Circulating Tumour Cells and Biomarkers in Neuroendocrine Tumours

by

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DECLARATION OF ORIGINALITY

‘I, Mohid Shakil Khan, 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.’

……

M S Khan

‘When a plant goes to seed, its seeds are carried in all directions’[1]

Stephen Paget, 1889

ABSTRACT

Neuroendocrine tumours (NETs) are heterogeneous with respect to biological behaviour which ranges from indolent to highly aggressive. Consequently the prognosis is variable and biomarkers that are able to predict the rate of tumour progression or survival are required to inform clinical management. In practice, histological grade has proved to be one of the best available indicators of prognosis. The histopathologial biomarkers, Ki-67 proliferation index and mitotic count, are used to assign grade in a three-tiered grading system proposed by international NET guidelines. Agreement between Ki-67 and mitotic count is implied in guidelines but in a series of 131 metastatic pancreatic and 136 metastatic midgut NETs, I demonstrate a discordance of 44% and 38%, respectively, when assigning grade. Multivariate analysis of this data suggest Ki-67 is a superior prognostic marker, and the additional value of mitotic count is questionable.

Although Ki-67 offers prognostic information, biomarkers detected in blood have the advantage in being obtainable by relatively non-invasive methods and in being easily repeated during the disease course. Current biomarkers, used in routine clinical practice, include plasma chromogranin A (CgA) and 24-hour urinary 5-hydroxy indole-acetic acid (5-HIAA) but there is a lack of prospective trials evaluating these as prognostic and predictive biomarkers in NETs. Circulating tumour cells (CTCs) have been investigated in other cancers and found to provide prognostic and predictive information. Detection of CTCs using the Cellsearch™ platform requires the expression of epithelial cell adhesion molecule (EpCAM) which has not been systematically evaluated in NETs. By immunohistochemistry I have demonstrated EpCAM expression in the majority of NETs and, for the first time, detected CTCs patients with metastatic NETs. In 175 patients prospectively recruited, one or more CTCs was detected in 51% of midgut and 36% of pancreatic NETs. In patients commencing a new treatment for metastatic NET, one or more CTCs at baseline was an independent poor prognostic factor, offering better prognostic value than existing markers including grade and CgA. Furthermore, a change in CTCs at 3 to 5 weeks after commencing therapy was predictive of response to treatment and survival, suggesting CTCs could provide an opportunity to assess response, and to change therapy at an early time-point than with conventional imaging.

I have also evaluated circulating free DNA (cfDNA) as a potential molecular biomarker. After extraction from plasma and quantification using chip-based capillary

electrophoresis for the first time, cfDNA was demonstrated in patients with NETs. A higher concentration of cfDNA was found in in a series of 88 patients with metastatic NETs compared to healthy controls and there was a correlation between quantity of cfDNA and CTCs. However, since cfDNA was only detected in 25% of cases, more sensitive methods of detecting cfDNA may be required before further studies are conducted to validate cfDNA as a biomarker and to analyse mutations in cfDNA.

The hypervascular nature of NETs and their response to anti-angiogenic therapy such as sunitinib suggested that circulating endothelial cells (CECs) might also be informative in this tumour group. The presence of CECs was demonstrated in a series of 55 patients with NETs, using immunomagnetic separation and phenotyping with CD105. Although not significantly elevated, there was a wider range of CECs in NETs compared to healthy controls, possibly reflecting the underlying angiogenic process. Although no definite conclusions can be made, further studies investigating the relationship with markers of angiogenesis, and changes with anti-angiogenic therapy could prove valuable.

Given the increasing number of treatment options available and varied survival, it is unclear what treatments to offer, in which patients, and in which sequence. Grade according to Ki-67 offers some prognostic information at the time of diagnosis but my research suggests that circulating biomarkers, specifically CTCs, provide additional and better prognostic and predictive information repeatable at numerous time-points during the disease course. Furthermore, detection of CTCs and cfDNA in NETs allows the possibility of future studies into their molecular analysis which may enhance our understanding of NET pathogenesis and metastasis.

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COMMUNICATIONS

Peer Reviewed Publications

Directly arising from research

Khan MS, Tsigani T, Hartley J, Caplin M, Meyer T. Circulating Tumour Cells are Prognostic Markers in Neuroendocrine Tumours. *Journal of Clinical Oncology* 2012 (In Press)

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Other peer reviewed publications arising from thesis

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'Ki-67 is a better biomarker than mitotic count', Digestive Diseases Week (American Gastroenterology Association), Chicago May 2011

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LIST OF ABBREVIATIONS

AGA	American Gastroenterology Association
AI	Allelic Imbalance
AJCC	American Joint Committee on Cancer
APC	allophycocyanin or adenomatosis polyposis coli
APUD	Amine Precursor Uptake Decarboxylation
ASCO	American Society of Clinical Oncology
bp	base pairs (DNA)
BSA	Bovine Serum Albumin
BSG	British Society of Gastroenterology
C_q	Quantification Cycle (in qPCR)
CEC	Circulating Endothelial Cell
CEK	Circulating Epithelial Kit
cfDNA	Cell-free DNA
CgA	Chromogranin A
CgB	Chromogranin B
CHD	Carcinoid Heart Disease
CI	Confidence interval
CK	cytokeratin
CMV	Cytomegalovrus
CPK	Circulating Profile Kit
CT	Computed Tomography
CTC	Circulating Tumour Cell
DAB	Diaminobenzidine
DAPI	4', 6-diamidino-2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNES	Diffuse Neuroendocrine System
DOTATATE	DOTA-0-Tyr3-octreotate
dsDNA	double-stranded DNA
ECMC	Experimental Cancer Medicine Network

EDTA	Ethylene Diamine Tetraacetic Acid
EGFR	Epithelial Growth Factor Receptor
ENETS	European Neuroendocrine Tumours Society
EPC	Endothelial Progenitor Cell
EpCAM	Epithelial Cell Adhesion Molecule
ESA	Epithelial Specific Antigen
FBS	Foetal Bovine Solution
FCS	Foetal Calf Solution
FDA	Food and Drug Administration (USA)
FCiSt	5-Fluorouracil Cisplatin Streptozocin (chemotherapy)
FFPE	Formalin Fixed Paraffin Embedded
FITC	Fluorescein Isothiocyanate
g	grams
g	gravity
GEP-NET	Gastroenteropancreatic Neuroendocrine Tumour
GIST	Gastrointestinal Stromal Tumour
GSTP1	glutathione-S-transferase-π
5-HIAA	5-hydroxyindole acetic acid
HIF-1	hypoxia-inducible factor-1
hMLH1	human mutL homolog 1
hpf	High Power Fields
HR	Hazard Ratio
ICC	Intraclass Correlation Coefficient
kDa	KiloDaltons
K_W	Weighted Kappa
L	litres
LOH	loss of heterozygosity
MDR1	multidrug resistance 1
MEM	Minimal Essential Medium Eagle
mg	milligrams
µg	micrograms
mL	millilitres
µl	microlitres

MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
mTOR	mammalian Target of Rapamycin
NANETs	North American Neuroendocrine Tumour Society
NCRI	National Cancer Research Institute
NET	Neuroendocrine Tumour
NSE	Neurone Specific Enolase
NT pro-BNP	N-terminal pro-brain natriuretic peptide
OS	Overall survival
PABAK	Prevalence-adjusted Bias-adjusted κ
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PD	Progressive Disease
PDGFR	platelet derived growth factor receptor
PE	Phycoerythrin
PFS	Progression-free survival
PGP9.5	Protein-gene Product 9.5
PRRT	Peptide Receptor Radionuclide Therapy
PTEN	Phosphatase and Tensin Homolog
PTGS2	prostaglandin-endoperoxide synthetase 2
PVA	Poly Vinyl Alcohol
QC	quality control
QCP	QC primer
QCT	QC template
qPCR	quantitative (real-time) polymerase chain reaction
RASSF1a	Ras association domain family 1 isoform A
REMARK	REporting recommendations for tumor MARKer prognostic studies
RIA	Radioimmunoassay
RECIST	Response Evaluation Criteria In Solid Tumours
RFA	Radiofrequency Ablation
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RR Risk	Reduction

RT-PCR	Reverse Transcription Polymerase Chain Reaction
s-VEGFR	soluble VEGF receptor
SD	Stable Disease
SEER	Surveillance, Epidemiology and End Results (SEER) Program
SLE	systemic lupus erythematosis
SPSS	Statistical Package for the Social Sciences
TACE	Transarterial Chemoembolisation
TACSTD	Tumour-Associated Calcium Signal TransDucer
TAE	Trans-arterial Embolisation
TNM	Tumour Node Metastases (Staging)
TS	Thymidylate Synthase
TUNEL	Terminal deoxynucleotodyl transferase dUTP Nick End Labelling
UCL	University College London
UICC	Union for International Cancer Control
UKINETS	UK & Ireland Neuroendocrine Tumour Society
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF receptor
WHO	World Health Organisation

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Chapter 1. Introduction to Neuroendocrine Tumours (NETs) and Biomarkers

1.1. Background of NETs

1.1.1. Epidemiology

Originally thought to be rare, incidence rates in the 1980's reported fewer than 2 per 100,000 per year.[2] Recent data, however, suggests an incidence of 5.25 per 100,000[3]. This increase, particularly in gastroenteropancreatic NETs (GEP-NETs), probably reflects changes in detection, better pathological expertise and awareness, incidental findings on imaging/endoscopy rather than increasing burden since GEP-NETs were found in up to 1% of necropsies[4], more than expected (see Figure 1.1).

NETs are a heterogeneous group of tumours arising from midgut, pancreas, stomach, lungs, or colorectum, exhibiting diverse biological behaviour from relatively indolent to highly aggressive cancers. Given heterogeneity in survival, it is not surprising that recent prevalence rates have been reported up to 35 per 100,000, more common than that of most gastrointestinal cancers including hepatobiliary, oesophageal and pancreatic carcinomas[3].

Survival rates vary depending on grade and site of tumour. Pancreatic NET 5-year survival from the SEER registry was only 37.6%, but within this group, survival heterogeneity existed[5]. Survival ranged from 30% for somatostatinomas to 95% for insulinomas at 5 years. 5-year survival for other GEP-NETs were 68.1% for midgut NETs, 64.7% for gastric NETs, 81.3% for appendix NETs and 88.6% for rectal NETs.

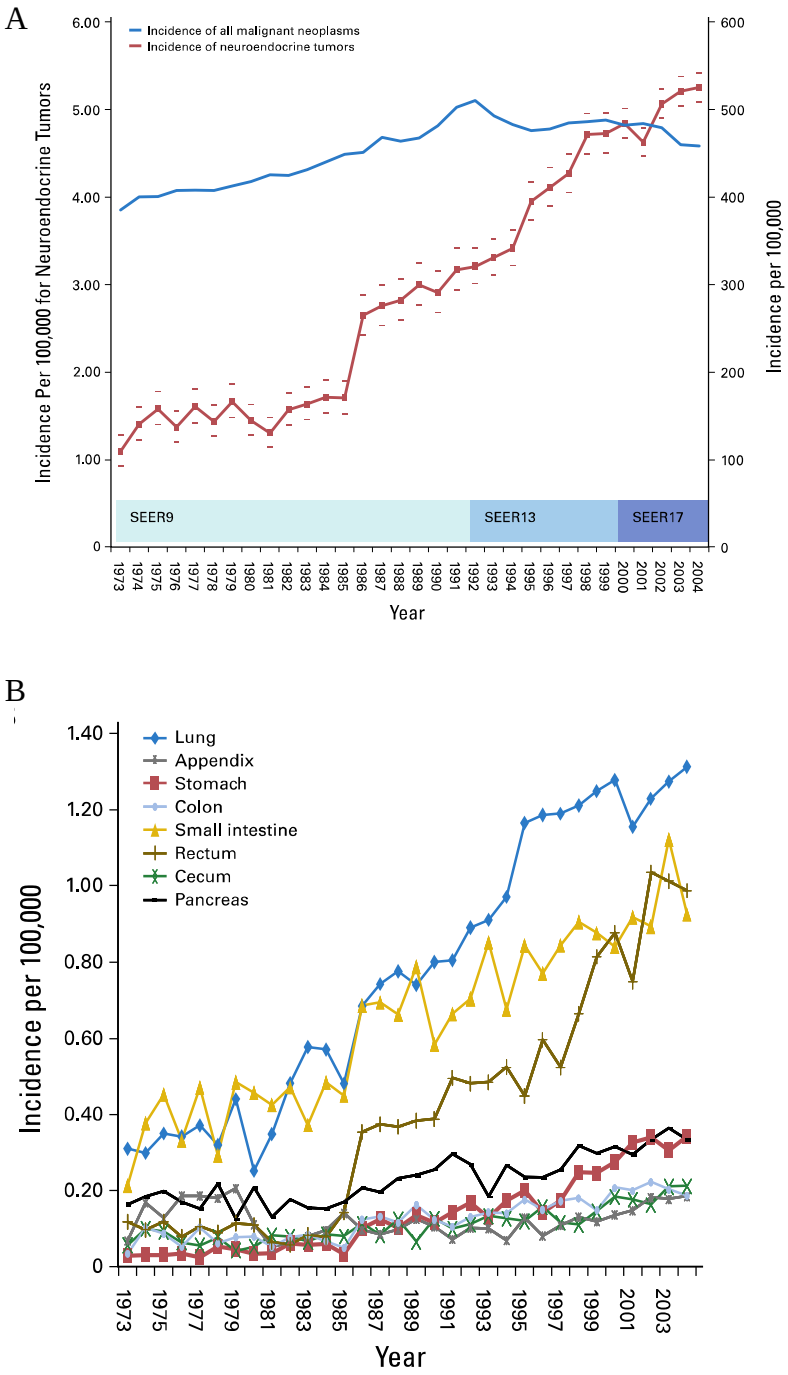


Figure 1.1 Incidence of neuroendocrine tumours (NETs) over time, by site and by disease stage. (A) Annual age-adjusted incidence of NETs by year (1973 to 2004) expressed as the number of tumours per 100,000 (95% CIs) age-adjusted. (B) Time-trend analyses of the incidence of NETs by primary tumour site (1973 to 2004). Statistically significant increases in incidence at all stages are shown ($P < 0.001$). [From Yao et al. One Hundred Years After "Carcinoid": Epidemiology of and Prognostic Factors for Neuroendocrine Tumors in 35,825 Cases in the United States. *JCO* 26(18)3063-72. Reprinted with permission. © American Society of Clinical Oncology. All rights reserved.]

1.1.2. Origin, Nomenclature and Aetiology

Neuroendocrine tumours (NETs) are malignant transformations of cells of the diffuse neuroendocrine system (DNES), the collection of various neuroendocrine cells scattered throughout the body[6]. Nomenclature through history has varied. Initially described in 1888 by Lubarsch as 'little carcinomata' in the distal ileum[7], the established term, carcinoid (or 'karzinoide'), was introduced by Obendorfer in 1907.[8] It was originally postulated that these various tumours throughout the body arise from common embryological tissue derived from the neural crest. This theory was supported by the Amine Precursor Uptake Decarboxylation (APUD) theory; the common ability of such cells to take up amine precursor molecules and to undertake decarboxylation of these substances resulting in the production of peptide hormones and biogenic amines.[9, 10] The APUD theory is still valid but the origin of NETs are increasingly thought to be the DNES, from cells of the pancreas and GI tract[11] and whether they are similar to other epithelial cancers remains controversial. Wnt signaling is required for renewal of intestinal stem cells and carcinogenesis in the gastrointestinal epithelium[12]. Wnt has also recently been reported to take part in the process of neuroendocrine differentiation[13] and thus this pathway may play a role in the development of NETs.

Through most of the 20th century, these tumours were labelled as 'carcinoid' tumours and confusingly 'carcinoid syndrome' was applied to clinical features of diarrhoea and flushing due to serotonin metabolite secretion from metastatic tumours derived from a midgut primary. By the start of the 21st century, terms such as 'neuroendocrine tumour' or 'endocrine tumour' were used in the WHO guidelines[14] and most recently, 'neuroendocrine neoplasms'[15] to encompass tumours of all behaviours.

The risk of NET in an individual with one affected first degree relative has been estimated to be approximately four times that of the general population; increasing to 12 times with two affected first degree relatives[16]. Although GEP-NETs are usually sporadic, familial syndromes including von Hippel-Lindau (vHL), tuberous sclerosis, multiple endocrine neoplasia 1 (MEN-1) syndrome and neurofibromatosis (NF-1) may be associated with pancreatic and proximal intestinal NETs.[17]

MEN-1 is an autosomal dominant disorder classically comprising of primary hyperparathyroidism (95%), pancreatic NETs (25-75%) and pituitary tumours (25-30%)[18]. NF-1 is an autosomal dominant disorder where loss of heterozygosity of NF-1 gene results in mTOR activation and tumour development[19]. It is usually diagnosed clinically, characterized by café au lait spots, cutaneous neurofibromas, optic gliomas

and iris harmatomas. vHL syndrome is caused by mutations in the VHL tumour suppressor gene (3p25-26) involved in regulating hypoxia-induced cell proliferation and angiogenesis. Clinical features include retinal or central haemangioblastomas, clear cell renal carcinomas, phaechromocytomas and pancreatic cysts. Pancreatic NETs occur in 15% so this should be screened for in cases[20].

Although the genetic basis of NETs has been difficult to elucidate due to heterogeneity, recently exome sequencing of sporadic pancreatic NETs resulted in the discovery of mutations of the MEN1 gene in 44%, and DAXX/ATRX genes (involved in chromatin remodelling) in 43% of cases[21]. These were associated with better prognosis. The p16/MTS1 tumour suppressor gene was also found to be homozygously deleted in 42% and methylated 58% of gastrinoma and non-functioning pancreatic NETs[22].

1.1.3. Classification

Since NETs exhibit a diverse spectrum of pathology, different classification systems have been devised to provide useful information for descriptive and prognostic purposes. However, many of these classification systems differ in criteria for grading and staging and also with regards to nomenclature and terminology.

Since the 1960's, NETs have been divided into fore-, mid- and hind-gut groups disregarding the mixed cell types of the foregut which include stomach, pancreas, duodenum and bronchial. Modern day practice involves classifying cases by primary site e.g. pancreatic, rectal or gastric NET. However, the term 'midgut NETs' is still used to identify tumours of the ileum or proximal colon.

Guidelines produced by the World Health Organisation (WHO) and European Neuroendocrine Tumour Society (ENETS) incorporate staging by tumour-node-metastasis (TNM) criteria for NETs of the gastrointestinal tract[23, 24, 25, 26]. Separate classification systems are in use for bronchial, thyroid and thymic NETs.

Classification is made according to site of primary tumour, size, invasion to muscularis propria and histological grade. Grade is particularly useful prognostically. It utilises mitotic count per 10 high power fields (HPF) or Ki-67 proliferation index to group NETs (Table 1.1). Ki-67 is a nuclear antigen associated with ribosomal RNA transcription, recognised by the MIB-1 antibody [27]. It is found in active phases of the cell cycle, in G1, S, G2 and M phases but not in resting G0 cells[28]. It is thus associated with cell proliferation and the percentage of cells staining for Ki-67 indicates the growth fraction. The higher the Ki-67 index, the more aggressive the tumour.

Grade	Ki67 (%)	Mitotic Count/10hpf
G1 (Low)	≤2	<2
G2 (Intermediate)	3-20	2-20
G3 (High)	>20	>20

Table 1.1 Grading classification of NETs according to the European Neuroendocrine Tumour Society (ENETS) Consensus Guidelines; hpf (high power fields)

Ki-67 proliferation index should be assessed in 2000 tumour cells in areas where the highest nuclear labelling is observed and mitoses in at least 40 high power fields.

The primary site and grade of NET affects survival. 5-year survival rates for pancreatic NETs are 94%, 63%, and 14% for low, intermediate and high-grade tumours respectively.[29] For midgut NETs, the figures are 95%, 82% and 51%.[30]

1.2. Diagnosis

GEP-NETs can be asymptomatic, diagnosed incidentally on imaging but may produce specific symptoms. Symptoms may relate to physical compression or obstruction of viscera by the tumour causing pain, nausea, vomiting or as a consequence of bioactive hormones released by the tumour. The syndromes described below are typically seen in patients with secretory pancreatic tumours[31] (Table 1.2).

Tumour/Syndrome	Symptoms
Insulinoma	Confusion, sweating, dizziness, weakness, unconsciousness, relief with eating
Gastrinoma	Zollinger-Ellison syndrome of severe peptic ulceration and diarrhoea
Glucagonoma	Necrolytic migratory erythema, weight loss, diabetes mellitus, stomatitis, diarrhoea
VIPoma	Werner-Morrison syndrome of profuse watery diarrhoea with marked hypokalaemia
Somatostatinoma	cholelithiasis; weight loss; diarrhoea and steatorrhoea. Diabetes mellitus
Non-syndromic pancreatic neuroendocrine tumour	Symptoms from pancreatic mass and/or liver metastases

Table 1.2 Syndromes associated with functioning pancreatic NETs

'Carcinoid syndrome', characterised by diarrhoea and flushing, is commonly a result of metastases to the liver, usually from a midgut NET with release of hormones such as serotonin and other vasoactive compounds, directly into the systemic circulation. In addition, midgut NETs may be associated with desmoplasia manifesting as intestinal and ureteric obstruction or heart failure associated with cardiac valve fibrosis.

The diagnosis of NET is confirmed on histology but the diagnosis of carcinoid syndrome is based on clinical symptoms, hormone profile, radiological and nuclear medicine imaging together with histology. NETs are often diagnosed with advanced disease after numerous years of vague symptoms typical of irritable bowel syndrome.

1.3. Imaging and Nuclear Medicine

Primary midgut NETs, being small, may be difficult to identify on imaging. Frequently, however, a lymph node metastasis with surrounding desmoplasia can be demonstrated as a 'mesenteric mass'. Pancreatic NETs and some NET liver metastases can be diagnosed on contrast-enhanced CT or MRI and are typically hypervascular in the arterial phase[32].

Endoscopic Ultrasound (EUS) can be performed to assess local invasion of gastric and duodenal NETs and for identifying and aspirating pancreatic lesions for tissue diagnosis (mean sensitivity 90%).[33, 34] The sensitivity of EUS may be reduced with extra pancreatic gastrinomas (80% of gastrinomas in MEN1 are found in the duodenum) for which an upper gastrointestinal endoscopy and CT or MRI should be performed.[35]

Most NETs express somatostatin receptors (SSTRs) of which there are five SSTR subtypes (SSTR 1-5) with SSTR-2 and SSTR-5 expressed in at least 80% and 77% of gastrointestinal NETs respectively.[36, 37] With the exception of insulinomas (only 50% express SSTR2), somatostatin receptor imaging e.g. Octreoscan™ or ^{68}Gallium-DOTA-Octreotate PET is the mainstay of staging and may assist in localising primary lesions in GEP-NETs.[38, 39]

Unlike adenocarcinomas, PET with 18-fluorodeoxyglucose ([18F]FDG) is often negative in low or intermediate grade NETs. However PET radionuclides such as ^{68}Gallium can be conjugated to somatostatin analogues for SSTR imaging. ^{68}Gallium-DOTA-Octreotate and ^{68}Ga-DOTA-Octreotide and more recently ^{68}Ga-DOTANOC have been found to be sensitive for NETs due detection of more SSTR subtypes and enhanced affinity compared to OctreoScan™.[40] These imaging modalities can help characterise metastases, assess extent of disease and locate primary lesions.

As with other cancers, monitoring the response of the tumour to therapy is currently achieved by measuring changes in size of target tumour lesions on interval imaging. The criteria has been standardised for most cancers by use of RECIST (Response Evaluation Criteria In Solid Tumours).[41] However, this may not be an ideal method of assessing response in NETs due to poor sensitivity in identifying necrotic lesions and also due to the slow-growing nature of most NETs.[42] Monitoring response in NETs with PET imaging is unproven and is undergoing evaluation[43].

1.4. Management in brief

Wherever possible, surgery should be attempted to achieve curative resection. In some cases with liver metastases, where the primary is resectable, resection of the liver metastases +/- ablation of non-resectable lesions may be considered as a curative approach.

Metastases are often present at the time of diagnosis e.g. in 60% of midgut NETs[44], where curative resection is usually not possible but surgery can be undertaken for palliation in selected cases[45].

The aim of medical treatment is thus to control tumour growth, prolong survival and improve symptoms (including those from excess hormone secretion) and quality of life. Treatment choice depends on site of primary, grade, co-morbidities, patient tolerability and availability of options. Management is guided by guidelines produced by the European Neuroendocrine Tumour Society (ENETS)[45, 46, 47] although the evidence base contains very few randomised-controlled or prospective studies.

Somatostatin analogues are the mainstay of treatment in low and intermediate grade metastatic midgut NETs. Until recently, this treatment was only indicated in functioning midgut NETs with 'carcinoid syndrome', symptoms of diarrhoea and cutaneous flushing as a consequence of secretion of serotonin and active metabolites from the metastases of a 'functioning' midgut NET into the circulation. The somatostatin analogues, octreotide and lanreotide in short and long-acting forms, have shown consistent benefit in alleviating the symptoms of carcinoid syndrome[48, 49]. However, a recent study suggests their use can be extended to non-functioning midgut NETs to prolong progression-free survival[50]. This placebo-controlled, double blind study in well-differentiated metastatic midgut NETs demonstrated that monthly injections of octreotide LAR prolongs time-to-progression. Median time to tumour progression in the treatment and placebo groups were 14.3 and 6 monthly respectively (HR=0.34 95%CI 0.2 to 0.59).

Interferon-α was introduced as a treatment for GEP-NETs in the early 1980's and exerts an anti-proliferation and anti-secretory effect. The usual dose employed is 3 to 5 million units subcutaneously, 3 to 5 times a week and symptomatic and biochemical responses have been noted in approximately 50% of patients with disease stabilisation in 60-80% at a follow up of 4 years[51, 52]. However, significant tumour reduction only occurs in 10-15%. Limitations in use of interferon include its side effects which include flu-like

symptoms, bone marrow suppression, thyroid disorders, psychiatric phenomenon and chronic fatigue syndrome. Therefore, it may be considered as second line therapy.

