient	.042		.079
	Sig. (2-tailed)	.712		.483
HER4	Correlation Coefficient	.047	.079	
	Sig. (2-tailed)	.681	.483	
Survival	Correlation Coefficient	.010	**.272(*)**	.194
	Sig. (2-tailed)	.937	**.032**	.130

Table 3.3 Correlation Table for a Cohort of 82 Patients with Neuroendocrine tumours. * shows significant correlation between HER-3 positive tumours and longer survival. ** significant negative correlation between expression of HER-2 and HER-4.

3.4 Discussion

This study demonstrated that HER-2 is not expressed in NETs, whilst EGFR and HER-4 are frequently and HER-3 infrequently expressed in NETs. Furthermore, HER-3 is correlated with better prognosis.

Studies examining EGFR expression have noted significantly worse prognosis in NETs expressing EGFR rather than those not [116]. This does not appear to be the case in this study, with over 80% of cases expressing EGFR and these tumours did not show a worse prognosis. A study by Atkins et al, has demonstrated that immunohistochemical expression of EGFR can vary with age of tissue samples [182], however, in this study the percentage of EGFR positive tumours did not differ between samples more or less than 2 years old.

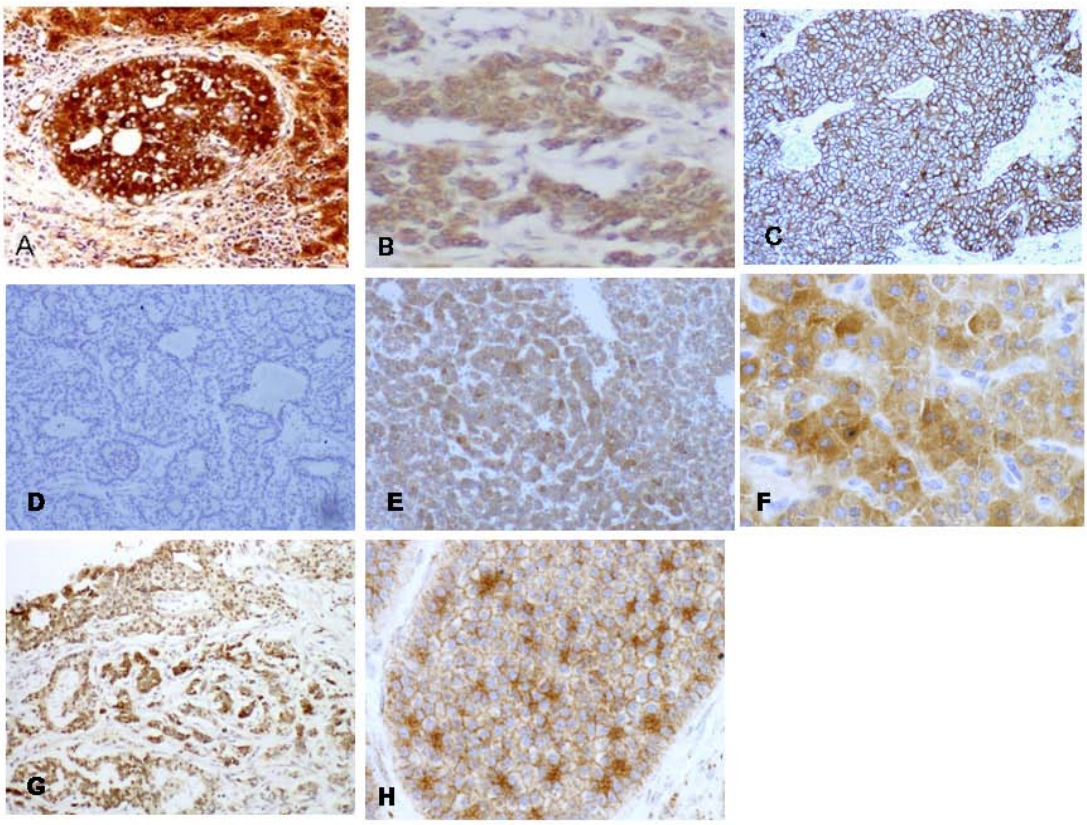


Figure 3.2. Immunohistochemical staining of HER receptors in NETs. A) EGFR staining of NET, predominantly cytoplasmic and membranous staining, x200 magnification. B) EGFR staining x400 magnification. C) HER-2 staining of breast tumour, predominantly membranous staining, x200 magnification. D) HER-2 in NET, no evidence of staining, x200 magnification. E) HER-3 predominantly cytoplasmic with some membranous staining in NET, x200. F) HER-3 staining of ileal NET, x400 magnification. G) HER-4 with predominantly membranous and some cytoplasmic staining in NET, x200 magnification. H) HER-4 staining predominantly membranous pattern, x400 magnification.

HER-2 receptor expression has been demonstrated in intestinal but not gastric NETs by Yamaguchi et al [180], this study used the same Dako antibody though the secondary antibody and duration of incubation of primary antibody is not reported. The reasons for this difference in staining are unclear and may be related to the age of the slides or the scoring system used to interpret the slides. Other studies have also demonstrated variable expression of HER-2 in NETs using immunohistochemical and PCR techniques [179;183]. The negative expression of HER-2 immunohistochemistry in this study could be due to a number of reasons; possibly related to low levels of receptor expression in these specimens which were below the threshold of detection by immunohistochemistry.

HER-2 was not expressed in any cases, which is of interest since cell line studies have shown that HER-2 is the preferred dimer partner for other receptors [184]. The positive controls in this study were breast cancer, which all demonstrated strong grade 3 staining, with minimal background staining. Therefore, confirming that the lack of staining was not due to technical reasons or the antibody. Even though HER-2 does not act as a receptor for EGF, it can decrease the rate of ligand dissociation from the cognate receptor, EGFR [185]. Thus, resulting in stronger and more prolonged activation of the EGFR signalling network [170]. Furthermore in cell line studies, mitogenic signalling appears to be stronger via HER-2 containing heterodimers than any other heterodimers [170;186]. All these factors lead to a stronger more prolonged signalling response following activation of HER-2 receptors. It would be interesting to examine HER-2 receptor expression using RT-PCR since this may be able to detect lower levels of receptor expression than immunohistochemistry.

Wang et al, demonstrated HER-3 expression in 6 of 98 (6%) malignant GEP NETs [179]. This study identified HER-3 positivity in 6% of GEP NETs (3 of 52 cases) and 50% (3 of 6 cases) of paragangliomas. This confirms that HER-3 is infrequently expressed in NETs. HER-3 expression correlated with improved survival, however, only 7 cases showed expression of HER-3; furthermore 3 of these cases were in paragangliomas which generally have a more indolent course than GEP NETs. HER-3 over expression has been associated with improved outcome with breast cancer in one study [187]. Further HER-3 positive cases need to be evaluated to confirm whether this is a consistent finding. Interestingly 50% of paraganglioma cases expressed HER-3, again a study of more paraganglioma cases needs to be performed to confirm this finding.

Studies performed looking at HER family of receptor expression in other cancers, have often found that HER-4 expression is associated with positive prognostic survival. This study has not demonstrated expression of HER-4 to be associated with an improved prognosis. Currently the role of HER-4 in NET biology is not understood and with further understanding of its interactions with other members of the HER family and downstream signalling effects one may be able to develop better understanding.

Co-expression of EGFR receptors appears to vary from different tumour groups; however, EGFR and HER-4 co-expression was identified in 2.3% of colonic adenocarcinoma [188] and 13.6% of non-small cell lung carcinomas [140]. There is currently no evidence that co-expression of EGFR and HER-4 is associated with alteration in prognosis.

Expression of only a single receptor was uncommon, with only 4 (4.8%) cases expressing EGFR alone and HER-4 was expressed in ten (8.2%) cases. One reason for

this may be that receptor expression may have been below the threshold level of immunohistochemical detection. HER-3 was not expressed alone, which is unsurprising since it has no intrinsic tyrosine kinase activity [189]. Co-expression of EGFR and HER-4 has been demonstrated in other tumours [140]. HER-2 co-expression is often linked with HER-3 expression, in this study HER-2 expression was absent in these tumour samples and HER-3 was rarely expressed.

This study demonstrates that EGFR, HER-3, HER-4 are expressed in neuroendocrine tumours. HER-3 expression was associated with better survival, though the number of cases was small and also paragangliomas have a different prognosis than GEP NETs. The lack of expression of HER-2 may in part explain the less aggressive clinical course of these tumours. Recent development of pan-HER receptor inhibitors may provide possible therapeutic options in NETs.

Chapter 4: Circulating Angiopoietin-1 and Angiopoietin-2 in the serum of patients with neuroendocrine tumours

Abstract

Introduction: Angiogenesis is an essential process in development and growth of tumours. There are a large number of angiogenic mediators including the angiopoietin family and vascular endothelial growth factor which play an important role in both physiological and pathological angiogenesis. This study examines serum concentrations of Ang-1 and Ang-2 in patients with neuroendocrine tumour compared to healthy controls.

Methods: ELISA for Angiopoietin-1 and Angiopoietin-2 was performed in 47 patients with histologically proven NETs and 44 healthy controls. Immunohistochemical staining for Ang-2 was performed in patients to demonstrate cellular location of Ang-2.

Results: Serum Ang-2 concentrations were significantly elevated in patients compared to controls (median 4756 vs. 2495 pg/ml, $p<0.001$), whereas there was no significant difference in Ang-1 concentrations. The ratio of Ang-2: Ang-1 was significantly elevated in patients compared to controls (0.13 vs. 0.066, $p<0.001$). Serum Ang-2 concentrations were significantly elevated in patients with distant metastases compared to those without metastasis (median 5080 pg/ml vs. 3360 pg/ml, $p=0.01$) and there was also a significant correlation between Ang-2 concentrations and volume of liver metastases, ($p<0.014$). Time to disease progression was more rapid in patients with serum Ang-2 concentrations >4756 pg/ml ($p=0.04$).

Conclusion: Serum Ang-2 but not Ang -1 is elevated in NET patients. Ang-2 may be a useful serum marker for monitoring disease progression and assessment of prognosis in patients with NETs.

4.1 Introduction

There are a large number of angiogenic mediators including the angiopoietin family and vascular endothelial growth factor which play an important role in both physiological and pathological angiogenesis [190-192]. To date four angiopoietins have been identified; termed Ang 1-4 [193]. Of these Angiopoietin-1 (Ang-1) and Angiopoeitin-2 (Ang-2) are the most widely studied and function as ligands for Tie-2, which is a receptor to tyrosine kinase specifically expressed on endothelial cells [194;195].

Ang-1 binds specifically to Tie-2 causing activation by phosphorylation. Ang-1 is produced by endothelial cells and pericytes and is widely expressed in adult tissue, where it appears to have a stabilizing effect on blood vessels [196]. The role of Ang-1 in tumour development is complex and studies have shown both pro- and anti-angiogenic effects with this growth factor. Ang-2 is expressed at sites of vascular remodelling [197] and promotes vessel destabilization [198]. This appears to be accomplished by Ang-2 binding to Tie-2 and therefore blocking Ang-1 binding. Ang-2 appears to be a non-signal transducing ligand and therefore disrupts normal Tie-2 activation [199]. The endothelial cells are then acted upon by various angiogenic factors such as VEGF, which leads to proliferation [135].

In tumours a shift in the balance between pro- and anti-angiogenic factors is thought to occur ; termed the ‘angiogenic switch’ resulting in an angiogenic phenotype [200;201]. It has been proposed that a change in the ratio of Ang-1: Ang-2 in favour of Ang-2 might play a role in this switch [202]. Support comes from animal studies in colonic and gastric tumours transfected with Ang-2 which grew larger and heavier in nude mice compared to those transfected with Ang-1 [203;204].

The serum concentrations of angiopoietins have not been evaluated in NET patients. The aim of this study was to measure serum Ang-1 and Ang-2 concentrations in patients with NETs and assess their interrelationship and clinical significance.

4.2Materials and Methods

4.2a Patients

We prospectively enrolled 47 patients with NETs, between July 2007 and March 2008. All patients had histological confirmation, including assessment of morphology and immunohistochemical analysis for neuron specific enolase, CgA, synaptophysin and PGP9.5. All cases were well or moderately differentiated tumours and no patients enrolled in the study had a poorly differentiated NET. The presence of necrosis, number of mitoses and Ki-67 index was recorded. Tumours were graded using the system proposed by European Neuroendocrine Tumour Society (ENETS) consensus group [19;21;163]. Using this classification low grade tumour was regarded as mitotic count <2 per 10 high power fields (HPF) and Ki67 ≤2%, intermediate grade as having a mitotic count 2-20 per 10 HPF and Ki67 3-20%. All patients underwent imaging with CT or MRI and appropriate nuclear medicine imaging including somatostatin receptor scintigraphy using ^{111}In-DTPA-Phe I-Pentetreotide (Octreoscan), within two months of blood sampling to enable staging of disease. Serum CgA was measured in all patients at the same time as serum collection for the study. All previous and current therapies that patients had received were recorded from patient records. The demographics of both groups are shown in table 4.1.

Survival data and time to progression of disease was identified in all cases. Time to progression was assessed using radiological evidence of disease progression according

to RECIST criteria [205] and was calculated in days from time of blood collection. Patients underwent cross sectional imaging for re-staging of their disease on a 3-4 monthly basis.

As control subjects, 44 healthy volunteers who were age and sex matched were enrolled in the same period, see Table 4.1. Most subjects in the control group were healthy relatives of patients and the remainder were volunteers. All control patients had no previous history of cancer.

The study was approved by the Local Ethical Committee and all participants gave written informed consent prior to obtaining samples.

4.2b Enzyme-Linked immunosorbent assays

Serum samples were obtained from each individual, immediately placed on ice and allowed to stand for 30 minutes. Following this the samples were centrifuged at 3000rpm for 15 minutes. Sera were stored at -80°C. Enzyme linked immunoabsorbent assays (ELISA) were used to measure Ang-1 and Ang-2 (Quantikine, R&D systems, Minneapolis). Serum samples were diluted as appropriate prior to being added to separate microplates, each containing a specific antibody for Ang-1 or Ang-2. The mixtures were then incubated for 2 hours on an orbital microplate shaker. Plates were then washed four times to remove unbound antigen. Enzyme-linked polyclonal antibodies specific for each angiogenic factor were then added and the mixture incubated for 2 hours. Plates were then washed four times prior to the substrate solution being added to the wells. The colour was allowed to develop following which the stop solution was added. The optical density of each well was determined at 540nm.

	NET	Control
Subject number	47	44
Age	61.4 (34-80)	59.65 (24-86)
Males	26	23
Females	21	21
Primary tumour		
Bronchial	3	
Pancreatic	17	
Jejunal	2	
Ileal	20	
Unknown	5	
Functional types		
Carcinoid syndrome	18	
Glucagonoma	1	
Non-functional	28	
Histological grade		
Low	24	
Intermediate	23	
Biochemical markers		
Chromogranin A >60	35	
Chromogranin A 0-60	12	
CgA (pMol/L) (Range)	Median 99 (range 17->1000)	
Concurrent SST analogues	29	
Previous Therapies		
Previous chemotherapy	4	
Radiotargetted therapy	4	
Surgery	19	

Table 4.1. Demographic data. All therapies are previous treatments that patients had undergone, except somatostatin (SST) analogues which all patients were currently on at time of study. Histological grade was classified using the ENETs proposed system for grading [19]. The classification was expanded to include bronchial and tumours of unknown primary site. Chromogranin A measurements were made using commercial RIA kit (Roche), which measures the pancreastatin fragment of chromogranin A, normal range is 0-60 pMol/L.

Chromogranin A was measured using a commercial RIA (Roche) which measures the pancreastatin fragment of chromogranin A.

4.2c Immunohistochemical analysis of Angiopoeitin-2

Formalin-fixed paraffin-embedded tumour tissues were available from 9 patients with a histologically confirmed diagnosis of neuroendocrine tumour in whom serum samples had been collected for Ang-2 analysis. All cases had been assessed for grade of tumour; including Ki67 proliferation index and/or number of mitoses per 10 high power fields were available in all cases. The study population comprised of 6 primary pancreatic NETs and 3 midgut NETs.

Four micrometer sections of tumour tissue were dewaxed three times in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation in 0.5% hydrogen peroxide and diluted in acetone, for 10 minutes. Slides were microwaved for 10minutes at 600w in citrate buffer and then transferred to a humidity chamber. Slides were incubated overnight in primary monoclonal Ang-2 antibody at a dilution 1:50 (Santa Cruz product no. Sc-74403). Slides were washed with Post Primary Block (Novolink^{TM} Max Polymer RE7280 detection kit) for 30 minutes in a humidity chamber at room temperature. Slides were washed in TBS-t and left for 5 minutes. Novolink^{TM} Polymer (RE7280-K) was added for 30 minutes in a humidity chamber for 30 minutes, following which slides were washed in TBS-t. The sections were then developed with Novolink DAB (3, 3'-diaminobenzidine tetrahydrochloride) solution for 5 minutes by adding 50µl of DAB chromagen to 1ml of Novolink DAB substrate buffer. The slides are counter-stained with Mayer's haematoxylin for 3 minutes. Positive controls for Angiopoeitin-2 were renal tissue; negative controls included substitution of the primary

antibody with normal sera. An experienced histopathologist (TV.L) performed the interpretation of immuno-histological staining for the antibody studied.

4.2d Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. The values were generally not normally distributed, so non-parametric tests were used to compare marker concentrations between groups, i.e. the Mann- Whitney *U* test (for two groups) or Kruskal-Wallis test (for several groups). Multivariate linear regression analyses were used to examine Ang-1 and Ang-2 (on a logarithmic scale) in relation to several other factors. Spearman correlation of rank coefficient was used to analyze correlations between parameters. Time to disease progression curves was plotted using the Kaplan-Meier method and the log-rank test applied.

We also evaluated the screening performance of the markers using the detection rate (sensitivity), defined as the proportion of NET cases that had concentrations above a specified cut-off, and the false-positive rate, defined as the proportion of controls that had concentrations above the same cut-off. The likelihood ratio quantifies the 'power' of the marker and is the detection rate divided by the false-positive rate; the higher the value the better the marker.

4.3 Results

4.3a Serum Ang-1 and -2 concentrations in Patients with NETs

Figure 4.1 shows scatterplots of the distributions of Ang-1, Ang-2, and the ratio of the two. Patients with NETs had significantly higher serum Ang-2 concentrations than the

control group (median 4756 vs. 2495 pg/ml), $p<0.001$. There was still a highly statistically significant difference when allowing for age and sex ($p<0.001$). Median serum Ang-1 concentrations were not different between the two groups (39135 pg/ml controls vs. 39405 pg/ml cases; $p= 0.60$). The ratio of Ang-2 to Ang-1 was significantly increased in patients (median 0.133 compared to 0.066 in controls $p<0.001$), but this probably largely reflects the difference associated with Ang-2. There was no evidence of a correlation between serum Ang-2 and Ang-1 ($r=0.11$, $p=0.49$); Ang-1 and plasma CgA ($r=-0.22$, $p=0.13$); or Ang-2 and plasma CgA ($r=0.03$, $p=0.8$). We conducted a multivariate analysis to look at the association between each of Ang-1, Ang-2 and the ratio, and three prognostic factors: histological grade, CgA and stage. There was some evidence of an association between histological grade and Ang-2, even after allowing for stage and CgA ($p=0.003$); the geometric mean was 4140 and 6668 pg/ml in the low and intermediate grades respectively. There was no evidence of an association between either Ang-1 or Ang-2: Ang-1 ratio and the prognostic factors.

To confirm that there was no significant daily variation in Ang-2 concentrations we obtained two matched serum samples from 17 control patients separated by 24 hours. There was no significant difference between Ang-2 concentrations ($p=0.143$) or Ang-1 ($p=0.662$).

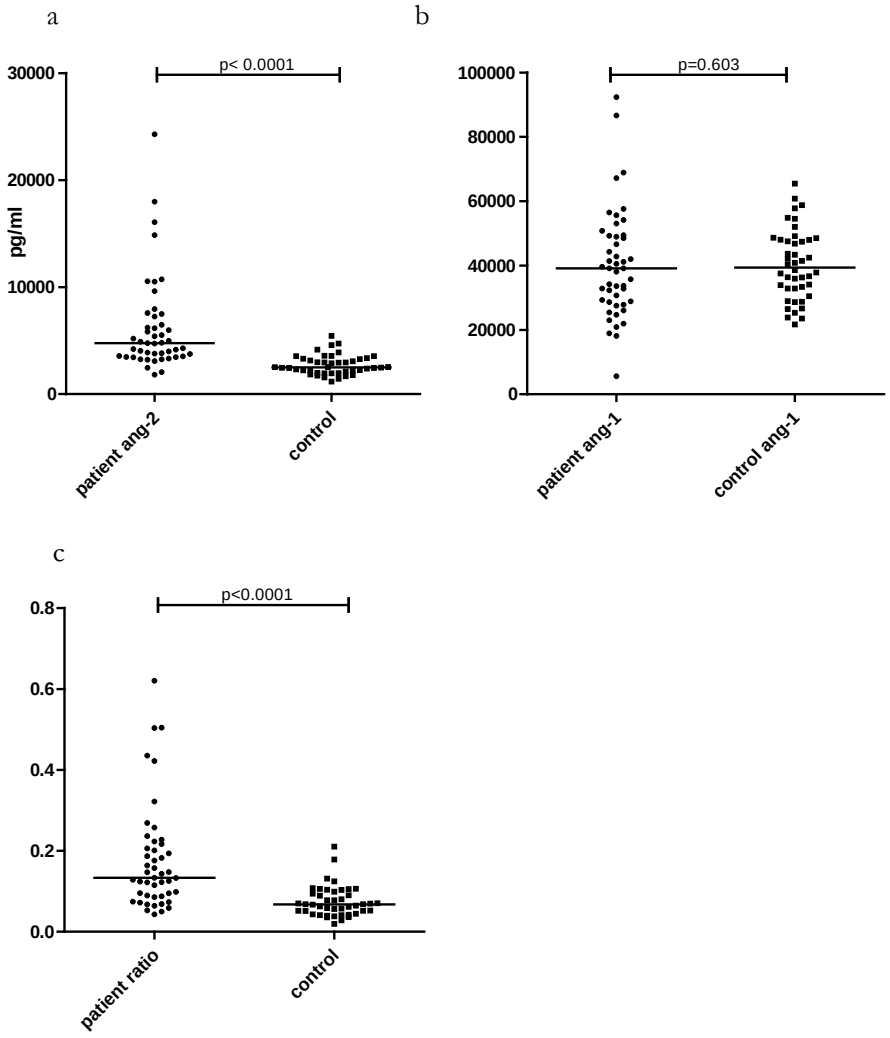


Figure 4.1. Comparisons of Ang-2, Ang-1 and Ang-2: Ang-1ratios in patients with NETs (n=47) and controls (n=44). a) Ang-2 concentrations b) Ang-1 concentrations c) ratio of Ang-2 to Ang-1

4.3b Angiopoietin concentrations and type of neuroendocrine tumour

Compared to controls (median 2495 pg/ml), serum Ang-2 was significantly elevated in both midgut NETs (median 4790 pg/ml, p< 0.001) and foregut primary NETs (median 4900 pg/ml, p<0.001). When comparing concentrations between foregut and midgut primary tumours, there was no evidence of a difference in serum Ang-2 (median 4900 pg/ml vs. 4790 pg/ml, p=0.59), serum Ang-1 (median 39100 pg/ml vs. 33750 pg/ml,

p=0.28); or the ratio of Ang-2 to Ang-1 (median 0.133 vs. 0.147, p=0.68). There was no significant difference in Ang-2 concentrations between patients with functional tumours (n=19) and non-functional tumours (n=28) (median 4503 pg/ml vs 4867 pg/ml, p=0.58). Furthermore, there was no difference in serum Ang-2 concentrations between the 29 patients currently on somatostatin analogues and 18 patients who were not (median 4755 pg/ml vs. 4500 pg/ml, p=0.82). Similarly for Ang-1 concentrations (median 40550 pg/ml vs. 33900 pg/ml, p=0.24).