Systemic chemotherapy is widely used but its precise role is not known due to studies including various grades, sites, and inconsistent response criteria. Thus there is no standard regimen. Systemic chemotherapy has been the standard treatment for pancreatic NETs based on the data from Moertel *et al* with an objective response of 69%[53]. This study used one of the first combinations with streptozocin (STZ) and 5-fluorouracil (5-FU). For well-differentiated pancreatic NETs, chemotherapy is associated with a response rate of 6-70% but survival benefit is uncertain. A recent series (n=79) combined 5-FU, cisplatin and STZ (FCiSt) in chemo-naive patients with metastatic or locally advanced NETs[54]. Response rates were 38% for pancreatic and 25% for non-pancreatic sites with median time to progression 9.1 months and median overall survival 31.5 months with an acceptable toxicity profile and an advantageous one-day outpatient administration.

The use of chemotherapy in midgut and hindgut NETs has a much lower response rate, with <20% of patients deriving benefit, which may only last 6-8 months[55, 56]. The alkylating agent temozolomide, in combination with thalidomide in a Phase II trial, induced a response rate of 25% with median duration of response 13.5 months[57]. A more recent retrospective analysis found temozolomide monotherapy achieved radiological response in 14% and stable disease in 53%[58]. Temozolomide might also be used in pancreatic NETs. In a retrospective series of chemotherapy-naïve patients with metastatic pancreatic NETs, the combination of temozolomide and capecitabine resulted in an objective radiological response in 70% with 92% 2-year survival rate and only 14% grade 3 or 4 adverse events[59]. Temozolomide is generally well tolerated with minimal side effects including leucopenia, nausea and abdominal pain. Its response rate and duration of effect are similar to those of other established regimens. For poorly differentiated or high grade NETs, chemotherapy is first line with a response rate of approximately 70% but with rapid relapse and poor survival[60].

Sunitinib and Everolimus might be considered first line options for G1 and G2 pancreatic NETs with progressive disease[61, 62]. Sunitinib is an oral tyrosine kinase inhibitor with action against all VEGFR, PDGFR, stem cell factor receptor, and FMS-like tyrosine kinase-3. The recent phase III study of sunitinib vs placebo in slowly progressing pancreatic NETs (n=171) was halted due to the interim analysis showing

significant benefit with progression-free survival (PFS) 11.4 months for Sunitinib and 5.5 months with placebo[61].

Mammalian target of rapamycin (mTOR) is a threonine kinase and part of the phosphatodylinositol-3-kinase (PI(3)K)/Akt/mTOR pathway which is crucial in regulation of cell survival and proliferation. RADIANT-3 is a randomized double-blind, placebo-controlled, multicentre phase III study of everolimus, an mTOR inhibitor, plus best supportive care versus placebo and best supportive care in patients with progressive advanced pancreatic NETs. Results from the latter have recently been published (n=410) and demonstrate prolonged PFS with median PFS of 11.0 months with RAD001 (Everolimus) compared to 4.6 months with placebo[62]. Similarly, in patients with progressing NETs and symptoms of carcinoid syndrome, in the phase III study, RADIANT-2, everolimus and octreotide prolonged PFS compared to placebo and octreotide (median PFS 16.4 vs 11.3 months).[63]

If first line treatment fails or there is progression after an interval period, second line therapy includes peptide receptor radionuclide therapy (PRRT). This involves directing radioactivity internally to the tumour site delivered by a radionuclide, such as ^{90}Yttrium (^{90}Y) or ^{177}Lutetium (^{177}Lu), coupled to a somatostatin analogue. In a recent study of ^{90}Y-labelled DOTA-0-Tyr3-octreotate (DOTATATE) radiological partial response occurred in 23%, stable disease in 77% at 6 months with symptomatic response in 72%[64].

Other options for second-line therapy include re-challenging with chemotherapy, radiofrequency ablation, and trans-arterial hepatic embolisation. Ideally, all therapeutic options should be discussed within a dedicated NET multidisciplinary team.

Randomised trials of radiofrequency ablation (RFA) are lacking in NET metastases but series indicate patients with bilobar metastases less than 5 in number with diameter less than 5cm may benefit in terms of relief from the symptoms of NET liver metastases and in achieving local control of the metastases. It may also be considered in combination with resection with a better survival rate than with RFA alone[65]. In one large series, 34 patients with a total of 234 NET metastases were treated with RFA[66]. 80% experienced complete or significant relief from symptoms lasting an average of 10 months and 41% showed no evidence of progression. Another series found similar results with 69% demonstrating relief from tumour-related symptoms[67].

Embolisation of a hepatic artery branch is indicated for those with multiple non-resectable and hormone-secreting liver metastases. The intention is to reduce tumour bulk and thus hormone output which may improve quality of life and survival. It can be effective in both symptom control and as an anti-proliferative treatment. The mechanism is to induce ischaemia in tumour cells thus reducing their hormone output. Symptomatic response is achieved in 40-80%, biochemical response in 50-60% with overall 5-year survival 50-60% post-embolisation[68, 69, 70]. Obliterating agents include polyvinyl chloride and gel-foam powder. It appears that ischaemia may increase the sensitivity to chemotherapeutic agents, hence the rationale behind trans-arterial chemoembolisation (TACE) utilising concomitant doxorubicin or cisplatin[71]. Mortality has been quoted as 2-6% with adverse events in 8-17%, the most common being post-embolisation syndrome (nausea, fever, abdominal pain).

Due to the prolonged course of disease, many patients undergo several lines of therapy at different time-points. The optimal sequence has yet to be elucidated. The assessment of overall survival (OS) due to an intervention may be difficult to assess in clinical trials due to the prolonged disease course. Progression-free survival (PFS) or time-to-progression (TTP) may be more reliable as an endpoint and is used a number of recent clinical trials. However, response or progression as assessed by RECIST may not reflect the underlying tumour biology, and requires waiting for an arbitrary interval before post-therapy imaging is undertaken. PFS may not relate to OS and therefore, a biomarker that could be used as a surrogate endpoint for OS would be highly desirable.

1.5. Biomarkers in general

The definition of a biomarker is: a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes, or responses (pharmacologic or otherwise) to a therapeutic intervention[72].

Biomarkers can be considered in the following categories:

- Predisposition biomarkers: to identify individuals at risk of developing cancer
- Early detection biomarkers: to screen patients for the presence of cancer
- Diagnostic biomarkers: to determine the presence or absence of cancer and to assess the cancer type
- Prognostic biomarkers: to identify the likely clinical disease course (predicting survival outcomes) and to help select appropriate therapy

- Predictive biomarkers: to predict response to therapy and monitor drug effects to maximise the likely benefit from specific therapies i.e. as surrogate endpoints

There is a general lack of prospective trials to validate prognostic and predictive biomarkers in most cancers including NETs.

Given that most NETs present with advanced disease, the development of early detection and diagnostic biomarkers are important. Even though the majority of NETs have a more prolonged survival than other cancers, they are extremely heterogeneous in terms of survival[3]. Consequently, prognostic biomarkers are helpful for providing treatment recommendations. Additionally, predictive biomarkers are important to predict response to treatments in a clinical setting, as many patients will have numerous courses of treatments over their disease course. Predictive biomarkers would be valuable in a trial setting since use of biomarkers as surrogate endpoints for PFS or OS may accelerate trials that would otherwise need very prolonged follow-up to report survival outcomes. There is little data on the relationship between PFS and OS in NETs but Delea et al. demonstrated a correlation between PFS and OS in pooled data from 22 trials but did not find that risk reduction (RR) in PFS correlated with RR in OS[73].

1.6. Circulating markers specific to NETs

1.6.1. Chromogranin A

The neuroendocrine cells that give rise to NETs secrete a variety of bioactive products that define their role. The best 'general' marker for NETs is Chromogranin A (CgA) which is elevated in approximately 90% of GEP-NETs[74] and its measurement can help in NET diagnosis. It is measured commonly in plasma (and sometimes in serum) prepared from blood in a heparinised tube, chilled once collected, and centrifuged within 30 minutes in a non-fasting state. Common commercial kits include detection by enzyme-linked immunosorbent assay (ELISA) or radio-immunoassays[75].

CgA is an acidic, hydrophilic protein (~460 amino acids) with a molecular mass of ~70-85 kDa.[76] Alongside other members of the granin family, it is a precursor protein that can be processed at multiple cleavage sites to produce a large number of small bioactive peptides with a wide range of biological activity, although questions on physiological function remain. These include roles in the regulation of parathormone secretion,[77] inhibition of insulin secretion,[78] regulation of catecholamine secretion,[79] regulation of vascular tone,[80] and roles in the inflammatory response.[81]

Evidence suggests that CgA is the most useful general circulating marker for NETs and this is significant considering the poorly understood extensive DNES. CgA is a member of the granin family together with Chromogranin B (CgB), Chromogranin C (CgC), secretogranins III, IV, V, VI and VGF. These are proteins, found as components of dense-core secretory granules in NE cells and are secreted by these NE cells in a regulated fashion.

There are also individual amines and peptide hormones which are specific to certain NETs including insulin (insulinoma), gastrin (gastrinoma), glucagon (glucagonoma), and serotonin (midgut NET).

There have been numerous studies looking at CgA in NET diagnosis. Plasma CgA is raised in GEP-NETs, bronchial NETs, and phaeochromocytomas amongst other NETs. Excluding medullary thyroid carcinomas and paragangliomas, CgA has a sensitivity of over 60%. Sensitivity and specificity vary depending on the type on NET and burden. In a recent large study, sensitivity was 85.3%, specificity 96% when compared with healthy controls.[82]

Although there have been several retrospective studies, there are few prospective studies investigating the prognostic or predictive value of CgA in NETs and these are mostly where biomarkers are incorporated as part of a larger clinical trial.

These studies can be categorised into:

1) those correlating CgA to clinicopathological features
2) those investigating the prognostic value of CgA and investigating utility of CgA in detecting recurrence
3) those using CgA to monitor response to therapy (as a predictive marker)

1.6.1.1. Correlation of CgA to Clinicopathological Features

Several studies have shown plasma CgA correlates with tumour stage or burden in NETs. In a retrospective study, Campana et al. demonstrated CgA was higher in patients with diffuse disease than with localised or hepatic disease.[82] Nehar et al. showed that there was a difference in CgA levels between those with metastases compared to localised disease (sensitivities 73% and 26% respectively)[83] with similar results reported by Bajetta et al. (78% and 37% respectively).[84] All these studies were in heterogeneous populations of NETs but mainly with midgut or pancreatic primaries. When measuring tumour burden (albeit with a different methodology) in midgut NETs, Janson et al. found CgA levels were higher in those with >5 liver metastases compared

to those with <5 or lymph node metastases.[85] Similarly, in a large study (n=143) CgA levels were higher in cases with 3 or greater 'localisations' of tumour[86]. When hepatic tumour burden was classified by percentage of liver involved in another study, this also correlated with CgA[87].

CgA has also been found to inversely correlate with quality of life as determined by a standardised questionnaire.[88] However, it does not seem to correlate with symptoms of carcinoid syndrome.[89]

1.6.1.2. Prognostic Value of CgA

Plasma CgA, as a prognostic marker, has been adopted into clinical practice but in several retrospective studies, evidence is not consistent in proving its prognostic value. These studies followed on from utility of CgA in a different cancer, neuroblastoma.[93] Baseline serum CgA was found to be a significant predictor of PFS but the authors did not perform multivariate analysis and a cut-off of 190ng/ml was derived from preliminary data.

Several retrospective studies are summarised in Table 1.3. On univariate analysis of a large (n=324) retrospective series of pancreatic NETs, plasma CgA (elevated more than three times the upper normal limit) was a significant predictor of shorter overall survival but was not on multivariate analysis[90]. A baseline CgA >1000 μg/L (but not urinary 5-HIAA) was found to be associated with worse overall survival in a prospective phase II trial of long acting octreotide[88] although this cut-off seemed to be arbitrarily produced and only univariate analysis performed. A cut-off of CgA>5000 μg/L (levels fixed arbitrarily) was found to be an independent predictor of OS in another retrospective study of 301 patients[85].

One of the landmark prospective randomised control trials, looking at the effect of octreotide LAR in non-functional midgut NETs (PROMID), did not find elevated CgA to be prognostic or predictive and did not find any significant reduction in CgA, with either placebo or active treatment at 6 months[50]. This was despite a highly significant difference in time to progression (TTP) between the two arms of the study. Similar results were found in a large multi-centred, retrospective series of midgut NETs with liver metastases (n=360) where Ki-67 and age were independent predictors of survival, but CgA was not.[91]

Author	No. Patients	CgA Cut-off	PFS		OS	
			Univ	Multiv	Univ	Multiv
Ekeblad[90]	324 (pancreatic)	3XULN	ND	ND	2.6	N
Korse[88]	30 (midgut)	1000ug/L	ND	ND	Y	ND
Janson[85]	301 (midgut)	5000ug/L	ND	ND	4.5	ND
Rinke[50]	85 (midgut)	ULN not specified	N	ND	N	N
Ahmed[91]	360 (midgut)	'increasing levels'	ND	ND	N (but increasing CgA was)	N
Oberg[92]	207 (pancreatic)	2 X ULN (73ng/mL)	N	ND	ND	ND
Turner[54]	79 (mixed)	ULN (60 U/L)	ND	2.77	ND	6.77

Table 1.3 Summary of studies evaluating CgA as a prognostic marker. If prognostic, of PFS or OS, hazard ratios shown else Y/N. ULN=upper limit of normal; ND=not done; Y=Yes, prognostic; N=No, not prognostic; Univ=univariate analysis; Multiv=multivariate analysis; PFS=progression-free survival; OS=overall survival

The prognostic value of CgA has been assessed in other prospective clinical trials. In the RADIANT-3 trial, serum CgA and NSE were evaluated monthly in patients given oral everolimus or placebo with best supportive care in pancreatic NETs[92]. Although this was a subgroup analysis, elevated NSE at baseline was associated with shorter progression-free survival (PFS). Although suggested otherwise in the report there was no significant difference in median PFS between those with elevated versus normal CgA. This contradicts results from RADIANT-1 trial, the earlier phase II study, where elevated baseline CgA and NSE were associated with shorter PFS and overall survival (OS)[94]. In a separate study looking at patients undergoing chemotherapy with Fluorouracil, Cisplatin and Streptozocin, elevated baseline CgA (above upper limit of normal) was found to be a prognostic factor although was not predictive of response[54].

1.6.1.3. CgA in Monitoring Response

Use of CgA as a predictive marker is more controversial. In a large study of 344 patients, an increase in CgA was associated with tumour progression and shorter

survival in a heterogeneous group undergoing surveillance or treatment.[87] Nehar et al. investigated serial measurements of CgA during the follow up of 42 patients.[83] A concordance of 80% was found between rising CgA levels and progression (and diminishing CgA levels and response) but the 'change' in levels of CgA was not defined. In a study looking at CgA response to a variety of treatments, the authors concluded that CgA changes corresponded to tumour response or progression but this was only found in a subset of 10 out of 33 patients.[95] In a small series of 24 pancreatic NETs, although the authors concluded that levels of CgA correlated with progression or response, neither response rates nor statistical analysis were reported[96]. In another prospective study of octreotide long-acting release (LAR) in 40 patients with carcinoid syndrome, no correlation was found with response[89].

In one of the few prospective biomarker studies where a response was defined, CgA, urinary 5-HIAA, and NSE were evaluated at unspecified intervals but only CgA was assessed as a marker predicting response in a subgroup of 46 patients.[84] An arbitrary change in >25% from baseline was significantly associated with response to a variety of therapies in a heterogeneous NET sample but there was a 20% discordance. In a subgroup analysis of the RADIANT-2 trial, of placebo and octreotide vs. everolimus and octreotide, monthly serum CgA and urinary 5-HIAA were evaluated.[97] These markers were considered elevated if above normal reference range. A biochemical response was defined as a normalisation or >50% reduction. Patients on both treatments had higher biochemical response rates than the single treatment arm (46% vs. 29% CgA, 61% vs. 47% 5-HIAA) and greater reductions in both biomarker levels. Since there was a better PFS in the double treatment arm, it was concluded that reductions in these biomarkers correlate with response. In the phase II study of everolimus in pancreatic NETs, an early reduction in CgA or NSE was associated with a longer PFS compared to those without a reduction[98].

Twenty-two patients, in another study, who had cytoreductive surgery for neuroendocrine hepatic metastases had CgA measure before and after surgery[99]. A reduction of >80% in CgA was predictive of symptomatic and radiological response but this was a small study.

Response to treatments apart from somatostatin analogues has also been studied. In a study investigating the effect of FCiSt chemotherapy, neither CgA or CA19-9 was found to be predictive of response but AFP was.[54] Desai et al. demonstrated that changes (>20%) in plasma pancreastatin (derivative of CgA) before and 2-weeks after

TACE corresponded with radiological response.[100] However, very few patients died or progressed. CgA has also been shown to rapidly diminish or normalise after radical resection of primary lesion and metastases.[101] Although this study had few cases (n=7), median follow up was 36 months with recurrence or progression signified by a progressive increase in CgA levels. CgA may also be useful in monitoring for recurrence after 'curative' resection of midgut NETs with a sensitivity of 85-91%.[102, 103]

In addition to monitoring response in midgut and pancreatic NETs, there is some utility of CgA (and gastrin) in monitoring response in phaeochromocytomas[104] and type I gastric NETs[105].

To summarise, various retrospective and few prospective studies have investigated CgA as a prognostic or a predictive marker with inconsistent results. This is not surprising as some only include univariate analysis, some are small studies and are based on a heterogeneous population undergoing many treatments. Additionally, cut-off levels or definitions of 'response' or 'change' in CgA vary between studies and are often arbitrarily chosen. The majority have explored the relationship of CgA with overall survival and not progression-free survival.

Furthermore, pitfalls exist with measuring plasma CgA due to false-positive elevation in impaired renal function,[106] steroid treatment,[107] chronic atrophic gastritis,[108] and treatment with proton pump inhibitors (PPIs).[109] Additionally, there is no recognised international standard for the CgA assay. Stridsberg et al. compared the three commercially available assays and demonstrated sensitivities varying between 67% and 93% in NET patients[75]. To conclude, although adopted into clinical practice, there is a need for properly designed prospective studies evaluating CgA as a biomarker.

1.6.2. Chromogranin B

Chromogranin B (CgB) (also known as secretogranin I) is a protein of 76kDa and co-localises with CgA in the secretory granules of NE cells. It is considered to have a role in regulating secretion and is a major granin of the human adrenal medulla. There is a scarcity of clinical studies incorporating CgB, possibly due to a lack of commercially available assays, but preliminary studies have found patients with elevated plasma CgB may have a worse prognosis than with elevated CgA[110]. Additionally, it may be a more sensitive marker for phaeochromocytomas[111]. In patients with

phaeochromocytoma, CgB concentrations in plasma correlated with the content in tumour tissue and declined to normal levels after tumour resection[112].

1.6.3. Urinary 5-HIAA

Neuroendocrine tumours arising from the midgut may result in symptoms due to the secretion of serotonin or other peptides i.e. carcinoid syndrome, signified by diarrhoea and flushing. The urinary breakdown metabolite of serotonin is 5-hydroxyindole acetic acid (5-HIAA) and is usually measured over a 24-hour collection. 24-hour urinary 5-HIAA as a marker in NETs has not been investigated to the same extent as CgA.

The overall sensitivity and specificity of 5-HIAA in carcinoid syndrome is 70% and 90% respectively[113, 114] but it may be normal in non-metastatic midgut NETs.

1.6.3.1. Prognostic Value of 5-HIAA

A summary of retrospective studies evaluating 5-HIAA as a prognostic marker is shown in Table 1.4. Urinary 5-HIAA levels of >20mmol/mol creatinine were found to be associated with worse survival on univariate and multivariate analyses in a study of 76 patients with NETs.[115] However, in another retrospective study of 301 patients, urinary 5-HIAA (dichotomised arbitrarily >300 μmol/24hours) was a prognostic factor on univariate but not multivariate analysis[85].

In a retrospective study of a heterogeneous group of 314 patients with midgut NETs, urinary 5-HIAA >250 μmol/24hours was associated with shorter median survival but only univariate analysis was undertaken.[116] Interestingly in this study, levels of urinary 5-HIAA diminished following resection of primary lesions despite liver metastases. In an older study of 50 patients, elevated 5-HIAA above normal was a prognostic factor on univariate analysis.[117] In a more recent national retrospective study of 316 midgut NETs, increasing urinary 5-HIAA was prognostic for worse survival on univariate but not multivariate analyses[91].

Raised baseline urinary 5-HIAA levels greater than 2 times the upper limit of normal (corresponding to median) was a predictor of survival on both univariate and multivariate analyses in a retrospective series of 119 metastatic midgut NETs[118].

In a retrospective study of a heterogeneous group of 139 midgut NETs, elevated urinary 5-HIAA, above normal reference range, correlated with extent of metastases and was associated with poorer survival on univariate but not on multivariate analyses.[119]

Author	No. Patients	5-HIAA Cut-off	PFS		OS	
			Univ	Multiv	Univ	Multiv
Van der Horst-Schrivers[115]	76	20mmol/mol creatinine	ND	ND	3.33	1.007
Janson[85]	301	200umol/24h	ND	ND	Y	N
Hellman[116]	314	250umol/24h	ND	ND	Y	ND
Agranovich[117]	50	8mg/24h	ND	ND	Y	ND
Ahmed[91]	316	Unclear	ND	ND	Y	N
Formica[118]	119	2X ULN (median)	ND	ND	1.87	2.36
Turner[119]	139	42umol/24h	ND	ND	Y	N

Table 1.4 Summary of studies exploring prognostic value of urinary 5-HIAA. If prognostic, of PFS or OS, hazard ratios shown else Y/N.ND=not done, Y=Yes, prognostic; N=No, not prognostic; Univ=univariate analysis; Multiv=multivariate analysis; ULN upper limit of normal; PFS=progression-free survival; OS=overall survival

1.6.3.2. Monitoring Response to Treatment

A subset on 52 patients were treated with somatostatin analogues in one study but reduction in urinary 5-HIAA did not correlate with response to treatment[119].

The above prognostic studies were conducted in mixed populations undergoing various treatments. However, on univariate analysis of patients undergoing trans-hepatic arterial embolisation (TAE), over half of patients had a 50% reduction in 5-HIAA levels. A correlation was found with survival and on multivariate analysis, percentage change in 5-HIAA was an independent predictor of survival.[68, 102]

In a more focussed study, 15 patients with midgut NETs, carcinoid syndrome and progressive disease were treated with hepatic artery embolization (HAE) or radiofrequency ablation of liver metastases.[120] Reduction in urinary 5-HIAA was associated with a symptomatic response to treatment but sample size was small.

Somatostatin analogues, being the mainstay of therapy in GEP-NETs and in carcinoid syndrome, have been demonstrated to reduce levels of urinary 5-HIAA and in some cases this occurs alongside symptomatic improvement.[121, 122, 123]. In an open-label study of prolonged release lanreotide (n=71), urinary 5-HIAA was useful in monitoring response.[124] Reductions in urinary 5-HIAA were seen alongside symptomatic

improvement but follow up was short. Although CgA correlated with survival and quality of life in patients treated with somatostatin analogues, urinary 5-HIAA did not.[88]

In one of the largest series (n=111) investigating treatment with interferon (75% midgut NETs) demonstrated >50% reduction of urinary 5-HIAA in 42% of cases (termed a biochemical response).[125] However, although implied, the relationship between this reduction in markers and objective radiological response was not mentioned.

Since approximately 20% of patients with carcinoid syndrome have carcinoid heart disease, 5-HIAA has been evaluated in this situation. Correlations have been demonstrated between 5-HIAA and severity of carcinoid heart disease[126] and between rising urinary 5-HIAA and progression of carcinoid heart disease.[127]

1.6.3.3. Surveillance

Reduced levels of 5-HIAA were seen after radical resection of primary lesion and metastases in a small study.[101] However, unlike rising CgA indicating tumour progression during post-operative surveillance, urinary 5-HIAA levels did not increase. In surveillance following radical resection of midgut NETs, serial measurements are a relative insensitive method to detect recurrence compared to plasma CgA (18% vs. 85%).[102]

1.6.3.4. 5-HIAA Overview

In summary, as with CgA, various retrospective and few prospective studies have investigated 5-HIAA as a prognostic or a predictive marker and results are inconsistent. Many studies only include univariate analysis while others have studied heterogeneous populations undergoing numerous treatments. Cut-off levels or definitions of ‘response’ or ‘change’ in 5-HIAA vary between studies, often arbitrarily chosen.

The measurement of 5-HIAA is also subject to confounding factors including compliance with 24-hour urine collection. Patients are required to adhere to a strict diet for 3 days prior to collection. This is as a result of tryptophan-rich foods including plums, pineapples, bananas, aubergines, tomatoes, avocados, and walnuts increasing urinary levels. Additionally, certain drugs increase levels e.g. acetanilide, phenacetin, glyceryl guaiacolate (found in cough syrups), cisplatin, fluorouracil (used in NET treatment), and melphalan. Other medications may reduce levels including

chlorpromazine, heparin, tricyclic antidepressants, levodopa, monoamine oxidase inhibitors, and promethazine.

False negative results occur in renal impairment and dialysis. Falsely raised levels occur in malabsorption of coeliac disease, tropical sprue, Whipple's disease, and cystic fibrosis where increased tryptophan metabolites are encountered in urine.

This has led to the European Neuroendocrine Tumour Society (ENETS) guidelines stating that urinary 5-HIAA is not a consistently reliable prognostic factor in this disease[128].

1.6.4. Other Markers

In a large retrospective series, elevated α-fetoprotein and human chorionic gonadotropin-β were found to be associated with worse overall survival but only 10-12% of patients had elevated levels.[129] Interestingly, these also correlated with CgA.

Turner et al. demonstrated neurokinin A (NKA) correlated with extent of metastatic disease and was independently prognostic of survival.[119] Reduction in levels also correlated with response to treatment with somatostatin analogues.

Fasting gut hormones have a role as diagnostic markers in functioning pancreatic NETs as shown in Table 1.2. Their role as prognostic or predictive markers is unclear. Levels of gastrin reduced significantly after resection of gastrinomas but there was a low sensitivity and specificity of detecting tumour progression or stabilisation[130]. Using vasoactive intestinal peptide (VIP) to monitor response to therapy is limited to case reports and series[131]. There is evidence to suggest Calcitonin is useful in medullary thyroid cancer. Persistently elevated calcitonin after thyroidectomy can signify residual disease[132]. The probability of local or distant metastases is strongly related to calcitonin levels; patients with calcitonin levels >150 ng/L, systemic imaging of the thorax, liver and bones is indicated, and the probability of detecting distant metastases increases as the calcitonin levels increase[133].

Neurone specific enolase (NSE) has been mentioned above but it is less sensitive than CgA (sensitivity less than 40% in NETs)[86] and subgroup analysis has suggested a relationship to survival but not as a marker predictive of response[84, 92].

Markers of angiogenesis in serum such as angiopoietin-2 have been found to elevated in NETs compared to controls and also associated with reduced time-to-progression[134, 135].

Screening of carcinoid heart disease (CHD) in patients with midgut NETs has been revolutionised by use of circulating N-terminal pro-brain natriuretic peptide (NT-pro-BNP)[136] although this is not a NET-specific marker and is utilised in left sided cardiac failure. Using NT-pro-BNP as a screening marker compared to regular echocardiography, the number of patients needed to diagnose one case of CHD reduced from 5.1 to 1.4 with elevated levels in those with CHD.

Novel markers are being discovered by new techniques. Gene expression profile analysis has suggested paraneoplastic antigen Ma2 autoantibody as such a marker and this has been validated as a marker of PFS and recurrence-free survival, albeit in a small study of 36 patients[137, 138]. Proteomic analysis of secretomes of cell lines have revealed Mac-2-binding protein (MAC-2BP) as another marker. Using ELISA, serum levels are increased compared to controls[139]. Finally, using real-time polymerase chain reaction (rtPCR), circulating mRNA of certain transcripts have been found to be elevated in some NETs[140].

1.6.5. Statement on Circulating Biomarkers

In conclusion, CgA is currently the best circulating marker in NETs but there are other promising markers in development. However, these need proper validation in large prospective studies with agreed and defined cut-off levels together with standardised methods of assay measurement.

1.7. Histopathological Markers

Neuroendocrine cells are characterised by uniform nuclei and clear granular cytoplasm. Originally, to assist in characterisation of NETs, silver staining was used (hence the term, ‘Argentaffinoma’) but immunohistochemistry superseded this using specific antibodies to relevant cellular products. Several general neuroendocrine markers are used to identify NETs including chromogranin A (CgA), neurone specific enolase (NSE), synaptophysin and protein-gene product 9.5 (PGP9.5). Immunohistochemical staining is used to identify the peptides and biogenic amines produced by the NET cells specific to the tumour type such as serotonin or insulin. None of these have been shown to have prognostic value.

Historically, GEP-NETs were separated into well differentiated and poorly differentiated, according to the degree to which the neoplastic cells resemble their normal cell phenotype. Well-differentiated NETs have nesting, trabecular or gyriform patterns of tumour cells, and produce neurosecretory granules with strong expression of

neuroendocrine markers such as Chromogranin A and synaptophysin on immunohistochemistry. Poorly differentiated NETs often lose expression of neuroendocrine immunohistochemical markers and have a much worse outcome.[141, 142]

The ‘grade’ of a NET refers to the biological aggressiveness of the tumour. Classifications of low- versus high-grade tumours have not been uniformly based on consistent pathologic characteristics but high-grade tumours often have worse outcomes. Some authors have utilised presence of necrosis, atypia, mitotic count[14, 90, 143] and others focussed on Ki-67 proliferation index[23, 24].

Several authors including the European Neuroendocrine Tumour Society (ENETS) have suggested a 3-tiered prognostic grading system for GEP-NETs based on similar tiered classification systems of bronchial and thymic NETs that correlate with survival [143, 144] These systems, assign grade by evaluation of certain pathological markers. Ki-67 proliferation index, using immunohistochemistry with the MIB-1 antibody, and counting cells undergoing mitosis are routinely used to classify NETs as low, intermediate and high grades[26].

The use of Ki67 and mitotic count as prognostic markers have mainly been evaluated retrospectively on NET specimens but these 3-tiered grading systems have been adopted into clinical practice to aid clinical decision making. Ki-67 and mitotic counts as prognostic markers are discussed further in chapter 2.

Several studies have investigated the prognostic and predictive utility of expression of novel markers on NET tissue. Akt, p53, human mutL homolog 1 (hMLH1), and hypoxia-inducible factor-1 (HIF-1) were associated with overall survival on tissue prior to chemotherapy and Akt, hLMH1, phosphatase and tensin homolog (PTEN), thymidylate synthase (TS) and CA9 correlated with treatment response.[145]

Downstream targets of the mTOR pathway have been shown to be associated with poorer survival including overexpression of p-S6K with strong expression of mTOR associated with higher proliferative capacity.[146]

1.8. Rationale for Studying Biomarkers in NETs

There is an unmet need for validated biomarkers for use in clinical practice using translational research methods. The disease course is frequently long and choice of treatments may be informed by historical information from a diagnostic biopsy.

Circulating biomarkers could provide real-time information on the biology of the tumour that can be repeated at several time-points throughout the disease. This would also have the advantage over tumour biopsies as repeated blood tests are more acceptable to patients than repeated biopsies and may be more representative of current tumour biology. Furthermore, progress of clinical trials in NETs tends to be slow due to prolonged survival endpoints compared to other cancers and a surrogate marker of survival would help development of new therapies.

1.9. Novel Biomarkers

Traditionally, biomarkers in cancer and other disease has been limited to circulating peptides and amines and histological markers on archival tissue. However, there is increasing research into tumour-derived cells and nucleic acids in the circulation which may better reflect tumour biology. Hundreds of biomarkers have shown initial promise yet have often yielded inconsistent conclusions or contradictory long-term studies[147].

1.9.1. Circulating Tumour Cells

Circulating Tumour Cells (CTCs) were first described in a patient with cancer by Ashworth in the nineteenth century where cells were found in peripheral blood similar to that of cells of the tumour[148]. The general model of the metastatic process is tumour growth, angiogenesis, epithelial to mesenchymal transition, local invasion, active intravasation, dissemination, circulating in the blood (CTC), attraction to specific organs, active extravasation, mesenchymal to epithelial transition and proliferation[149]. Thus, CTCs represent a great opportunity to investigate this process with access from a simple blood test. They have been detected at very low concentrations at a ratio of approximately $1:10^9$ normal peripheral blood cells and it is only relatively recently that technological advances have enabled their isolation and enumeration.

Techniques to detect CTCs are required to be highly sensitive, specific and reproducible. To date, several methods have been used including immunocytochemistry, reverse-transcription-PCR (RT-PCR), PCR, and flow cytometry[150, 151]. However, the majority of studies have utilised immunomagnetic separation where CTCs are captured by magnetic beads coated with antibodies typically against carcinoma antigens. The carcinoma antigen which has been commonly employed is Epithelial Cell Adhesion Molecule (EpCAM), a common antigen in epithelial cancers[152].

Immunomagnetic separation using EpCAM is the principle behind CTC enrichment by the CellSearch™ System[153]. This is a semi-automated platform that uses which captures CTCs and with a separate staining system enables microscopic identification and enumeration. Its high sensitivity, specificity, and reproducibility led to it being approved by the Food and Drug Administration (FDA) to monitor metastatic breast, colorectal and prostate cancers. Using this system, CTCs have been validated as prognostic and predictive markers in these cancers[154, 155].

Despite a significant proportion of NETs being diagnosed with advanced disease, CTCs have not been demonstrated in NETs and the systematic analysis of EpCAM expression in NETs has not been performed to date.

1.9.2. Circulating Nucleic Acids

Cell-free DNA (cfDNA) is defined as extracellular DNA found in the circulation of patients with cancer and other disease, in either plasma or serum. There is increasing interest in the utility of cfDNA as a diagnostic, prognostic and predictive marker. In major cancers, levels of cfDNA are higher than in healthy subjects with changes in quantity of cfDNA after treatment correlating with response[156, 157].

cfDNA, originating from tumour cells, has the advantage of carrying genetic alterations from the primary tumour, some of which may be tumorigenic. Thus mutations in tumour suppressor genes or oncogenes can be detected in blood with levels having a prognostic value and in some cases, value in predicting response to therapy[158]. Epigenetic phenomenon have also been studied in cfDNA with some aberrant methylation of tumour suppressor promoter regions found in serum or plasma as well as in tumour tissue[159]. Tumour derived cfDNA, therefore, has great potential and versatility as a biomarker. However, cfDNA has not yet been demonstrated in NETs.

1.9.3. Circulating Endothelial Cells

Circulating endothelial cells (CECs) have been found to be increased in the peripheral blood of patients in a number of diseases e.g. sickle cell anaemia[160], myocardial infarction[161], and endotoxaemia[162]. Since angiogeneisis and co-option of pre-existing blood vessels are important steps in cancer progression and metastasis[163], it has been hypothesised that CECs may have a role to play in these processes.

New blood vessels are formed by endothelial cells derived from the tumour microenvironment and it has also been shown that bone-marrow derived endothelial

progenitor cells mobilised by tumour signals may contribute to vasculogenesis[164]. The main surface marker used to detect CECs by immunomagnetic separation or by fluorescence-activated cell sorting (FACS) is CD146.

CECs are increased in a number of cancers compared to healthy subjects and may be associated with progressive disease[165]. Furthermore, numbers of CECs are affected by treatment and may be useful as a predictive marker[166]. Neuroendocrine tumours are considered to be hypervascular tumours but it is not known whether CECs are detectable in NETs.

1.10. Aims of This Thesis

Currently, tumour grade provides the best method of defining prognosis and is used in clinical practice routinely, adopted into international guidelines. My first aim (chapter 2) was to assess whether the current methods of assigning tumour grade with either Ki-67 or mitotic count were comparable and of equal prognostic value. Additionally, I define new thresholds for Ki-67 proliferation index which provide better prognostic value than current grading thresholds.

Given that tumour grade can be based on a small biopsy specimen which may have been taken years before a treatment decision needs to be made, and with the lack of prospectively validated biomarkers, I went on to explore the novel biomarkers in patients with NETs.

In chapter 3, I investigated whether NETs express EpCAM and hence, whether CTCs can be identified in the blood of patients with CTCs. Once identified, I investigated CTCs as prognostic biomarkers in chapter 4. I also studied CTCs as predictive biomarkers, monitoring response to therapy.

With traditional circulating biomarkers in NETs based on detection of peptides and amines, my aim in chapter 5 was to determine whether another form of circulating biomarker, cfDNA, is detectable in blood of patients with NETs. This would be the first step in determining whether NET DNA is detectable in blood, and whether it has any prognostic value as a biomarker. Since some authors suggest circulating nucleic acids are a surrogate marker of CTCs, I also explored this association.

Lastly, with the prior chapters dealing with non-peptide circulating biomarkers derived from the tumour itself, I focussed on CECs. Since NETs are though to be hypervascular

with anti-angiogenic agents, including Sunitinib, having proven clinical effect, I determined whether CECs are detectable and of prognostic use in NETs in chapter 6.

This thesis thus defines new thresholds utilising Ki67 proliferation index for NETs as well as demonstrating how CTCs can be utilised for prognostication and potentially for understanding the native tumour in an individual.

Chapter 2. Grading of NETs using Ki-67 Proliferation Index and Mitotic Count

2.1. Introduction

As discussed earlier, histopathological markers previously studied in NETs and used clinically include mitotic count and Ki-67 proliferation index. Gastroenteropancreatic (GEP)-NETs are largely indolent low-grade neoplasms with few aggressive cancers. Originally, GEP-NETs were separated into well-differentiated and poorly-differentiated tumours. Whether some well differentiated NETs behave as high grade tumours is controversial[167] but generally poorly differentiated NETs behave like high grade tumours.

Classifications of low- versus high-grade tumours have not been uniformly based on histological characteristics. Some authors have used presence of necrosis, atypia, mitotic count and others focus on Ki-67 proliferation index.

The Ki-67 nuclear protein is one of several cell-cycle-regulating proteins, most commonly demonstrated by immunohistochemistry with the mouse anti-human monoclonal MIB-1 antibody[168]. Ki-67 is a DNA-binding protein that is expressed in all phases of the cell-cycle except in G0 and is widely used to assess tumour proliferation[169]. Despite numerous studies as a prognostic biomarker, its exact function is yet to be elucidated.

Some authors question the use of Ki-67 proliferation index as an independent prognostic indicator[170]. The controversy of Ki-67 is not limited to NETs; proliferative markers have been used particularly in breast and prostate cancers.

Similar to NETs, there are a number of treatments available for prostate cancer with a proportion of cancers just requiring surveillance where mitoses are not frequently seen[171]. In a large study from the Trans-Atlantic Prostate Group (n=693), Ki-67 staining was a significant negative prognostic factor of survival on multivariate analysis in conservatively treated patients[172]. Interestingly, prognostication based on crude (semi-quantitative) estimation of Ki-67 proliferation index by a histopathologist was not inferior to formal quantitative assessment which could save arduous counting of cells.

In breast cancer, Ki-67 has become the most widely used method for comparing proliferation between tumour samples. Although studies suggest Ki-67 is a prognostic marker in breast cancer, cut-off thresholds vary considerably[173]. Recently, a consensus group was convened by co-chairs of the Breast International Group and North American Breast Cancer Group Biomarker Working Party[174]. They recognised, similarly to NETs, that many studies validating Ki-67 are retrospective with heterogeneous groups of patients treated by many methods. They were unable to suggest thresholds despite staining levels of 10-20% being the most common used to dichotomise populations but concluded that Ki-67 is the most robust prognostic immunohistochemical marker. In addition to a prognostic marker in breast cancer, it has been studied as a predictive marker[175], and as an endpoint for suppression of proliferation in clinical trials of neoadjuvant endocrine therapy[176].

Advantages of Ki-67 include the ability of most histopathology departments to perform immunohistochemistry on small quantities of paraffin-embedded tissue with verifiable control tissue.

Similarly, the prognostic value of mitotic counts is well established in breast cancer. In a series of 364 patients with invasive ductal breast cancer, mitotic activity was expressed per 10 hpfs and optimal thresholds (13 and 35 mitoses per 10hpf) for a three-tiered grading system were evaluated, clearly distinguishing prognostic groups based on survival[177].

Where tumour tissue is limited it may not be possible to perform an accurate mitotic count and in these cases, Ki-67 may provide a more accurate proliferative index. The NANETS guidelines still suggest that where adequate tissue is available, Ki-67 provides no additional information.

In breast cancer, investigators have incorporated Ki-67 into a panel of immunohistochemistry biomarkers to produce an prognostic algorithm[178]. However, the single measurement of mitotic activity was superior to a complex prognostication program[179]. Predictive models have been suggested in NETs but have not been systematically clinically validated[180].

Several authors have suggested a three-tiered grading system for GEP-NETs based on similar tiered classification systems of bronchial and thymic NETs that correlate with survival[143, 144]. In each system, the low and intermediate grades are related closely

with distinction made by proliferative rate or necrosis[26]. The criteria that define each category are not, however, perfectly matched between each proposed system.

The intermediate grade prognostic category is supported by various studies. Tomassetti *et al* found that a Ki-67 >2.6% was a negative prognostic factor in well-differentiated ileal NETs[181]. Various other groups also found that a cut-off of approximately 2% stratified different prognostic groups in pancreatic and midgut NETs[182, 183, 184]. Prior to these studies, subdividing well-differentiated NETs into low and intermediate grade based on mitotic count was also suggested[185, 186].

Other studies did not find low and intermediate grade subdividing of well-differentiated NETs to be prognostic. Durante looked at numerous candidate pathological factors but did not find a grading system (with intermediate grade Ki-67 of 2-10%) to be prognostic in 215 metastatic GEP-NETs[187].

However, it is difficult to compare these studies as they are mixture of retrospective and prospective series in heterogeneous populations with heterogeneous treatments.

The European Neuroendocrine Tumour Society (ENETS) have proposed a 3-tiered grading system for foregut, midgut and hindgut NETs based on findings by Rindi *et al*[23, 24]. This classifies NETs into low (G1), intermediate (G2) and high (G3) grade tumours according to Ki-67 ‘and/or’ mitotic count as detailed in Table 2.1. There has been inconsistency between a number of different grading/ staging systems and guidelines throughout the history of NETs with but the major guidelines have all been updated recently and are more comparable than with previous versions.

Grade	Ki-67 (%)	Mitoses per 10 hpf
G1	≤2	<2
G2	3-20	2-20
G3	>20	>20

Table 2.1 Grading of NETs as proposed by ENETS[23]. Hpf = high power fields

In 2009, the AJCC/UICC (American Joint Committee on Cancer/ Union for International Cancer Control) introduced TNM staging of gastrointestinal and pancreatic NETs which differ in several aspects from the ENETS guidelines[188]. High grade NETs are not included and there are differences in the primary tumour classification (T of TNM) for pancreatic and appendiceal NETs, a discrepancy studied

specifically by Liszka *et al*[189]. The guidelines do concede that Ki-67 is a useful prognostic marker. In 2010, the North American Neuroendocrine Tumours Society (NANETS) provided guidelines for clinical management and refer to diagnosis by either ENETS and AJCC/UICC systems but states that it should be indicated which is used[170, 190, 191, 192, 193].

The recent 2010 WHO guidelines suggest the AJCC/UICC system should be used but briefly mentions the ENETS TNM system and for the first time, a grading system that resembles that suggested by ENETS based on Ki-67 or mitotic count[26].

The 3-tiered grading systems have been adopted into clinical practice to aid clinical decision-making. The ENETs proposal implies that there is a clear agreement between grade according to Ki-67 index and grade according to mitotic count based on the above cut-off values. Definitive data do not exist to determine whether this assumption is correct or whether the cut-off values used to distinguish the 3 grades are optimal.

Strosberg *et al* found complete agreement (apart from one isolated case) between grading according to a 2-tiered system based on Ki-67 above and below 20% and mitotic count above and below 10 mitoses per 10 HPF[194]. Although this was a study of 83 GEP-NETs, a proportion were hindgut NETs and the categories were not the same as the 3-tiered system proposed by ENETs which has been adopted into routine practice throughout Europe.

To date, there has been no study systematically investigating the agreement between Ki-67 and mitotic count when using the ENETS grading system. I therefore investigated the agreement between grade according to Ki-67 index and grade according to mitotic count per 10 HPF at the time of diagnosis in midgut and pancreatic NETs. Additionally, I explored the prognostic value of each in terms of progression-free and overall survival in order to assess the validity of the 3-tiered classification.

This analysis provides a comparator for the assessment of novel biomarkers such as circulating tumour cells (CTCs) discussed in subsequent chapters.

2.2. Materials and Methods

2.2.1. Clinical Specimens

Prior to investigating a new biomarker, I wished to assess the clinical validity of these established biomarkers and grading system which have been internationally adopted. I have obtained Ki-67 immunohistochemical analyses on NET specimens as well as mitotic counts per 10 HPF as part of routine pathological evaluation.

Patients with metastatic gastroenteropancreatic NETs diagnosed between January 1989 and October 2009 were identified from a database at the Neuroendocrine Tumour Unit, Royal Free Hospital. Patients were diagnosed as having a NET morphologically and immunohistochemically with presence of metastatic disease measurable by RECIST 1.1[41]. Diagnostic tissue from either biopsy or surgical specimen prior to commencement of treatment had been fixed in formalin, then processed and embedded in paraffin. Sections were reviewed by a pathologist with expertise in NETs to establish diagnosis, degree of differentiation (well or poorly differentiated) and mitotic count. On light microscopy, mitotic figures (per 10 high power fields (HPF)) were evaluated in at least 40 fields of highest mitotic activity.

2.2.2. Immunohistochemistry

Sections from tumours were submitted for immunohistochemical examination to evaluate Ki-67 proliferation index. Three micrometer sections of tumour tissue were deparaffinised in xylene and rehydrated in graded alcohols. Endogenous peroxidase was blocked with 0.5% H_2O_2 in methanol for 10 minutes. Thereafter, sections were subjected to 3 minute pressure cooking heat-mediated antigen retrieval (HMAR). Immunohistochemical staining was performed with the NovoLink™ Polymer detection system (Novocastra, Newcastle-upon-Tyne, UK). Sections were incubated with MIB-1 antibody detecting Ki-67 (DAKO, Cambridgeshire, UK) at a dilution of 1:200 for 1 hour at room temperature, post-primary block for 30 minutes, followed by Novolink™ polymer for 30 minutes. Reaction products were visualised with application of diaminobenzidine substrate chromogen solution. Slides were counterstained in haematoxylin and mounted.

2.2.3. Assessing Ki-67 Proliferation Index

The Ki-67 proliferation index was determined by assessing the percentage of positively staining tumour cell nuclei in 2000 neoplastic cells in areas with highest degree of nuclear labelling where possible.

2.2.4. Assigning Grade

Histological grading was assigned to each case according to TNM classification proposed by the European Neuroendocrine Tumours Society (ENETS) [23, 24]. Low-grade (G1) NETs were defined by a Ki-67 index of 2% or less, or a mitotic count of less than 2 per 10 HPF; intermediate-grade (G2) by Ki-67 between 3 and 20%, or a mitotic count between 2 and 20 per 10 HPF; and high-grade (G3) by Ki-67 greater than 20%, or a mitotic count greater than 20 per 10 HPF (Table 2.1). Each case was assigned two grades, one grade according to Ki-67 proliferation index and one grade according mitotic count.

Cases were then reclassified into grades with alternative cut-offs, firstly defined by tertiles, and then into Ki-67 thresholds suggested by Scarpa *et al.*[29] G1:1-5%, G2:6-20%, and G3: >20%. These alternative classifications were analysed similarly to existing classifications.

2.2.5. Interobserver Reliability

To assess reliability, 44 H&E stained sections (for mitotic count) and 44 Ki-67 stained sections (for Ki-67 proliferation index) were reviewed by a second independent expert pathologist blind to the initial assessments. Sections were chosen to distribute low and intermediate grades evenly with a small proportion of high grade sections, reflecting clinical practice. Mitotic count and Ki-67 were assessed as above. Grade was assigned using both indices.

2.2.6. Clinical Data

Pre-treatment biochemical data obtained at the time of diagnosis included plasma Chromogranin A (CgA), and for midgut NETs, 24-hour urinary 5-hydroxy-indoleacetic acid (5-HIAA).

Patients underwent CT or MRI scans to monitor for disease progression. Imaging was interpreted by an independent radiologist using RECIST criteria. Progression-free survival (PFS) was recorded as the time from diagnosis to radiological disease

progression or death. Overall survival (OS) was recorded as the time from diagnosis to the patient's death.

2.2.7. Statistical Analysis

Statistical analyses were performed using SPSS for Windows (SPSS Inc., Chicago, IL) where *P* values of <0.05 were considered significant. Pancreatic and midgut NETs were analysed separately. Correlation between grades assigned by Ki-67 and mitotic count was assessed using non-parametric correlation. Agreement between grades was assessed with weighted kappas (κ_W) with significance of values stated in Table 2.2. Interobserver reliability was assessed using weighted kappas. Since CgA was not normally distributed (even when transformed onto a logarithmic scale) this was analysed in two groups: above and below twice the upper limit of normal (120 pmol/L)[92].

Weighted Kappa (κ_W)	Agreement
0-0.2	Poor
0.21-0.4	Fair
0.41-0.6	Moderate
0.61-0.8	Good
0.81-1.00	Very good

Table 2.2 Significance of weighted kappas as a measure of agreement between categorical variables

Survival was estimated using Kaplan-Meier methodology stratified by both grading systems and differences in survival between groups analysed by log-rank testing. I explored potential markers that were prognostic in terms of PFS or OS. Grading assigned by either index were analysed as categorical variables. Since CgA was not normally distributed (even when transformed onto a logarithmic scale) this was analysed in two groups: above and below 2 times the upper limit of normal (120 pmol/L)[92].Urinary 5-HIAA was analysed in two groups: above and below the median[118]. Cox-proportional hazards regression analysis was used to obtain univariate and multivariate hazard ratios for PFS or OS.

2.3. Results

2.3.1. Patient Characteristics

A total of 285 cases of GEP-NETs, 144 (51%) with primary tumour site in the pancreas, 141 (49%) of midgut origin were identified. 131 and 136 of these, respectively, had complete data and were included in the analysis. Patient characteristics are shown in Table 2.3.

Primary Site	**Pancreatic**	**Midgut**	**Total**
Number	131	136	267
Age at Diagnosis			
median years (range)	51.5 (21-81)	56 (22-84)	54 (21-84)
Gender			
Male	65	70	135
Female	66	66	132
Grade according to Ki-67			
Low	34	68	102
Intermediate	68	58	126
High	29	10	39
Grade according to Mitotic Count			
Low	65	84	149
Intermediate	55	50	105
High	11	2	13
Chromogranin A in pmol/L			
≤120	90	68	158
>120	41	68	109
Urinary 5-HIAA (µmol/24hr)			
≤96			
>96	-	61	-
Missing	-	60	-
	-	15	-
Subsequent Therapy			
None	1	5	6
Surgical resection	49	70	119
Chemotherapy	78	28	106
Somatostatin analogues	33	84	118
Interferon	5	3	8
Radiofrequency ablation	2	1	3
Embolisation	6	10	16
Radionuclides	14	40	54

Table 2.3 Background characteristics of patient group

2.3.2. Inter-observer Agreement

44 cases (22 pancreatic and 22 midgut NET) had both H&E and Ki-67 stained sections evaluated by a second expert pathologist. Inter-observer reliability was assessed between the grade assigned (according to ENETS) according to both indices, rather than the absolute mitotic counts or Ki-67 index. The matrix of this grading agreement is shown in Table 2.4 and Table 2.5.