4.3c Angiopoietin concentrations and stage of disease

Median Ang-2 concentrations were elevated in patients with metastatic disease compared to those with localised disease without distant metastases (median 5081 pg/ml vs. 3359 pg/ml, p=0.01); see Figure 4.2.The ratio of Ang-2 to Ang-1 was also somewhat higher in patients with distant metastases (0.147 vs. 0.105, p=0.019) (Figure 4.2).

From radiological assessment of CT or MRI scans performed within 2 months of blood sampling, visual assessments were made of the volume of liver metastases present. Four categories were created; localized disease without metastases, low volume liver metastases (involving <25% of liver), intermediate (involving 25-50% of liver) and large volume liver metastases (>50% of liver), see Figure 4.4.

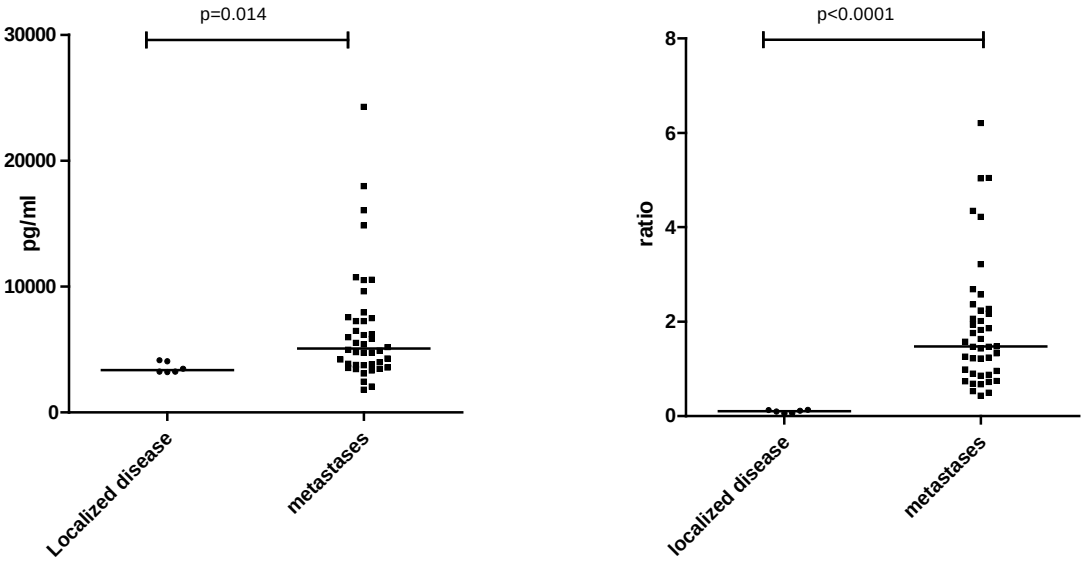


Figure 4.2. Comparison of serum Ang-2 concentrations (left figure) or the ratio of Ang-2 to Ang-1 (right figure) in patients with liver metastases (n=41 cases) and those with localised disease (n=6).

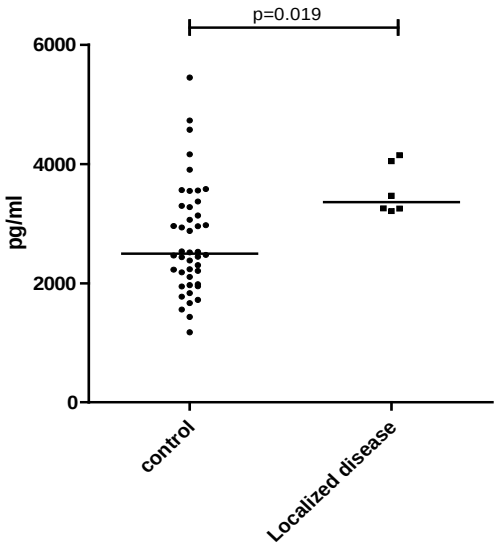


Figure 4.3. Serum Ang-2 concentrations in patients vs controls. Serum Ang-2 concentrations are significantly raised in patients with localized non-metastatic disease (n=6 cases) compared to healthy controls (n=44).

Ang-2 concentrations were significantly higher in patients with localised non-metastatic disease (n=6), compared to healthy controls (3360 vs. 2495 pg/ml, p=0.02) (Figure 4.3).

There was a statistically significant difference between the groups -when comparing localized disease, low, intermediate and large volume liver metastases- (p=0.014, one way ANOVA test), with a suggestion that increased Ang-2 is associated with increased tumour burden, Figure 4.4.

4.3d Serum Ang-2 as a marker of disease

ROC curves for serum Ang-2 were constructed to determine the cut-off values for specificity and sensitivity of Ang-2 and Ang-2: Ang-1 ratio. The area under the curve for serum Ang-2 was 0.88 and was greater than Ang-1 (0.53), (Figure 4.5). Detection rate, false positive rate and likelihood ratio for Ang-2 and the ratio of Ang-2 to Ang-1 are shown in Table 4.2. Serum Ang-2 was a better marker than the ratio, with a sensitivity of 85%, for a false positive rate of 22.7%. The chromogranin A assay has been previously validated and a cut off of 60 pmol/ml has been defined. Using this cut off in our study chromogranin A had a sensitivity of 80.9%.

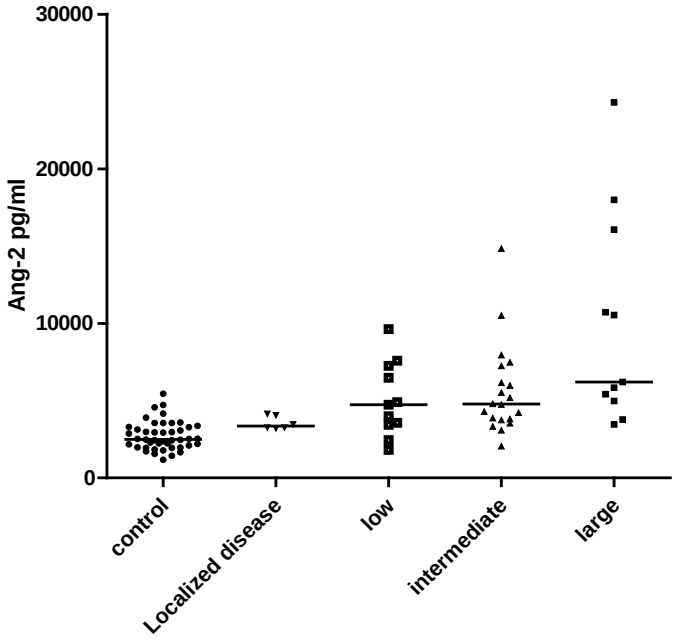


Figure 4.4. Serum Ang-2 concentrations in controls (n=44) and patients with different volumes of tumour load. Patients without metastatic disease (n=6) and those with low (<25% liver metastases) (n=11), intermediate (25-50% liver metastases) (n=19) and large volume (>50% liver metastases) (n=11) liver metastases.

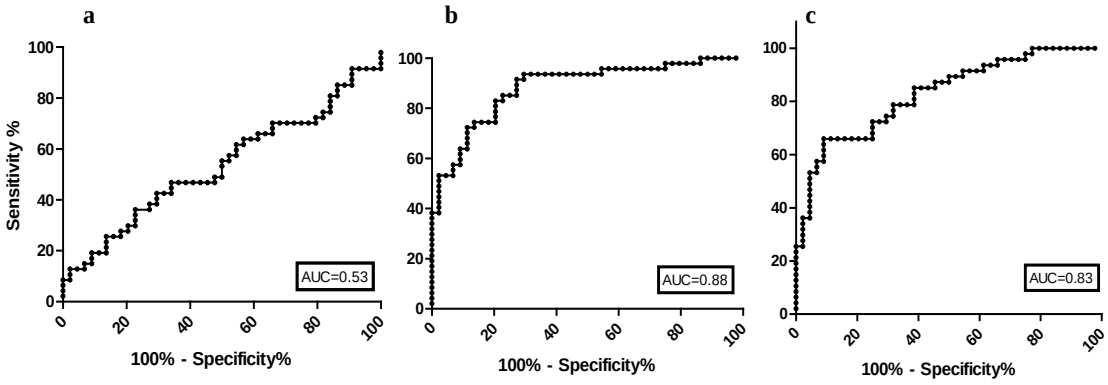


Figure 4.5. Receiver Operator Curves for Ang-1 (a), Ang-2 (b) and the ratio of Ang-2 to Ang-1 (c). AUC: area under the curve

Detection rate (sensitivity)	Ang-2				Angiopoeitin-2:1 ratio			
	Cut-off	FPR (%)	C.I (%)	L.R	Cut-off	FPR (%)	C.I (%)	L.R
70%	>3770	11.4	3.6-24.6	6.1	>0.095	25.0	13.2-40.3	2.8
75%	>3565	15.4	5.2-27.4	4.9	>0.088	31.8	18.6-47.6	2.4
80%	>3447	21.5	9.8-35.3	3.7	>0.074	38.6	24.4-55.6	2.1
85%	>3313	22.7	11.5-37.8	3.7	>0.071	38.4	24.4-52.2	2.3
90%	>3172	27.3	15.0-42.8	3.3	>0.064	55.6	38.8-69.4	1.7
95%	>2447	55.8	38.8-69.6	1.7	>.0527	65.9	50.1-79.6	1.5

Table 4.2. Detection rate (sensitivity), false positive rates and likelihood ratio for Ang-2 and the ratio of Ang-2 to Ang-1 in NETs. The cut-off values for Ang-2 are in pg/ml. FPR, is false positive rate; C.I, is confidence interval, L.R, is likelihood ratio.

4.3e Immunohistochemical staining

Staining for Ang-2 was identified in 5 of the 9 tumour samples. In all cases with positive Ang-2 staining, the corresponding serum Ang-2 concentrations were greater than the control population, see table 4.3. Of the 4 samples negative for Ang-2 staining the serum Ang-2 was raised above the >4756 pg/ml (median Ang-2 value for patient cohort) in 3 cases. The staining pattern was predominantly cytoplasmic, with clear tumour staining present. There was no staining of the background liver tissue and staining was localized only to tumour cells and endothelium (Figure 4.6). In one case there was strong staining in 10% of tumour cells metastatic to the gallbladder however only moderate 1% staining of the surrounding tumour in liver metastases, see Figure 4.6. Ang-2 staining was seen in low and intermediate grade tumours as well as pancreatic and midgut tumours. The

immunohistochemical studies demonstrate there is intra-tumoural variation in staining patterns for Ang-2.

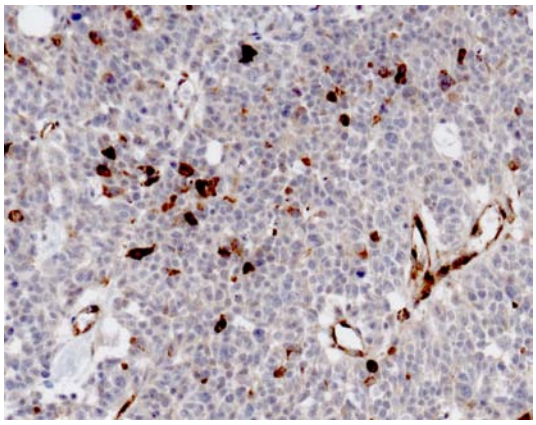
Primary	Grade	Functional	Biopsy site	Ang-2 (pg/ml)	Staining pattern
Midgut	Low grade	Yes	Liver	6800	Negative
Midgut	Low grade	Yes	Liver	13708	Moderate staining granular staining 10% and strong staining in cytoplasm 1% of cells
Midgut	Low grade	Yes	Liver	6205	Negative
Pancreas	Low grade	No	Pancreas	5983	Negative
Pancreas	Low grade	No	Liver	6152	Moderate staining 1% cells
Pancreas	Low	Yes	Liver	3815	Strong positivity in 1% cells
Pancreas	Intermediate	Yes	Liver	10540	Strong positivity in 1% cells
Pancreas	Low	No	Liver	3752	Strong positivity in 1% cells
Pancreas	Intermediate	No	Gallbladder/ liver	14846	5% strong positive in gallbladder tumour, 1% positive staining in liver

Table 4.3. Immunohistochemical staining for Ang-2 performed in 9 patients. Table shows site of primary, location and grade of tumour biopsy and staining pattern for Ang-2.

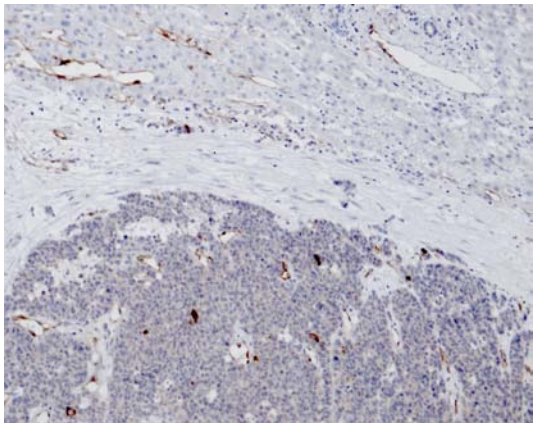
4.3f Survival data

Due to the indolent nature of these tumours, survival data alone is difficult to obtain (only 4 patients died during the 8 month follow-up). We therefore examined time to disease progression. Patients with progressive disease within 2 months prior to blood sampling were excluded from progression analysis. The median serum Ang-2 concentration in the patient group as a whole was used to divide the patients into two groups (cut-off value was 4756 pg/ml). The median follow-up period was 6 months, range 4-8 months. NET patients with serum Ang-2 concentrations >4756 pg/ml (n=14) had a worse prognosis than those with Ang-2 concentrations $\leq$4756 pg/ml (n=22),

$p<0.05$ (Figure 4.7). Both groups had similar previous treatments and a similar number of patients on somatostatin analogues (see Figure 4.7b).



A)



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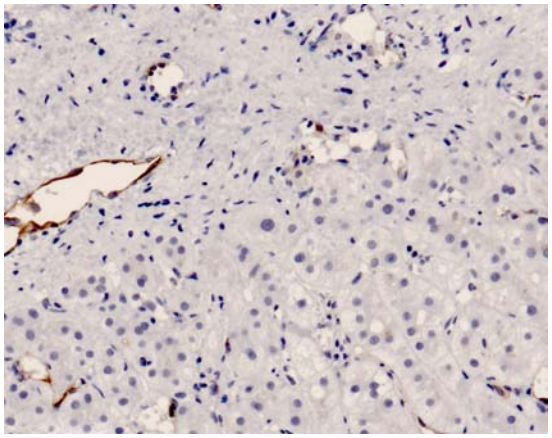


C)

Figure 4.6. A) Ang-2 staining in NET invading the gallbladder, shows strong Ang-2 positivity in tumour cells and endothelial cells (internal control), x20 magnification. B) the top part of the image is normal liver with negative Ang-2 staining, the lower part of the image is liver metastatic NET, with scattered Ag-2 positive staining, x10 magnification. C) Normal liver showing only endothelial positive staining for Ang-2, x20 magnification.

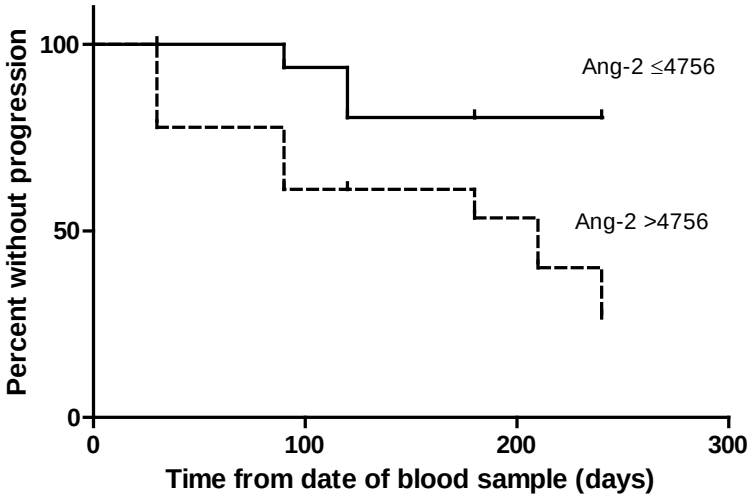


Figure 4.7a)

Therapy	**Ang-2 ≤4756 (n=22)**	**Ang-2 >4756 (n=14)**
Concurrent SST analogues	14 (63.6%)	8 (57.1%)
Prior Therapy		
Chemotherapy	2 (9.1%)	3 (21.4%)
Interferon	2 (9.1%)	0 (0%)
Radiotargeted therapy	5 (22.7%)	2 (14.3%)
Right hepatectomy	4 (18.2%)	1 (7.1%)
Other surgery	3 (13.6%)	5 (35.7%)
None	3 (13.6%)	1 (7.1%)

Figure 4.7b)

Figure 4.7. a) Kaplan-Meier curves of time to progression of NETs according to Ang-2 concentrations. Log rank p=0.03). Demonstrates significantly shorter time to progression for patients with an Ang-2 concentration of >4756pg/ml ($p<0.05$). b) Treatments undergone by patients in the two groups during the course of their illness and the number of patients on current somatostatin analogue therapy.

4.4 Discussion

This is the first study to demonstrate elevated serum Ang-2 concentrations and Ang-2: Ang-1 ratio in patients with NETs compared to healthy controls. Similar findings have been reported in a number of studies looking at serum Ang-2 concentrations in other

cancers including colonic, lung and ovarian cancer [192;206-209]. Furthermore, Ang-2 mRNA concentrations are elevated in the tumour tissue of a number of other cancers, including HCC, prostate cancer and breast cancer [204;207;210-214]. The findings suggest that Ang-2 was superior to Ang-1 for differentiating NET patients from control samples, and distinguishing patients with distant metastasis from those without and appears to increase in relation to increasing disease burden. In addition, our multivariate analysis suggests that high concentrations of Ang-2 are associated with higher grade tumours.

NETs often progress at a relatively slow rate; however, occasionally they may grow rapidly. During periods of growth, or progressive disease there may be a shift in proangiogenic factors including Ang-2. This could be a reflection on the biology and natural course of NETs. Interpretation of Ang-1 concentrations is difficult due to the wide range of values expressed and the complex behaviour of this growth factor. Interestingly high concentrations of Ang-1 were not correlated with increased Ang-2 concentrations. However, the higher ratio of Ang-2 is more commonly associated with high tumour load, i.e. >50% liver metastases rather than low volume disease or patients without metastasis. Our results support the hypothesis that elevation of Ang-2 being an important aspect of tumour angiogenesis.

This study has shown Ang-2 as a marker, with high sensitivity, however lacking specificity for use as a single marker for NETs since it is raised in a number of cancers and patients with cirrhosis. However, it could potentially have a role as an additional marker for monitoring development of recurrent disease or distant metastases, though, further validation of this marker will be required. Since CgA lacks sensitivity in cases with low tumour volume [29], Ang-2 concentrations could be useful since there was a significant difference in Ang-2 concentrations in controls and patients without metastatic

(i.e. low volume) disease, however, the number of patients with localized disease were small (n=6).

In our study, while Ang-1 did not have a significant prognostic implication, serum Ang-2 was more promising as a potential prognosticator; with concentrations >4756 pg/ml associated with earlier time to disease progression. The exact value for the cut-off will require prospective validation in another data set. Whether the concentration of serum Ang-2 is also a predictor of survival will require re-evaluation after prolonged follow-up.

Currently there are no accurate biochemical markers to aid in the diagnosis of progressive disease in patients with NETs. Even though CgA has been reported to have prognostic value especially in midgut carcinoid tumours it is not 100% sensitive [27]. Therefore, the use of Ang-2 in patients with NET could serve as a marker indicating likelihood of developing progressive disease.

There was no significant difference in Ang-2 concentrations between patients treated with SST analogues and those not. Since SST analogues are thought to inhibit angiogenesis through a number of pathways the lack of difference between the groups at first may seem surprising. However, there may be a number of reasons no difference is seen, most importantly is the heterogeneity of the groups. The tumour load of patients on somatostatin analogues is often greater than those not on SST analogue therapies, since these patients all have metastatic tumours and hence syndromic. We have demonstrated patients with large volume disease generally had high Ang-2 concentrations.