The weighted kappa for agreement on grade assigned by mitotic count was 0.83 (95% CI 0.68-0.99). The weighted kappa for agreement on grade assigned by Ki-67 was slightly higher at 0.87 (95% CI 0.74-1.00). Although not perfect agreement, both these signify very good agreement. The four disagreements were between G1 and G2.

		Observer B			
		G1	G2	G3	
Observer A	G1	21	4	0	**25**
	G2	0	18	0	**18**
	G3	0	0	1	**1**
		21	**22**	**1**	**44**

Table 2.4 Interobserver Agreement of Grade Assigned by Mitotic Count

		Observer B			
		G1	G2	G3	
Observer A	G1	17	3	0	**20**
	G2	1	18	0	**19**
	G3	0	0	5	**5**
		18	**21**	**5**	**44**

Table 2.5 Interobserver Agreement of Grade Assigned by Ki-67 Proliferation Index

2.3.3. Agreement Between Grading According to Ki-67 Proliferation Index and Mitotic Count

Of 267 cases, 242 were well differentiated and 25 poorly differentiated NETs. Of the 21 pancreatic NETs that were poorly differentiated, one was graded as G2 and 20 as G3 according to Ki-67; one G1, 11 G2, and 9 G3 according to mitotic count. Four midgut NETs were considered to be poorly differentiated. These were all designated as G3 according to Ki-67 and all as G2 according to mitotic count.

There was a moderate (approaching strong) correlation between absolute Ki-67 index and mitotic counts ($\rho=0.65$ $P<0.001$ for pancreatic and $\rho=0.59$ $P<0.001$ for midgut NETs) (Figure 2.1). There was agreement between grade assigned by Ki-67 and grade assigned by mitotic count in 74 of 131 (56%) pancreatic NET cases; and in 84 of 136 (62%) of midgut NETs (Table 2.6 and Table 2.7 respectively). Thus there was a discordance of 44% and 38% respectively when assigning grade using Ki-67 or mitotic count. This corresponded with weighted kappas of 0.41 (95% CI 0.30-0.53) and 0.35 (95% CI 0.22-0.48) respectively (moderate and fair agreement respectively).

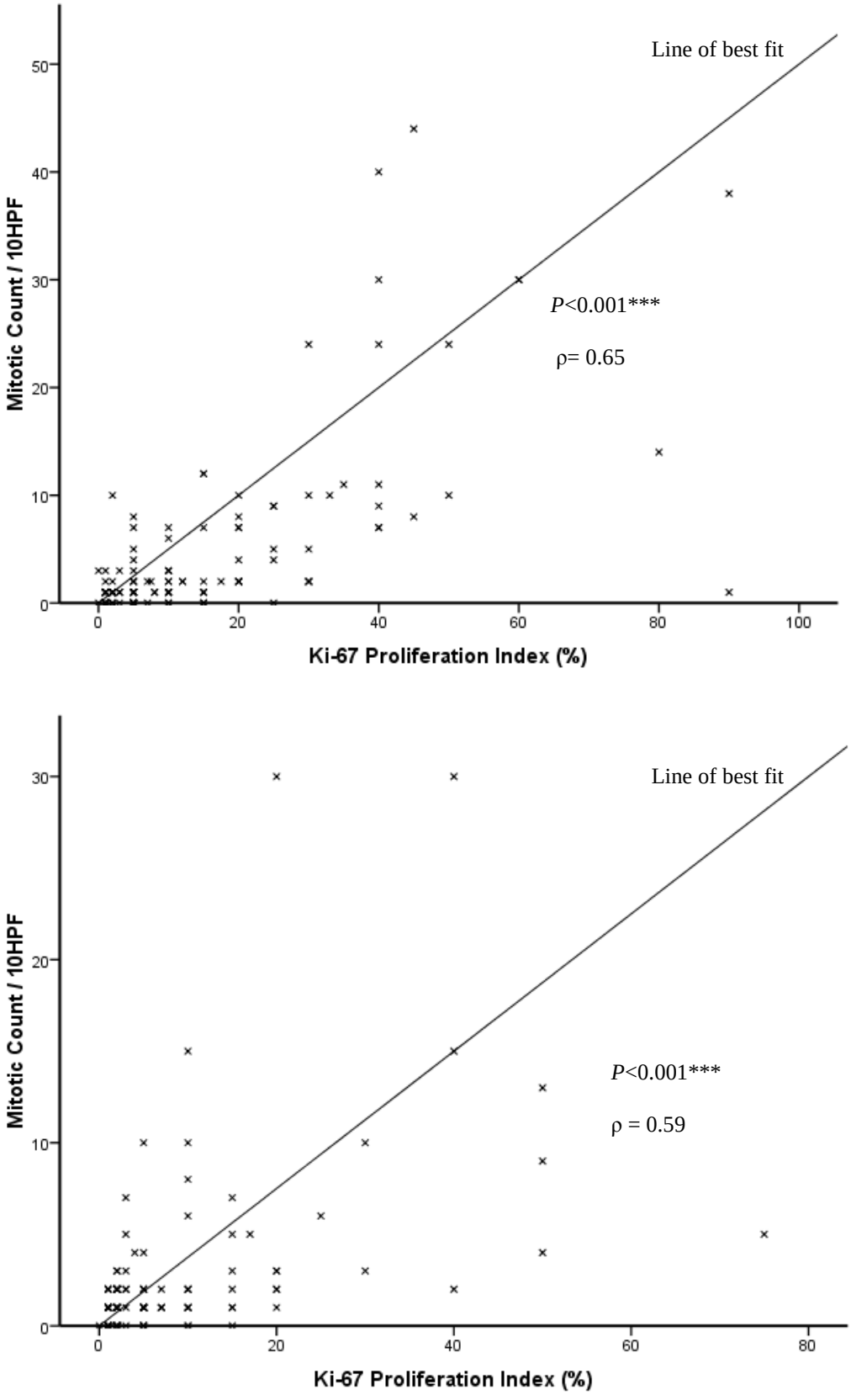


Figure 2.1 Correlation between Ki-67 and Mitotic Counts in **A** Pancreatic NETs **B** Midgut NETs

PANCREATIC		Grade according to mitotic count/10HPF			
		G1	G2	G3	
Grade according to Ki-67	G1	**29**	5	0	**34**
	G2	34	**34**	0	**68**
	G3	2	16	**11**	**29**
		65	**55**	**11**	**131**

Table 2.6 Agreement between grade assigned by Ki-67 and mitotic counts for pancreatic NETs (agreement in 74/131 cases)

MIDGUT		Grade according to mitotic count/10HPF			
		G1	G2	G3	
Grade according to Ki-67	G1	**55**	13	0	**68**
	G2	29	**28**	1	**58**
	G3	0	9	**1**	**10**
		84	**50**	**2**	**136**

Table 2.7 Agreement between grade assigned by Ki-67 and mitotic counts for midgut NETs (agreement in 84/136 cases)

2.3.4. Survival

Patients were followed up for a median of 46 months (pancreatic) and 42 months (midgut).

For pancreatic NETs, median PFS was 33 months and median OS 84 months. For midgut NETs, median PFS was 42 months, median OS 82 months. 1-, 3-, 5-, and 10-year survival data is shown in Table 2.8.

Pancreatic	**1Yr (%)**	**3Yr (%)**	**5Yr (%)**	**10Yr (%)**
PFS	76.9	44.8	29.3	13.4
OS	89.6	78.6	58.8	35.8

Midgut	**1Yr (%)**	**3Yr (%)**	**5Yr (%)**	**10Yr (%)**
PFS	85.4	57.5	37.3	14
OS	92.7	73.8	61.3	36.4

Table 2.8 PFS and OS for Pancreatic and Midgut NETs

The three-tiered grading systems according to either Ki-67 or mitotic counts were able to distinguish significantly different groups prognostically in pancreatic NETs (Figure 2.2). There was some overlap of Kaplan-Meier curves, however, between G1 and G2 in estimating OS using mitotic count.

When analysing midgut NETs, however, only grade according to Ki-67 was able to distinguish the 3-tiered prognostically different groups in terms of PFS and OS (Figure 2.3). Grade according to mitotic count was not able to distinguish G1 from G2 in terms of OS or PFS.

On multivariate analysis, there was evidence to suggest that a higher grade according to Ki-67 was a independent prognostic indicator of both PFS and OS in both pancreatic and midgut NETs (Table 2.9 and Table 2.10 respectively). Grade according to mitotic count, however, was found not to be prognostic. CgA above 120 was significantly associated with worse OS in pancreatic NETs only.

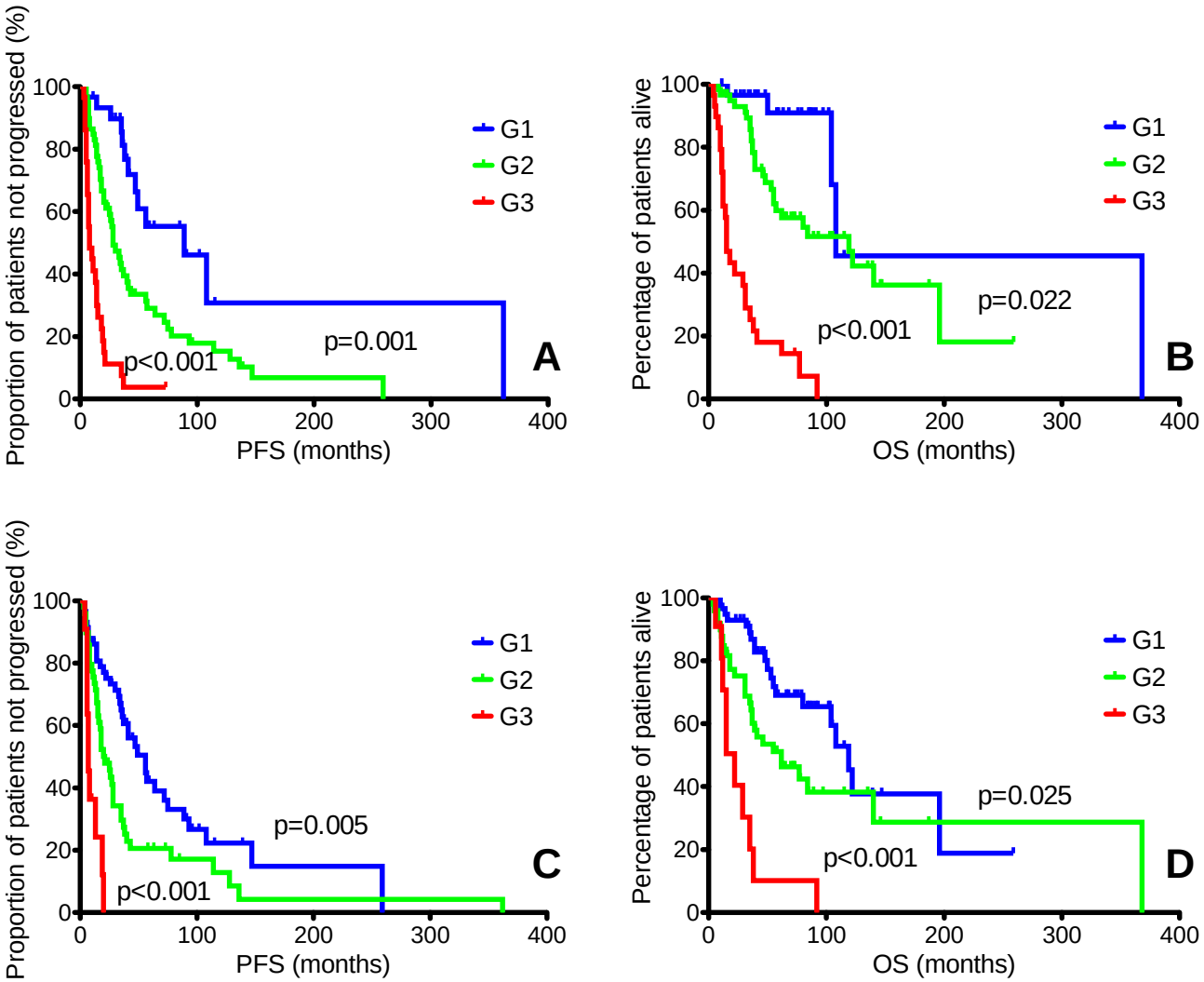


Figure 2.2 Survival curves for PFS and OS of pancreatic NETs using grade according to Ki-67 (A and B) or grade according to mitotic count (C and D); blue (G1), green (G2), red (G3)

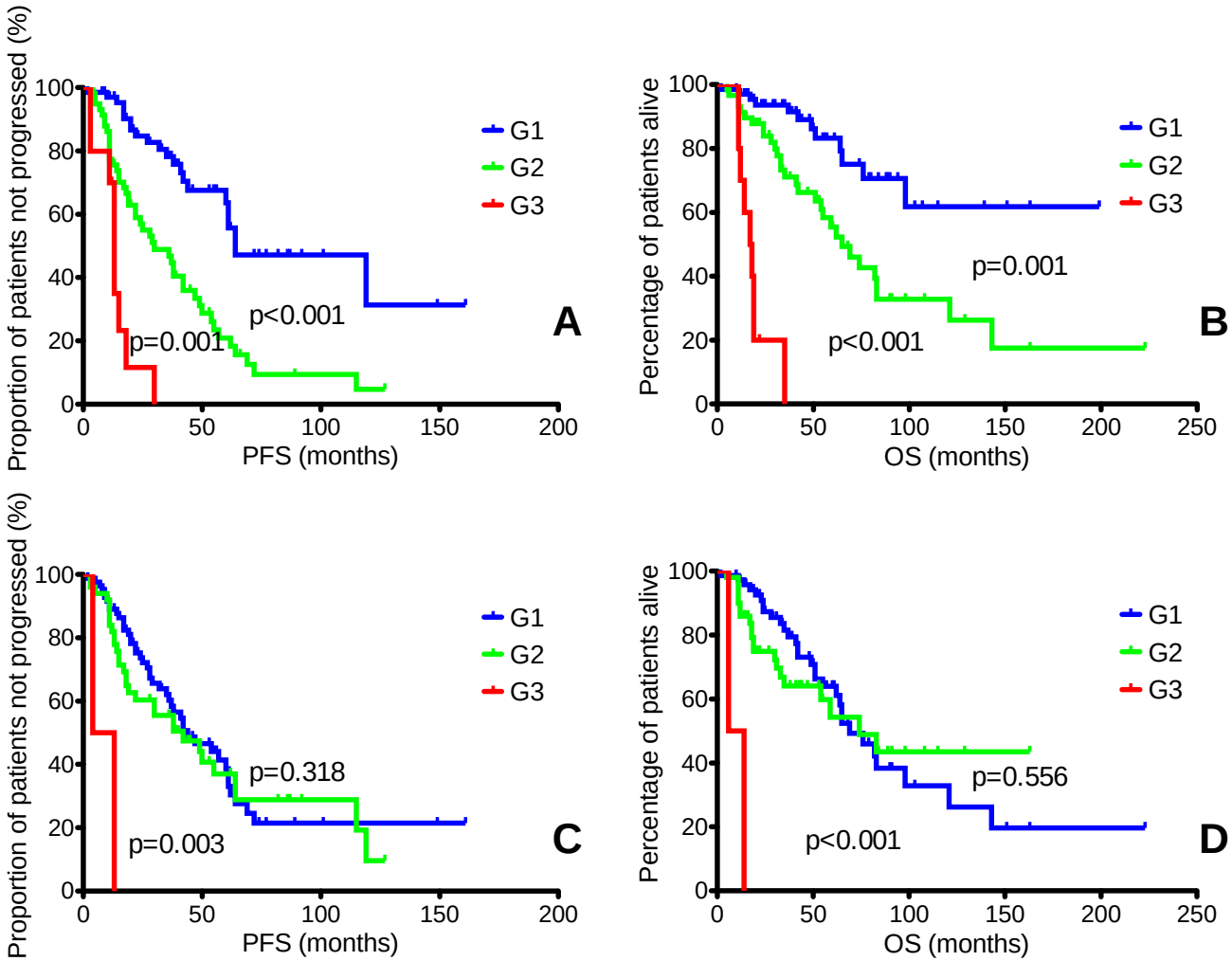


Figure 2.3 Survival curves for PFS and OS of midgut NETs using grade according to Ki-67 (A and B) or grade according to mitotic count (C and D); blue (G1), green (G2), red (G3)

Risk Factor	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Differentiation				
Well	1.00		1.00	
Poorly	1.026 (0.68-1.56)	0.906	1.237 (0.79-1.95)	0.359
CgA				
CgA≤120	1.00		1.00	
CgA>120	1.662 (0.97-2.84)	0.062	2.66 (1.42-4.99)	0.002
Grade (Ki67)				
1	1.00		1.00	
2	2.37 (1.22-4.62)	0.011	2.24 (0.75-6.75)	0.151
3	7.21 (2.55-20.4)	<0.001	9.84 (2.48-39.04)	0.001
Grade (mito)				
1	1.00		1.00	
2	1.35 (0.81-2.24)	0.253	0.914 (0.46-1.81)	0.795
3	1.66 (0.97-2.84)	0.099	1.19 (0.39-3.64)	0.764

Table 2.9 Multivariate analyses of independent prognostic factors for Pancreatic NETs

Risk Factor	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Differentiation				
Well	1.00		1.00	
Poorly	1.84 (0.73-4.62)	0.194	0.82 (0.33-2.05)	0.675
CgA				
CgA≤120	1.00		1.00	
CgA>120	1.55 (0.8-3.02)	0.451	1.37 (0.61-3.09)	0.45
Urinary 5-HIAA				
5HIAA≤96	1.00		1.00	
5HIAA>96	0.81 (0.43-1.54)	0.522	1.69 (0.73-3.92)	0.22
Grade (Ki67)				
1	1.00		1.00	
2	2.87 (1.67-4.92)	<0.001	2.25 (1.1-4.59)	0.025
3	11.74 (3.61-38.3)	<0.001	27.13 (7.07-104)	<0.001
Grade (mito)				
1	1.00		1.00	
2	0.735 (0.43-1.27)	0.271	0.676 (0.33-1.37)	0.278
3	0.989 (0.1-9.95)	0.993	0.719 (0.06-8.04)	0.789

Table 2.10 Multivariate analyses of independent prognostic factors for midgut NETs

2.3.5. Alternative Thresholds for Grade Classification (according to tertiles)

Tumour grades were reassigned using alternative cut-offs, designated by tertiles. For pancreatic NETs, this was:

- G1: mitotic count 0-1/10 high power fields (hpf) or Ki-67<5%
- G2: mitotic count 2-3/10 hpf or Ki-67 5-15%
- G3: mitotic count >3/10hpf or Ki-67>15%

For midgut NETs, this was:

- G1: mitotic count 0-1/10hpf or Ki-67<3%
- G2: mitotic count 2-3/10hpf or Ki-67 3-5%
- G3: mitotic count >3/10hpf or Ki-67>5%

Agreement between grades assigned by these new thresholds are shown in Table 2.11 and Table 2.12. Weighted kappas were 0.66 (good agreement) in pancreatic NETs and 0.38 (fair agreement) in midgut NETs. This is slightly better agreement than with the ENETS guidelines. An inevitable consequence of making the G2 Ki-67 threshold higher is that many more cases are G1 using Ki-67 but still G2 using mitotic count.

Univariate analyses with survival curves for this new grading classification are shown in Figure 2.4 for pancreatic NETs and Figure 2.5 for midgut NETs. For both pancreatic and midgut NETs, Ki-67 was able to distinguish three different prognostic groups better than with mitotic count where there was overlapping with survival curves. Although this was a similar result to that when using the ENETS thresholds, the G2 curve appears closer to G3 with these new thresholds.

Mutlivariate analyses are shown in Table 2.13 and Table 2.14 respectively which confirms Ki-67 as an independent prognostic marker. Hazard ratios for grade using Ki-67 were lower using tertiles compared to the ENETS thresholds. This suggests that these new thresholds are not as good as original ENETS thresholds.

PANCREATIC		Grade according to mitotic count/10HPF			
		G1	G2	G3	
Grade according to Ki-67	G1	**37**	30	2	**69**
	G2	5	**14**	8	**27**
	G3	1	9	**29**	**39**
		43	**53**	**39**	**135**

Table 2.11 Agreement between grades assigned according mitotic count and Ki-67 but with thresholds assigned by tertiles in pancreatic NETs; weighted kappa 0.66 (95% CI 0.54-0.77)

MIDGUT		Grade according to mitotic count/10HPF			
		G1	G2	G3	
Grade according to Ki-67	G1	**55**	17	13	**85**
	G2	13	**6**	12	**31**
	G3	0	5	**16**	**21**
		68	**28**	**41**	**137**

Table 2.12 Agreement between grades assigned according mitotic count and Ki-67 but with thresholds assigned by tertiles in midgut NETs; weighted kappa 0.38 (95% CI 0.26-0.51)

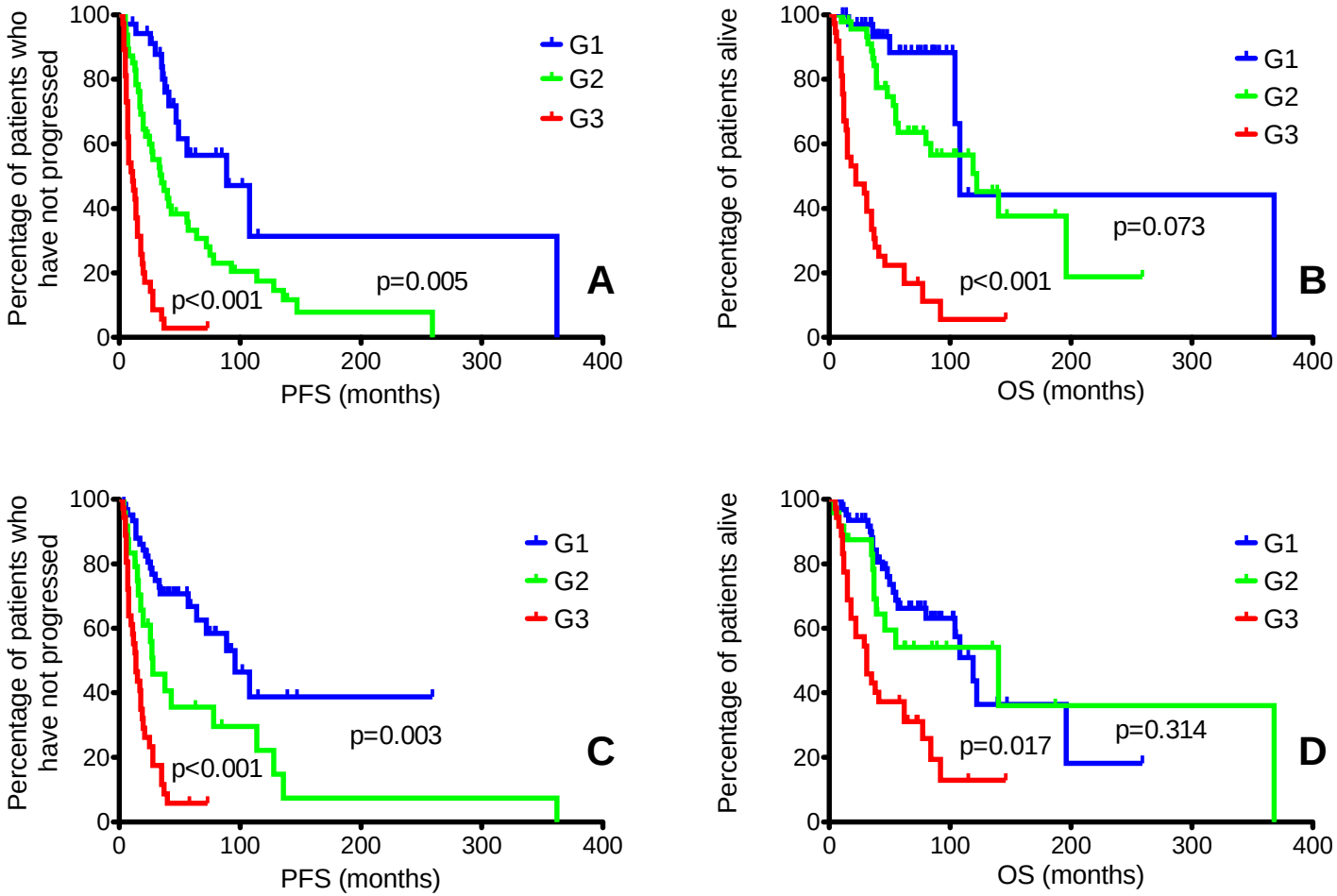


Figure 2.4 Univariate anlayses (Kaplan-Meier curves with log-rank tests) demonstrating survival in pancreatic NETs (A) PFS and (B) OS using Grade according to Ki-67 divided into teritlles; (C) PFS and (D) OS using Grade according to mitotic count divided into tertiles