We have demonstrated by immunohistochemistry that a minority of NET cells strongly express Ang-2 in contrast to the normal liver. We did not find any clear correlation

between tumour staining and serum concentrations. There are three explanations for this; firstly there may be heterogeneity of expression within the tumour, as we have demonstrated clearly in one case, and one small sample many not be representative. Secondly the Ang-2 serum concentration appears to be related to tumour burden. Hence a small tumour with high Ang-2 expression my result in a lower serum concentration than a bulky metastatic tumour with a lower Ang-2 expression. Finally we cannot exclude the possibility that the Ang-2 arises from another source but its release is associated with tumour.

There were limitations with our study; firstly, the study incorporated a rather diverse group of patients including patients with NETs with a foregut, midgut and those with unknown primaries. Secondly the study group had a number of different previous treatments and some patients had stable disease without any evidence of radiological progression, whilst others had progressive disease at time of serum collection. This may in part explain the heterogeneity identified with Ang-1 and Ang-2 concentrations.

In conclusion, this is the first study reporting that serum Ang-2 is elevated in patients with NETS and that it may serve as a useful marker in NETs for monitoring and prognostication. A greater understanding of the role of Ang-1 and -2 in the pathogenesis of NETs may provide an opportunity for the development of effective therapy.

Chapter 5: Characterization of proteins from the plasma of neuroendocrine tumour patients using 2-D gel electrophoresis and tandem mass spectrometry

Abstract

Introduction: The purpose of this study was to develop techniques for identifying cancer biomarkers in human plasma using proteomics. 2-D gel electrophoresis is a recognized technique for identifying potential biomarkers in biological tissue.

Material and Methods: Plasma samples from five patients and five healthy controls were collected. Samples were fractionated using the immunoaffinity column. Protein concentrations of all fractionated samples were calculated using a Bradford assay. 2-D gel electrophoresis was performed and the gels fixed prior to staining with colloidal brilliant dye stain. Image analysis was performed using ProGenesis SameSpots software (non-linear dynamics). Proteins of interest were manually excised and analysed using LC-MS/MS. The proteins were identified using Mascot software.

Results: A total of 4153 protein spots were identified in the 10 gels. Following image analysis using ProGenesis Samespots software two protein spots of interest were identified between the patient and control gels. These were identified using LC/MS/MS as being albumin and pre-albumin. However, the albumin concentrations between the patients and controls were the same when concentrations were checked in serum.

Discussion: A large number of protein spots were present in each gel; however, the majority of these were the abundantly expressed proteins. Due to the limitations of 2-D

gel electrophoresis in identifying proteins expressed below 2.5-10 micromolar concentrations this technique may not be suitable for identifying proteins that are not abundantly expressed in plasma.

Experimental work

Sample collection, sample fractionation, 2-D gel electrophoresis was performed by R. Srirajaskanthan.

LC/MS/MS was performed by N. Beaumont.

5.1 Introduction

Neuroendocrine tumour cells synthesise peptides and amine products [215]. In addition to secreted hormones other proteins are secreted from these tumours. These secreted proteins are thought to have autocrine, paracrine and systemic effects, and may be involved in cell stabilization and oncogenesis [12]. A number of these proteins may be released into the systemic circulation and can be measured in sera or plasma, for example Chromogranin A, Chromogranin B, gastrin and pancreatic polypeptide [216]. There is a need for other accurate markers to aid in the identification of these tumours and aid in the management of these patients.

New proteomics technologies, notably development of more sensitive and accurate mass spectrometry (MS) techniques, have improved the ability to discover new disease biomarkers [217;218]. Plasma proteomics enables the identification of proteins present within plasma; this technology has been used successfully to identify markers in a variety of cancers [219;220]. A range of different proteomic techniques is available. These involve a variety of techniques each with its own merits. This study used 2-D gel electrophoresis and LC-MS/MS. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) [221], in which proteins are separated according to charge (pI) by isoelectric focusing (IEF) in the first dimension and according to size (molecular weight) by SDS-PAGE in the second dimension, has a capacity for the resolution of complex mixtures of proteins, permitting the simultaneous analysis of hundreds or even thousands of gene products [222].

A major problem in profiling plasma proteins by 2-D electrophoresis is that the 5 to 10 most abundant plasma proteins comprise over 85% of total serum protein [223;224]. The

proteins that are likely to be of interest are present at significantly lower concentrations. The most abundant serum proteins make it difficult to run 2-D gels reproducibly and also limit the amount of serum that can be loaded onto 2-D gels. They can also mask the differential expression of lower abundance proteins with similar molecular weights and iso-electric points [224]. To combat this problem the plasma samples are fractionated prior to loading to remove >90% of the 10 most abundant proteins.

The primary objective of this study was to identify either an individual or a panel of proteins that are present in the plasma of patients with metastatic neuroendocrine tumours. A pilot study focussed on patients with metastatic midgut carcinoid tumours between the age range 30-75. This pilot study was performed on 5 patients and 5 healthy controls. Once the proteins of interest were identified they will be validated by performing ELISA in the plasma of a large cohort of fifty pateints.

5.2 Materials and Methods

5.2a Study design

Plasma samples were obtained from thirty patients and twenty healthy controls. Controls were either partners of the patients, who had no co-morbidities or healthy volunteers. The control group had no history of cancer or other co-morbidities. The two groups were age and sex matched. For the pilot study to identify potential protein markers, 2-D electrophoresis was performed on the plasma of 5 patients and 5 healthy controls. Patient demographics of the control group are shown in Table 5.1. Protein spots of interest have been identified using LC/MS/MS and these proteins were validated in the plasma of the large cohort of 30 patients and 20 controls. The study was approved by the Local Ethical

Committee and all participants gave written informed consent prior to obtaining samples.

5.2b Plasma sample extraction

Thirty patients and twenty control subjects were fasted for a minimum of 6 hours following which they had blood samples taken. Venesection was performed by the same individual in all cases, using a 16G vacutainer system. The samples were then immediately placed in ice and centrifuged (3000rpm for 10 minutes) within 30 minutes of obtaining the sample. The plasma was aliquoted into eppendorfs and stored in a -80°C freezer.

5.2c Plasma fractionation

High abundance proteins were removed using the Aurum serum protein mini-kit (Bio-Rad). The Aurum serum protein mini kit contains columns filled with a mixture of Affi-Gel Blue and Affi-Gel protein A. This resin blend allows for the simultaneous removal of both albumin and immunoglobulin (Ig) from plasma samples. This column enables loading 60µl of plasma and removes over 90% of albumin and immunoglobulin.

Following washing the protein columns with buffer, and centrifuging the column to dry the resin bed, 60µl of plasma diluted in 180µl of buffer was added. This was repeatedly vortexed gently prior to centriguation. Further buffer was added following which the sample was centrifuged. The residual fractionated sample was then collected in a collection tube and aliquoted into eppendorfs prior to storage in -80°C freezer.

5.2d 1-D gel electrophoresis

Prior to performing 2-D gel electrophoresis a 1-D gel electrophoresis was performed to identify whether there was different protein expression (molecular weight) in 1-D. Sixty micrograms of fractionated plasma was added to 15 µl of sample buffer.; run on SDS-page gel over 16 hours. The gel was fixed in a solution of (methanol 20%, acetic acid 10%) for 1 hour, then stained in commasie blue for 24hours. The gels were extensively destained in a solution of 10% methanol for 24 hours, prior to being imaged using BioRad GS800 scanner (Figure 5.1).

5.2e 2-D gel electrophoresis and imaging

Protein concentration of all fractionated samples were calculated using a Bradford assay.

The fractionated plasma samples (160µl) were added to sample buffer (7M urea; 1 M thiourea; 4% chaps; 2% ampholytes; 50 Mm DTT) and passive hydrated into immobilized pH gradient (IPG) strips (11cm pH 5-8 IPG strips) over a period of 16 hours. The protein in the IPG strips was focused using an Amersham IPGphor apparatus for a minimum of 30,000 volt hours. Following IEF focusing the sample strips were equilibrated in a loading buffer (7M urea; 1M thiourea; 2% SDS) with 50 mM DTT for 15 minutes then equilibrated in buffer containing 50 mM iodoacetamide for a further 15 minutes. This procedure reduces any disulphide bonds that might cross-link proteins and then alkylation prevents re-oxidation of thiol groups during electrophoresis. The samples were then separated in the second dimension on 12% Tris-glycine gels, which were run overnight.

The gels were then immersed in a fixing solution (Methanol 40%, 10% acetic acid) for 1 hour. The samples were then stained in colloidal brilliant blue dye stain (Sigma) for

24 hours then replaced with fresh dye for a further 24 hours. The gels were extensively destained in 20% methanol and digitally imaged on a Bio-Rad GS800 scanner using PDQuest 7.3 software.

5.2f Image analysis

Spatial irreproducibility between 2-D gels by the conventional method of 2-D PAGE is an inherent problem. To help overcome this issue the images of the gels can be warped to enable them to be overlaid and analyzed using ProGenesis SameSpots software (Non-Linear Dynamics). Once these gel-images had been overlaid and warped the gels from the two groups were compared. Differences in optical density of less than two-fold can be identified using this software.

5.2g Analysis of proteins

Protein spots of interest were defined as spots that were consistently different between the two groups of gels as analyzed using ANOVA. These spots were then manually removed from the gel and proteins identified using LC/MS/MS. The protein spots were then destained and subsequently digested with trypsin (Promega Gold) in 100 mM ammonium bicarbonate. The samples were then dried using a SpeediVac, and proteins were extracted with sequential washes of 0.1% trifluoracetic acid (TFA); 10% acetonitrile in 0.1% TFA; 40% acetonitrile in 0.1% TFA. The extracted peptides (typically less than 40 µl) were then analyzed using LC-MS/MS. A data-determined acquisition was performed on MicroMass Q-ToF Micro (Waters) using ProteinLynx 4 software. Proteins were identified from the .pkl files using Mascot software (Matrix Science) [225]. Protein identifications were only accepted with a Mowse score in excess of 40, incorporating the identification of at least two peptides.

5.3 Results

The pilot group consisted of 5 patients average age 49 (range40-64). There were three males and two females. All patients had midgut neuroendocrine tumours with large volume (>25% of liver volume) liver metastases. The 1-D gel showed no difference in protein bands between the patients and control groups (Figure 5.1).

	Patient	Control
Age (median) in yrs	49	45
Range in yrs	40-64	29-69
Males	3	3
Females	2	2
years since diagnosis	5 (3-8)	
Chromogranin >60pg/ml	5	
somatostatin analogues	5	
Previous surgery	1	

Table 5.1. Demographic data of patient and control group used in pilot study.

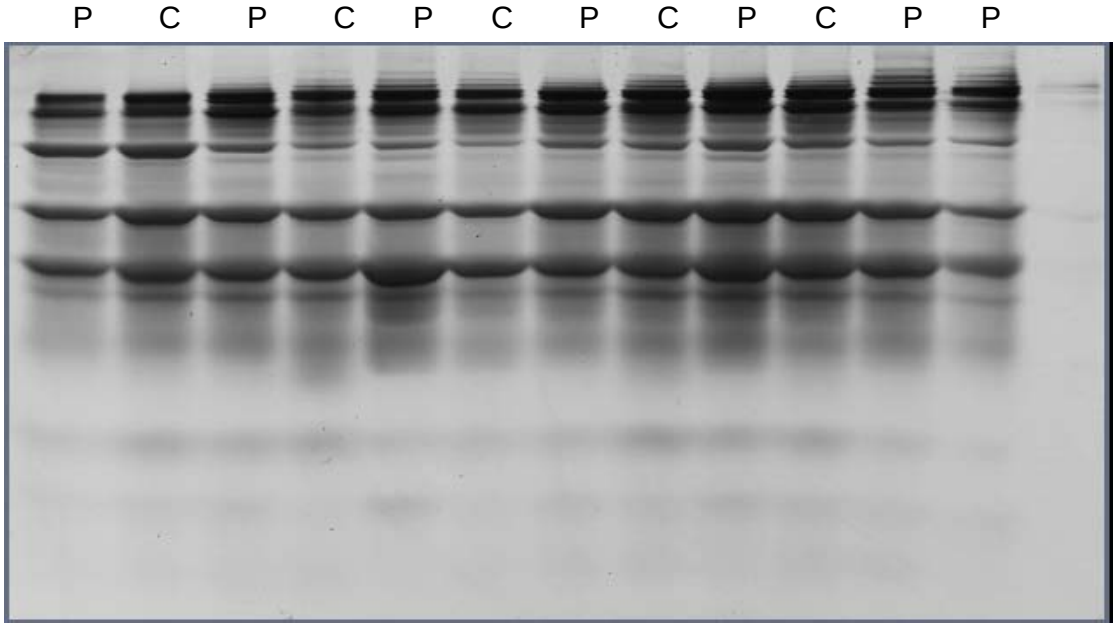
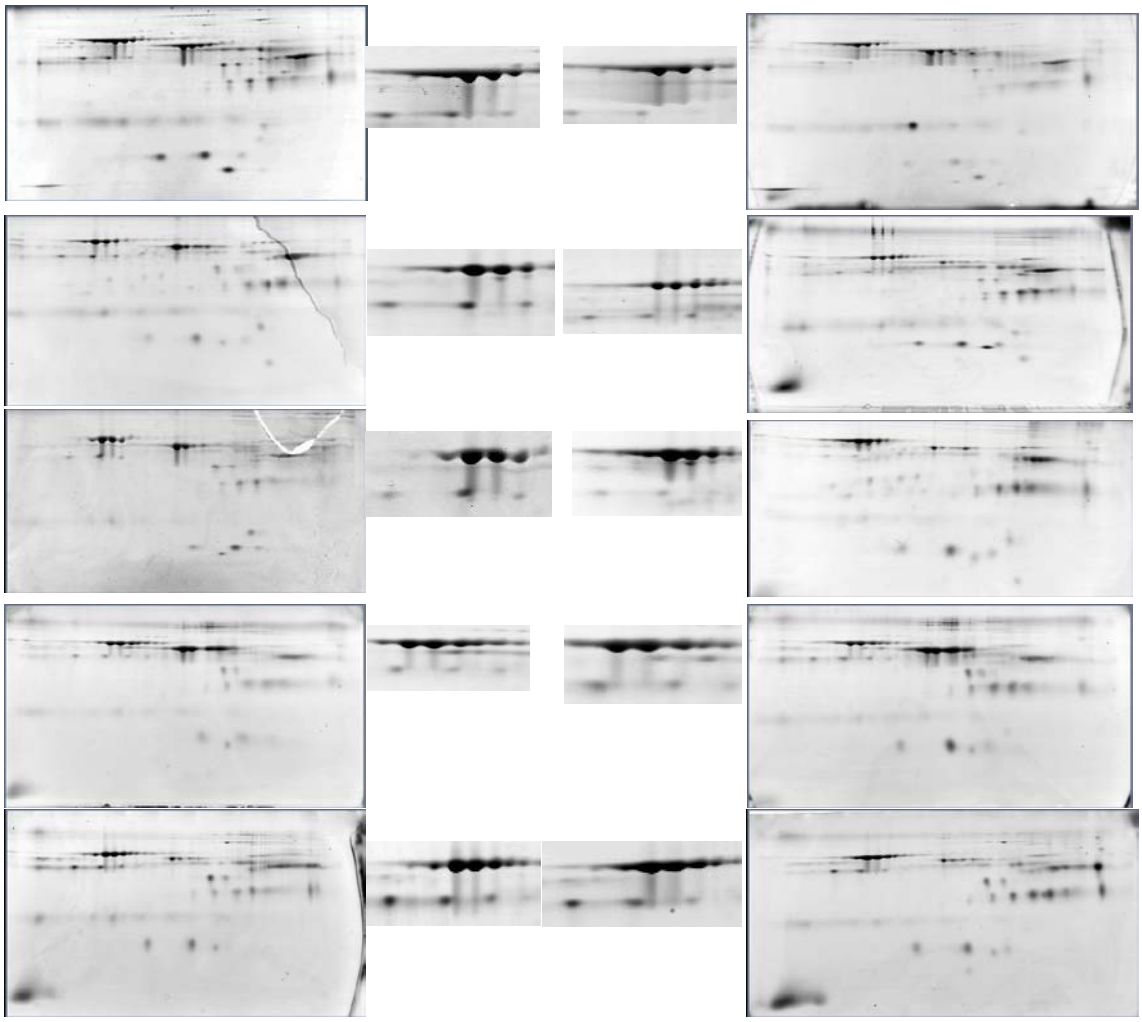


Figure 5. 1. SDS-PAGE of 6 patients and 5 controls. 1-D electrophoresis from 6 patients (labelled P) and 5 control samples (labelled C). No difference in proteins bands identified between the two groups, when analysed using PQ quest software.

Over four hundred protein spots were identified in each gel when analysed with SameSpots software. The total number of protein spots in all the pilot gels was 4153. Figure 5.2, illustrates the 2-D gel images from the patients and control samples. Following image analysis using Samespots software, three protein spots of interest were identified (see Figure 5.3). However, upon closer inspection of the gels, one of these spots were actually related to protein trains left by the original protein spot, rather than separate proteins. The remaining two spots were upregulated in patient groups. These protein spots were excised and identified using LC/MS/MS. The two proteins were identified as being albumin and pre-albumin. Upon reviewing the patients biochemical data there was no difference in the albumin concentrations in the sera of the patients in the two groups.



PATIENTS CONTROLS

Figure 5. 2. 2-D gel electrophoresis gels from the pilot study. Shows the gels of 5 patients and 5 controls from the initial pilot study. The proteins of interest are shown in the enlarged image next to each gel.

Due to the lack of significant proteins expressed in the pilot study, the following alterations were made to the experimental protocol:

1. Increase in the size of pilot group to 10 patients all with metastatic midgut NETs. This did not identify any new proteins, however, it did not demonstrate the increased expression of pre-albumin or albumin identified in the original pilot study.
2. Increase in protein load to 180µl, then 200µl to the IEF strip. This led to the loss of separation of proteins in the 1-D dimension and produced poorer quality gels.
3. Decreased protein load 140µl and 120µl to the IEF strip, led to similar gels seen in the initial pilot study, however, it identified a weaker signal from expressed proteins and no new protein spots.
4. Different pH strips for IEF focussing, pH 3-10 and pH 7-10 were used to see if increased proteins were identified using a broader pH range (pH 3-10) strips. This however, led to poor separation of proteins which were predominantly expressed in the pH 5-8 range (see Figure 5.4).
5. Different staining techniques instead of colloidal brilliant blue were trialled, including deep purple and florescent pink, however, these did not lead to increased visualization of less abundant proteins (see Figure 5.5).

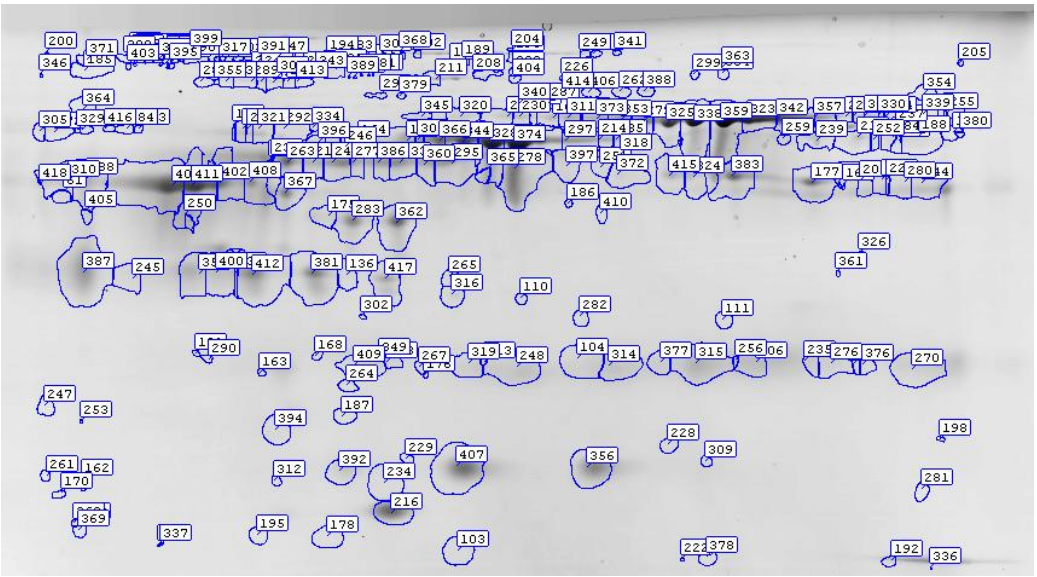
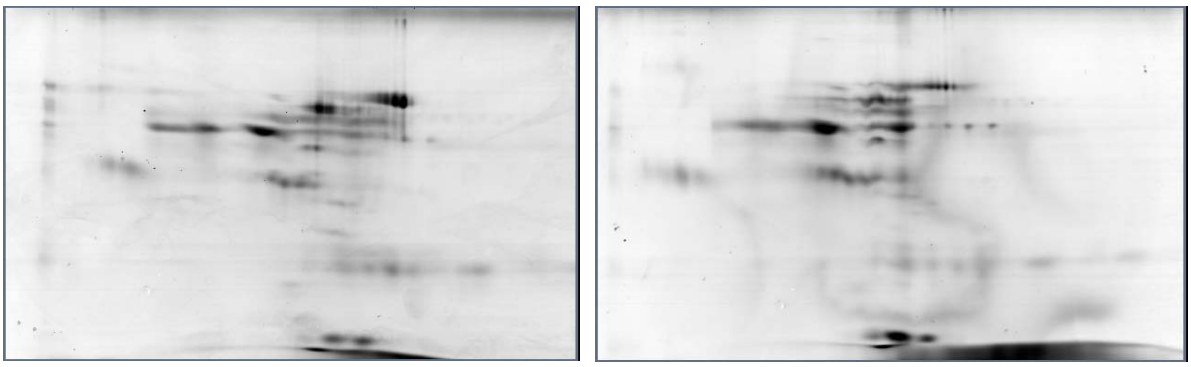


Figure 5. 3. Illustrates the protein spots and areas of interest identified using the SameSpots software. These protein spots are then compared to the other 11 gels to find areas of interest.



control patient

Figure 5. 4. 2-D electrophoresis gels, the first dimension was performed using pH 3-10 strips. Most proteins are located within the pH range of 5-8. No difference in protein expression was identified between patient and control groups.