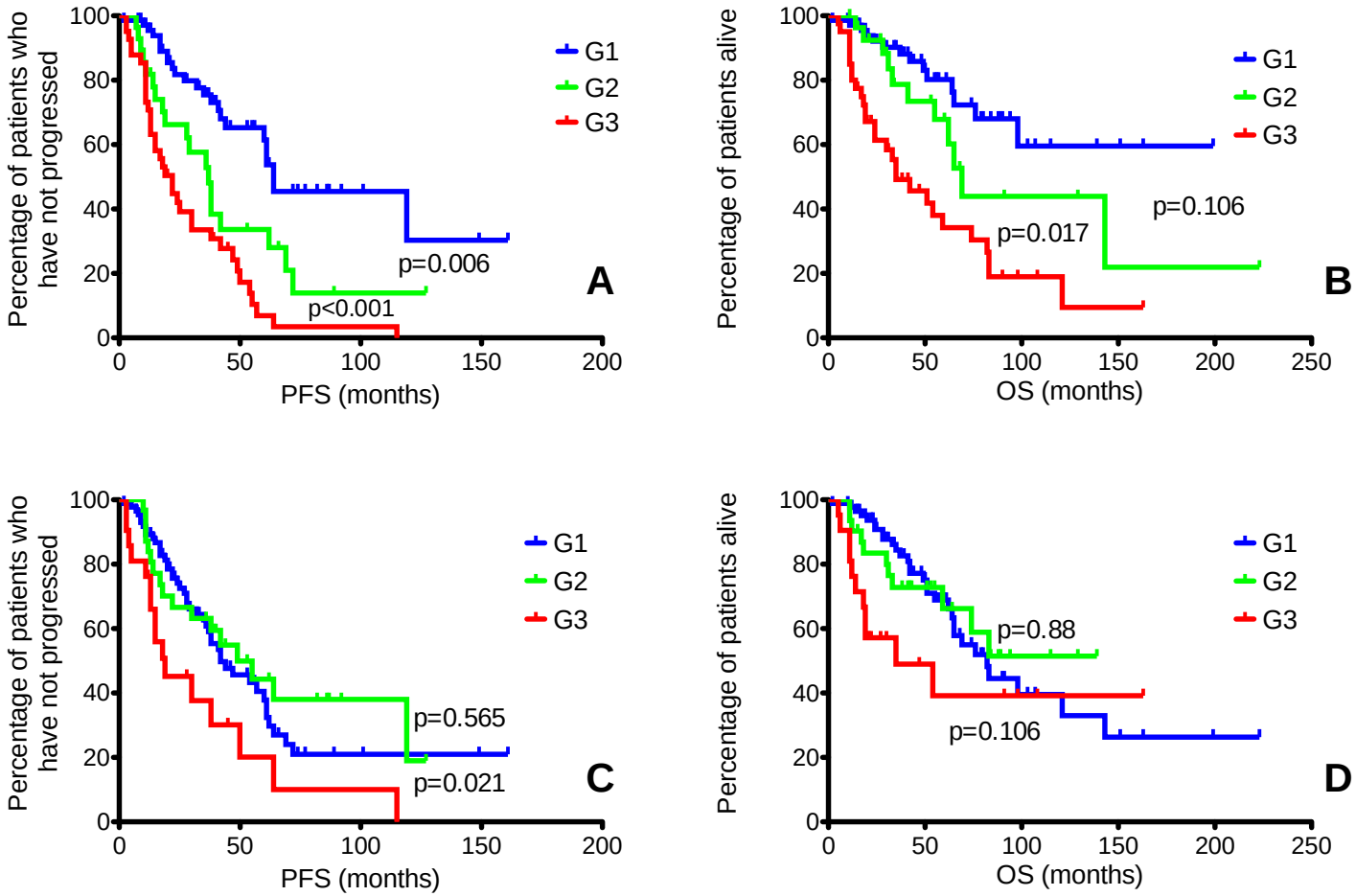


Figure 2.5 Univariate anlayses (Kaplan-Meier curves with log-rank tests) demonstrating survival in midgut NETs (A) PFS and (B) OS using Grade according to Ki-67 divided into teritlles; (C) PFS and (D) OS using Grade according to mitotic count divided into tertiles

??????Risk Factor	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Age (for every 10 years)	1.01 (0.99-1.03)	0.137	1.02 (0.99-1.05)	0.119
CgA				
CgA≤120	1.00		1.00	
CgA>120	1.35 (0.83-2.21)	0.226	2.60 (1.37-4.92)	0.003
Grade (Ki67)				
1	1.00		1.00	
2	2.48 (1.27-4.89)	0.008	2.13 (0.68-6.67)	0.193
3	9.71 (3.95-23.85)	<0.001	24.6 (6.15-98.5)	<0.001
Grade (mito)				
1	1.00		1.00	
2	1.02 (0.52-2.02)	0.947	0.32 (0.10-1.00)	0.052
3	1.43 (0.65-3.14)	0.368	0.50 (0.16-1.54)	0.228

Table 2.13 Multivariate analyses of prognostic factors for pancreatic NETs, with grade according to Ki-67 and mitotic count thresholds according to tertiles

Risk Factor	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Age (for every 10 years)	0.99 (0.83-1.20)	0.991	1.13 (0.89-1.44)	0.325
CgA				
CgA≤120	1.00			
CgA>120	1.49 (0.91-2.45)	0.111	1.85 (0.99-3.43)	0.052
Grade (Ki67)				
1	1.00		1.00	
2	2.14 (1.13-4.05)	0.019	1.76 (0.75-4.12)	0.192
3	4.40 (2.36-8.20)	<0.001	4.37 (2.08-9.19)	<0.001
Grade (mito)				
1	1.00		1.00	
2	0.77 (0.43-1.37)	0.370	0.81 (0.40-1.66)	0.572
3	0.97 (0.49-1.93)	0.940	1.01 (0.46-2.21)	0.979

Table 2.14 Mutlivariate analyses of prognostic factors in midgut NETs with grade according to Ki-67 or mitotic count assigned using thresholds divided into tertiles

2.3.6. Alternative Thresholds for Grade Classification (according to Scarpa *et al.*[29])

Tumour grades were again reassigned with alternative cut-offs for Ki-67 suggested by Scarpa *et al.* for Ki-67[29]. This group validated this in pancreatic NETs but I applied this system to both pancreatic and midgut NETs.

- G1: Ki-67≤5%
- G2: Ki-67>5% and ≤20%
- G3: Ki67>20%

Univariate analyses with survival curves for this new grading classification are shown in Figure 2.6 for pancreatic and midgut NETs. Ki-67 using these alternate thresholds appeared to separate three prognostically different groups with no overlap (which there was with the ENETS thresholds in pancreatic NETs with OS). Mutlivariate analyses are shown in Table 2.15 and Table 2.16 where Ki-67 was again confirmed as an independent prognostic indicator. The hazard ratios, particularly for G2, were higher than with ENETS thresholds, suggesting that these alternate thresholds may be more optimal when prognosticating.

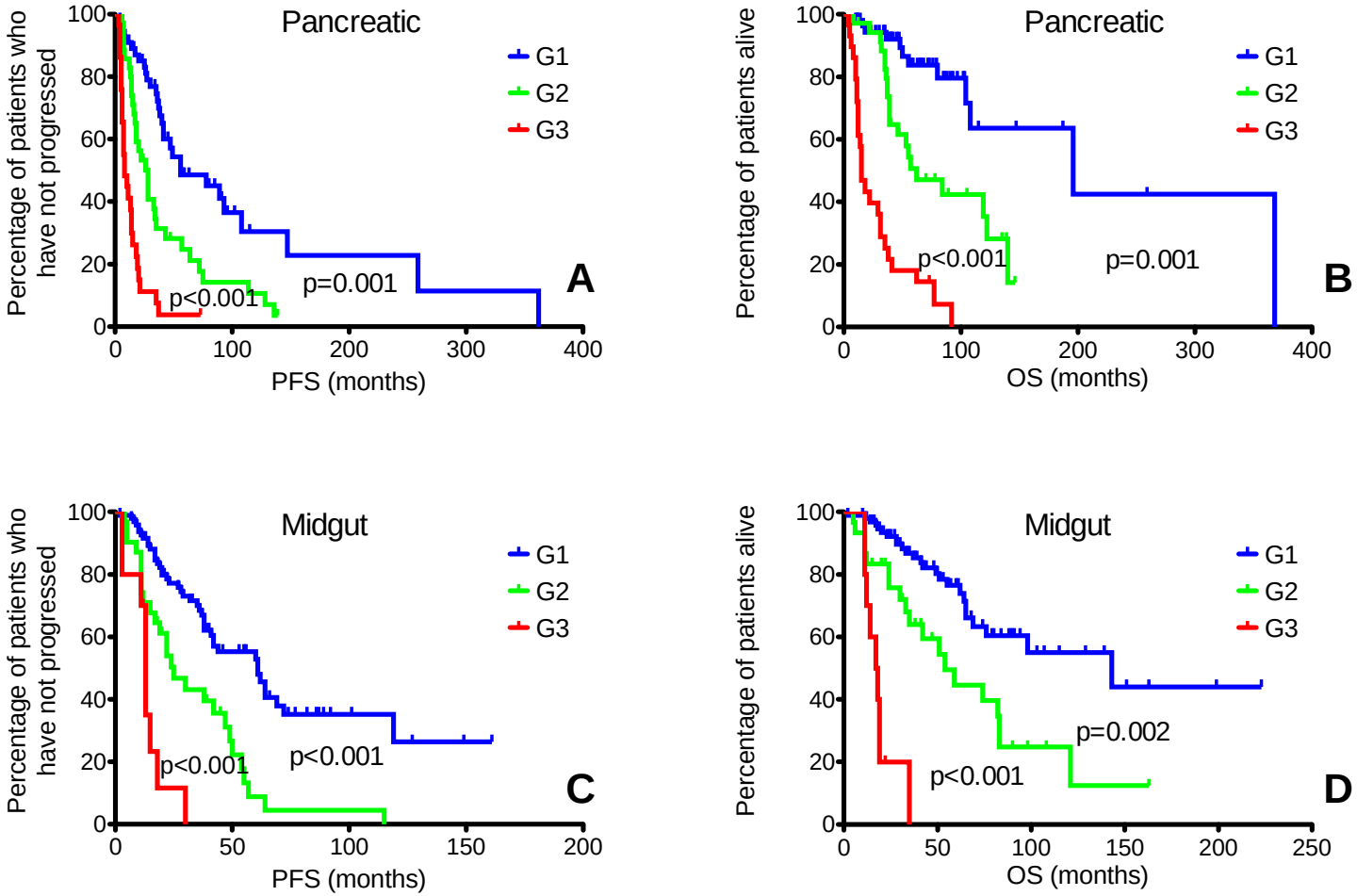


Figure 2.6 Univariate anlayses (Kaplan-Meier curves with log-rank tests) demonstrating survival in pancreatic NETs (A) PFS and (B) OS; midgut NETs (C) PFS and (D) OS with grade (G1,G2,G3) according to Ki-67 classifications according to Scarpa et al[29]

????Risk Factor	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Age (for every 10 years)	1.01 (0.99-1.03)	0.164	1.022 (0.99=1.05)	0.103
CgA				
CgA≤120	1.00		1.00	
CgA>120	1.10 (0.66-1.84)	0.715	2.09 (1.08-4.06)	0.029
Grade (Ki67)				
1	1.00		1.00	
2	2.86 (1.58-5.17)	0.001	5.97 (1.97-18.14)	0.002
3	8.97 (3.90-20.6)	<0.001	33.8 (9.50-120)	<0.001
Grade (mito)				
1	1.00		1.00	
2	1.32 (0.76-2.30)	0.330	0.72 (0.32-1.58)	0.409
3	1.68 (0.62-4.59)	0.311	0.91 (0.29-2.88)	0.872

Table 2.15 Multivariate analyses of prognostic factors in pancreatic NETs with grade (G1,G2,G3) according to Ki-67 classifications according to Scarpa et al[29]

Risk Factor	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Age (for every 10 years)	0.99 (0.82-1.19)	0.887	1.12 (0.89-1.43)	0.001
CgA				
CgA≤120	1.00		1.00	
CgA>120	1.64 (1.02-2.62)	0.040	2.03 (1.12-3.68)	0.020
Grade (Ki67)				
1	1.00		1.00	
2	3.09 (1.80-5.32)	<0.001	3.02 (1.58-5.75)	0.001
3	10.34 (3.95-27.1)	<0.001	22.1 (7.12-68.4)	<0.001
Grade (mito)				
1	1.00		1.00	
2	0.71 (0.41-1.22)	0.210	0.61 (0.30-1.21)	0.158
3	1.48 (0.28-7.93)	0.650	1.57 (0.27-9.21)	0.620

Table 2.16 Multivariate analyses of prognostic factors in midgut NETs with grade (G1,G2,G3) according to Ki-67 classifications according to Scarpa et al[29]

2.4. Discussion

2.4.1. Survival Compared to Other Studies

Although difficult to assess, our overall survival data appears to be comparable to previous series. In a Swedish single-centre series of 324 pancreatic NETs, 5- and 10-year survival was 64% and 44% respectively (median follow-up 54 months) compared to 59% and 36% in our series (median follow up 46 months)[90]. However, the patients in our series were more advanced: only 180 of 324 in the Swedish series had metastatic disease compared to all of the patients in our series. In addition, a more recent series has reported 5- and 10-year survival rates 35% and 17% respectively in a metastatic subgroup[29].

In an American series based on Surveillance, Epidemiology and End Results (SEER) data, the 5-year survival of all small intestinal NETs was 61% for all stages, and 50% for metastatic disease. This is slightly worse than our series (61% 5-year overall survival) for metastatic midgut NETs. Although a higher 5-year survival rate was quoted with more recent SEER data (68%), a breakdown of stage was not given[5]. A higher 5-year survival rate (83%) was experienced in a recent European series but this did include a number of slow growing hindgut NETs[30]. PFS cannot be compared due to lack of consistency in definitions of progression and surveillance protocols throughout the literature.

2.4.2. Inter-observer Agreement Between Pathologists

In my subset of 44 cases, the agreement between independent pathologists for assessing Ki-67 and mitotic count was very good defined by weighted kappas as a measure of agreement, although not perfect with 9% discordance. I recognise that this is not a large validation subset but the make-up is divided evenly between midgut and pancreatic NETS and this is a larger sample than in previously published data[195]. Despite studies in a variety of cancers consistently demonstrating its utility as a biomarker, poor inter-rater agreement between pathologists in assessment of Ki-67 in lymphoma samples has been demonstrated in a small sample (n=36)[195] although not assessed in other tumour types. Although there were not many high grade NETs in my subgroup, this reflects the infrequency of G3 NETs. Additionally, the distribution of this subgroup was divided evenly between low and intermediate grade NETs. Regardless of the

absence of complete agreement, grading was prognostic in our survival data below and using one of a panel of expert NET pathologists, this reflects clinical practice.

2.4.3. Agreement (Discrepancy) in Grading

There is a correlation between absolute Ki-67 index and mitotic count. This is as expected since both are markers of cell division. However, when using these indices to assign a grade, there was 44% and 38% discordance in pancreatic and midgut NETs i.e. moderate and poor agreement defined by weighted kappas. This is clinically important since if the ENETS guidelines are followed, either mitotic count or Ki-67 can be used to assign the grade. In practice, most centres provide both indices and this could provide conflicting information about grading which can impact on clinical management since intermediate grade NETs may be treated in a more aggressive manner than low grade NETs. When a case is G2 by one index and G1 by another, it is unclear which grade to use and hence confusion arises as to when and how to treat these cases. What is apparent from my data is that some cases can be classified G2 or G3 depending on which index is used. This can have major implications as G3 NETs are usually treated aggressively, with chemotherapy first line.

My findings conflict with the findings by Strosberg *et al*[194], who demonstrated complete agreement between grade by Ki-67 and mitotic count. They, however, used a two-tiered rather than three-tiered grading system which is a simplification of and not a true representation of the ENETS grading classification.

2.4.4. Prognosis Using Grade According to Ki-67 and Mitotic Count

Since there was disagreement between grade assignment depending on whether Ki-67 or mitotic count was used, I investigated which index was more clinically valuable by analysing the prognostic value of each.

In pancreatic NETs, although grade according to mitotic count was prognostic (in terms of PFS and OS) on univariate Kaplan-Meier analyses, it was not an independent prognostic factor on multivariate analyses. Only grade according to Ki-67 was prognostic in univariate and multivariate analyses in both pancreatic and midgut NETs. Baseline CgA>120 at the time of diagnosis was the only other risk factor found to be associated with worse OS on multivariate anlaysis, but only in metastatic pancreatic NETs.

This suggests that given the discrepancy in grade assignment above, grade according Ki-67 is a better prognostic variable than grade according to mitotic count.

One of the reasons why there is a discrepancy in grade assignment and why grade according to mitotic count does not hold the same prognostic value as Ki-67 could be that mitotic count is affected by pre-analytical or analytical factors such as delay in tissue fixation[196, 197], problems in identification of a mitotic figure[198], selection of measurement area[199, 200], or assessment of mitotic cells in relation to tumour tissue in the sample[201]. However, similarly, this also applies to Ki-67 with pre-analytical factors such as time to fixation, type of fixation, time in fixative, and storage; and analytical issues including assessment of staining. Furthermore, the ENETS guidelines stipulate that Ki-67 is assessed in areas of highest proliferative activity whereas mitoses are expressed by ten separate high power fields over an average of 40 high power fields and many fields may not have any activity. In NETs, Ki-67 assessment has been standardised to 2000 cells with highest activity whereas the breast cancer consensus is 500 to 1000 cells and assessment of 'hot spots' being less consistent[174].

Another reason for the discrepancy in grading and prognostic value is that the grading thresholds may not be optimal. The 2% threshold was derived from previous data[181]. However, the thresholds may not apply to all populations of NETs studied as there is heterogeneity in terms of primary tumour, stage of disease and subsequent treatments. I have separated midgut and pancreatic NETs in the analyses and also attempted to make the sample homogeneous by recruiting cases with measurable metastatic disease. It is this population which makes up the majority of clinical practice and also the population where clinical management pathways are still debated. However, I acknowledge that this is still a heterogeneous group in terms of subsequent treatments and timing of treatments. This makes the prognostic data for OS more valuable than PFS. Another limitation is that this was a retrospective study in a single tertiary centre and larger prospective multi-centred studies are required.

The grading system has been validated in retrospective series. In foregut NETs (n=158), Pape *et al* found survival of G1 tumours better than that of G2 tumours, and in turn better than G3[202]. However, the authors opted to use Ki-67 and not mitotic count and also included patients without metastases. More recently, Scarpa *et al* also opted to use grading according to Ki-67 in a series of 237 pancreatic NETs and found the cut-offs of 2% and 20% unable to distinguish G1 and G2 tumours according to survival on

multivariate analysis but did distinguish G3 from G1 NETs[29]. However, the authors found a modified grading system with cut-offs of 5% and 20% to be significantly prognostic, incorporating modified TNM staging into the multivariate model. Pape *et al* found another modified grading system with cut-offs of 5 and 10% and to be prognostic in a heterogeneous series of 239 NETs but again did not use mitotic count[203]. However, Jann *et al* demonstrated the original grading system to be prognostic in midgut and hindgut NETs (n=189)[30]. Due to these differing thresholds in the literature, I went on to analyse my data using alternate thresholds.

2.4.5. Alternate Thresholds for Grading

When the grading strata thresholds were divided into tertiles rather than the ENETS classification, there was moderate and fair agreement when assigning grade according to Ki-67 or mitotic count in pancreatic and midgut NETs respectively. However, with survival analyses, this grading system was only prognostic of PFS and generally, the Kaplan-Meier curves were not as separable as with the ENETS classification.

Using thresholds of 5% and 20% for Ki-67 grading, Scarpa *et al* validated an alternate grading stratification but this was a study of pancreatic NETs and the sample did not contain any midgut NETs[29]. I used these thresholds to assign grade according to Ki-67 in both midgut and pancreatic NETs (and grade according to mitotic count using the ENETS thresholds). In both midgut and pancreatic NETs, on univariate and multivariate analyses, this was the best prognostic grading system in terms of both PFS and OS.

2.4.6. Conclusions

Here I have attempted to clinically validate the grading system proposed by ENETS guidelines in patients with NETs with metastatic disease, a more homogenous group than the large published pathology datasets. Since the majority of patients present with metastases at the time of diagnosis, this is a clinically relevant population. My data suggests that the grading guidelines suggested by ENETS, should not assume agreement between Ki-67 and mitotic count and needs to suggest which index is used. I also conclude that grade according to Ki-67 is more clinically valuable in predicting prognosis than mitotic count. Furthermore, the alternate grading strata suggested by Scarpa *et al.*[29], were found to prognostic in both metastatic pancreatic and midgut NETs and more optimal than ENETS guidelines. In conclusion, with mitotic count adding no additional information, and both these indices measuring proliferation, clinical grading in NETs should use Ki-67 and make a departure from mitotic count.

Although grade according to Ki-67 is a valuable prognostic factor, it is far from ideal as it is commonly based on a specimen at the time of diagnosis which, given the variable survival of NETs, may be many years in the past. Therefore, it may not reflect current tumour biology which is a dynamic process. For dynamic prognostication throughout the course of the disease, repeatedly evaluating markers in blood or urine is a more acceptable form of sampling than repeated biopsy. However, the established biomarkers, plasma CgA and urinary 5-HIAA are not thoroughly validated prognostic or predictive biomarkers as explained in the introductory chapter. Therefore there is a requirement for prospectively validated biomarkers.

Chapter 3. EpCAM Expression and Enumerating Circulating Tumour Cells (CTCs) in Neuroendocrine Tumours

3.1. Introduction

Recent technological advances have enabled enumeration and characterisation of circulating tumour cells (CTCs) by different methods. One such method is the CellSearch™ System. This is a semi-automated platform that uses immunomagnetic enrichment of CTCs from blood and has been shown to detect CTCs with high sensitivity, specificity, and reproducibility[153]. Large studies have demonstrated that the number of CTCs in patients with metastatic breast cancer to be an independent predictor of progression-free and overall survival[154, 204]. These results have been reproduced in metastatic prostate and colorectal cancer[205, 206] and the system has been approved by the United States of America (USA) Food and Drug Administration (FDA) to monitor metastatic breast, colorectal and prostate cancers.

The CellSearch™ system requires Epithelial Cell Adhesion Molecule (EpCAM) expression to isolate CTCs. EpCAM is a 39-42 kDa transmembrane epithelial glycoprotein[207] and is overexpressed in human carcinomas[152]. Its exact function is yet to be elucidated but its expression enables the CellSearch™ platform to enrich CTCs via immunomagnetic separation with iron particles coupled to EpCAM antibodies. The platform consists of a semi-automated system that enriches the sample for cells expressing EpCAM by immunomagnetic separation. The system incubates samples with ferrofluids coated with epithelial cell-specific EpCAM antibodies and a magnetic field isolates CTCs by immunomagnetic separation. The system also labels the cells with the fluorescent nucleic acid, DAPI. Fluorescently-labelled monoclonal antibodies specific for leukocytes (CD45–allophycocyanin) and epithelial cells (cytokeratin 8,18,19–phycoerythrin) are used to distinguish epithelial cells from any leukocytes which may inadvertently isolated (see Figure 3.1).

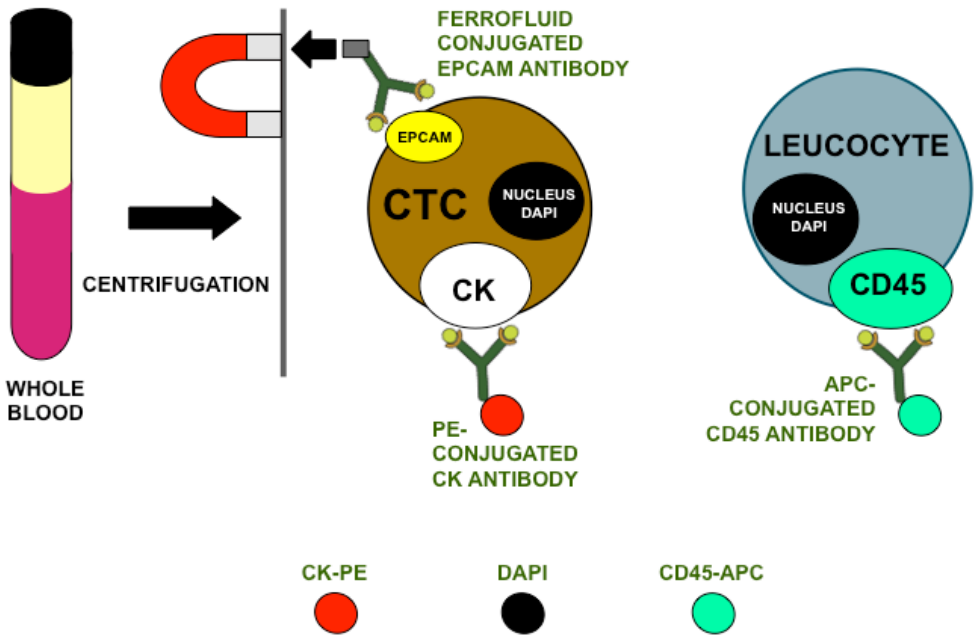


Figure 3.1 Schematic representation of immunomagnetic separation and immunofluorescent staining employed by the CellSearch platform to enrich CTCs from peripheral blood; CTC Circulating Tumour Cell; EPCAM Epithelial Cell Adhesion Molecule CK-PE Cytokeratin-Phycoerythrin; CD-45APC CD-45 Allophyocyanin DAPI 4', 6-diamidino-2-phenylindole

Although some series have reported a small neuroendocrine subset of lung cancers and insulinomas to overexpress this epithelial marker[152, 208], the systematic analysis of EpCAM expression in NETs has not been undertaken. Since NETs were originally thought to be derived from cells of the neural crest sharing secretory and histological properties with neural cells, it has been debated whether NETs are epithelial in origin.[10, 11] It has thus been assumed NETs do not express EpCAM and in one study, NET patient samples were used as 'negative' controls for CTC detection using the Cellsearch™ system[209, 210, 211, 212].

I therefore performed an initial study to determine the level of EpCAM expression in NETs. Having demonstrated widespread expression, I proceeded to investigate whether circulating NET cells could be detected in patients.