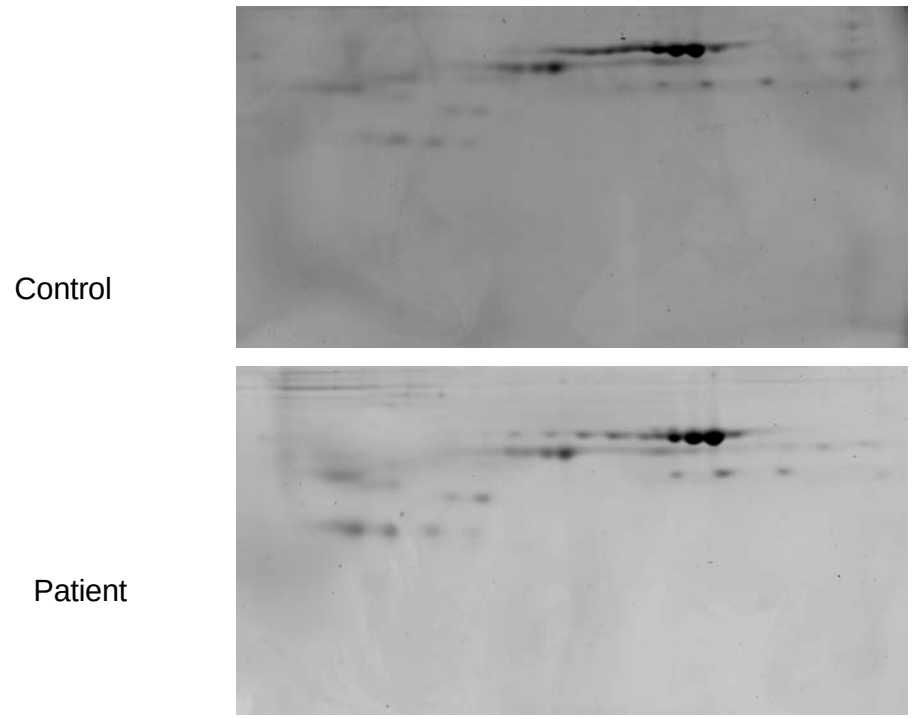


Figure 5. 5. Deep purple staining of 2-D gel electrophoresis. Shows the staining obtained using deep purple stain, an alternative staining agent to commasie blue.

5.4 Discussion

In total 4153 protein spots were identified in the pilot study, of which three were differentially expressed in the patient group when compared to the control group. Identification of these three proteins showed that the spots were albumin and pre-albumin. The likely cause for this difference is related to a variation in albumin expression which occurs between gel samples. No other differences in protein spot expression between healthy controls and patients with neuroendocrine tumours were identified. It is known that chromogranin A is raised in the plasma of patients with NETs. The concentration of expression of this protein in some of our patients was

>1000picomolar. Yet due to limitations of the 2-D technique we were unable to identify chromogranin A expression in plasma.

Upon reviewing the literature there are a number of studies identifying novel biomarkers in a number of different cancers. The most commonly used technique is high-throughput analysis using SELDI [226]. This system has limitations with reproducibility or sensitivity and does not identify specific proteins [227;228]. 2-D electrophoresis with LC/MS/MS to identify novel protein markers in cancer has been reported in a number of studies [229]. Annexin-1 is one marker of prostate cancer that has been well validated following identification using 2-D gel electrophoresis. Studies in pancreatic, lung and ovarian cancer have identified other markers which may be useful prognostic indicators [230]. To date there are no studies describing identification of protein markers in the plasma of patients with NETs.

Plasma is an attractive biological fluid in which to attempt to identify biomarkers since obtaining it is minimally invasive and it is relatively abundant. Unfortunately there are a number of limitations to 2-D electrophoresis which have been previously described [231;232]. Firstly, the technique is unable to identify proteins which are below the threshold of detection, which is 2.5-10micromolar. Plasma is known to have between a seven to ten fold difference in concentration of expression between the least and most abundant proteins. Therefore this difference of expression is difficult to be seen using 2-D gel electrophoresis [233]. This was evidenced by the fact that even following fractionation the most heavily expressed proteins were albumin, pre-albumin and immunoglobulin. This patient to patient variation is a common problem, which makes the correct validation of these proteins so important. This variability could be explained by the fact albumin and pre-albumin were identified as being increased in the patient group. Alternatively,

differences in effectiveness of albumin depletion with the fractionation kit, could explain the findings.

To improve the resolution of protein spots different staining methods were trialled, including florescent staining. However, these did not provide any improvement in gel resolution. To ensure that proteins expressed outside of the pH 5-8 range were not excluded, gels were performed using broad range pH strips 3-11 and also pH strips 7-11. The resultant gels did not express as many proteins of interest and again there was no difference between controls and patients.

Further work is needed to examine whether removal of the 50 most highly expressed proteins enables identification of less commonly found proteins. However, whether this will resolve the issue which remains regarding the wide range of level of expression remains to be seen. Due to the technical difficulties and complexities of plasma, the use of tumour tissue may enable identification of new markers. There are a number of studies which have successfully identified new biomarkers in tumour tissue from a variety of cancers. Some of the proteins identified from tumour tissue have been identified in serum and consequently validated as serum biomarkers.

In conclusion plasma proteomic analysis using 2-D gel electrophoresis has numerous practical limitations and whilst removal of more abundant proteins may potentially enable less abundant proteins to be identified the use of other tissues or cell lines may provide a better way to identify potential biomarkers.

Chapter 6: Novel biomarkers for Neuroendocrine tumours identified from analysis of proteomes secreted from NET cell lines.

Abstract

Introduction: Neuroendocrine tumours display diverse tumour biology; however, most secrete into the circulation peptides consistent with their neuroendocrine origin, such as chromogranin A. This study sought to identify other potential markers for NETs by analyzing the secreted proteomes of three neuroendocrine cell lines.

Methods: BON-1, NCI-H727 and SHP-77 cells were grown in serum-free media, and the secreted proteins were separated by SDS-PAGE and identified by LC-MS/MS. Validation studies of potential markers involved western blotting of NET cell lines, immunohistochemistry of NET tumour samples and serum ELISA of NET patients and healthy controls.

Results: Two hundred and five proteins were identified from the 3cell lines, of which 61 were secreted by two or more of the cell lines and 19 by all three lines. Mac-2 binding protein (Mac-2BP) was found to be secreted by all three cell lines, and this was confirmed by Western blotting. Immunohistochemical analysis found 29 of 33 NET cases, from different primary sites, to be positive for Mac-2BP. Serum Mac-2BP was significantly elevated in NET patients, compared with healthy controls ($p<0.001$).

Discussion: In this study, it is shown that analysis of the secreted proteomes of neuroendocrine cell lines can identify potential biomarkers for NETs. Mac-2BP may

have a role in the pathophysiology of NETs and may potentially be a biomarker for use in the diagnosis and management of this disease.

Experimental work

The sample collection, sample fractionation, 2-D gel electrophoresis, western blotting, serum ELISA and immunohistochemistry was performed by R. Srirajaskanthan. LC/MS/MS and western blotting was performed by N. Beaumont.

6.1 Introduction

The incidence of neuroendocrine tumours (NETs) is 2-5 per 100,000, although recent epidemiological data suggests this is rising [8;154]. The five year survival rate for patients with midgut metastatic disease is presently 40% [234].

NETs most commonly arise from the gastroenteropancreatic system, however they can originate in other organs [216;234]. NETs of the gut are thought to arise from cells of the diffuse endocrine system, which are characterized by the secretion of a variety of hormonal peptides and other bioactive molecules. Chromogranin A (CgA) is the biochemical marker in the circulation currently used for monitoring and screening of NETs [12]. However, this marker is neither 100% specific nor sensitive, especially for patients with low volume disease [29]. Further markers are needed to aid the screening and management of NETs.

Proteins from cancer cells are secreted into the circulation. The serum concentrations of signature proteins may increase in particular cancers, and correlate with cancer progression and proliferation. Consequently, secreted proteins are important as serum biomarkers for some cancers. Examples include carcinoembryonic antigen (CEA) for colon cancer, CA-125 for ovarian cancer [235] and prostate-specific antigen for prostate cancer [236].

A number of studies have investigated the secreted proteomes of cultured cells in the search for marker proteins of different [237;238]. This approach is based upon the assumption that the proteins secreted by the cell line will be representative of tumor cells *in vivo*. Studies have been performed using cell line models of prostate cancer, breast

cancer [237] and head and neck cancer [239]. Some putative markers were subsequently validated using immunological methods on limited numbers of clinical samples. However, some of these markers did not prove to be consistently linked with the disease when studied in additional clinical samples [237]. This strategy appears to be justified for NET because these cells are known to secrete biologically-active amines and proteins into the circulation.

The aim of this study was to identify putative protein markers, particularly those that could indicate the metastatic spread of NETs. We have analyzed the secreted proteomes of three neuroendocrine cell lines and assessed a new putative NET marker using patient tissue and serum samples.

6.2 Materials and Methods

6.2a Cell lines

BON-1, a pancreatic neuroendocrine cell line, and two neuroendocrine lung cancer cell lines (NCI-H727 and SHP-77) were used. BON-1 cells were maintained in Dulbecco's modified Eagle's medium/ F-12 medium (1:1) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. NCI-H727 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin and streptomycin. SHP-727 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. All cells were cultured in 75cm^2 flasks in a humidified incubator at 37°C and 5% CO_2.

6.2b Cell culture

Approximately 10^6 cells per cell line were seeded into 75 cm^2 tissue culture flasks. After approximately 48 hours when more than 80% confluent, the media were removed. Monolayers were washed in serum-free media and then incubated with 8 ml of serum-free media. After 24 hours, the conditioned media (CM) were collected. Trypan Blue exclusion indicated >99% viability of the attached cells. Media with 10% FBS was added to the flasks and incubated for a further 48 hours to ensure the attached cells were still viable. The CM were centrifuged at 2000 x *g* for 5 min to remove any cell debris and concentrated using a 10 kDa Centricon Mini-Prep (Millipore) to 200 µl. Concentrated CM samples were aliquoted and stored at -80°C.

6.2c SDS-PAGE and digestion

Samples of 30 µl of concentrated CM were fortified to 50 mM DTT and 10 µl of 4 x Laemmli buffer were added. Samples were separated by SDS-PAGE using 12% Tris-glycine gels. Samples were stained in colloidal brilliant blue dye (Sigma) for 24 hours, then with fresh dye for a further 24 hours. Gels were extensively destained in water and digitally imaged on a Bio-Rad GS800 scanner using Quantity One software (Biorad). Gels were reduced in 50 mM DTT for one hour and then alkylated in 30 mM iodoacetamide for one hour. Each lane of proteins in the gel was cut into 50 bands. These were dried *in vacuo* prior to digestion with alkylated trypsin (Promega) overnight at 37°C in 30 mM ammonium bicarbonate. The resulting mixture of peptides was extracted in 20% acetonitrile containing 0.1% trifluoroacetic acid.

6.2d Mass spectrometry

Peptides were separated by reverse-phase HPLC: a 45-min linear gradient was developed from 3% to 50% acetonitrile in 0.1% formic acid on a C18 column (Dionex PepMap100, 100mm x 75 µm) using an Ultimate 3000 system (Dionex). Data-determined acquisition was performed on a MicroMass Q-ToF Micro (Waters) using ProteinLynx 4 software. Peak lists were generated by the MassLynx 4.0 PeptideAuto program using default parameters, and used by Mascot 2.205 (Matrix Science, UK) to interrogate the human sequences (148148 sequences) of the MSDB 20060831 non-redundant sequence database annotated 31st August 2006 [225]. Search parameters allowed not more than a single missed cleavage site, all cysteine residues to be modified by carbamidomethylation, and variable oxidation of methionine residues. Precursor and fragment ion mass tolerance were set to 1.2 and 0.6 Da respectively. Peptide identity

were accepted provided that their Mowse scores were above 38 ($p < 0.05$). The assignments were manually verified by checking that there were several consecutive y or b-ions. Proteins were accepted on the basis of the combined scores from more than one peptide ion; except for one protein identification which was accepted on the basis of data from a single peptide because it had eight out of ten consecutive y-ions.

6.2e Analysis of identified proteins

Mascot accession numbers were uploaded as CSV files to Protein Centre software (Proxeon) for the analysis of identified proteins. This software uses a knowledge base derived from the published literature to relate gene products to each other based on their interaction and function. The cellular localization of proteins in each sample was identified and examined in greater detail for proteins expressed in two or more cell lines.

6.2f Validation of proteins

For validation of possible marker proteins the following assessment was performed: western blot analysis of the CM was performed to confirm that the correct protein had been identified, immunohistochemical analysis was performed on sections prepared from paraffin embedded NET tissue, and serum analysis was performed on samples prepared from a cohort of NET patients and controls.

6.2g Western blotting

Samples of 30 µl of concentrated CM were separated by SDS-PAGE; proteins were transferred to PVDF membranes (Invitrogen iBlot) and soaked in blocking solution (5% (w/v) skimmed milk powder in TBS-T (20mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1%

(v/v) Tween-20) for 2 hours at room temperature. One membrane was incubated with 1% (w/v) skimmed milk powder in TBS-T, whilst a duplicate was incubated in the same buffer incorporating 1.5 µg/ml anti-Mac-2BP polyclonal mouse antibody (Bender Systems). Both membranes were incubated for 16 hours at 4°C. Membranes were washed with TBS-T three times for 10 min each and subsequently incubated in horseradish peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1: 5,000 in blocking solution) for 120 min at room temperature. After another three washes in TBS-T, immuno-reactive bands were detected using the enhanced chemi-luminescence reaction (ECL, General Electric).

6.2h Immunohistochemistry

The study population included all major NET subtypes including: 9 foregut, 16 mid-gut, 2 paragangliomas and 6 NETs of unknown primary. Sections of 3 µm of tumor tissue were de-waxed three times in xylene and rehydrated in ethanol. Slides were submersed in 10mM citric acid (pH 6.0) for 2 min in a pressure cooker, cooled immediately in water, and transferred to fresh water at room temperature for 5 min. All slides were blocked in normal horse serum containing avidin and biotin for 40 min, before incubation with anti-Mac-2BP rabbit polyclonal antibody (Bender Systems) at a dilution of 1:400 for one hour at room temperature. Biotinylated secondary antibody was incubated with slides for 30min. Bound antibody was visualized using a DAB peroxidase substrate kit. Sections were counterstained with Mayer's haematoxylin for 3.5 min. As Mac-2BP expression is upregulated in gastric carcinomas [240], these were selected as positive controls. Negative controls included substitution of the primary antibody with normal horse serum.

6.2i Tumor classification

Tumours were classified according to their site of origin, degree of differentiation and initial mitotic index. Two examiners (R.S. and J.W.) independently interpreted each immunohistological result. Discordances were jointly reviewed to reach agreement or determine an average value for disputed sections. Slides were scored on the basis of intensity of staining whereby 0 = negative, 1 = weakly positive, 2 = moderate, 3 = strongly positive. The extent of tumor staining was also scored, whereby 10 random high power fields were assessed to determine the average percentage of positive staining cells in which: 1 = <25%, 2 = 25 - 75% and 3 = >75%. The product of the density of staining and the percentage of tumor cells staining positive was used as the histological score, giving final values of 0,1,2,3,4,6,9 [181].

6.2j Blood analysis

For Mac-2BP, serum was obtained from 47 patients with proven diagnosis of NETs and 24 healthy controls. Control subjects had no history of cancer and were age and sex matched. The study was approved by the Local Ethics Committee and all participants gave written informed consent prior to obtaining samples. The cohort of NET patients included 3 paragangliomas, 15 pancreatic, 25 small bowel, 1 large bowel and 3 bronchial NETs. Serological measurement of Mac-2BP was performed using an enzyme linked immunosorbance assay (ELISA), from Bender Systems.

CgA analysis was performed on blood samples from patients using a radio-immuno assay (RIA) kit (Roche). As this assay has previously been validated and is currently used in clinical practice, no samples were run on normal healthy controls.

6.2k Statistical analysis

Statistical software (Graph Pad Prism Software, San Diego, CA) was used for the analysis. All values are given as median (plus range). As the values did not fit a standard distribution non-parametric analysis was performed. The Mann-Whitney *U* test was used to compare patients and control groups, and the Kruskal-Wallis test was used to compare multiple groups. Spearman correlation of rank coefficient was used to analyze correlations between parameters. Spearman correlation was also used to analyze correlation between Mac-2BP immunohistochemistry and other parameters.

6.3 Results

6.3a Identification of CM proteins using SDS-PAGE and mass spectrometry

Using similar amounts of total protein from each CM (Figure 6.1), and after contaminants including porcine trypsin, and major human hair and skin keratins had been excluded, a total of 205 different proteins were identified in CM preparations from the three cell lines. 82 proteins were identified in the BON-1 CM, 102 proteins from NCI-H727 CM, and 101 from SHP-77 CM. 42 proteins were identified in the CM from two lines only, while 19 proteins were identified in the CM from all three cell lines (Figure 6.2).

Sixty one proteins were present in CM preparations from two or more cell lines (Table 6.1), and 24 of these are known to be extracellular and not permanently bound to cells. The number of unique peptides identified from each of these proteins is also shown. All of the 205 proteins were identified from two or more unique peptide identifications, range 2-20 (mean 6). For the 61 proteins in Table 6.1 the ion score range was from 37 to 106 (mean 67), and the Mascot score ranged from 40 - 421 (mean 102).

Reference Protein Accession	Protein	Cell line	Loc'n	Mass	Score	% coverage	No. peptides	Ion score
NP_033562	14-3-3 protein epsilon	BNS	C	29043	171	34	12	67
NP_006817	14-3-3 protein theta/tau	NS	C	27633	129	13	4	101
NP_663723	14-3-3 protein zeta/delta	BS	C	27614	73	12	3	60
NP_001605	Actin gamma	BNS	C	41662	151	5	6	106
NP_006399	AGR2	BS	E	19848	46	14	4	37
NP_000286	Alpha-1-antitrypsin	NS	E	46737	49	4	3	41
NP_001624	**Alpha-1-microglobulin**	BS	E	38999	77	5	4	56
NP_001123476	Alpha-actinin 1	BNS	C	105438	90	6	6	80
NP_004915	Alpha-actinin-4	BNS	C	104723	90	6	9	80
NP_000475	Alzheimer's disease amyloid beta protein	BS	M,E	86944	141	11	12	75
NP_001002858	Annexin A2	BS	C,E	40280	70	7	4	68
NP_001145	Annexin A5	BNS	C,E	35806	55	8	5	56
NP_004351	**Cadherin 1**	BS	M,E	97456	212	8	13	67
NP_001009566	Calsyntenin 1	BNS	M,E	109662	135	6	9	89
NP_006126	CapZ alpha-1	BN	C	32792	87	14	5	64
NP_001864	**Carboxypeptidase E**	BNS	E	53151	115	13	8	50
NP_001279	Chloride intracellular channel protein 1	NS	M	26792	63	27	9	63
NP_001266	**Chromogranin A**	BNS	E	50688	95	3	4	64
NP_001810	Chromogranin B (secretogranin 1)	BNS	E	78276	72	5	5	72
NP_003460	Chromogranin C (secretogranin 2)	BNS	E	70810	97	7	5	81
NP_005498	Cofilin-1	BS	C	18371	94	27	7	52
NP_001814	Creatine kinase B	BN	C	42513	120	8	2	90
NP_000090	**Cystatin C**	BS	E	15749	128	19	3	72
NP_001952	Elongation factor 2 (EF-2)	NS	C	95207	92	3	2	93
NP_001419	Enolase, alpha	BNS	C	47038	97	4	2	97
NP_001967	Enolase, beta	NS	C	46801	167	10	6	100
NP_001966	Enolase, gamma	BNS	C	47138	84	7	4	84
NP_000025	Fructose-bisphosphate	BNS	C	39289	49	9	2	49

	aldolase							
NP_000166	Glucose-6-phosphate isomerase	NS	C,E	63016	103	7	6	66
NP_036545	Glutaminyl-peptide cyclotransferase	BS	C	40887	87	25	6	56
NP_002037	Glyceraldehyde-3-phosphate dehydrogenase	BNS	C	35922	49	6	4	49
NP_004855	Growth differentiation factor 15	BS	E	34009	90	10	6	86
NP_002131	Heterogeneous nuclear ribonucleoprotein K transcript variant			50897	63	22	8	58
NP_005336 NP_002146 NP_006588	Hsp 70 protein 1A/6/8	NS	C	69921 70897 70767	193	27	16	69
NP_005338	Hsp 70 protein 5	NS	C	72202	133	14	9	79
NP_003290	Hsp 90 beta 1 (endoplasmin)	NS	C	92469	131	14	10	61
NP_031381	HSP90AB1 protein	BNS	C	83133	65	6	5	61
NP_001017963	HSP90AA1 protein	BNS	C	98030	421	25	20	99
NP_002256	Importin beta, chain B	NS	C	97040	40	3	1	40
NP_002207	Inter-alpha-trypsin inhibitor heavy chain 2	BS	E	106333	102	15	10	69
NP_002264	Keratin 8	BS	C	53573	71	6	3	61
NP_002291	L-lactate dehydrogenase, chain H	NS	C	36507	54	17	7	49
NP_005557	L-lactate dehydrogenase, chain M	BS	C	36558	126	23	12	69
NP_005558	**Mac-2-binding protein**	BNS	E	65200	114	8	8	47
NP_005908	Malate dehydrogenase	BN	C	36295	155	29	11	64
NP_002108	MHC class I antigen; HLA-C	BS	M	40649	50	13	3	50
NP_002406	MIF	BS	E	12345	76	9	3	52
NP_006175	Nucleobindin 1	NS	C	53748	77	2	2	77
NP_002565	Peroxiredoxin 1	BS	C	21979	67	18	3	50
NP_005800	Peroxiredoxin-2	BS	C	21761	45	5	2	45
NP_005013	Profilin	BS	C	14923	209	23	4	90
NP_000468	**Serum albumin**	BNS	E	69368	115	3	2	68

NP_006746	TALDO1	NS	C	37409	56	8	6	46
NP_003237	Thrombospondin 1	BS	E	129383	208	8	12	70
NP_003245	TIMP1	BS	E	23189	50	5	2	50
NP_000356	Triosephosphate isomerase	BS	C	26538	89	11	7	70
NP_002760	**Trypsin (PRSS1)**	BS	E	26558	43	12	4	43
NP_003339	Ubiquitin-protein ligase E2N	BS	C	17007	64	13	2	65
NP_079493	**UL16 binding protein 2**	BS	M,E	27237	51	11	4	51
NP_009057	VCP	NS	C	89191	51	18	11	47
NP_003369	**VGF, NGF-inducible**	BNS	E	67275	152	22	18	97

Table 6. 1. Proteins secreted by two or more cell lines.61 proteins were identified from two or more cell lines. Proteins found reproducibly in three BON-1 CM analyses are shown in bold. Cellular location (Loc'n) was identified using Protein Center and extensive literature searches (C, cytoplasmic; E, extracellular and not constitutively membrane-bound; M, membrane-bound); in many cases more than one cellular location has been reported for proteins. Cell lines from which proteins were identified are indicated (B, BON-1; N, NCI-H727; S, SHP-77). Reference Protein Accession numbers are from the NCBI Protein database (National Center for Biotechnology Information, USA); Mass is the predicted mass of the precursor in Daltons; Score is the Mowse score; Ion score is the highest Mowse score for individual peptides.

Of the 61 proteins identified in CM preparations from two or more cell lines, Chromogranins A, B and C were detected in the CM from all three cell lines. CgA secretion is consistent with previous studies which had demonstrated that this protein is secreted by all three cell lines [237;241-244]. Chromogranin A was used as the positive controls for the analysis of each preparation and each cell line during this investigation.

The reproducibility of each protein identified in a secreted proteome was assessed using the BON-1 cell line: repetition of the entire procedure demonstrated that 81% of the 82 BON-1 proteins in Table 6.1 could be reproducibly identified. However when approx.

50% less protein was analyzed in a third series of experiments, only 46% of the initial set of proteins could be identified. In addition to establishing the importance of sample loading, variation in the presence of cytosolic, membrane and extracellular matrix (ECM) proteins was also apparent in different CM preparations, indicating empirical variations in cell detachment and incorporation of ECM.

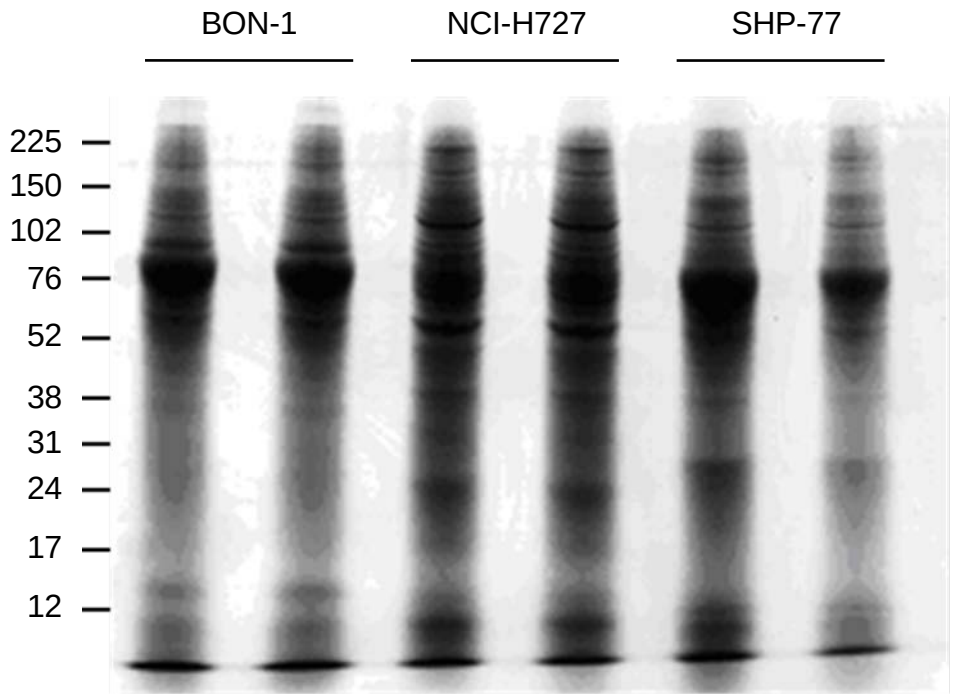
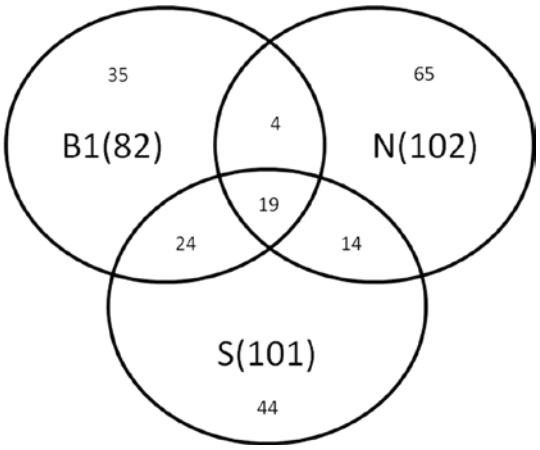


Figure 6. 1. SDS-PAGE separation of CM preparations. Duplicate aliquots of CM from each cell line were fractionated by SDS-PAGE and stained with Coomassie Blue. Marker protein positions and molecular weights (kDa) are indicated.

A



B

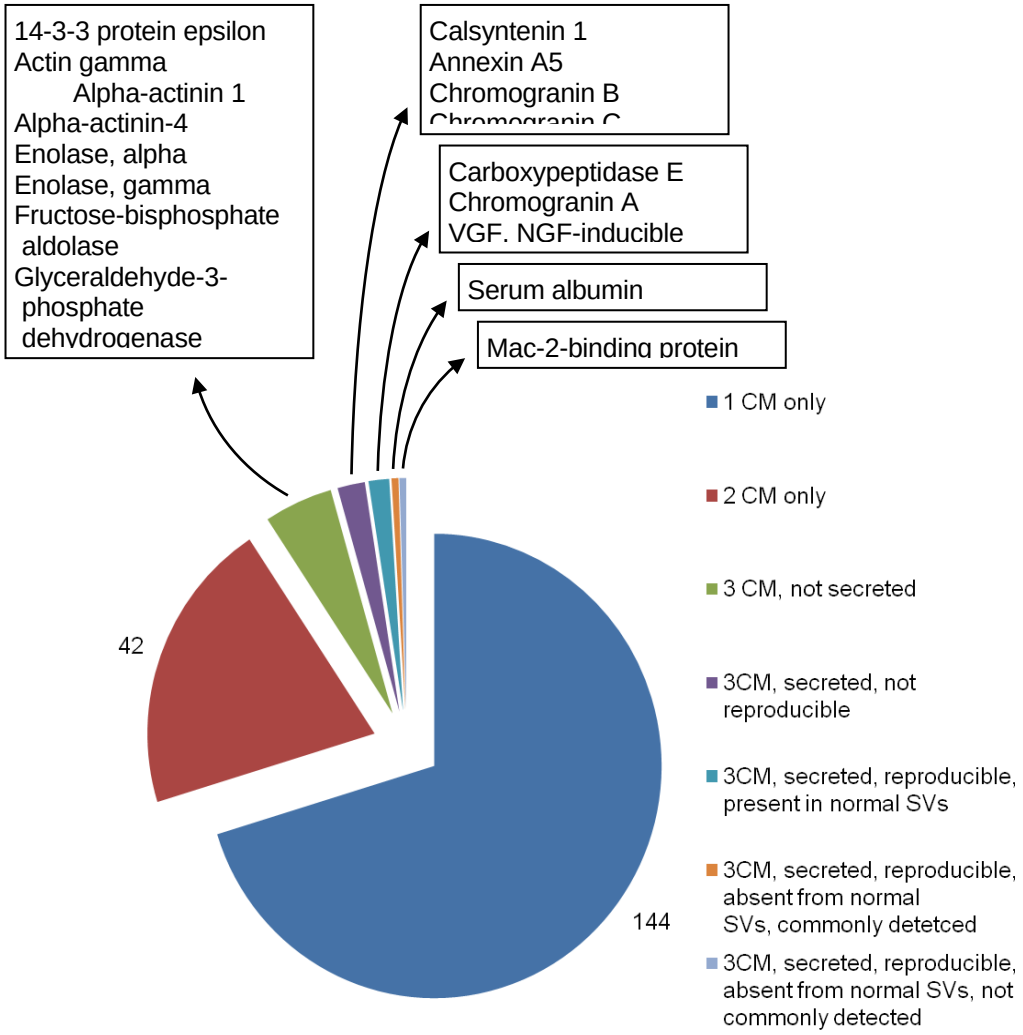


Figure 6. 2. Distribution of proteins secreted by three neuroendocrine cell lines. (A) Venn diagram showing in parentheses the total number of different proteins identified in the CM for each cell line (B, BON-1; N, NCI-H727; S, SHP-77), as well as the number of proteins identified exclusively in one, two or all three cell lines. (B) Pie chart illustrating the progressive selection of candidate NET markers from the 205 CM proteins exclusively present in CM preparations from one (1 CM), two (2 CM) or three (3 CM) cell lines.

6.3b Cellular location of CM proteins

The cellular location of each protein identified using Protein Centre and NCBI databases, revealed that many proteins have been reported to be present in more than one location. Verification and further refinement of protein localization was based on extensive mining of the research literature, though we cannot exclude the possibility that intracellular and cell surface proteins or fragments thereof are secreted by the cell lines used in this study. Of the 19 proteins found in CM preparations from all three cell lines, 9 are known to be secreted (Table 6.1). Furthermore, of these 9 proteins, 5 were found in every run performed, including replicates (Figure 6.2a). These 5 proteins (carboxypeptidase E, CgA, NGF-inducible VGF, serum albumin and Mac-2BP) were therefore the most suitable for consideration as markers (Figure 6.2b).

Comparison between proteins secreted by NET cell lines and normal tissue

In this study, we opted to focus on proteins not detected in normal neuroendocrine secretory vesicles (SVs), as these may be more sensitive markers and may also be involved in tumor development or progression. We compared the soluble proteins from neuroendocrine SVs isolated from normal tissue [245] and the CM preparations in order to identify proteins that are not specific to transformed cells: 18 of the 205 proteins from NET cell line CMs were found in SVs. 10 of these proteins were present in the 61 proteins secreted by two or more NET lines, and 3 were present in the 5 secreted proteins found in every preparation (Figure 6.2b). Finally, we excluded proteins found by Lin et al. [246] to be commonly identified in comparative proteomic studies of a variety of different tissues. Accordingly, albumin was not studied further, leaving Mac-2BP as the best candidate marker.

6.3c Assessment of Mac-2BP as a marker for NET

Western Blotting

Mac-2BP has not previously been studied as a potential marker for NET and was selected for validation, initially by Western blotting. Blots were performed on the CM from all three cell lines in order to test for the presence of Mac-2BP, using a parallel blot which was not probed with primary antibody as a negative control (Figure 6.3). Both Mac-2BP was identified in the CM from all three cell lines, consistent with the identifications made by LC-MS/MS.

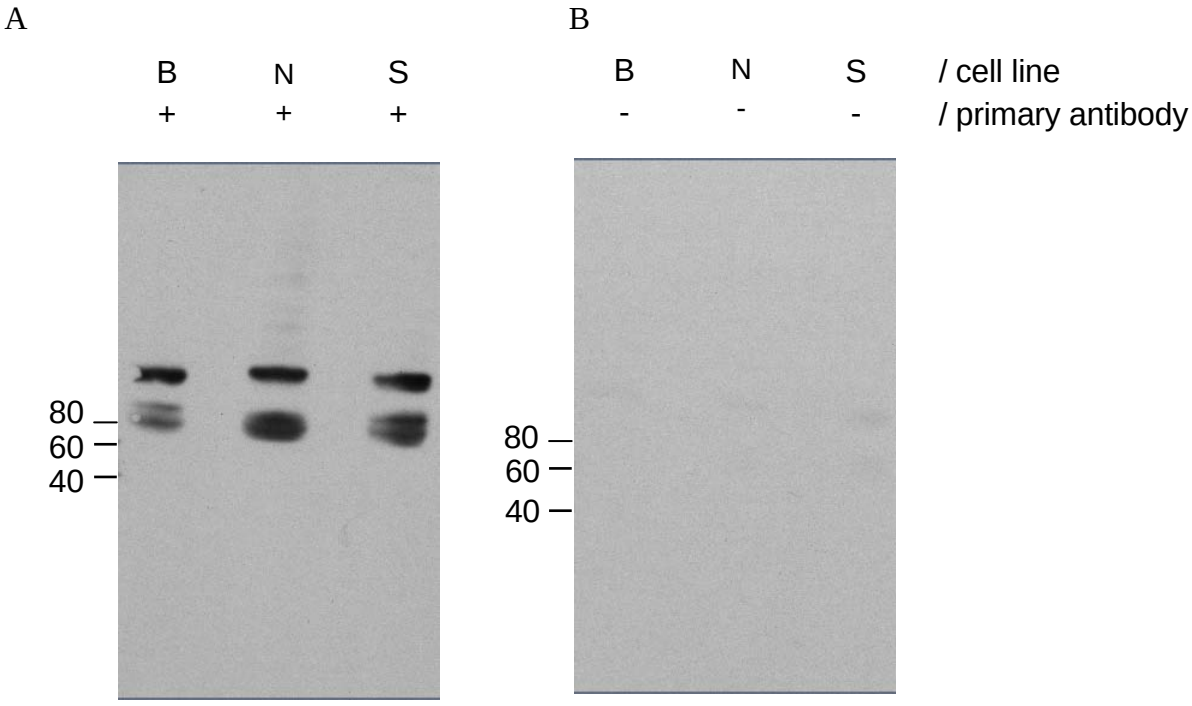


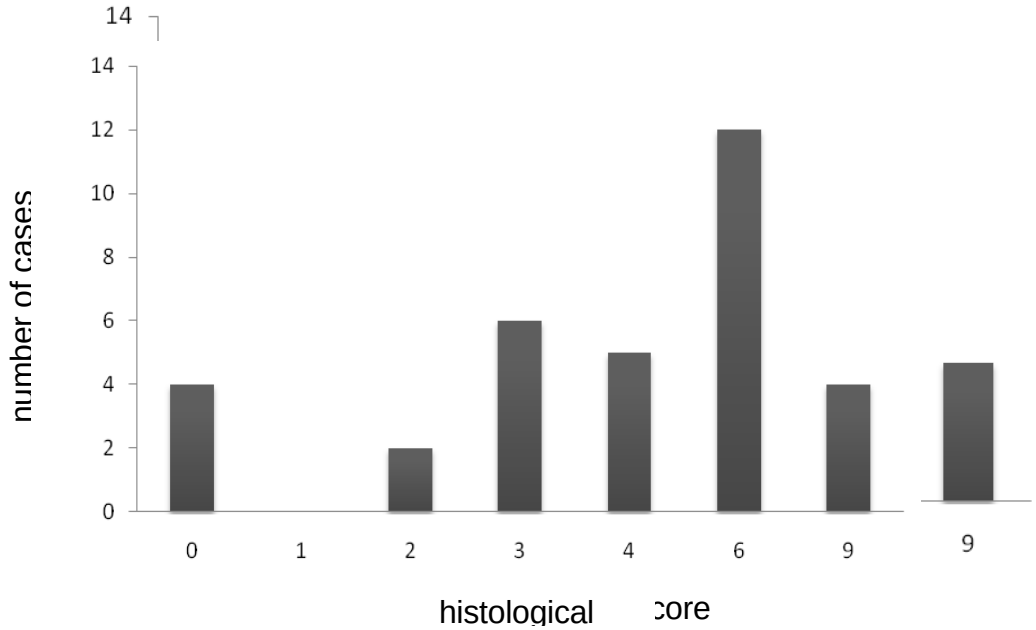
Figure 6. 3. Western blotting of conditioned media to confirm Mac-2BP expression by all NET cell lines. Western blots of equal loadings (30µl) of CM from each cell line (B, BON-1; N, NCI-H727; S, SHP-77) using (A) anti-Mac-2BP antibodies and (B) no primary antibody. Marker protein positions and molecular weights (kDa) are indicated.

6.3d Immunohistochemistry

In order to test whether Mac-2BP was elevated in different types of tumor and surrounding stromal tissue, immunohistochemistry was performed on biopsy samples from 33 patients with previously diagnosed NET. Twenty-nine of these samples had positive staining for Mac-2BP (Figure 6.4a) localized in each case to tumor areas. Staining was predominantly cytoplasmic with no nuclear staining apparent (Figure 6.5), and was seen in all types of neuroendocrine tumor examined (pancreatic, ileal, paraganglioma and tumours of unknown primary origin) (Figure 6.4b). The strength of

staining showed no apparent correlation with tumor type. The 4 cases with no detectable staining for Mac-2BP were all well-differentiated neuroendocrine carcinomas. We found no significant correlation between tumor grade and staining for Mac-2BP.

A



B

Site	Number of cases		Mac-2BP staining	
	Total	Positive	Intensity	Area
Pancreatic	9	9	1-3	1-3
Ileal	16	15	1-3	1-3
Paraganglioma	2	1	2	2
Unknown	6	4	1-3	2-3
Total	33	29		

Figure 6. 4. Immunohistochemical analysis of Mac-2BP expression in NET tissue

(A) Tumor samples from a panel of different NET types were scored according to the intensity of immunohistochemical staining for Mac-2BP (histological score). (B) Details of the intensity and scoring ranges are shown for each NET type, showing the number of cases in total, the number of cases with positive uptake (i.e. score >2), the intensity scored on a scale of 1 to 3, where 1 = weak, 2 = moderate and 3 = intense; and the area scored on a range of 1-3, where 1 = <25%, 2 = 25-75% and 3 = >75% positively stained.

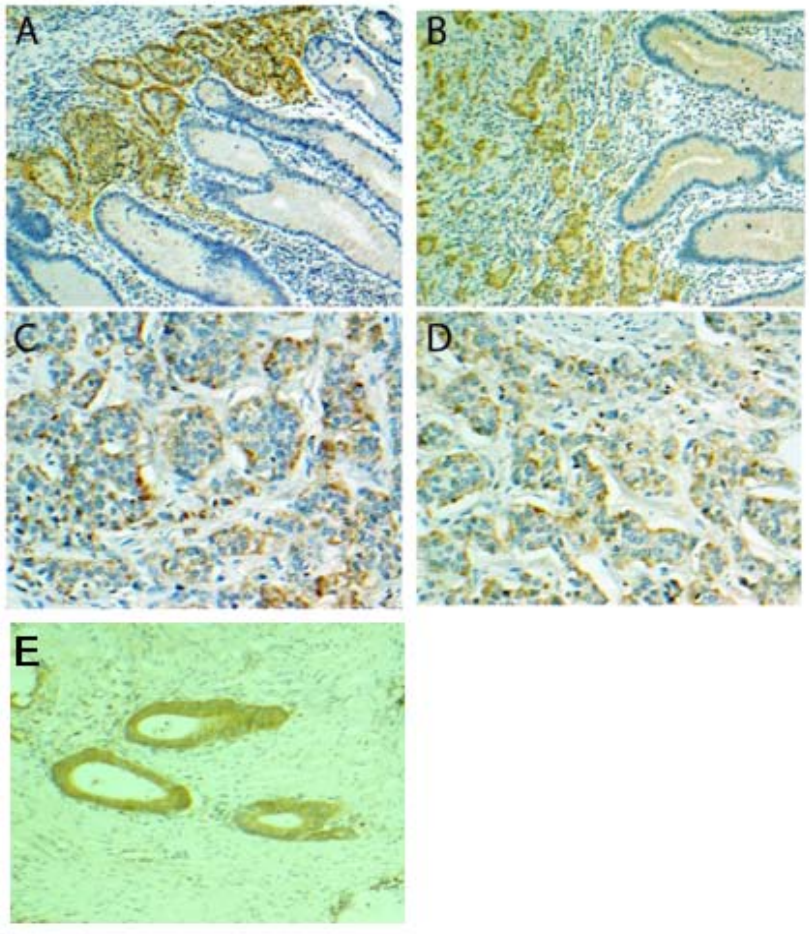


Figure 6. 5. Immunohistochemical staining of NET and normal tissue for Mac-2BP.
Fixed tissue sections were stained with anti-Mac-2BP antibody (brown) and nuclei counterstained with haematoxylin (blue). (A) Ileal NET tissue shows predominantly cytoplasmic staining of the clusters of NET cells. The surrounding mucosa appears normal with no visible staining; x200 magnification. (B) Midgut NET, clearly demonstrating staining of clusters of NET cells and negative staining of the surrounding tissue; x200 magnification. (C) Pancreatic NET showing cytoplasmic staining in the tumor cells; x400 magnification. (D) Ileal NET, clearly containing NET and normal cells. Normal tissue shows no evidence of epithelial staining while adjacent NET cells are clearly stained; x400 magnification. (E) Gastric cancer control showing staining moderate staining of gastric cancer cells, with minimal background staining; x 200 magnification.

6.3e Serum Mac-2BP concentrations in patients with NET

Mac-2BP concentrations were assessed in serum samples from 47 patients and 24 healthy control subjects (Figure 6.6a). The group of patients with NET taken as a whole (3.31 µg/ml, range 0.82-10.66 µg/ml) had a significantly higher serum Mac-2BP concentrations than the control group (2.30 µg/ml, range 1.16-3.56 µg/ml, $p<0.001$). When compared according to primary site of NET serum Mac-2BP was significantly elevated in NETs that originated from the midgut (3.34 µg/ml, range 0.82-10.66 µg/ml) compared to controls ($p< 0.001$)) as well as pancreatic NET (2.67 µg/ml, range 1.37-10.50 µg/ml) when compared to controls ($p<0.05$). There was no significant difference in Mac-2BP concentrations between patients with pancreatic or midgut primary tumours. There was a positive correlation between CgA and Mac-2BP in patients with mid-gut NETs using a Spearman rank correlation ($r = 0.36$, $p = 0.013$), but no such correlation in pancreatic NETs.