3.2. Materials and Methods

3.2.1. Cell Lines

Cells from a bronchial neuroendocrine tumour cell line, NCI-H727, were maintained in RPMI 1640 medium (PAA Laboratories, Somerset, UK) supplemented with 10% fetal

bovine solution (FBS), 2mM L-Glutamine, penicillin and streptomycin. A breast cancer cell line, MCF-7, was maintained in Minimal Essential Media Eagle (MEM) (PAA Laboratories, Somerset, UK) supplemented with 10% FBS, 2mM L-Glutamine, penicillin and streptomycin. All cells were cultured in 75cm^2 culture flasks (Corning, NY, USA) in a humidified incubator at 37°C and 5% CO2.

3.2.2. Cell Culture

Approximately 10^6 cells per cell line were seeded into 75cm^2 tissue culture flasks. After approximately 48 hours when more than 80% confluent, the media were removed. Trypan Blue exclusion indicated >99% viability of the attached cells. Sterile 12mm diameter coverslips were placed in wells of a 24 –well plate (BD Falcon, Oxford, UK). Approximately 2 X10^5 cells were seeded into each well and incubated at 37°C for 24 hours at 5% CO_2.

3.2.3. Immunofluorescence

Media were removed from each well and cells washed twice with Phosphate Buffer Solution (PBS) prior to fixing in 1mL of 1% formaldehyde for 10 minutes. Fixative was removed and cells were washed 3 times with PBS. 1mL 0.1M glycine was added to each well to neutralise for 5 minutes and cells were washed again 3 times with PBS. To permeabilise the cells, 0.1% Triton X-100 was added to each well for 10 minutes before 3 further washes with PBS. To prevent non-specific binding, cells were blocked with 0.1% Bovine Serum Albumin (BSA) for 30 minutes and then washed with PBS. Cells were incubated with the FITC-conjugated anti-synaptophysin mouse monoclonal antibody (Acris Antibodies, Herford, Germany) at a dilution of 1:20 or the Alexa Fluor 488-conjugated anti-CD56 mouse monoclonal antibody (BD PharMingen, San Diego, CA) at a dilution of 1:20 for 1 hour at room temperature. After further washing in PBS, coverslips were removed from each well and mounted onto slides with Vectashield (Vector Laboratories Ltd, Peterborough, UK) containing 4', 6-diamidino-2-phenylindole (DAPI) and sealed with clear nail varnish before visualisation. Negative controls included substitution of the primary antibody with normal horse serum. MCF-7 was used as a negative control for CD56 staining. Cells were visualised on a Zeiss LSM 510 meta confocal microscope Zeiss Axioskop 2, AxioImager Z1 microscope, Zeiss, Welwyn Garden City, UK) using Axiovision v4.3 software (Zeiss).

3.2.4. Immunohistochemistry

Consecutive blocks of formalin-fixed and paraffin-embedded tissue were available from 75 patients with a histopathological confirmed diagnosis of Neuroendocrine Tumour (NET). Three micrometre sections of tumour tissue were made. After de-paraffinisation in xylene and rehydration in graded alcohols, endogenous peroxidase in tissue sections was blocked with 0.5% H_2O_2 in methanol at room temperature for 10 minutes. Thereafter, sections were subjected to antigen retrieval for 10 minutes in 0.1% trypsin at 37°C (EpCAM retrieval) or subjected to 3 minute pressure cooking heat-mediated antigen retrieval (HMAR) (Ki-67 retrieval). Sections were incubated with mouse anti-EpCAM monoclonal antibody (ESA, clone VU-1D9, Novocastra, Newcastle-upon-Tyne, UK) at a dilution of 1:50 for 1 hour at room temperature or with MIB-1 antibody detecting Ki-67 (DAKO, Cambridgeshire, UK) at a dilution of 1:200 for 1 hour at room temperature. Immunohistochemical staining was performed with the NovoLink™ Polymer detection system (Novocastra, Newcastle-upon-Tyne, UK). Sections were incubated with post-primary block for 30 minutes, followed by Novolink™ polymer for 30 minutes. Reaction products were visualised with application of diaminobenzidine (DAB) substrate chromogen solution. Slides were counterstained in haematoxylin and mounted. Negative controls were performed by omitting the primary antibody. For negative and positive tissue controls, internal normal tissues with known EpCAM negativity/positivity were used.

Two examiners (T.L. and M.K.) performed the immunohistological scoring independently of each other with light microscopy. Any discordant results were reviewed together to reach agreement. EpCAM scoring was based on intensity of staining: 0=negative, 1=weakly positive, 2=moderate, 3=strongly positive. Extent of tumour staining was also scored, where 10 random high power fields (HPF) were assessed and the average percentage of positive staining cells was estimated: 1= <25%, 2= 25-75%, 3= >75%. The product of staining density and extent was used as the immunohistochemical score giving final values of 0, 1, 2, 3, 4, 6, and 9. Scores of 0 were counted as negative, 1-2 as weak staining, 3-4 as moderate staining, and 6-9 as strong staining. The Ki-67 proliferation index was determined by assessing the percentage of positively staining tumour cell nuclei in 2000 neoplastic cells in areas with highest degree of nuclear labelling where possible.

All cases were classified according to site of origin and had their tumour graded using the systems proposed by the European Neuroendocrine Tumour Society (ENETS)

consensus group and the World Health Organisation including Ki-67 proliferation index and/or mitoses per 10[23, 26]. Using this classification, a low grade tumour was regarded as Ki67≤2% or mitotic count <2 per 10 HPF; intermediate grade Ki67 3-20% or mitotic count 2-20 per 10 HPF; and high grade Ki67>20% or mitotic count>20 per 10 HPF; grade according to Ki67 proliferation index or initial mitotic count as above; and level of differentiation.

3.2.5. Patient Recruitment

176 patients were recruited between August 2009 and June 2011 for blood sampling and CTC evaluation. This study was approved by the Local Ethics Committee (Ref 09/H0704/44) and all patients provided written informed consent. All eligible participants had histologically proven NET and metastatic disease measurable by RECIST criteria. They were categorised according to the site of primary NET: midgut, pancreas, bronchopulmonary, hindgut or of unknown primary. Patients that had undergone chemotherapy, interferon, receptor-targeted radiolabeled therapy, or embolisation within the previous two months were excluded.

Radiological burden was assessed by quantification of hepatic tumour load from 4 to 6 slices of a CT/MRI scan with the most amount of disease by a semi-quantitative approach. Hepatic tumour burden was categorised as 25% or less, more than 25% but 50% or less, more than 50% but 75% or less, or more than 75%. Data were collected on primary site, duration of diagnoses, any previous treatment received, WHO performance status (PS) and whether the primary tumour had been resected. Grade of tumour according to Ki-67 proliferation index was recorded according to ENETs guidelines[23, 24].

CgA analysis was performed on plasma samples from patients using a radio-immuno assay (RIA) kit (Roche). 5-HIAA analysis was performed on 24-hour urine samples from patients using a manual in-house radio-immunoassay. As these assays have previously been validated and are currently used in clinical practice, 0 samples were run on normal healthy controls.

3.2.6. Immunomagnetic Separation, CTC Isolation and Enumeration on the Cellsearch™ Platform

7.5ml blood was drawn from each patient into CellSave tubes (Veridex LLC) containing EDTA and a cellular preservative. Samples were maintained at room temperature and processed within 96 hours using the Cellsearch™ (Veridex LLC) platform as previously

described for the isolation and enumeration of CTCs. Briefly, 7.5mL of blood were mixed with 6mL of buffer, centrifuged at 800 X g for 10 minutes and then placed on the AutoPrep part of platform. The instrument then added ferrofluids after aspirating the plasma and buffer layer. After the incubation and subsequent magnetic separation, unbound cells and remaining plasma were aspirated. The staining reagents were added together with a permeabilisation agent to fluorescently label the immunomagentically labelled cells. After incubation, excess staining reagents were aspirated and magnetic separation repeated. In the final step, cells were resuspended in the MagNest Cell Presentation device (Veridex LLC) which consists of a chamber and two magnets that orient the cells for analysis.

The identification and enumeration of CTCs on the display unit were performed with the use of the CellSearch™ Analyser II, a semi-automated fluorescence-based microscopy system that permits computer-generated reconstruction of cellular images of cells in the MagNest Cell Presentation device. All evaluations were performed without knowledge of the clinical status of the patients by 2 independent operators (M.K and T.T.). CTCs were defined as intact nucleated cells (DAPI+) lacking CD45 and expressing cytokeratin (see Figure 3.2). Any discordant results were reviewed together to reach agreement. Technical details of the CellSearch™ platform including accuracy, precision, linearity, CTC and reproducibility, have been described elsewhere[153]. The platform has been evaluated using the blood of healthy controls leading it to be approved by the FDA for evaluation in patients with carcinomas. I did not repeat these published experiments using healthy controls[153].

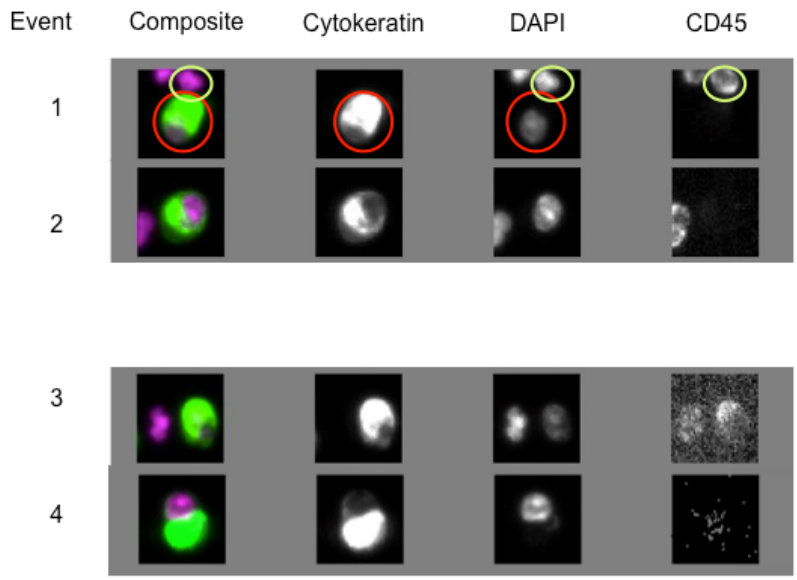


Figure 3.2 Image reconstruction from the CellSearch™ Analyser with identification of CTCs. Each horizontal 'event' is a possible cell or cells focused on by the automated microscope; there are 4 events depicted here. The columns refer to channels where the same event is imaged through different filters to view staining patterns: from right to left, CD45 (APC filter), DAPI (for nuclear staining), cytokeratin (PE filter) and a composite of all 3 stains. Event number 1 demonstrates a CTC (encircled in red for illustrative purposes) – a nucleus within a cytokeratin skeleton with absence of CD45 expression. In this same event, another cell circled in yellow for illustrative purposes is CD45 positive, DAPI positive, and represents a leucocyte rather than a CTC. Events 2-4 demonstrate further CTCs.

3.2.7. Characterising CTCs as Neuroendocrine on the Cellsearch™ Platform

The Cellsearch™ platform allows use of one additional marker processed simultaneously with CK-PE and CD45-APC antibodies on the platform to characterise CTCs. To confirm the neuroendocrine origin of CTCs, analysis of synaptophysin and CD56 expression on CTCs was performed with either FITC-conjugated anti-synaptophysin mouse antibody (Acris Antibodies, Herford, Germany) and Alexa Fluor 488-conjugated anti-CD56 mouse antibody (BD PharMingen, San Diego, CA) as additional markers. A selected patient's sample was processed alongside samples of healthy control blood spiked with 10^3 NCI-H727 and 10^3 MCF-7 cells as well as un-spiked healthy blood as a negative control. MCF-7 was used as a negative control for CD56 staining. The antibodies were substituted for PBS for antibody omission controls. The antibody concentrations were 100µg/mL for anti-synaptophysin and 12µg/mL for anti-CD56. The positivity of synaptophysin and CD56 of neuroendocrine CTCs was

evaluated by 2 independent operators using the research mode of CellSearch™ Analyser II with an integration time of 0.8 seconds.

3.2.8. Statistical Analysis

Analysis was performed using SPSS for Windows (SPSS Inc., Chicago, IL) and GraphPad Prism (GraphPad Software, San Diego, CA) where p values of <0.05 were considered significant. Differences in baseline characteristics between progressors and non-progressors were analysed with Fisher's exact, Mann–Whitney, and t tests. Associations between level of CTCs and clinicopathological data were evaluated with Mann-Whitney U or Kruskal-Wallis tests. Association between presence/absence of CTCs and clinicopathological data was analysed using Chi squared (or Fishers exact) tests.

3.3. Results

3.3.1. Immunohistochemical Interpretation of EpCAM Expression

In normal tissue, EpCAM was expressed mostly on the basal or basolateral cell membrane of small and large intestinal, appendiceal, and bile duct epithelia (Figure 3.3). Variable EpCAM expression, including some cytoplasmic staining, was seen in pancreatic islets, pancreatic acini, and gastric oxyntic glands. EpCAM was negative in hepatocytes, mesothelium, and gastric foveolar epithelium. Variable staining, including some cytoplasmic staining, was seen in pancreatic islets.

All ileal (n=26), pancreatic (n=16), unknown primary (n=2), and gastric (n=4) NETs showed strong (score 6–9) homogeneous membranous staining for EpCAM (Figure 3.3). Moderate to strong staining was seen in appendiceal (n=7) NETs. EpCAM expression was not affected by grade. Bronchopulmonary NETs (n=13) showed variable EpCAM expression from negative, weak to strong staining. EpCAM distribution was also variable in bronchopulmonary NETs: EpCAM was observed in cell membranes and in cytoplasm. EpCAM was not expressed in paraganglioma (n=1). Details can be found in Table 3.1.

3.3.1. Patients for CTC Evaluation

176 patients with metastatic NETs were recruited and had 7.5ml blood drawn for CTC evaluation. One sample was discarded due to haemolysis. Baseline characteristics are shown in Table 3.2.

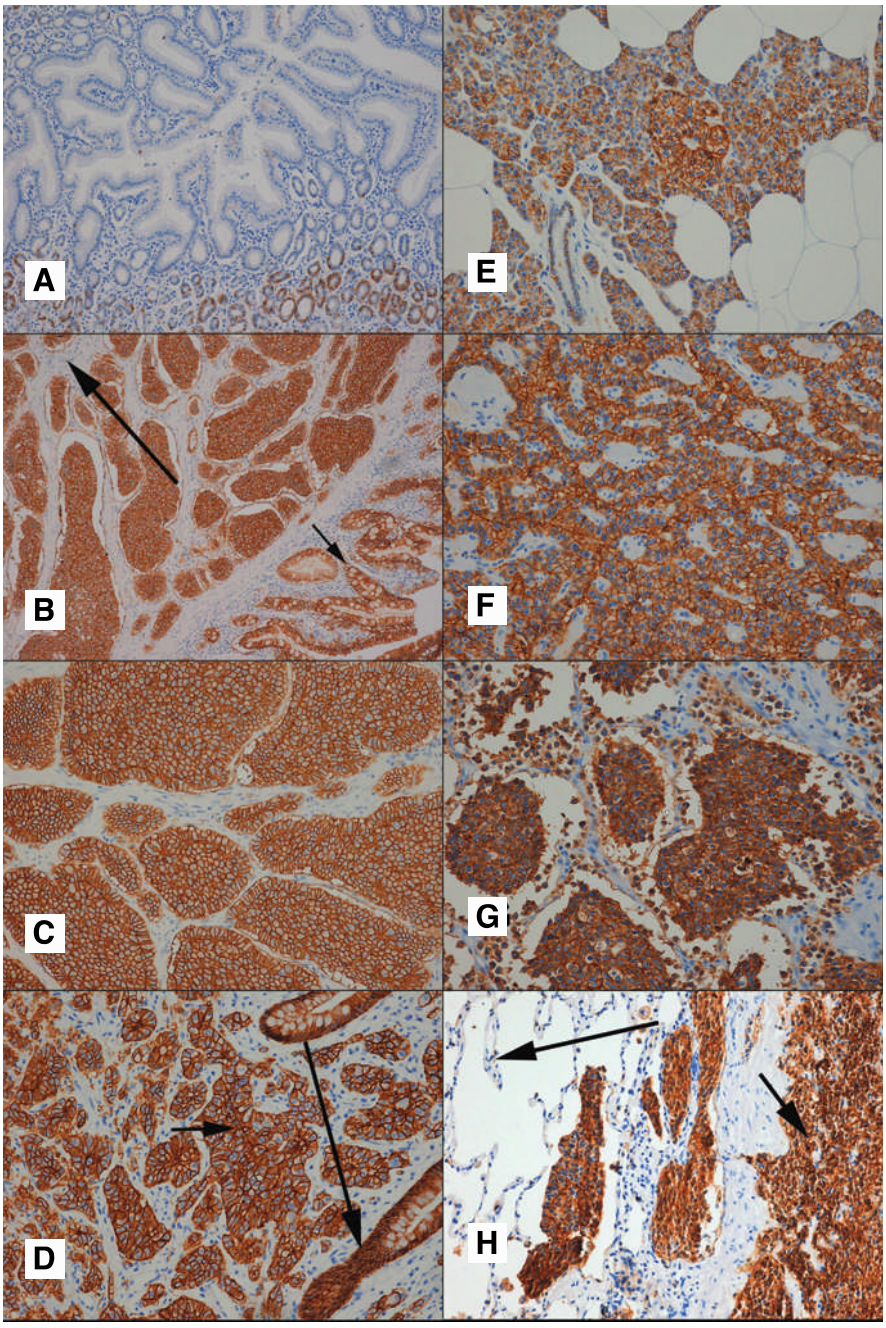


Figure 3.3 Immunohistochemistry with EpCAM. A) gastric mucosa with absent expression, apart from, in oxyntic cells. B) ileal NET resection with intense membranous staining of NET and of normal mucosa (short arrow) with negative stroma (long arrow). C) EpCAM positivity in ileal NET at high power. D) EpCAM-positive appendiceal NET (short arrow) with positive normal appendix mucosa (long arrow). E) normal pancreas with positive acini, islet, and duct. F) pancreatic NET with membranous staining at high power. G) poorly differentiated gastric NET with membranous and cytoplasmic staining. H) bronchopulmonary NET with cytoplasmic staining (short arrow) and negative lung parenchyma/ alveoli (long arrow).

NET Primary	Grade	Total Cases	EpCAM Staining		
			Positive n	Score	Staining Pattern
Bronchopulmonary	Typical	4	4	2-9	C+M
	Atypical	9	8	1-9	C+M
Gastric	Low	0			
	Intermediate	2	2	6-9	M
	High	2	2	6-9	M
Pancreatic	Low	7	7	9	M
	Intermediate	4	4	6-9	M
	High	5	5	9	M
Ileal	Low	15	15	9	M
	Intermediate	9	9	9	M
	High	2	2	9	M
Appendix	Low	6	6	4-9	M
	Intermediate	0			
	High	1	1	9	M
Rectal	Low	1	1	4	M
	Intermediate	1	1	9	M
	High	2	2	6-9	M
Unknown	Low	0			
	Intermediate	2	2	9	M
	High	0			
Nasal	Intermediate	1	1	6	M
Paraganglioma	Intermediate	1	0	0	
Total		74	72		

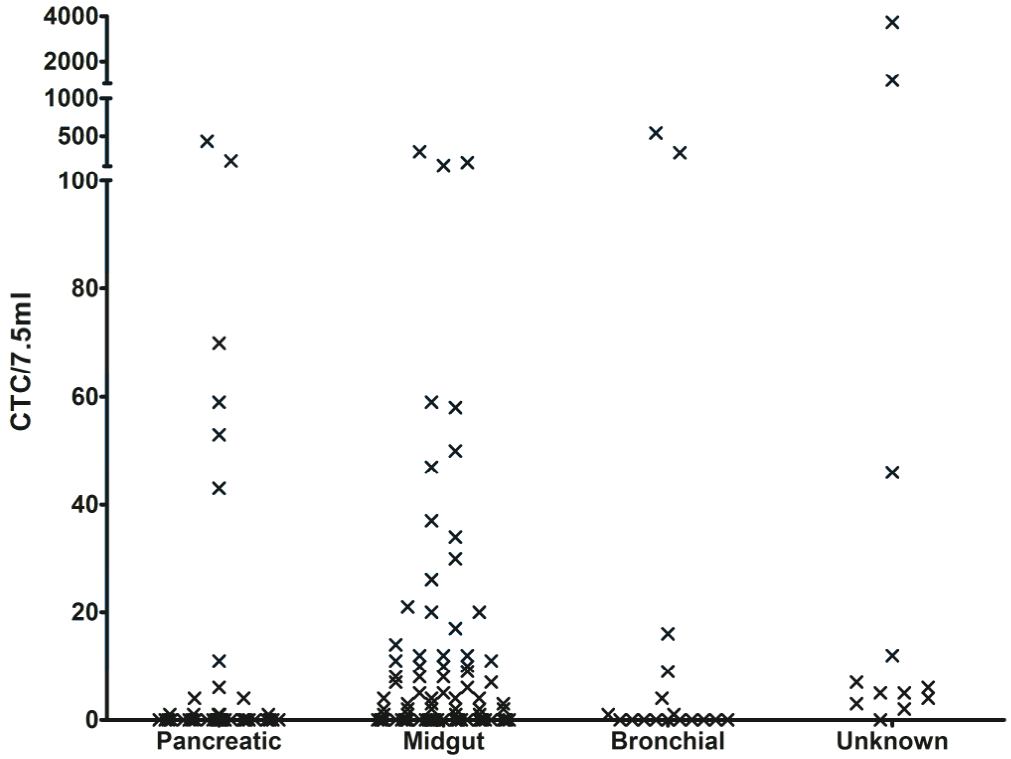
Table 3.1 EpCAM Expression in NETs (m=membranous; c=cytoplasmic)

	Pancreatic	Midgut	Broncho-pulmonary	Unknown Primary	Hindgut	Total
	n=42	n=101	n=17	n=12	n=3	n=175
Age, median years (range)	53 (23-87)	63 (34-85)	55 (30-80)	62.5 (31-78)	74 (43-75)	63 (23-87)
Sex						
Male	25	54	8	4	1	92
Female	17	47	9	8	2	83
Grade						
Low	17	59	6	1	0	83
Intermediate	10	36	8	7	2	63
High	15	6	3	4	1	29
Burden of Liver metastases						
≤25%	19	48	11	4	1	83
25%≤50%	15	36	4	4	0	59
50%≤75%	3	11	2	3	1	20
>75%	5	6	0	1	1	13
Duration of diagnosis, median months (range)	41 (2-166)	30 (1-134)	22 (9-287)	13 (1-67)	18 (5-22)	26 (1-287)
Performance Status						
0	29	60	12	6	1	108
1	13	34	5	5	2	59
2	0	6	0	0	0	6
3	0	0	0	1	0	1
4	0	1	0	0	0	1
Previous treatments						
Resection of primary	18	50	6	0	1	75
SST	13	59	3	3	1	79
Chemotherapy	18	10	4	6	1	39
TAE	3	13	0	2	0	18
Radionuclides	5	16	1	1	0	23
Interferon	3	2	0	0	0	5
Liver resection	7	10	0	0	0	17
Number of previous non-surgical treatments						
0	12	36	10	4	1	63
1	21	38	6	5	2	72
2	6	20	1	2	0	29
3	3	6	0	1	0	10
4	0	1	0	0	0	1

Table 3.2 Clinical Characteristics of NET Patient Sample

3.3.2. CTC Enumeration in Patients with NETs

Enumeration of CTCs according to primary NET is shown in Figure 3.4. Fifty-one per cent (51/101) of metastatic midgut NET patients had CTCs identified with a mean +/- SEM of 11.6 +/- 3.6 CTCs per 7.5ml whole blood (range 0-294). In comparison, 36% (15/42) of pancreatic NETs and 41% (7/17) of bronchopulmonary NETs had CTCs detected (mean 414.3 +/- SEM 233). In addition, 92% (11/12) patients with NETs of unknown primary had CTCs present. Hindgut NETs are not shown in the figure; 1 of these 3 cases had presence of CTCs (10/7.5ml). Number of CTCs were not normally distributed.



n	42	101	17	12
CTC ≥1	15 (36%)	52 (51%)	7 (41%)	11 (92%)
Mean CTCs (+/- SEM) 95% CI	20.4 +/-11.3 0-43	11.6 +/-3.6 5-19	49.6 +/-148 0-123	414.3 +/-233 0-1109
Median*	6	8	1	6
Range CTCs	0-430	0-294	0-542	0-3731

* median in those who had greater than 0 CTCs

Figure 3.4 CTC Enumeration According to Primary NET

3.3.3. Characterisation of CTCs as Neuroendocrine in Origin

Using immunofluorescence, NCI-H727 and MCF-7 both expressed synaptophysin. NCI-H727 expressed CD56 but MCF-7 did not. On the Cellsearch system™, spiked cell lines were easily identified as CTCs, being CK+DAPI+CD45-. When the fluorescent antibodies were translated to the CellSearch platform, spiked NCI-H727 was positive for synaptophysin and CD56; MCF-7 was positive for synaptophysin but negative for CD56. When the selected patient's sample was processed on the system, 82% per cent of the patient's CTCs were positive for synaptophysin and 21% for CD56 (Figure 3.5).

3.3.4. CTCs and Tumour EpCAM Expression

Archival histopathologic tissue was available from 26 of the patients who underwent CTC evaluation. EpCAM expression was evaluated in these samples which included 7 midgut, 2 liver metastases unknown primary, 8 pancreatic, 9 bronchopulmonary NETs.

All seven midgut NETs, and two liver metastases with unknown primary demonstrated strong EpCAM staining. 5 and 2 of these had CTCs present, respectively. All eight pancreatic NETs demonstrated strong EpCAM expression but only one had CTCs present. Five of the 9 bronchopulmonary NETs had none or weak EpCAM staining and none of these had CTCs. This is as expected since EpCAM expression was utilised to isolate CTCs. CTCs were only detected in those bronchopulmonary NETs displaying moderate to strong EpCAM expression.