From radiological assessment of cross-sectional imaging performed within 2 months of blood sampling, visual assessments (by R.S) were made of the volume of liver metastases present. Three categories were created, low volume liver metastases (involving <25% of liver), intermediate (involving 25-50% of liver) and large volume liver metastases (>50% of liver). When comparing volume of liver metastases and Mac-2BP concentrations using Kruskal-Wallis one way ANOVA test (Figure 6.6b), there was a significant difference between the groups ($p = 0.024$). This indicates that higher Mac-2BP concentrations occur in serum with greater tumor burden.

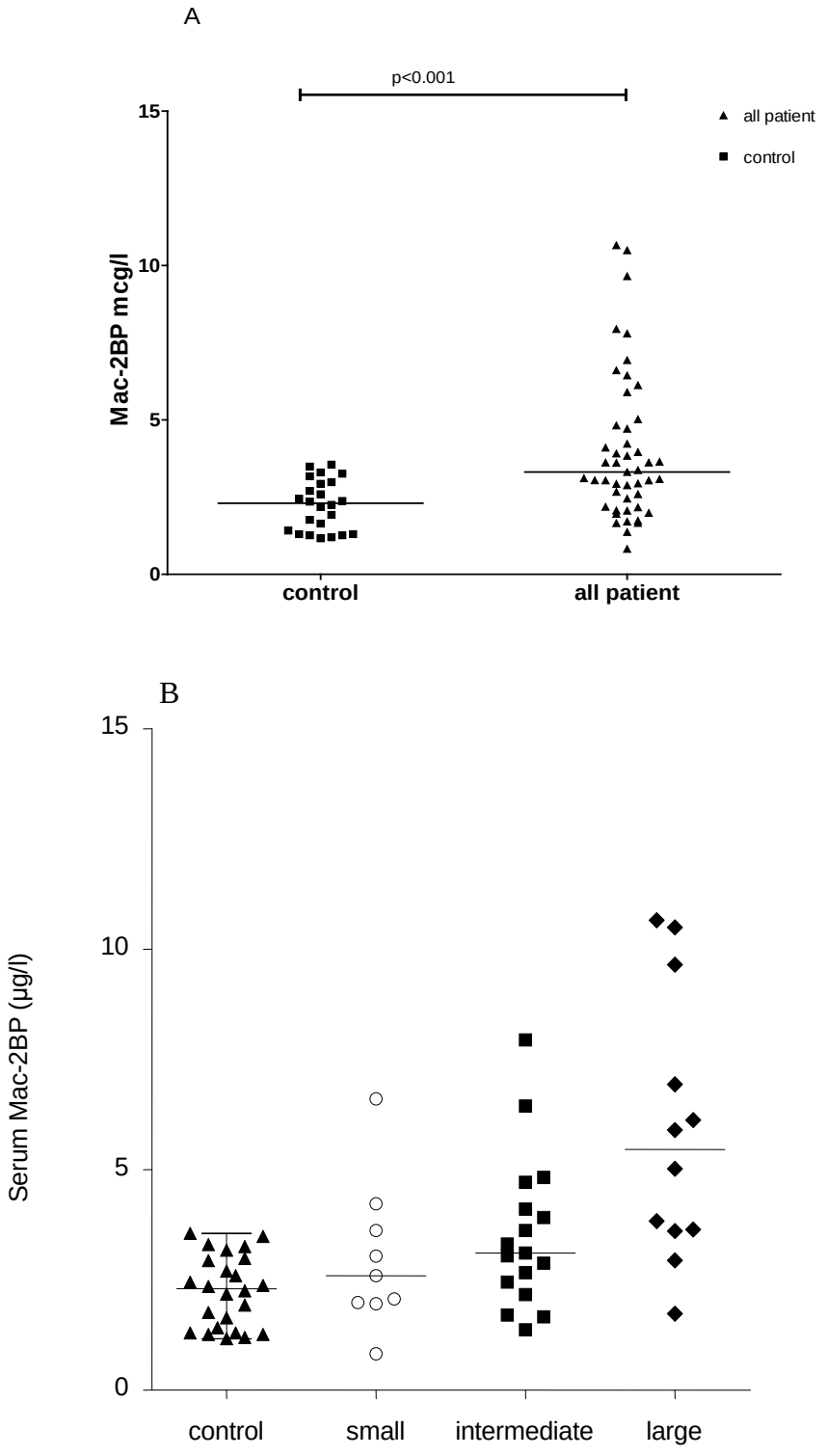


Figure 6. 6. Serum Mac-2BP protein concentrations in patients and controls. Serum concentrations of Mac-2BP were assayed using an ELISA test. (A) A Mann Whitney U test demonstrates that serum Mac-2BP concentrations are significantly higher in patients than controls ($p<0.001$). (B) A Kruskal Wallis one-way ANOVA test shows significant difference in Mac-2BP concentrations in NET patients with no metastases, small, intermediate and large volume liver metastases ($p=0.024$).

6.3f Assessment of the sensitivity and specificity of serum Mac-2BP as a marker for NET

To investigate whether concentrations of Mac-2BP in the circulation could be useful as a marker of NET, serum samples were taken from patients and control subjects. The concentrations of Mac-2BP were measured in the serum by ELISA. Receiver Operator Characteristic (ROC) curves for serum Mac-2BP were constructed to determine the cut-off values (for set sensitivity and associated specificity of the assay for Mac-2BP) for all NETs. ROC curves are a graphical method of assessing the characteristic of a diagnostic test, by plotting sensitivity (true positive rate) against 1 – specificity (false positive rate). These parameters are employed as measures of the proportion of positives and negatives, respectively, which are correctly identified.

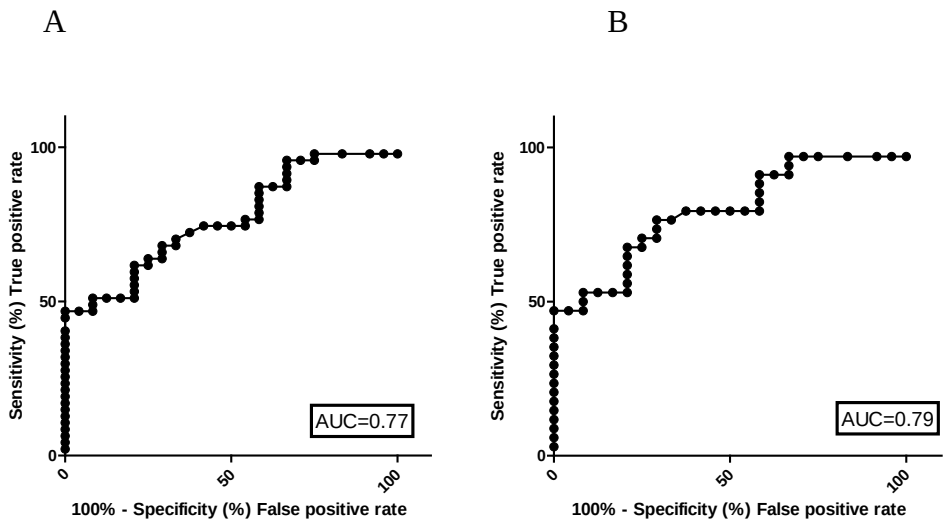


Figure 6. 7. Assessment of serum Mac-2BP as a NET marker. ROC curves and the corresponding area under the curve (AUC) are shown for (A) serum Mac-2BP concentrations in all types of NET, and (B) serum Mac-2BP from patients with midgut primary NETs.

Accuracy of the test is measured by the area under the ROC curve: an area of 1 represents a perfect test, while an area of 0.5 represents a random association, generally >0.75 is regarded as a good marker. If the AUC was 0.75, then on average a patient will have a more abnormal Mac-2BP than 75% of controls. If the test was perfect, then every patient would have a more abnormal test result than every control and hence the AUC would equal 1. The AUC for all NETs was 0.77 (Figure 6.7a), which shows Mac-2BP is a good marker of NET. Serum Mac-2BP ≥ 2.41 μg/ml, was a highly sensitive marker (≥75%) for NETs; the associated specificity relative to the control group was 59.3% (Table 6.2). For the detection of mid-gut NET, the area under the curve was slightly higher at 0.79 (Figure 6.7b) and, at ≥75% sensitivity, the specificity was 70.8% (cut-off value >2.91 μg/ml).

Since this marker is not proposed for population screening but for monitoring the disease status of NET patients, a high specificity in identifying patients with disease is more useful than a high sensitivity. Mac-2BP achieves 100% specificity for identifying disease in all of the NET patients when the cut-off value is increased to >3.58μg/ml, corresponding to a detection rate of 47% (Table 6.2).

Detection rate (Sensitivity)	CI (%)	Mac-2BP in all GEP NETs				Mac-2BP in mid-gut NETs			
		Cut-off µg/ml	FPR (%)	CI (%)	LR	Cut-off µg/ml	FPR (%)	CI (%)	LR
47%	32.1-61.9	>3.58	0	85.6-100		>3.59	0	85.6-100	
75%	59.7-86.0	>2.41	41.7	22.2-63.4	1.80	>2.91	29.2	12.2-44.6	2.56
80%	66.7-90.9	>2.06	58.3	37.4-78.2	1.37	>2.30	50.0	29.2-61.8	1.60
85%	71.7-93.8	>1.97	58.3	36.4-77.2	1.46	>2.02	58.3	36.4-77.9	1.46
90%	76.9-96.5	>1.72	66.6	45.3-84.3	1.35	>1.94	58.2	37.0-77.7	1.55
95%	85.5-99.5	>1.65	70.2	45.3-84.3	1.35	>1.70	66.6	44.7-84.3	1.43

Table 6. 2. Assessment of serum Mac-2BP as a marker for all gastro-enteropancreatic (GEP) NETs and mid-gut NETs. The detection rate (sensitivity) is the total true positive results divided by the true positive and false negative results; False positive rate (FPR) is 100%-specificity; Confidence interval (CI); Likelihood ratio (LR) is sensitivity divided by 1-specificity. At concentrations of >3.58 µg/ml Mac-2BP is 100% specific for NETs.

6.3g The combination of Mac-2BP and Chromogranin A as biochemical markers of NET

The CgA assay (at a cut-off >60 pg/ml) correctly identified 72.3% (34 out of 47) of the NET cases in our patient population. Serum Mac2-BP at a cut-off of >3.58 µg/ml has 100% specificity for NET (Table 6.2). Using this threshold, an additional five cases (10.6%) were identified as positive for NET. The combination of these two biochemical markers correctly identified 82.9% of the NET cases in this cohort of patients.

6.4 Discussion

In this study we analyzed samples of serum-free media that had been conditioned by three neuroendocrine cell lines: NCI-H727, BON-1 and SHP-77. Protein identifications were only accepted when at least two peptides from the protein produced a significant Mowse score, apart from one case where a convincing series of y-ions was identified. This stringency may have reduced the number of proteins identified, but increases the confidence of identifications. We identified 9 extracellular proteins including Mac-2BP that are secreted by all three cell lines. Mac-2BP is expressed in 88% of immunohistochemical samples and serum concentrations of Mac-2BP are significantly higher in patients than controls.

BON-1 is a pancreatic neuroendocrine cell line [243], and NCI-H727 and SHP-77 are bronchial neuroendocrine tumor cell lines. In the present study the established NET serum markers, chromogranins A, B and C, were identified in the CM of all three lines, consistent with the neuroendocrine phenotype of these cells. These proteins have not been reported in the secretomes of several non-NET cell lines [237;239;247-249], and were found in normal neuroendocrine secretory vesicles [245]. However, these proteins possess limitations as NET biomarkers.

Several other proteins identified here in the CM of NET cell lines have also been identified in other cancer cell lines and have postulated roles in cancer, including heat shock protein 70, heat shock protein 90, laminin alpha-5 chain, thioredoxin reductase, annexin A2, glutathione-S-transferase, Mac-2BP, and nerve growth factor (NGF) inducible VGF [239;250-252]. Consequently they are potential markers of different types of tumor. Furthermore, a number of these proteins may be involved in the

pathophysiological development or progression of tumours, including NETs. Amongst the 9 proteins of specific interest to this study because they were secreted by all 3 cell lines, we chose to focus on Mac-2BP for validation because it was reproducibly detected in replicate CM preparations, and was not found in normal neuroendocrine secretory vesicles or as a commonly occurring component of tissue proteomes.

Other proteins identified in this study may still prove of value as markers: a study by Rindi *et al*. [252] has previously established that NGF-inducible VGF is expressed in the majority of NETs, and it is likely that combinations of markers will prove more specific than individual markers. Future work will address the potential value of other proteins in this set, individually and in combination.

Mac-2BP is elevated in patients with a variety of different cancers, including breast, nasopharyngeal and lung cancer [253-255]. It is associated with poor survival and metastatic spread of liver and lung cancers [256;257]. This study indicates that Mac-2BP is also elevated in patients with NETs: (i) Mac-2BP concentrations were significantly elevated in both pancreatic and mid-gut NETs; (ii) elevation of serum Mac-2BP is related to volume of liver metastases; (iii). CgA is currently the most commonly used NET marker, but it does not offer accurate prognostic information and its accuracy is dependent on the assay used. Furthermore, CgA is labile and blood samples need to be collected on ice and stored frozen prior to analysis. Consequently, additional studies are warranted to assess a role for Mac-2BP as biochemical NET marker.

Using a threshold of >3.58 µg/ml Mac-2BP is a 100%-specific marker in identifying NETs in this study. The low likelihood ratio of Mac-2BP and its high false positive rate means that it currently would not be useful as a sole screening marker. We have found

that the serum concentrations of Mac 2-BP >3.58 µg/ml can identify additional cases of NET which would not have been identified using the standard assay for CgA. Mac-2BP could be used as part of a panel of markers for diagnosing NETs; although a large-scale study would be needed to validate this approach. The other role for serum concentrations of Mac-2BP could be in identifying patients with progressive disease or increases in tumor load, since Mac-2BP shows a significant trend with increasing tumor load.

Mac-2 BP, also known as 90K, is an oligomeric glycoprotein composed of subunits of approximately 90 kDa [258;258], and binds galectin-1, -3 and -7. Several lines of evidence support a role for galectins in tumor invasion and metastasis. These proteins have been reported to mediate apoptosis, cell proliferation and angiogenesis [251;259]. The mechanisms underlying the role of Mac-2BP and galectins in cancer may be related to the ability of these proteins to interact with and modulate cell-cell and cell-matrix adhesion and apoptosis [260;261]. Inohara and co-workers found that Mac-2BP can mediate homotypic cell adhesion and the formation of multi-cellular aggregates by cross-linking galectin-1 and -3 residues on adjacent tumor cells [262]. This process appears to be critical for cancer cell survival in the bloodstream and may be a vital step in metastatic diffusion. Moreover, Mac-2BP and galectins are also located in the ECM where they may play a role in cell attachment by binding to ß1 integrins, collagens, and fibronectin [261;263].

Further studies will be required to assess the suitability and reliability of Mac-2BP as a marker of NET. It will be necessary to determine whether there is diurnal variation of Mac-2BP by sampling serum from patient and controls subjects several times a day. A study to determine changes in serum Mac-2BP following different treatments such as chemotherapy, radiotargetted therapy or surgical resection would be of benefit to

identify if Mac-2BP is related to concentration of disease activity. Finally, larger studies could assess whether Mac-2BP would be useful as a prognostic marker in patients with NETs.

In summary, the identification of the secreted proteome of NET cell lines has established a panel of possible biomarkers for NET. Extensive studies will be required to rigorously assess the potential of Mac-2BP as a marker for the clinical management of NET and to elucidate the pathophysiological role of Mac-2BP. This study provides evidence for the utility of this combination of CM preparation, LC-MS/MS and immunological methods to identify putative serum markers from cultured cells, and their initial assessment using clinical samples. This combination is a powerful strategy for finding and evaluating potential serum markers for NETs and other types of cancer.

Chapter 7: The role of ^{68}Ga-DOTATATE PET in patients with neuroendocrine tumours and negative or equivocal ^{111}In-DTPA-octreotide scintigraphy

Abstract

Introduction: ^{111}In-DTPA-octreotide scintigraphy is currently the nuclear medicine imaging modality of choice for identifying NETs. However, there are cohorts of patients in whom scintigraphy is negative or equivocal. The aim of this study was to determine whether ^{68}Ga-DOTATATE PET identifies lesions and alters clinical management in patients with negative or weak positive ^{111}In-DTPA-octreotide scintigraphy.

Methods: Fifty-one patients with histologically confirmed diagnosis of neuroendocrine tumours and negative (35 patients) or equivocal (16 patients) uptake on ^{111}In-DTPA-octreotide scintigraphy underwent ^{68}Ga-DOTATATE PET. Findings were compared by using a region-by-region analysis. All findings were verified with CT and/or MRI. Following ^{68}Ga-DOTATATE PET, all cases were reviewed to determine whether the ^{68}Ga-DOTATATE PET findings resulted in any alteration in management, in terms of suitability for peptide receptor therapy, somatostatin analogues and surgery.

Results: Of the 51 patients, 47 had evidence of disease on cross-sectional imaging or biochemically. ^{68}Ga-DOTATATE PET was positive in 41 of these 47 patients (87.2%). No false positive lesions were identified. ^{68}Ga-DOTATATE PET detected 168 of the 226 lesions (74.3%) which were identified with cross-sectional imaging. ^{68}Ga-DOTATATE PET identified significantly more lesions than ^{111}In-DTPA-octreotide

scintigraphy ($p<0.0001$). ^{68}Ga-DOTATATE imaging changed management in 36 patients (70.6%), who were subsequently deemed suitable for peptide receptor targeted therapy.

Conclusion: In patients with negative or equivocal ^{111}In-DTPA-octreotide scintigraphy, ^{68}Ga-DOTATATE PET imaging identifies additional lesions and may alter management in the majority of cases.

Experimental work: Interpretation of Ga-68-DOTATATE PET and 111-In-DTPA-Octreotide scintigraphic images was performed by I. Kayani, A.M Quigley and J.Bomanji.

7.1 Introduction

Somatostatin receptor (SSTR) overexpression in NETs has enabled development of imaging with scintigraphy using radiolabelled somatostatin analogues [264;265]. To date, five SSTRs have been characterised, all of which are expressed in differing frequencies in NETs [266]. SSTRs 2 and 5 are expressed in 70–90% of NETs [165;266]. Radiolabelled somatostatin analogues can allow visualisation and staging of carcinoid tumours expressing SSTRs 2 and 5 [267]. The most commonly used radioligand is ^{111}In-DTPA-octreotide, which is commercially available as ^{111}In-pentetreotide. The expression of SSTRs in these tumours allows radionuclide therapy, subject to good uptake of somatostatin radiolabelled analogues. To date this has been assessed using ^{111}In-DTPA-octreotide scintigraphy [41;103].

^{111}In-DTPA-octreotide binds predominantly to SSTR2-positive cells and emits gamma rays which enable imaging of SSTR2-positive NETs [268]. There are limitations with this technique, and it has restricted ability to identify lesions of <1 cm and to obtain good spatial resolution, even when employing single-photon emission computed tomography (SPECT) [269]. In the last few years, studies using ^{68}Ga-DOTATOC PET in NETs have shown promising results, with a higher rate of lesion identification than is achieved with conventional ^{111}In-DTPA-octreotide scintigraphy [270-272]. This study used DOTA-DPhe, Tyr-octreotate (DOTATATE), a somatostatin 2 receptor analogue [273], labelled with ^{68}Ga, a positron emitter. ^{68}Ga is produced from a ^{68}Ge/^{68}Ga generator and therefore is not dependent on a cyclotron.

The study had three aims: first, to assess whether ^{68}Ga-DOTATATE PET imaging identifies SSTR2-positive disease in NET patients with negative or weakly positive ^{111}In-DTPA-octreotide scan; secondly, to establish whether there is any association

between tumour histology and lesion identification using ^{68}Ga-DOTATATE PET; and finally, to evaluate whether ^{68}Ga-DOTATATE PET alters clinical management in patients with weak or negative ^{111}In-DTPA-octreotide scan.

7.2 Materials and Methods

7.2a Study population

During the period from November 2006 to March 2008 a total of 312 patients underwent ^{111}In-DTPA-octreotide scintigraphy. Of these, 51 were referred for ^{68}Ga-DOTATATE PET owing to scintigraphy being negative or showing only low grade uptake of tracer (Krenning score <2). Images were reviewed prospectively and clinical data from the 51 patients (27 men, 24 females; age range 18-80 years, median age 55.5), who were imaged with ^{68}Ga-DOTATATE PET/CT between January 2007 and April 2008. Forty five cases underwent chest, abdomen, and pelvis CT scans, 2 had abdominal MRI scans and 4 had chest, abdomen, and pelvis CT scans and additional MRI of liver. All patients gave informed consent.

The primary tumour origin was as follows: medullary thyroid, 2; thymic carcinoids, 2; paraganglioma, 2; hindgut, 2; bronchial, 2; pancreatic, 13; midgut, 22; the remaining six patients had cancer of unknown primary origin (Table 7.1). All patients had a previous histological diagnosis of NET and all had undergone an ^{111}In-DTPA-octreotide scan within a median time of 4 months (range 1-8 months) of the ^{68}Ga-DOTATATE PET scans. The number of cases from each anatomical site is listed in Table7. 2. Histology was available in all cases and tumour grade was classified as low, intermediate or high, using the proposed European Neuroendocrine Tumour Society (ENETS) criteria for

grading Gastroenteropancreatic NETs [19;21]. The classification was extended to include tumours of other sites and cancer of unknown primary origin.

7.2b Image acquisition

^{68}Ga-DOTATATE PET: Images were acquired 1 h post injection of 120-200 MBq of ^{68}Ga-DOTATATE. No adverse effects were observed after the injection of ^{68}Ga-DOTATATE. Imaging was performed using a dedicated combined GE Discovery LS PET/CT unit (Michigan, USA); whole-body examinations (mid brain to mid thigh) were performed with the patient supine.