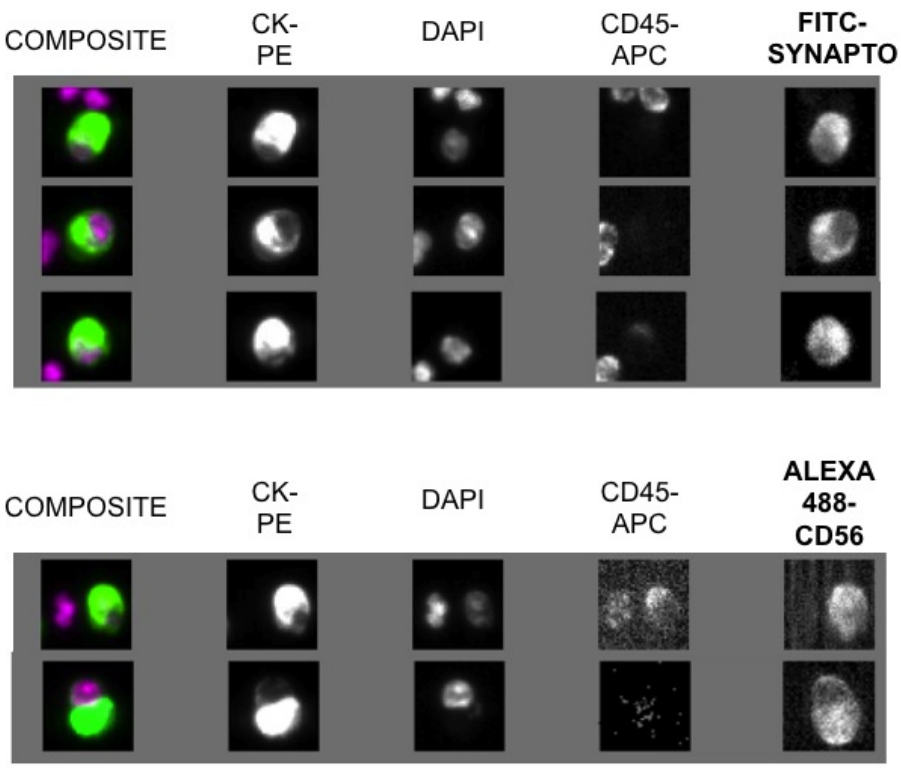


Figure 3.5 Diagram demonstrating CTCs expressing synaptophysin and CD56 from a patient's sample. Each horizontal event depicts the same cell(s) viewed with different filters (vertically). The top group of three demonstrates three CTCs positive for synaptophysin (in the FITC-SYNAPTO channel). The bottom two events show two CTCs positive for CD56 (ALEXA 488-CD56 channel).

3.3.5. Numbers of CTCs in NETs compared to other cancers

Numbers of CTCs from this study were compared to numbers of CTCs enumerated in other metastatic cancers using the same technology (Table 3.3)[153]. Previous studies have stated the median of cases with 2 CTCs and above. When taking all NETs regardless of primary, and using this criteria for comparison, the median CTC count in our study was 10/7.5ml. This is similar to metastatic breast and prostate, the two main cancers utilising this CTC technology, and greater than the other cancers. In fact, 42% of patients of NET patients had CTCs of two or above which is second only in frequency to metastatic prostate cancer.

Comparing midgut NETs to metastatic colorectal adenocarcinomas, NETs had a higher median CTC count (10 vs. 5 respectively) and greater percentage of cases (47% vs. 30%) with CTCs of 2 or above. Pancreatic NETs had a higher median (48) and percentage of cases with CTCs of 2 or above (24%) when compare to pancreatic adenocarcinomas (median 3.5 and 19% respectively), Levels of CTCs in metastatic bronchial NETs were similar to metastatic lung cancer.

	No. of patients	Mean CTC (SD)	Median with ≥ 2	No. (%) ≥ 2	No. (%) ≥ 5	No. (%) ≥ 10	No. (%) ≥ 50
Healthy[153]	145	0.1 (0.2)	N/A	0 (0)	0 (0)	0 (0)	0 (0)
Metastatic Cancer Type							
Breast[153]	422	84 (855)	10	489 (37)	340 (26)	256 (19)	129 (10)
Prostate[153]	123	75 (333)	13	107 (57)	77 (41)	61 (32)	27 (14)
Prostate[205] (progressive)	219				125 (57)		
Gastric[153]	9	24 (83)	3	4 (31)	1 (8)	1 (8)	1 (8)
Gastric[205] [213]	27	0.4 (0.7)	0	4 (14)			
Lung[153]	99	30 (178)	9	34 (20)	24 (14)	16 (10)	10 (6)
SCLC[214]	50	2915 (8115)	28				
Non-SCLC[215]	101			21 (21)	15 (15)		
Pancreatic adenocarcinoma[153]	16	2 (6)	3.5	4 (19)	1 (5)	1 (5)	0 (0)
Pancreatic adenocarcinoma[216]	32	17 (31)	0.5	11 (42)			
Colorectal[153]	196	4 (11)	5	99 (30)	56 (17)	30 (9)	5 (2)
All NETs	**175**	**45 (300)**	**10**	**74 (42)**	**53 (30)**	**39 (22)**	**15 (9)**
Midgut NET	101	12 (35)	10	47 (47)	32 (32)	24 (24)	6 (6)
Pancreatic NET	42	20 (72)	48	10 (24)	8 (19)	7 (17)	5 (12)
Bronchial NET	17	414 (1095)	16	5 (29)	4 (24)	3 (18)	2 (12)

Table 3.3 CTCs in NETs compared to other cancers, enumerated by Cellsearch™ method

3.3.6. CTC Correlation with Existing Markers and Clinicopathological Data

Associations between CTCs and clinicopathological data are shown in Table 3.4. The liver metastases burden categories, 25-50%, 50-75% and >75%, were combined due to small number of events in these categories (n=59, 20 and 13 respectively) compared to that <25% (n=83). PS scores of 1-4 were grouped together due to the few numbers compared to patients with PS of 0. Since CgA was not normally distributed (even when transformed onto a logarithmic scale) this was analysed in two groups: above and below twice the upper limit of normal (120 pmol/L)[92]. In addition to CTCs being analysed as a continuous variable, they were also analysed as a dichotomous variable: presence or absence of CTCs i.e. CTC≥1.

There were associations between presence of CTCs and grade (Chi-squared P=0.036), metastatic burden (Chi-squared P<0.001), CgA (Chi-squared P<0.001) (Figure 3.8) and presence of bone metastases (Chi-squared P=0.03). These associations were also present when CTCs were analysed as a continuous variable (Figure 3.6, Figure 3.7, Figure 3.10). CTCs were not associated with performance status (Figure 3.9).

There was a weak correlation between CTC levels and urinary 5-HIAA in midgut and unknown NETs where available (r=0.4, P=0.007, n=28) although 5-HIAA levels were higher in those with CTCs present (Mann-Whitney, P=0.04).

There was no significant difference in the presence (Chi-squared, P=0.61) or levels (Mann-Whitney, P=0.21) of CTCs between those on somatostatin analogues and those who were not. In addition, there was no association between CTCs and whether the primary tumour had been resected (Mann-Whitney, P=0.68).

	Percentage of patients with CTC≥1	CTCs as continuous variable (mean CTC)
Metastatic Burden		
≤25%	33	16
>25%	64	71
P value	<0.001	<0.001
Tumour Grade		
G1	40	11
G2	54	11
G3	66	218
P value	0.036	0.006
Performance Status		
0	49	25
≥1	50	469
P value	1.0	0.794
CgA Levels		
≤120	29	20
>120	64	64
P value	<0.001	<0.001
Bone metastases		
Present	45	38
Absent	66	75
P Value	0.030	0.028

Table 3.4 Summary of association of CTCs with existing NET clinicopathological factors; CTCs analysed as dichotomous variable (CTC≥1) and as a continuous variable

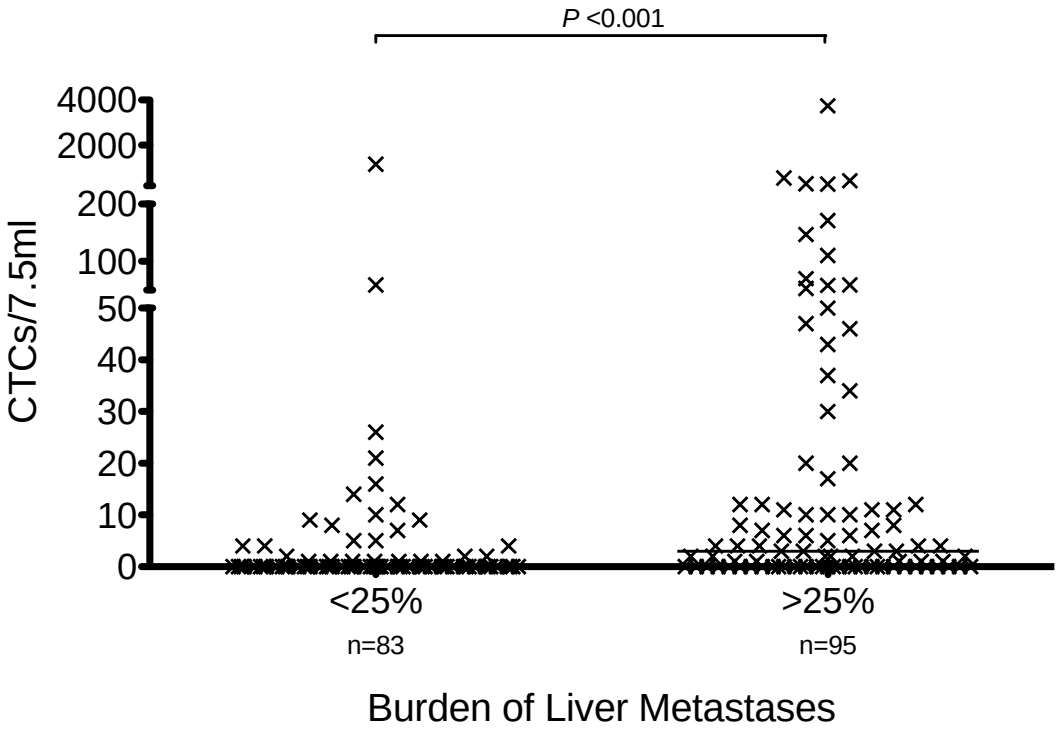


Figure 3.6 Association between Metastatic Burden and CTCs (Horizontal bars indicate median)

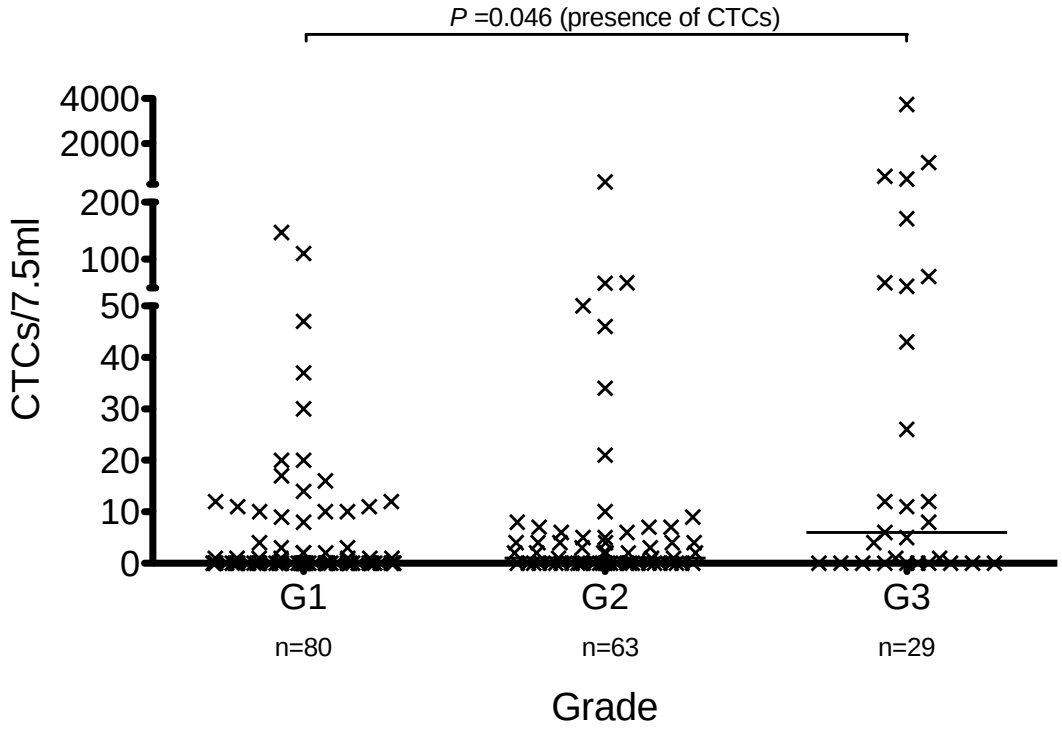


Figure 3.7 Association between Grade according to Ki-67 proliferation Index and CTCs G1=low grade; G2=intermediate grade G3=high grade. (Horizontal bars indicate median)

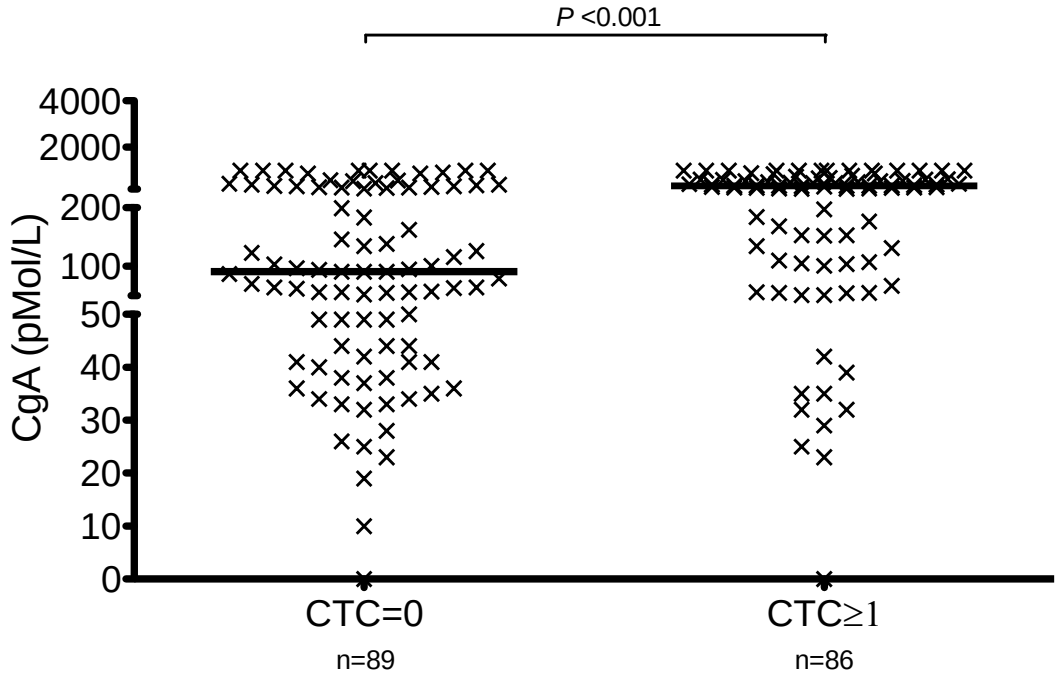


Figure 3.8 CgA levels according to absence or presence of CTCs (horizontal bars represent median)

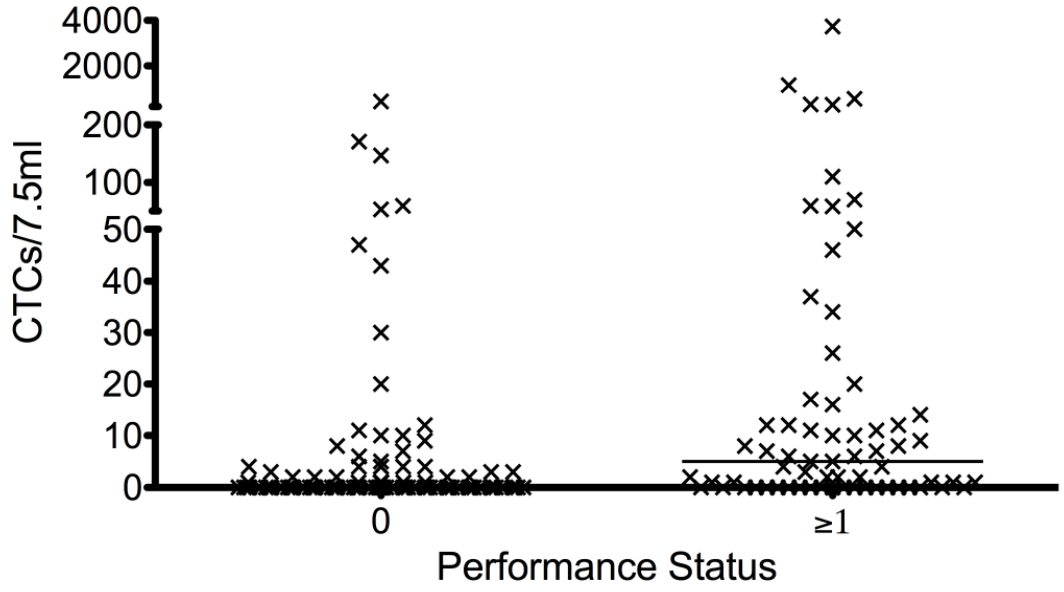


Figure 3.9 Association between performance status (WHO) and CTCs (Horizontal bars indicate median)

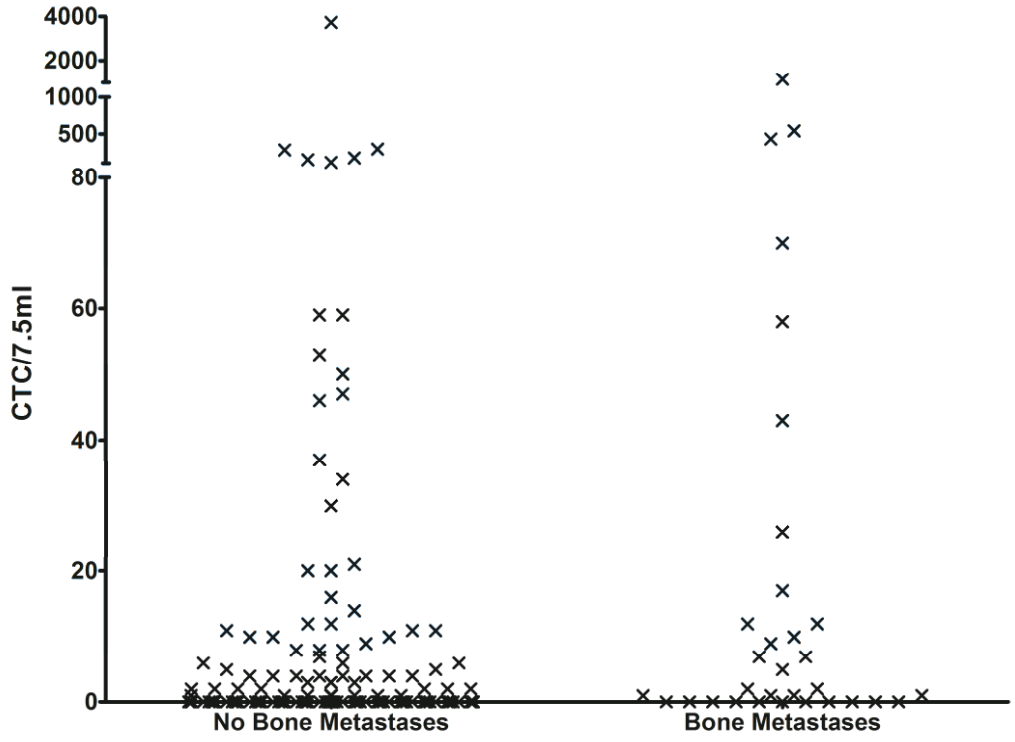


Figure 3.10 CTCs in the absence and presence of bone metastases

3.4. Discussion

This is the first systematic analysis of EpCAM expression and CTC detection in NETs. Regardless of grade and degree of differentiation, all GEP-NETs demonstrated strong EpCAM expression. Given the origin of NETs is still debated, the expression of this carcinoma-associated protein adds evidence to an epithelial origin rather than cells of the neural crest as once thought.[10, 11] Some of the proposed functions of EpCAM include regulating *c-myc* and cyclins, promoting cell cycling and enhancing proliferation[217, 218, 219, 220, 221]. Anti-EpCAM therapy has been trialled in metastatic breast, colorectal cancer and in malignant ascites[222, 223, 224, 225, 226, 227] and our results presents an opportunity for EpCAM directed therapy in NETs. However, due to normal pancreas and small intestine tissue expressing EpCAM, caution must be taken.

The Cellsearch™ method of CTC enrichment has been validated previously and only 0.3% of healthy controls and benign cases have 2 or more CTCs per 7.5mL of blood[153]. Hence, the absence of healthy controls in our study. I have demonstrated the neuroendocrine origin of the CTCs by using NET specific immunohistochemical markers (synaptophysin or CD56).

Fifty-one per cent of midgut NETs and 36% of pancreatic NETs had one or more CTCs. Numbers of CTCs from this study were compared to numbers of CTCs in other metastatic cancers using the same technology (Table 3.3). This is a limited comparison since the patient populations are invariably heterogeneous albeit all with metastatic disease. However, when analysing cases with CTCs of 2 or above (as done in other cancers), the median CTC count was 10 per 7.5ml which is similar to metastatic breast and prostate cancer, and more than the other cancers. 42% of patients of NET patients had CTCs of 2 or above which, as a proportion, is second only to prostate cancer.

Comparing midgut and pancreatic NETs to colorectal and pancreatic adenocarcinomas, NETs had a higher median CTC count and higher proportion of patients with CTCs. Levels of CTCs in metastatic bronchial NETs were similar to metastatic lung cancer.

This has important implications. The majority of CTC biomarker and downstream CTC research, to date, has been conducted in metastatic breast and prostate cancer because of the relatively high frequency of CTCs compared to other cancers and due to this research, led to approval by the FDA. The abundance of CTCs in NETs found in our

experiments lends itself to further research in CTCs similarly to that in those other cancers. Given the variable and often prolonged survival of NETs, CTCs may be incorporated into clinical trials as a biomarker to elucidate predictive and prognostic value. This has important implications especially if validated as a surrogate endpoint since the outcomes in NET trials i.e. PFS, OS are often much longer than other tumours.

Prognostic CTC cut-off levels have been determined in other cancers[205, 228] and further studies are required to define these in NETs.

Despite abundant EpCAM expression in pancreatic NETs, only a small proportion (36%) had CTCs detected compared to midgut NETs. Possible reasons include less shedding of CTCs i.e. less haematogenous spread, loss of EpCAM expression or unidentified factors particular to this sample of pancreatic NETs.

CTCs were only detected in bronchopulmonary NETs when the primary tumour expresses EpCAM. Absent EpCAM expression has been cited as a reason for absence of CTCs in metastatic breast cancer[229] but for the first time, I report synchronous CTC detection and differential EpCAM expression. EpCAM positive bronchopulmonary NETs had the highest levels of CTCs possibly due to absence of portal circulation which filters CTCs[210].

More patients in the higher burden group (>25% liver involvement) had CTCs present and CTC levels were higher in patients with greater burden. This suggests that in NETs, the finding of CTCs may reflect the burden of the underlying disease and higher metastatic potential, contradicting studies in breast cancer[228] but supporting a study in prostate cancer[230]. This may be due to the indolent nature of NETs compared to breast cancer.

There appeared to be an association between CTCs and the existing biomarker CgA. Together with the association between CTCs and 24-hour urinary 5-HIAA, this may be due to tumours being more metabolically active when CTCs are present or CTCs secreting serotonin and other vesicle products into the circulation. This relationship requires further investigation into the biochemical properties of CTCs.

Although there were associations between CTCs and tumour grade, and between CTCs and presence of bone metastases, caution must be taken in interpretation. Although *P* values were below the 0.05 level, there were multiple significance tests in this section, increasing the likelihood of a significant result occurring by chance. Thus, a large, homogenous trial is required to elucidate the validity of these associations.

Nevertheless, the suggestion of an association between bone metastases and higher CTC counts would support previous data in breast cancer[231]. The relationship with grade could suggest that more aggressive tumours shed more CTCs into the circulation. With grade based on a specimen that may have been taken many years ago, CTCs could be more representative of current tumour biology. However, a study with repeated tumour sampling, correlating with CTC enumeration is required.

This confirmation of CTCs in NETs offers an opportunity to molecularly characterise NETs without invasive biopsies, which may accelerate development of new therapies and allow the natural history of the metastatic process to be studied i.e. ‘the liquid biopsy’. In view of the increasing arsenal of treatment options [50, 54, 61, 62, 232, 233, 234] and the variable survival of NETs, CTCs could be used as a prognostic marker in NETs to stratify therapy, and in real-time monitoring of tumour growth or treatment response.

Chapter 4. Circulating Tumour Cells as Prognostic and Predictive Biomarkers

4.1. Introduction

In the first chapter, I established that there is a lack of prospectively validated biomarkers in neuroendocrine tumours (NETs). Given the variable survival and heterogeneity of NETs, clinically validated prognostic and predictive biomarkers are needed. The current most widely used circulating biomarker, chromogranin A (CgA), has not been robustly prospectively validated. The most widely used tissue biomarker, Ki-67, samples a small population of the tumour mass and is often performed at diagnosis which may be years from treatment decisions. Endpoints used in clinical practice and in clinical trials are based on radiological assessment but given the variable survival, this is often assessed over a long period of time, sometimes years, Some NETs are very stable over time, in terms of size, whereas others progress. Therefore, a biomarker which can predict progression and response to therapy at an early time-point would be useful as well as discriminating stable from progressive tumours.