CT was performed using the four 3.75-mm detectors, a pitch of 1.5 and a 5-mm collimation. The CT exposure factors for all examinations were 140 kVp and 80 mA in 0.8 s. Maintaining patient position, a whole-body PET emission scan was performed and covered an area identical to that covered by CT; scans were performed in 3D mode. All PET acquisitions were carried out in 3D mode (4 min per bed position), approximately 30 minutes scanning time. PET images were reconstructed using CT for attenuation correction. Transaxial emission images of 4.3 x 4.3 x 4.25 mm^3 were reconstructed using ordered subsets expectation maximization (OSEM) with two iterations and 28 subsets. The axial field of view was 148.75 mm, resulting in 35 slices per bed position. Long-acting somatostatin analogues were not stopped prior to imaging with PET or ^{111}In-DTPA-octreotide scintigraphy.

^{111}In-DTPA-octreotide SPECT: Patients were injected with 200 MBq of ^{111}In-pentetreotide IV (Covidien, Petten, Netherlands). Whole-body images were acquired at 24 h post injection on a dual-headed gamma camera (Picker Prism, Phillips Medical Technology, Cleveland, Ohio) using a medium-energy collimator. Patients were scanned

with a camera speed of 7.2 cm/min, matrix 256 x 1024. Photopeaks were set at 173 and 247 keV with +/- 10% windows with no offset. Abdominal images were obtained from the dome of the liver downwards. An additional chest and/or pelvic SPECT study was performed if warranted by the appearances of the whole body, or if abnormalities had been identified previously in other areas on cross-sectional imaging. Scan parameters were as follows: medium-energy collimator, 60 stops per head at 3º intervals, 25 s per stop, 64 x 64 matrix. The same energy window was used as with planar imaging. Images were processed and analysed at a workstation using Odyssey software (Phillips Medical Technology, Cleveland, Ohio). They were reconstructed using an iterative OSEM programme with six iterations and post filtered with a count-optimised Butterworth filter. Images were displayed as a full data set of orthogonal slices of 9-mm thickness.

The images from ^{68}Ga-DOTATATE PET/CT were reported in consensus by an experienced dedicated nuclear medicine physician and a dual accredited radiologist/nuclear medicine physician, who were unaware of the results of the previous ^{111}In-DTPA-octreotide study. Areas of abnormal focal uptake were documented. The relative uptake of ^{68}Ga-DOTATATE was evaluated on the basis of intensity of tracer uptake (SUV_{max}). The images from ^{111}In-DTPA-octreotide scintigraphy were reported independently by an experienced nuclear medicine physician. Lesions identified were graded using the Krenning score as follows: 0 = no abnormality, 1 = faint uptake; 2 = clear uptake in the tumour, similar to the liver; 3 = uptake higher than in the liver; 4 = increased accumulation in the tumour much greater than that in the liver [274].

7.2c Interpretation of data

The number of lesions that could be identified clearly as single foci was determined for each patient. To enable a methodical and consistent approach to lesion identification, lesions were grouped into three categories: organs (lungs, breast, liver, pancreas, gastrointestinal tract, kidney, spleen and pelvis/ovaries), nodal regions (thoracic, mesenteric, abdominal and pelvic (excluding mesenteric)) and musculoskeletal system (vertebrae, bony thorax, bony pelvis and limb bones). Owing to confluency and inability to clearly delineate single liver lesions in some cases, liver metastases were classified as one organ metastasis, independent of the number of liver metastases present.

To confirm the presence of lesions, all patients underwent cross-sectional imaging with CT or MRI scan. Patients who underwent ^{68}Ga-PET had combined PET/CT to confirm location of disease. Plasma chromogranin A concentrations were available for all patients, and, where appropriate, fasting gut hormone concentrations and urinary 5-HIAA were also measured.

7.2d Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. The values were generally not normally distributed, so non-parametric tests were used to compare marker concentrations between groups, i.e. the Mann- Whitney U test (for two groups).

7.3 Results

In total, 51 patients underwent ^{68}Ga-DOTATATE PET and ^{111}In-DTPA-octreotide scan. Of these, 47 had evidence of disease on cross-sectional imaging; the four remaining

patients had undergone previous curative surgical resection and were under surveillance imaging. In the 47 patients with evidence of disease, a total of 226 lesions were identified on cross-sectional imaging (including MRI, CT and PET/CT) (Table 7.2). Thirty-five cases were negative with ^{111}In-DTPA-octreotide scintigraphy, while in the remaining 16 cases a total of 27 lesions were identified with a Krenning score <2. ^{68}Ga-DOTATATE PET identified disease in 41 of 47 patients (87.2%) and detected 168 (74.3%) of the 226 lesions identified on cross-sectional imaging. The number of lesions seen in each organ with each modality is listed in Table 7.3.

7.3a Patient analysis

Negative ^{111}In-DTPA-octreotide scintigraphy

Among the 51 patients, ^{111}In-DTPA-octreotide was negative with no evidence of uptake (other than physiological uptake) in 35 cases. In three of these 35 cases, no disease was identified on cross-sectional imaging and patients had normal chromogranin A and fasting gut hormone concentrations. The three patients with no evidence of disease on all modalities are considered to be disease free currently; all of these cases had undergone previous surgical resection.

Demographics	**No.**
Male	27
Female	24
Mean age(years)	55.5
Age range (years)	18-80
Primary origin	
Bronchial	2
Thyroid	2
Thymic	2
Pancreatic	13
Midgut	22
Hindgut	2
Paraganglioma	2
Unknown	6
Histological grade	
Low	28
Intermediate	19
High	4
Treatment	
Current therapy with somatostatin analogues	27
Previous surgery	9
Previous chemotherapy	10

Table 7. 1. Characteristics of study population

The remaining 32 patients all had disease identified in cross-sectional imaging. Among these 32 patients, ^{68}Ga-DOTATATE was positive in 27 (Fig. 7.1 & 7.2). In total, 97 lesions were identified in the 27 patients with positive ^{68}Ga-DOTATATE PET, whilst cross-sectional imaging identified 125 lesions in the 32 patients with disease.

Five cases which were negative for both ^{68}Ga-DOTATATE PET and ^{111}In-DOTA-octreotide had evidence of disease on cross-sectional imaging. In these cases, 27 lesions were identified on CT +/- MRI (Table 7.2). These tumours were all metastatic in nature; the primary sites were ileal and pancreatic in one case each, while three were from

unknown primaries. Two patients had functional syndromes (both carcinoid) and the remaining three had no syndromic features. Histology from these five cases showed two low-grade tumours, two intermediate-grade tumours and one high-grade tumour.

Site	Histology	No. of cases	^{111}In-DTPA octreotide scintigraphy	^{68}Ga-DOTATATE PET	Cross-sectional imaging
Bronchial	Low	1	1	1	1
	Intermediate	1	0	14	14
Thyroid	Low	1	0	0	1
	Intermediate	1	0	1	1
Thymic	Intermediate	1	0	1	4
	High	1	1	1	1
Pancreatic	Low	9	12	22	25
	Intermediate	2	0	2	6
	High	2	1	4	5
Ileal	Low	12	7	40	48
	Intermediate	10	3	38	46
Paraganglioma	Low	1	1	17	17
	Intermediate	1	0	12	12
Unknown	Low	2	0	3	8
	Intermediate	3	0	7	17
	High	1	0	0	15
Hindgut	Low	2	1	5	5
Total lesions		51[b]	27	168	226

Table 7. 2. Total number of lesions identified in each type of NET with the different modalities. [a]Histological grade was determined using the proposed ENETS guidelines and extrapolated to include thymic carcinoid, unknown primaries and paraganglioma. [b] The total number of cases includes the four patients with no evidence of disease on cross-sectional imaging.

7.3b Low grade/faint uptake ^{111}In-DTPA-octreotide scintigraphy

In sixteen cases there was some uptake with ^{111}In-DTPA-octreotide scintigraphy; however, the uptake in the extra-hepatic lesion was low grade and notably less than that in the liver. Cross-sectional evidence of disease was identified in 15 of these cases. In one case ^{111}In-DTPA-octreotide scintigraphy identified very low grade uptake in the

mediastinum and left lung, which was commented on in view of the fact that the patient had medullary thyroid cancer, which had been previously resected. Subsequently, there was no residual disease on cross-sectional imaging or uptake on ^{68}Ga-DOTATATE PET, and plasma tumour markers plus chromogranin A were normal. This was the only false positive result seen among all 51 cases. Positive uptake with ^{68}Ga-DOTATATE PET was seen in 14 of the 16 cases with low grade uptake on ^{111}In-DTPA-octreotide. A total of 101 lesions were identified with cross-sectional imaging in the 15 cases with disease; of these, 71 (70.3%) were identified with ^{68}Ga-DOTATATE PET.

^{68}Ga-DOTATATE PET was false negative in one case, which was an intermediate-grade non-functional pancreatic tumour. In this case ^{111}In-DTPA-octreotide scintigraphy showed faint uptake in the region of the gallbladder/liver, corresponding to the site of liver metastases.

The sensitivity of ^{111}In-DTPA-octreotide scintigraphy was not determined in view of the bias of the sample population. The specificity of ^{111}In-DTPA-octreotide scintigraphy was 98%, since there was only one false positive result. The sensitivity of ^{68}Ga-DOTATATE PET was 87.2% and the specificity, 100%.

7.3c Lesion analysis

Evidence of NET disease was identified in 47 of the 51 patients in this study by cross-sectional imaging. A total of 226 lesions were identified with CT +/- MRI, whilst ^{111}In-DTPA-octreotide scintigraphy identified 27 lesions and ^{68}Ga-DOTATATE PET identified 168 lesions. ^{68}Ga-DOTATATE PET identified 74.3% of all lesions, which is a

significantly higher than was identified by ^{111}In-DTPA-octreotide scintigraphy ($p<0.001$).

The location of lesions (according to specific body region) identified by ^{68}Ga-DOTATATE PET and by ^{111}In-DTPA-octreotide scintigraphy was compared (Table 7.3). It is to be noted that SPECT scanning was only performed in the region of the chest and abdomen and therefore, skeletal metastases may not have been identified on planar imaging. Thirty-six bony lesions were identified by ^{68}Ga-DOTATATE PET and CT +/- MRI, of which 28 were located in the rib cage or pelvis. Eight bony metastases were identified in the long bones (limbs).

Organ/region	^{111}In-DTPA-octreotide scintigraphy	^{68}Ga-DOTATATE PET	CT +/- MRI
Lung	3	9	16
Breast	0	1	1
Pancreas	4	6	6
Liver	6	23	34
GI tract	0	7	7
Kidney	2	3	2
Spleen	0	0	1
Pelvis/ovaries	4	1	1
Thoracic nodes	0	17	17
Mesenteric nodes	1	19	37
Abdominal nodes	7	27	45
Vertebrae	0	19	23
Bones	0	36	36
Total	27	168	226

Table 7. 3. Location of lesions identified by ^{111}In-DTPA-octreotide scintigraphy, Ga-DOTATATE PET and cross-sectional imaging (CT +/- MRI).

7.3d ^{68}Ga-DOTATATE PET and histology

Histology was available in all cases. Table 7.4 shows the number of positive cases in patients with different grades of disease. The results in respect of ^{111}In-DTPA-octreotide are difficult to interpret in view of the selection of patients chosen. ^{68}Ga-DOTATATE PET showed good lesion recognition with low-grade tumours. The sensitivity of ^{68}Ga-DOTATATE PET decreased in the more aggressive and poorly differentiated tumours; however, this reduction did not reach significance.

Histology	No. of cases	Disease on cross-sectional imaging	^{111}In-DTPA-octreotide scintigraphy	^{68}Ga-DOTATATE PET
Low	28	24	11 (45.8%)	22 (91.7%)
Intermediate	19	19	3 (15.8%)	16 (84.2%)
High	4	4	2 (50%)	3 (75%)
Total	51	47	16	41

Table 7. 4. Number of positive* ^{111}In-DTPA-octreotide scintigraphy and ^{68}Ga-DOTATATE PET results in patients with different histological grades of disease. * Positive for ^{111}In-DTPA-octreotide scintigraphy means those with weak/ equivocal uptake.

7.3e Positive ^{68}Ga-DOTATATE PET in conjunction with initially negative CT

In two cases, patients had undergone initial scans with CT which had been reported as normal. One patient had normal biochemical markers and the other had raised chromogranin A concentrations. When these patients underwent ^{68}Ga-DOTATATE PET/CT, the PET identified multiple bony metastases; these were found to correspond with sclerotic foci on CT when PET/CT fusion images were reviewed.

7.3f Change in clinical management

To ascertain whether ^{68}Ga-DOTATATE PET altered the clinical management in patients who had already undergone ^{111}In-DTPA-octreotide scintigraphy, all the case notes were reviewed. Change in clinical management was classified into the following four categories: (1) consideration of ^{90}Y-DOTATATE radiotargeted therapy, (2) suitability for commencement of somatostatin analogues, (3) altered stage of disease and (4) consideration for surgical resection.

Among the 51 cases, management was altered in 36 (70.6%) following ^{68}Ga-DOTATATE PET. Of these 36 cases, 20 were considered suitable for peptide receptor radiotargeted therapy with ^{90}Y-DOTATATE in view of the strength of tracer uptake on ^{68}Ga-DOTATATE PET. A further four patients with negative results on both ^{68}Ga-DOTATATE and ^{111}In-DTPA-octreotide scans were excluded from peptide receptor therapy with ^{90}Y-DOTATATE. In seven patients without functional symptoms but with positive uptake on ^{68}Ga-DOTATATE PET, somatostatin analogues were commenced for their antiproliferative effects.

In four cases, surgery was regarded as a possible treatment option in view of the limited and resectable disease. In 3 cases this was regarding disease localised to abdomen and pelvis and one case of disease limited to liver. In these cases ^{68}Ga-DOTATATE PET changed clinical management by confirming that the disease present on cross sectional imaging corresponded with areas of tracer uptake with PET imaging; which had not been identified with ^{111}In-DTPA-octreotide scintigraphy. Curative resections were performed in two cases, and there was no evidence of recurrence at 12 and 14 months. Another patient had debulking surgery of >90% of the tumour load and the remaining disease was stable at 6 months post surgery. The fourth case the patient has not undergone surgical therapy to date and is considering other treatment options.

Finally, in one case in which there was no evidence of disease on CT scanning or biochemically, low-grade uptake in the mediastinum and left lung was observed on the ^{111}In-DTPA-octreotide scan. ^{68}Ga-DOTATATE PET did not reveal any evidence of abnormal uptake and the patient was regarded as disease free. In this case ^{68}Ga-DOTATATE PET confirmed cross-sectional imaging findings but altered management by identifying no evidence of disease uptake, which had been seen on ^{111}In-DTPA-scintigraphy.

7.4 Discussion

This study is the first to demonstrate that ^{68}Ga-DOTATATE PET imaging can identify disease in patients with NETs when conventional ^{111}In-DTPA-octreotide scan is negative or shows only faint uptake. Furthermore, ^{68}Ga-DOTATATE PET imaging changed the clinical management in 70% of the patients studied.

Although ^{68}Ga-DOTATATE PET identified disease in patients with negative or weakly positive ^{111}In-DTPA-octreotide scans, it did not map the full extent of disease. ^{68}Ga-DOTATATE PET identified 74.3% of lesions documented by conventional cross-sectional imaging. There were individual cases in which some lesions were ^{68}Ga-DOTATATE avid whilst others were not. This illustrates the variable SSTR expression and heterogeneity of NETs, especially with intermediate- and high-grade tumours.

Inherently, PET has superior spatial resolution to conventional SPECT imaging. Reubi et al have previously demonstrated that ^{68}Ga-DOTATATE bound SSTR-2 with a considerably higher affinity than ^{111}In-DTPA-octreotide [166]. The higher affinity of ^{68}Ga-DOTATATE for the SSTR-2 may enable smaller lesions to be visualised on the PET/CT. It is very likely that SPECT imaging of the entire body would have identified more lesions, especially skeletal metastases, but whole-body SPECT is neither practical nor comfortable for patients. It was also observed that in areas where ^{111}In-DTPA-octreotide SPECT and ^{68}Ga-DOTATATE PET were performed, the number of lesions detected with PET was greater. The ^{111}In-DTPA-octreotide scintigraphy protocol used in this study requires total scan time of 50mins to 1 hour, which for many patients is the most that can be tolerated. The SPECT imaging protocol in this study is similar to that

recommended in other guidelines [275]. Whilst longer whole body scanning times may enable smaller lesions to be visualised the target to background ratio will not be altered.

Studies using ^{68}Ga-DOTATOC PET have also shown higher sensitivity and specificity than ^{111}In-DTPA-octreotide scintigraphy [276]. A study by Buchmann et al. in 27 patients demonstrated an increase in lesion identification with PET compared to ^{111}In-DTPA-octreotide SPECT [270]. Another study by Gabriel et al., with a prospective design, compared ^{68}Ga-DOTATOC PET and ^{111}In-DTPA-octreotide SPECT in 84 patients and found that a significantly higher number of lesions were identified with ^{68}Ga-DOTATOC PET [271].

The purpose of our study was not to directly compare the performance of ^{111}In-DTPA-octreotide scintigraphy and ^{68}Ga-DOTATATE PET but to assess the clinical utility of ^{68}Ga-DOTATATE PET/CT in a specific clinical subset with negative or low grade uptake of ^{111}In-DTPA octreotide. In these patients we have clearly demonstrated that ^{68}Ga-DOTATATE PET imaging leads to changes in management. The role of ^{68}Ga-DOTATATE PET seems to be similar to that of ^{111}In-DTPA-octreotide scintigraphy, in that its main clinical uses are in localisation of disease and assessment of suitability for treatment with somatostatin analogues and radiotargeted therapy. In this study, 39% of patients were deemed suitable for radiotargeted therapy with on the basis of ^{68}Ga-DOTATATE PET imaging. To date 8 patients have received ^{90}Y-DOTATATE peptide receptor therapy, of which 7 have completed three cycles of therapy. Of these 7 cases, 5 have achieved disease stabilization by RECIST criteria and 1 partial response (duration of follow-up 6-22months). 1 case showed initial disease stabilization, however, has progressed at 12 months post therapy. No difference in treatment response to peptide receptor therapy was observed between patients who were positive on ^{68}Ga-

DOTATATE PET and negative on ^{111}In-DTPA-octreotide scintigraphy compared to patients who received such therapy on the basis of positive ^{111}In-DTPA-octreotide scintigraphy. However, long-term follow-up data are required to confirm this finding.

Suitability for somatostatin analogue therapy in patients with non-functional but receptor-positive tumours is important in view of the potential antiproliferative action of SST analogues. There is evidence of anti-tumour activity of SST analogues in patients with functional NETs [277]. It is postulated that patients with non-functional NETs and negative ^{111}In-DTPA-octreotide scintigraphy but positive ^{68}Ga-DOTATATE PET imaging may well benefit from somatostatin analogue therapy.

In our study, 20 (39%) patients were deemed suitable for radiotargeted therapy with ^{90}Y-DOTATATE on the basis of ^{68}Ga-DOTATATE PET imaging. Seven have completed therapy with Y-90 DOTATATE, 6 of whom achieved disease stabilization and one minor response (<25% reduction in liver metastases size). No statistical difference in treatment response to peptide receptor therapy was observed between patients who were positive on ^{68}Ga-DOTATATE PET and negative on ^{111}In-DTPA-octreotide scintigraphy compared to patients who received such therapy on the basis of positive ^{111}In-DTPA-octreotide scintigraphy. However, long-term follow-up data are required to confirm this finding. No studies have reported so far on response rates of patients who have ^{68}Ga-DOTATATE PET positive but ^{111}In-DTPA-octreotide scintigraphy NETs.

At the time of primary staging, ^{68}Ga-DOTATATE PET has an important role in the localisation of disease, especially in patients who are negative on ^{111}In-DTPA-octreotide imaging. From a surgical perspective, localisation of the primary lesion, nodal involvement and distant metastases is of primary importance. Our study has shown the

clinical utility of ^{68}Ga-DOTATATE PET in this setting, in that it provides accurate staging of disease.

SSTR expression decreases as the histological grade of tumour increases, and high-grade tumours have limited SSTR expression [278]. In this study, ^{68}Ga-DOTATATE PET had similar sensitivities in identifying disease in low-, intermediate- and high-grade tumours. For an accurate assessment of the role of ^{68}Ga-DOTATATE PET in patients with low-, intermediate- or high-grade tumours, a study looking at unselected NET patients would be of benefit. Within the literature there is evidence that ^{68}Ga-DOTATATE PET is of limited value in patients with intermediate- and high-grade tumours and that FDG-PET may be more suitable for these cases [273].

Other PET tracers have also been trialled for imaging of NETs, including ^{11}C-5-hydroxytryptophan and ^{18}F-fluoro-L-3,4-dihydroxyphenylalanine. These have shown promising results, with higher detection rates than those achieved with ^{111}In-DTPA-octreotide SPECT and CT [279]. However, currently these substrates do not provide any therapeutic options, unlike somatostatin analogues, and therefore have limited clinical roles.

In conclusion ^{68}Ga-DOTATATE PET imaging detects 74% of lesions in patients with negative or faint uptake (Krenning score <2) on ^{111}In-DTPA-octreotide scintigraphy and changes the clinical management in 70.6% of these patients

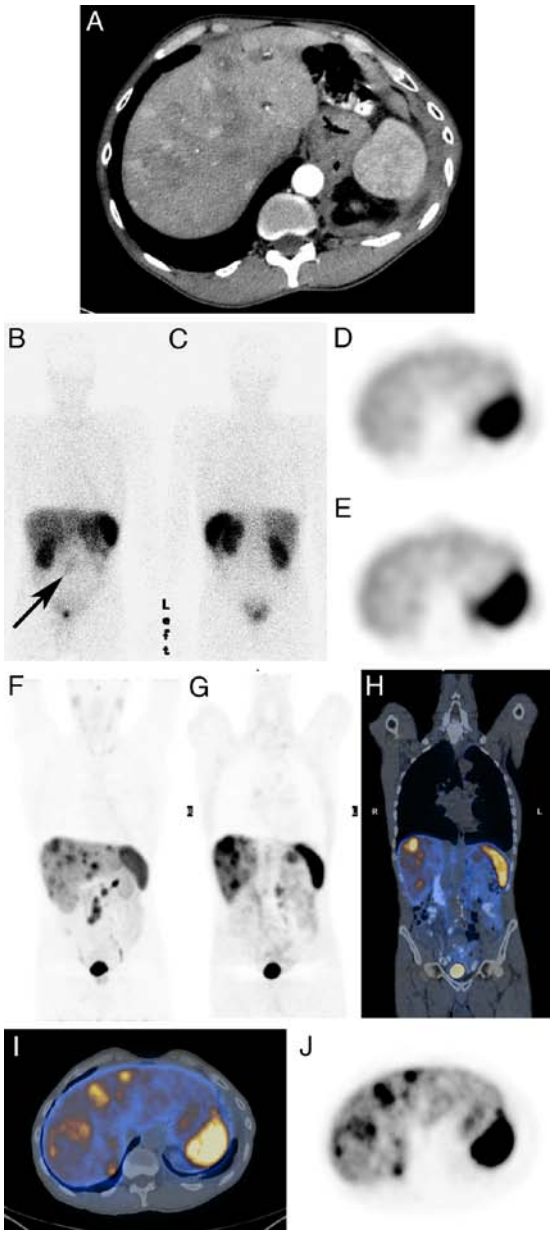


Figure 7. 1. Combined images demonstrating lesions using CT/ PET and 111In-DTPA-Octreotide scintigraphy. A 69 year old male patient with a low grade metastatic midgut NET, A) Arterial phase CT scan identifying multiple arterial enhancing and low attenuation liver metastases. B & C) Anterior and posterior whole body ^{111}In-DTPA-Octreotide scintigraphy showing low grade (Krenning score =1) mesenteric metastases (see arrow) but no liver metastases. D & E) SPECT axial images at level of spleen with heterogenous liver uptake and no discernable liver deposits. F) Maximum intensity projection (MIPS) image with ^{68}Ga-DOTATATE PET showing multiple deposits in the liver and mesentery. G) ^{68}Ga-DOTATATE PET coronal section anterior to the kidney shows multiple liver metastases. H) Fused ^{68}Ga-DOTATATE PET/CT image of figure 1G. I) Fused axial ^{68}Ga-DOTATATE PET/CT image at level of spleen showing multiple liver metastases. J) ^{68}Ga-DOTATATE PET axial image at level of spleen showing multiple liver metastases.

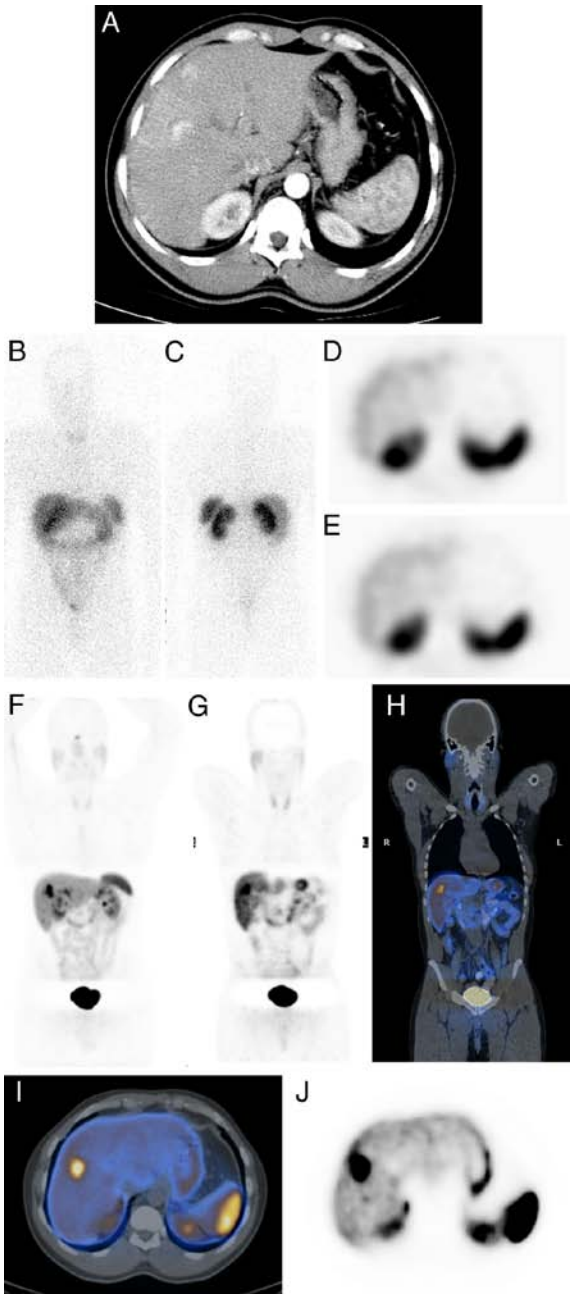


Figure 7. 2. Combined images demonstrating lesions using CT/ PET and 111In-DTPA-Octreotide scintigraphy. A 42 year old male patient with intermediate grade metastatic midgut NET. A) Arterial phase CT at level of splenic hilum, showing two arterially enhancing liver metastases. B & C) Anterior and posterior whole body ^{111}In-DTPA-Octreotide scintigraphy showing physiological uptake only. D & E) SPECT axial ^{111}In-DTPA-Octreotide images at level of the spleen, no discernable liver lesions identified. F) MIPs image identifying liver metastases. G) ^{68}Ga-DOTATATE PET coronal section showing liver metastases. H) Fused PET/CT image of figure 2G. I) Fused axial ^{68}Ga-DOTATATE PET/CT image showing large metastatic liver deposit. J) Axial ^{68}Ga-DOTATATE PET image showing large metastatic liver deposit.

Chapter 8: Overview and Discussion

NETs can arise from almost any organ within the body and demonstrate a diverse range of tumour behaviour; ranging from benign to highly malignant poorly differentiated tumours. Whilst many differences are present in tumour biology, there appear to be some similarities in terms of receptor expression and cellular pathways involved in oncogenesis.

Chapter 2

Somatostatin receptor expression in NETs is a well recognised feature of these tumours and provides the basis for nuclear medicine imaging with radiolabelled somatostatin analogues. It is known that low grade well differentiated tumours have high SSTR-2 and SSTR-5 expression, however, high grade tumours which are usually poorly differentiated loose expression of SSTR receptors. In chapter 2, we demonstrated a significant decrease in SSTR-2 and SSTR-5 receptor expression between low and high grade tumours, using immunohistochemistry. D2R expression had previously been demonstrated in NETs using RT-PCR, our experiments demonstrated for the first time expression of D2R expression in NETs using immunohistochemistry. There is a strong correlation of D2R and SSTR-2 expression, in all grades of tumours; which means patients with positive somatostatin scintigraphy are likely to have tumours that express both SSTR-2 and D2R receptors.

Dimerization of membrane receptors following response to external signal is a well recognised phenomenon [280] and there is also evidence of hetero-oligomerization of somatostatin and dopamine receptors [281]. Studies in human SSTR5 and D2R receptors tranfected in CHO-K1 cell lines have shown evidence of hetero-oligomerization following ligand binding to either receptor [281]. Following receptor activation, of either

receptor, there is hetero-dimerization and these results in enhanced function of the receptor. Rocheville et al, illustrated that binding of D2R receptor agonist, quinpirole, increased binding affinity of somatostatin by 3000% [281]. Recently it was demonstrated that human D2R and SSTR2 also operate as functional heterodimers following ligand stimulation with enhanced functional activity [148].

Chimeric agents that bind both SSTR and D2R receptors have been developed and shown activity in patients with pituitary adenomas and also in *in vitro* studies in NET cell lines [64;168;169]. Recent studies using the dopamine-somatatin chimeric compound BIM-23A760 has demonstrated antiproliferative effects in non-functional pituitary tumours [282]. New chimeric agents such as BIM-23A387 may have a role in symptom control for patients with carcinoid syndrome who have become resistant to therapy with somatostatin analogues. Clinical studies are currently being designed to evaluate this agent in patients with NETs.

The development of chimeric agents provides a promising avenue to treating patients whom have become resistant to therapy with standard somatostatin analogues, and these new agents may also have anti-proliferative effects. This study demonstrates that co-expression of SSTR and D2R receptors and therefore, patients who are SSTR-2 positive on ^{111}In-DTPA-octreotide scintigraphy are likely to express both SSTR-2 and D2R and could potentially be suitable for therapy with chimeric molecules.

Chapter 3

The EGFR family of G-protein coupled receptors are involved in tumour development of a number of different cancers, including breast, colon and head and neck squamous cell carcinomas. The expression of EGFR in NETs has been previously demonstrated using immunohistochemistry and RT-PCR [115;116]. Other studies have demonstrated expression

of HER-2 in some NETs. However, to date no studies have examined expression of all four HER family receptors in NETs. In Chapter 3, expression of the EGFR receptor family (HER-2, HER-3 and HER-4) was studied in NETs using immunohistochemistry. The results obtained were analysed in conjunction with a previous study which had assessed EGFR expression in the same cases. The EGFR staining from the previous study was re-assessed and identified expression in 86.5% of cases. EGFR expression has been correlated with worse prognosis in other cancers. However, this was not demonstrated in our study. HER-2 was not expressed in any of the tumours. This finding is different from other studies which have demonstrated HER-2 expression in small number of cases using immunohistochemistry[180]. The reason for different findings is unclear, the positive control in this study stained positively for HER-2 with strong membrane staining in a HER-2 positive breast cancer. Therefore, it is unlikely that technical limitations were responsible for the different in staining identified in this study compared to other previously published studies. The other studies were specific to pancreatic NETs and therefore, it may be selection bias, or the intensity of the scoring system used in between studies that has led to this variation in results. It would be interesting to assess for receptor expression in these samples using other techniques including RT-PCR, since the threshold of detecting receptor expression would be lower. HER-3 was expressed 8.5% (7 of 82 cases) of which 3 were paragangliomas. There appeared to be a survival benefit in patients expressing HER-3, however, since paragangliomas have a better outcome than other NETs, it is unclear whether HER-3 can be regarded as a possible prognostic factor. Further studies need to be performed in patients with NETs to fully evaluate whether HER-3 expression is of prognostic benefit. HER-4 was expressed 91.5% of cases, there was no association of prognosis with HER-4 expression. EGFR expression was not correlated with HER-3 or HER-4 expression. EGFR and HER-4 were co-expressed in 79.3% of cases.

This is the first study to demonstrate co-expression of the EGFR family of receptors. Importantly the high expression of EGFR may provide a possible therapeutic target for anti-EGFR therapy with chimeric monoclonal antibodies [283]. The preliminary results from phase II clinical studies looking at Gefitinib in NETs showed initial progression free survival, however, no objective clinical response [117;284]. It has been postulated that the low response is due to the fact that other signalling pathways are activated following inhibition of EGFR receptor [285]. There is evidence that strength of EGFR expression does not correlate to response to EGFR inhibitors [286], furthermore, EGFR negative tumours have been shown to be responsive to EGFR inhibitors [285;287].

With the development of HER-4 monoclonal antibody therapy, the high expression of this receptor in NETs may provide a possible role for molecular targeted therapy. However, the actual role of HER-4 in tumourogenesis is unclear, with some evidence supporting its role as an anti-tumoural receptor, with over-expression associated with positive prognostic value [171;174]. Studies in breast cancer have shown conflicting results with some reports associating HER-4 expression with short survival and others with longer survival [143;174;288]. Further work needs to be done to understand the downstream signalling that occurs following activation of HER-4.

Chapter 4

Tumour angiogenesis is integral to tumour growth and development of tumour metastases. A number of pro- and anti-angiogenic factors are produced by the body during normal development. In malignancy there is dysregulation of angiogenic factors with a predominance of pro-angiogenic factors. We demonstrated elevated Ang-2 in the serum in NETs compared to controls. Ang-2 was shown to be a sensitive marker (85.7%

sensitivity with Ang-2 >3313pg/ml), however not a specific marker for NETs since it is elevated in a number of other malignancies. Ang-1 concentrations were not elevated in patients with NETs compared to controls. Therefore, Ang-1 concentrations are not of benefit in identifying patients with NETs. It has been postulated that a shift in the Ang-2: Ang-1 ratio may in part be responsible for promoting angiogenesis. In this study examining the ratio of Ang-2: Ang-1 did not appear to be as a sensitive marker of disease as Ang-2 concentration alone. There was a significant trend in Ang-2 concentrations with volume of liver metastases. Ang-2 concentrations >4756pg/ml, were associated with shorter time to disease progression. This finding has not been demonstrated in NETs previously and requires further investigation to determine whether Ang-2 can be used to monitor response to different therapies. These initial findings of raised Ang-2 concentrations in NETs and its possible role as a prognostic marker has opened up a number of other potential studies and further work. We did not demonstrate that patients on SST analogues have a lower Ang-2 concentration than those not receiving SST analogues. Due to the heterogeneity of the groups studied further studies to see whether SST analogues reduce Ang-2 concentrations would be useful.

The vascular nature of NETs and the findings demonstrated in this study does raise the question whether analysis of other pro-angiogenic markers such as VEGF may offer a role as a prognostic marker. Currently it is difficult to measure a response to therapy in patients receiving different palliative therapies, since radiologically the majority of patients demonstrate no radiological response to most molecular targeted and chemotherapy agents. Reduction in CgA has been demonstrated in patients receiving biotherapy with somatostatin analogues though other markers would also be of value in demonstrating a response to therapy. There may be a role for Ang-2 in demonstrating a

response to therapy. Further studies assessing Ang-2 response to TACE or chemotherapy would be interesting.

Molecular therapies targeted to VEGF have shown promising results in NETs [119;289;290]. It would be interesting to see if inhibiting Ang-2 or the Tie-2 receptor would lead to inhibition of tumour progression. Ang-2 could potentially be an interesting molecular target for NET therapy.

Chapter 5

The second part of the thesis was aimed at looking at techniques to enable identification of NETs and also to monitor disease progression. Tumour markers are generally proteins that can be identified in the tumour or biological fluids. In NETs these tumour markers are either secreted proteins released from neuroendocrine cells, for example chromogranin A, or proteins involved in tumour growth and angiogenesis.

Chromogranin A is one of the best validated general markers for NETs currently available. However, it is not a 100% sensitive or specific and is dependent on the assay provided. The pancreastatin fragment of chromogranin A is one of the commercially available assays currently used, however, this is not marker is not as accurate as measuring the whole chromogranin A. Currently there are no biochemical markers that offer accurate measure of tumour load. Elevated chromogranin A has been proven to be a negative prognostic marker in patients with NETs [28]. To determine whether we could identify any new biochemical markers for identifying NET we performed proteomic analysis of plasma samples from patients with NETs and compared them to healthy controls.

2-D protein electrophoresis is a well established technique for identification of proteins from various media. In Chapter 5, we analysed the fractionated plasma from patients with NETs and compared the protein signatures on gels to those of healthy controls. Whilst over 200 protein spots were identified in each gel, a total of 4153 protein spots were identified in all gels. Using warping software which enables mapping the gels of patients and controls, three proteins of interest were identified; these identified using mass spectrometry and found to be albumin and pre-albumin. The most likely explanation for this is the fact even though >90% of albumin is removed by fractionation prior to gel electrophoresis, however this is not accurately standardised, Consequently, a slight increase in amount of albumin in the sample can cause a marked difference in expression on the gel. The main limitation with the use of plasma in 2-D electrophoresis is the large dynamic range of protein expression in plasma. Even with fractionation prior to electrophoresis, which removes >90% of the 20 most common proteins the expression of high abundance proteins is still 3-5 fold higher than that of low expressed proteins. Fractionation itself also causes problems in that proteins that may be bound to albumin are also lost from the sample. Other proteomic techniques have been used to analyse plasma samples, which avoid the use of 2-D gel electrophoresis.

Ideal media to perform 2-D electrophoresis is a one which contains the proteins of interest expressed at a moderate to high intensity so that it leaves a clear protein signature. Tumour tissue or cell lines are often a good source for identification of tumour markers, since the concentrations seen will be at the highest concentration in the tumour. Furthermore, the number of other proteins that are expressed in the serum or plasma such as albumin will not be as abundant in tissue. Therefore, a further study which may be of benefit will be to perform 2-D electrophoresis in resected tumour tissue from patients with NETs. In other cancers, tumour tissue based proteomic analysis has led to

the identification of a number of potential biomarkers using the 2-D gel electrophoresis technique [291;292].

Proteomics as a field is expanding and the technological advances in equipment is enabling a faster and higher throughput of material. The main issues with proteomic analysis are re-producability of results, which is why good proteomic analysis and validation of identified biomarkers, is required. In addition to the technique we employed (2-D gel electrophoresis and LC/MS/MS) a number of other proteomic techniques are available including Surface-enhanced laser desorption/ionization (SELDI) in conjunction with TOF/MS. This enables identification of protein peaks of interest between patient and control samples. The benefit of this system is that larger number of samples can be run however; further analysis is required to identify the proteins of interest.

Chapter 6

In Chapter 6, proteomic analysis was performed on three known NET cell lines, BON-1 a pancreatic cell line and two NET lung cancer cell lines(NCI-H727 & SHP-77). A total of 266 different proteins were expressed in the three cell lines, and 17 were expressed in all three cell lines. These 17 proteins included chromogranin A, chromogranin B, VGF-nerve inducible factor and Mac-2BP. We performed western blotting to confirm presence of Mac-2BP and Chromogranin A in NET cell lines. Validation of Mac-2BP as a potential marker for NETs was performed using immunohistochemistry of tumour tissue and ELISA of patient serum. 90% of cases demonstrated positive immunohistochemical staining for Mac-2BP in NET tissue. Serum analysis showed significantly elevated Mac-2BP in patients compared to controls.

Proteomic analysis of NETs cell lines to identify potential serum markers of NETs has not been previously described. Using this technique it may be possible to identify a number of potential markers which can be used aid in the diagnosis of NETs.

This study has demonstrated for the first time the potential role of Mac-2BP as a biomarker in NETs. Other markers of interest are VGF-nerve inducible factor and GDF-15. Of these VGF-nerve-inducible factor has been previously assessed in NET tissue using IHC [252]. However, no studies to date have been performed to assess whether this is a suitable plasma marker. GDF-15 has been proposed to be involved in tumourogenesis in other studies [293]; though its role in NETs has never been demonstrated.

The pathogenesis of desmoplasia is poorly understood, it is thought to be mediated via kinins as well as other proteins. Examination of NET secretory cell lines, looking for small secreted particles could potentially identify these proteins. From our study, the small proteins <10kDa are yet to be studied, further studies will be aimed at precipitating these proteins from the culture free serum and then identifying them using LC/MS/MS.

Further studies to elucidate the role of Mac-2BP in tumour progression in NETs would be of interest. Studies to date have identified its role appears to be in tumour metastases. However, a volume of research has also focussed on its role in HIV patients. It appears to be an integral protein in tumourgenesis with its expression being identified in a number of other cancers including breast, colo-rectal and lung cancer [251;253;258;294]. Mac-2BP also known as 90K is a glyco-protein that binds to galectins. It would be of interest to see whether inhibition of Mac-2BP from binding to galectins and beta-1 integrin has anti-proliferative effects.

Chapter 7

Imaging of NETs is essential for staging of disease, monitoring response to treatment and also with NETs to determine whether tumours are likely to be suitable for biotherapy or peptide receptor targeted therapy. Radiological imaging of NETs is comprised of conventional cross-sectional imaging (CT and MRI) plus nuclear medicine imaging (^{111}In-DTPA-octreotide scintigraphy). Over the last decade PET imaging has become integral to management of patients with certain types of cancer, however, its role in NETs is unclear. A number of different radio-ligands have been trialled in NETs. We performed a study to examine ^{68}Ga-DOTATATE PET imaging is of benefit in identifying tumours and altering clinical management in patients with negative or equivocal uptake of ^{111}In-DTPA-octreotide scintigraphy. Traditionally, patients with negative uptake on ^{111}In-DTPA-octreotide scintigraphy would not be considered for peptide receptor targeted therapy, since it would be thought their tumours expressed a low concentration of SSTR-2 receptors and so the radiolabelled agents would not to take up into tumour tissue.

In our study we demonstrated that ^{68}Ga-DOTATATE PET identified significantly more lesions than ^{111}In-DTPA-octreotide scintigraphy. Disease was identified in 41 of 47 patients using ^{68}Ga-DOTATATE PET all of whom had evidence of disease on cross-sectional imaging. Management was altered in 70.6% of cases; we concluded that ^{68}Ga-DOTATATE PET is a useful imaging modality in patients with negative or equivocal uptake on ^{111}In-DTPA-octreotide scintigraphy.

This study had limitations with regards to the study population, in that we selected a very select subset of patients in whom ^{111}In-DTPA-octreotide scintigraphy was weak or negative. To truly compare ^{111}In-DTPA-octreotide scintigraphy and ^{68}Ga-DOTATATE

PET, the study population should be unselected patients with similar primary sites. Furthermore, SPECT imaging should be performed of the chest and abdomen to enable more accurate lesion detection. For planar imaging ideally slower speed of imaging could potentially enable higher lesion detection. If this study is performed on a large group of patients then sensitivity, specificity and accuracy can be determined for both types of imaging.

Other studies of interest will be to see which PET tracer is best for imaging NETs. 68Ga-DOTATOC, 68Ga-DOTANOC, Fluorodeoxyglucose (FDG) and [11C] 5-hydroxy-L-tryptophan (5-HTP) have all been evaluated in NETs, with very different findings dependent in part on the tumour biology and primary site. It could well be that for tumours of different primary site and histological grade different radiotracers enable optimal disease localization.

Finally, another study of interest will be to see whether the (standard uptake value) SUVmax is reduced between pre- and post- therapy images. This may offer a way to assess outcome following therapy, since often there is no radiological change in lesion size using cross sectional imaging.

In conclusion NETs are regarded as rare tumours, however, due to their increasing incidence and indolent nature, their prevalence is much higher than many other cancers. Furthermore, due to their late presentation, palliative measures provide the mainstay of management. Therefore, novel treatments other than chemotherapy are required to enable symptom control and improve survival. This research has contributed to enabling further methods to image these tumours radiologically as well as opening potential avenues for monitoring patients with serological markers including Ang-2 and Mac-

2BP. Through immunohistochemical studies we have gained a better understanding of NET pathophysiology by demonstrating the expression of HER family of receptors as well as the co-expression of D2R, SSTR-2 and SSTR-5 receptors.

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