4.1.1. CTC detection and other technologies

The presence of CTCs was first described by the Australian physician, Thomas Ashworth, in 1869[148]. Recent technological advances in CTC detection and enumeration have made routine evaluation of CTCs feasible. In most cases, there are two steps: isolation-enrichment and detection. The CellSearch™ platform employed in my study, and explained in chapter 2, is just one of many technologies used but it is the only method which has been approved by FDA. Other methods include immunomagnetic enrichment, multiplex reverse transcription quantitative PCR (RT-qPCR)-based methods, microfilter and microchip devices or a combination of these. The main analytical approaches are shown in Table 4.1.

A disadvantage of the anti-EpCAM antibody-based methods is that some tumour cells do not express EpCAM as shown by a study looking at ‘normal-like’ breast cancer cell lines[235]. However, a combination of anti-CD146 and anti-EpCAM has been proposed to improve CTC detection[236].

The CTC filtration devices listed in Table 4.1 are only those which have been evaluated on peripheral blood samples. Isolation by size of epithelial tumour cells (ISET) is based on individual isolation of epithelial tumour cells by filtration due to their larger size compared to leukocytes[237]. FISH and PCR-based genetic analyses have been applied to ISET-isolated cells[237]. A microfluidic device, called the CTC-chip[238], has been developed to capture CTCs through use of EpCAM antibody-coated micro-posts with the same group recently developing the herringbone chip which is a high-throughput microfluidic mixing device[239].

Other methods to detect surrogate markers of CTCs include molecular assays to detect circulating free DNA or RNA in serum or plasma. These allow high throughput but are dependent on looking for particular sequences of mRNA or DNA (If known to be specific for a tumour) and cannot be used to enumerate CTCs or for morphological analysis. Assays to detect cytokeratin-19 by RT-qPCR have been used on clinical samples. Numerous circulating DNA/RNA markers have been studied mainly in breast cancer, in particular as part of a multiplexed PCR-coupled liquid bead array which utilised CTC targets *SCGB2A2* (mammaglobulin), melanoma antigen A, *TWIST1* and *KRT19*.

Assay	Enrichment	Detection	Advantages	Disadvantages	References
CellSearch™	Immunomagnetic positive selection (EpCAM antibody)	Markers: CK-8/9/19, CD45, DAPI	FDA approved Visual confirmation Clinical relevance	EpCAM dependent Limited markers	Allard *et al*[153]
CTC Chip	EpCAM antibody covered micro-posts	Markers: CKs, CD45	High detection rate Visual confirmation	Subjective analysis EpCAM dependent	Nagrath *et al[238]*
CTC-filtering devices	Size (filters)	Markers: CKs	Capture and analysis platform Multiplexed imaging Genetic analysis	CTCs can be heterogeneous in size Contamination	Vona *et al*[237] Zheng *et al[240]*
Laser-scanning cytometer	EpCAM antibodies coupled to columns	Fluorophore-conjugated anti-epithelial antibody	Automated microscope 10000-fold enrichment	EpCAM dependent	Pachmann *et al[241]*
EpiSpot assay	Negative selection (CD45)	Detection of secreted proteins: CK19, mucin-1, cathepsin-D	Only viable cells detected	Clinical relevance not studied	Alix-Panabieres *et al*[242]
RT-PCR	Ficol gradient centrification	Markers: CK19, HER2, h-MAM, CEA, GABA A	High sensitivity Only viable cells detected	No morphological analysis	Slade *et al*[243] Reinholz *et al[244]*
RT-qPCR	Ficol gradient centrification	CK19, BST1, PTPRC	High sensitivity Only viable cells detected	No morphological analysis	Stathopoulou *et al[245]* Sieuwerts *et al[246]*
RT-qPCR	OncoQuick enrichment and RNA pre-amplification	EpCAM, hMAM, PPIC, SLC6AB, CCNE2, EMP2	High sensitivity Only viable cells detected	No morphological analysis	Obermayr *et al*[247]
Multiplex RT-PCR Adnatest	EpCAM and MUC1 antibodies coupled to ferrofluids	Multiplex PCR for MUC1, HER2, EpCAM	High sensitivity Only viable cells detected Saves time	No morphological analysis EpCAM dependent	Aktas *et al*[248]
Liquid bead array	Ficol gradient centrifugation and EpCAM antibody ferrofluids	Multplex PCR for CK19, HER2, MAGE-A3, hMAM, TWIST1	On viable cells detected Multiple markers	No morphological analysis EpCAM dependent	Markou *et al[249]*

Table 4.1 Overview of analytical methods for detection and characterisation of CTCs. EpCAM Epithelial cell adhesion molecule; CK-19 Cytokeratin-19; CKs cytokeratins; HER2 human epidermal growth factor receptor-2; h-MAM human mammaglobulin; CEA Carcinoembryonic antigen ; GABA-A γ-aminobutyric acid; BST1 bone marrow stromal cell anitgen; PTPRC protein tyrosinase phosphatase, receptor type, C; PPIC peptidylprolyl isomerase C; SLC6AB soluble carrier family 6 (neurotransmitter transporter, creatine); CCNE2 cyclin E2; EMP2 epithelial membrane protein 2; MAGE melanoma associated antigen

4.1.2. Clinical Application and Characterisation of CTCs

It has been hypothesised that CTC counts could reflect on-going metastasis[230] and accumulating evidence correlating CTCs with clinically relevant outcomes such as progression-free (PFS) and overall survival (OS) supports this[204, 250].

Using the CellSearch™ platform, prospective studies in patients with metastatic breast cancer about to undergo new systemic therapy, have demonstrated patients with ≥5 CTCs at baseline having significantly shorter median PFS and OS than those with <5 CTCs[154, 204]. After first follow-up, those patients with persistent elevated counts or that exceeded the threshold of 5 CTCs were associated with an adverse outcome. CTC counts have demonstrated utility as a predictive biomarker with Nakamura *et al* demonstrating those with no change or a reduction in CTCs at 3-4 and 12 weeks after treatment onset as showing better outcome than those with an increase[251].

In metastatic prostate cancer, similar results were shown with those ≥5 CTCs having significantly worse PFS and OS than those with <5 CTCs. As with breast studies, pre- and post-treatment CTC counts were prognostic and predictive and had superior predictive value compared to PSA[205]. Similarly, Cohen *et al* prospectively investigated 430 patients with metastatic colorectal cancer[206]. Their results demonstrated patients with ≥3 CTCs had significantly worse PFS and OS compared to those with <3 CTCs. Furthermore, persistence of CTCs after 'curative' resection was associated with high incidence of relapse and worse relapse-free survival[252]. With another method, using circulating mRNA, expression of four mRNA markers were associated with significantly shorter disease-free survival in this cancer type[253]. Moreover, a meta-analysis concluded that despite inter-study heterogeneity, CTC detection should be considered as a surrogate prognostic marker[254]. Other studies in lung cancer have had similar findings[215].

CTCs are very rare cells and downstream analysis presents difficult analytical and technical challenges. However, CTCs have the potential to provide a 'liquid biopsy' that can facilitate personalised, stratified therapy. Molecular and cytogenetic analyses of CTCs have been reported. Using FISH, chromosomal amplification of androgen receptor, rearrangement of *ERG* gene, loss of *PTEN*, and relative gain in *MYC* were detected in CTCs from patients with prostate cancer[255]. Other studies may have implications for therapy. CTC HER2 status may be different to that of the primary tumour which may have clinical relevance for selecting patients who would benefit

from molecular targeted therapy. Molecular analyses of CTCs have also been studied recently. The presence of an *EGFR*-activating mutation in CTCs was observed in 92% of patients while it was present in 33% responding to tyrosine-kinase inhibitors and 64% who had clinical progression[256]. Thus the authors concluded that this mutation in CTCs might be relevant to drug resistance. mRNA and miRNA expression in CTCs has also had promising initial results despite the high background of leukocytes[257].

4.1.3. Aims

Having detected CTCs in NETs using the CellSearch™ platform, and considering the clinical utility of CTCs in other cancers, I set out to demonstrate the clinical relevance of CTCs in NETs. My aims were:

- to investigate the relationship between CTC detection and radiological progression
- to evaluate CTCs as a prognostic biomarker in terms of PFS and OS
- to evaluate CTCs as a predictive biomarker i.e. investigate their utility in predicting response to treatment
- to evaluate CTCs as biomarkers in comparison to grade and chromogranin A (CgA)

4.2. Materials and Methods

4.2.1. Patient Recruitment and Laboratory Measurements

This study was approved by the local ethics committee (Ref 09/H0704/44) and patients provided written informed consent.

In order to investigate the relationship between CTCs and progression of disease and to justify prospective studies, an initial pilot series (n=63) comprising of patients undergoing surveillance and treatment was analysed. In this initial dataset, progression was assessed retrospectively. In order to classify a patient as having progressive or non-progressive disease, target lesions (according to RECIST 1.1) were defined using CT or MRI within six weeks of blood sampling and compared retrospectively with the previous imaging.

Patients (n=138) about to commence a new treatment for metastatic NET were prospectively recruited between August 2009 and August 2011. Additionally, patients (n=37) were prospectively recruited if undergoing surveillance without any change in management. The combined dataset (n=175), comprising of treatment and surveillance groups were prospectively studied for survival analysis.

All eligible participants had histologically proven NET and metastatic disease measurable by RECIST criteria. They were categorised according to the site of primary NET: midgut, pancreas, bronchopulmonary, hindgut or of unknown primary. New treatment was defined as NET-specific therapy not given to the patient previously and patients who had treatment in the previous 4 months (except for somatostatin analogues) were excluded.

All patients had blood samples taken at baseline (within 4 weeks prior to treatment for the treatment cohort). For the treatment cohort, further samples were taken at 3-5 weeks after commencing treatment (first post-treatment sample) and at 10-15 weeks (second post-treatment sample). Blood was processed for CTC enrichment and enumeration as described in chapter 3, section 3.2.6. CTC enumeration was performed by two independent observers blinded to clinical endpoints.

Plasma CgA was also evaluated at the same time-points using a radio-immuno assay (RIA) kit (Roche). 5-HIAA analysis was performed on 24-hour urine samples from patients using a manual in-house radio-immunoassay. As these assays have previously

been validated and are currently used in clinical practice, no samples were run on normal healthy controls.

Quantification of hepatic tumour load (from 4 to 6 slices of a CT/MRI scan) was used to assess radiological burden by a semi-quantitative approach. Hepatic tumour burden was categorised as less than 25%, more than 25% but less than 50%, more than 50% but 75% or less, or more than 75%.

Data were collected on primary site, duration of diagnoses, any previous treatment received, WHO performance status and whether the primary tumour had been resected. Grade of tumour according to Ki-67 proliferation index was recorded according to ENETs guidelines[23, 24]. The study design met the REMARK (REporting recommendations for tumor MARKer prognostic studies) criteria[258].

4.2.2. Treatments

Treatments were prescribed as clinically indicated and listed in Table 4.2.

Treatment	Number of patients (n=138)	Details	Timing of post-treatment scan
Somatostatin analogues (SST)	34	Daily subcutaneous octreotide, or monthly Octreotide LAR/Somatuline Autogel	6 months+
Chemotherapy	29	Based on previous data for 5-fluorouracil, cisplatin and streptozocin (FCiSt)[54]	after completion
Peptide Receptor Radionuclide Therapy (PRRT)	40	3 doses of intravenous ^{90}Yttrium-DOTA-Octreotate as per hospital protocol	after completion
Trans-arterial Embolisation (TAE)	18	Selective cannulation of a branch of the hepatic artery and injection of polyvinyl chloride microparticles	6 months*
Radiofrequency Ablation (RFA)	2	Percutaneously under image guidance	3 months*
Sunitinib	4	Oral dose of 37.5mg a day	6 months+
Interferon alpha	4	3 million units subcutaneously three times a week	6 months+
Surgical resection of primary/metastases	7		6 months*

Table 4.2 List of treatments undertaken in treatment cohort. *after procedure/therapy. +after commencement of therapy

4.2.3. Outcomes, Endpoints and Statistical Analyses

Association between presence of CTCs and radiological status in the pilot series (n-63) was assessed with Fisher's exact test. For the combined dataset (n=175), PFS and OS were estimated using Kaplan-Meier methods from date of baseline sample to date of radiological progression (RECIST 1.1); and date of death (due to neuroendocrine cancer) or last follow-up. Survival curves were compared using log-rank testing. Cox-proportional hazards regression analysis was used to obtain univariate and multivariate hazard ratios for PFS or OS.

In order to stratify patients into those with favorable and unfavorable prognosis, results (in terms of differences in PFS) obtained for the first 90 patients enrolled (training set) were used to select a cut-off level of CTCs. This cut-off level was then validated with the 85 patients subsequently enrolled in the study (validation set).

For the treatment cohort (n=138), PFS and OS were analysed according to baseline CTC count and changes in CTCs analysed at first and second post-treatment time-points separately. In order to assess therapy-induced changes in number of CTCs, data were split into tertiles comparing post-treatment CTCs to baseline: those with a reduction of 65% or more from baseline, those with less than a 65% reduction to less than 33% increase from baseline (including no change), and those with a greater than 33% increase over baseline. Those patients with zero CTCs at baseline and zero CTCs at post-treatment time-points were used as a reference group for comparison. These groups were compared for association with response to therapy, and with survival outcomes.

With a median PFS in the worst and best prognostic groups, as defined by CTC cut-off, of 6 and 12 months respectively, a minimum sample size of 142 was required with 80% power and an alpha of 0.05 (hazard ratio 2.0).

To assess response to therapy, CT or MRI after therapy (according to timing listed in Table 4.2) was compared with baseline imaging to assess whether a patient had progressive or non-progressive (stable disease or minor/partial response) disease. Comparisons were performed by an independent radiologist who also recorded percentage changes in target lesion dimension and classified them according to RECIST 1.1.

4.3. Results

4.3.1. The Relationship between CTCs and Progressive Disease using Pilot Data

In order to explore the relationship between CTCs and growth of tumours, patients (n=63) from an initial pilot, who had undergone serial imaging of tumours, were selected. All patients had imaging within 6 weeks of sampling and a previous scan for comparison evaluated by an independent radiologist. Bronchopulmonary NETs were excluded from this analysis because of variable EpCAM expression.

Eighteen of 19 (95%) patients who had progressive disease according to RECIST had CTCs detected compared with 9 of 44 (20%) patients who had non-progressive disease (Figure 4.1). This was a significant difference (χ^2=31.4, P<0.001), with no statistical difference in other factors between progressors and non-progressors (Table 4.3).

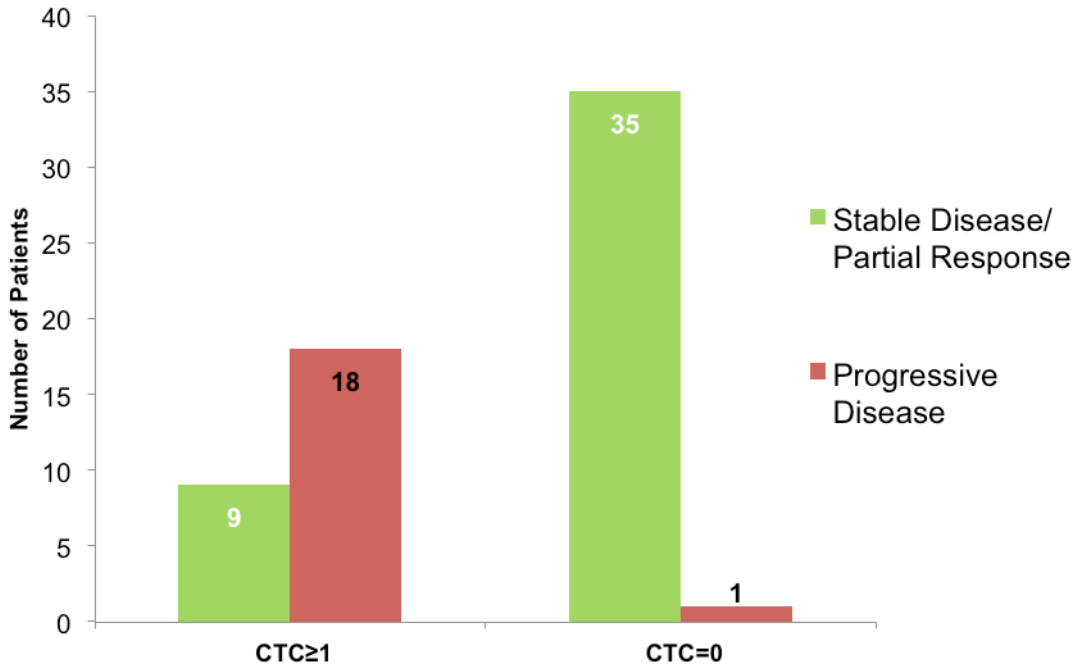


Figure 4.1 Presence of CTCs and Progressive Disease

Characteristic	Non-Progressive Disease n=44	Progressive Disease n=19	Test *P* value
Age median years (range)	59.5 (38-79)	56.1 (30-77)	U=0.36 *P*=0.37
Grade Low Intermediate High	22 19 3	8 9 2	Fishers exact *P*=0.57
Burden of Liver metastases,% <25 25≤50 >50	24 16 4	7 10 2	Fishers exact *P*=0.39
Chromogranin A in pmol/L, mean (range)	297.8 (33-1000)	359.4 (34-1000)	t=0.46 *P*=0.65
Resection of primary Yes No	16 28	8 11	Fishers exact *P*=0.99
Duration of diagnosis, median months (range)	58.5 (5-278)	55 (10-108)	U=343 *P*=0.99
On-going somatostatin analogue therapy Yes No	22 12	11 8	Fishers exact *P*=0.77
Interval to last scan, median weeks (range)	18 (12-56)	18 (10-30)	U=347 *P*=0.76
CTC **CTC=0** **CTC≥1**	**35** **9**	**1** **18**	Fishers exact ***P*<0.001**

Table 4.3 Characteristics of cases with non-progressive vs. progressive disease in patients with metastatic midgut, pancreatic or unknown primary

When absolute changes in target lesions were assessed, 23 of 27 (85%) patients with detectable CTCs had growth of tumour lesions, and 31 of 36 (86%) patients without CTCs had no growth or spontaneous shrinkage (Figure 4.2). There was no association between the number of CTCs and absolute increase in tumour size.

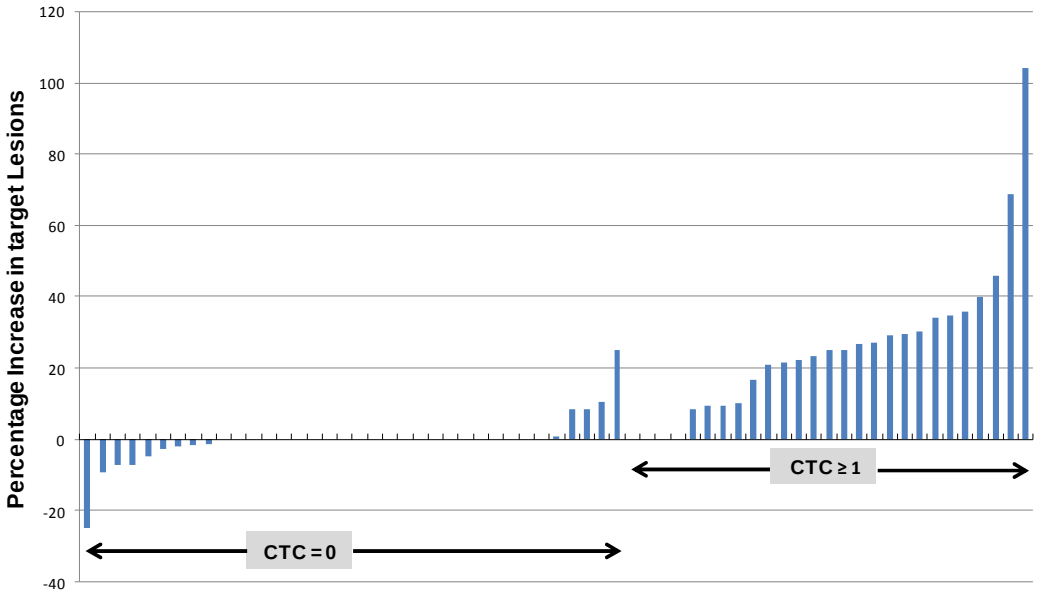


Figure 4.2 Percentage Increase in lesions on imaging grouped by the presence of CTCs. Each bar represents an individual case. Cases without CTCs are grouped on the left-hand side of the chart (CTC=0); cases with CTCs detected on the right-hand side (CTC≥1)

4.3.2. The Combined Dataset of Surveillance and Treatment Groups

Having established that CTCs are associated with progression of tumour when assessed retrospectively, it was hypothesised that CTCs are associated with a poor prognosis. The combined dataset of surveillance and treatment groups (n=175) from chapter 3 was prospectively studied for this purpose.

The background characteristics of the combined dataset are shown in chapter 3, Table 3.2.

4.3.3. Training and Validation Sets to Establish CTC Prognostic Threshold

I aimed to select a level of CTCs that most clearly distinguished patients with slow progression or stable disease from those with rapid progression. To achieve this, increasing thresholds of baseline CTC levels were systematically correlated with PFS for a 'training set' comprising of 90 patients from the combined dataset (n=175). Specifically, Kaplan-Meier survival curves were plotted comparing patient groups above and below each threshold and log-rank testing performed to obtain significance levels of difference across groups. Thresholds were tested commencing at CTCs of one and above, increasing by 1 CTC until 50 CTCs. Thereafter, CTC thresholds between 50 and 1000 were tested at increments to include the next CTC count sequentially (Table 4.4).

Survival differences were greatest at a CTC level of one i.e. those without CTCs (CTC=0) and those with CTCs (CTC≥1). Cox-proportional hazards also demonstrated that survival difference was greatest at this level. Thus a cut-off of 1 CTC per 7.5ml was chosen to distinguish patients with an unfavourable prognosis from patients with a favourable prognosis. The same optimal cut-off was also identified when OS was analysed instead of PFS.

Groups (CTC Cut-off)	Numbers in each group	12 months PFS (%)	Survival Difference	Hazard Ratio	*P* Value
0	35	88			
≥1	50	50	38	4.3	0.001
0-1	40	87			
≥2	50	50	37	4.3	0.002
0-2	46	85			
≥3	44	55	30	3.3	0.002
0-3	49	78			
≥4	41	52	26	3.3	0.002
0-4	54	77			
≥5	36	49	28	3.4	0.001
0-5	55	76			
≥6	35	45	31	3.1	0.002
0-6	58	74			
≥7	32	45	29	2.8	0.004
0-7	60	70			
≥8	30	51	18	2.4	0.013
0-8	62	71			
≥9	28	47	24	2.4	0.012
0-9	64	71			
≥10	26	54	17	2.4	0.015
0-10	66	71			
≥11	24	51	20	2.8	0.005
0-11	68	71			
≥12	22	50	21	2.5	0.015
0-12	69	71			
≥13	21	48	23	2.3	0.023
0-13	69	71			
≥14	21	48	23	2.3	0.023
0-14	69	71			
≥15	21	48	23	2.3	0.023
0-15	69	71			
≥16	21	48	23	2.3	0.023
0-16	70	71			
≥17	20	48	23	2.3	0.023
0-17	71	72			
≥18	19	45	27	3.0	0.004
0-18	71	72			
≥19	19	45	27	3.0	0.004
0-20	72	73			
≥21	18	41	32	3.3	0.001
0-21	73	73			
≥22	17	37	36	3.7	0.001
0-22	73	73			
≥23	17	37	36	3.7	0.001
0-23	73	73			
≥24	17	37	36	3.7	0.001
0-24	73	73			
≥25	17	37	36	3.7	0.001
0-25	73	73			
≥26	17	37	36	3.7	0.001
0-26	74	72			
≥27	16	39	33	3.3	0.002
0-27	74	72			
≥28	16	39	33	3.3	0.002
0-28	74	72			
≥29	16	39	33	3.3	0.002
0-29	74	72			
≥30	16	39	33	3.3	0.002

0-30 ≥31	74 16	72 37	35	3.6	0.001
0-31 ≥32	74 16	72 37	35	3.6	0.001
0-32 ≥33	74 16	72 37	35	3.6	0.001
0-33 ≥34	74 16	72 37	35	3.6	0.001
0-34 ≥35	74 16	72 37	35	3.6	0.001
0-35 ≥36	74 16	72 37	35	3.6	0.001
0-36 ≥37	74 16	72 37	35	3.6	0.001
0-37 ≥38	76 14	71 34	37	3.9	0.001
0-38 ≥39	76 14	69 31.5	37.5	3.9	0.001
0-39 ≥40	76 14	69 31.5	37.5	3.9	0.001
0-40 ≥41	76 14	69 31.5	37.5	3.9	0.001
0-41 ≥42	76 14	69 31.5	37.5	3.9	0.001
0-42 ≥43	76 14	69 31.5	37.5	3.9	0.001
0-43 ≥44	76 14	69 31.5	37.5	3.9	0.001
0-44 ≥45	76 14	69 31.5	37.5	3.9	0.001
0-45 ≥46	76 14	69 31.5	37.5	3.9	0.001
0-46 ≥47	77 13	69 31.5	37.5	3.9	0.001
0-47 ≥48	77 13	69 31.5	37.5	3.9	0.001
0-48 ≥49	77 13	69 31.5	37.5	3.9	0.001
0-49 ≥50	77 13	69 31.5	37.5	3.9	0.001
0-50 ≥51	79 11	63 27	36	4.1	0.001
0-53 ≥54	80 10	63 30	33	3.9	0.001
0-58 ≥59	81 9	63 33	30	3.8	0.002
0-59 ≥60	83 7	61 29	32	4.1	0.002
0-70 ≥71	84 6	65 33	32	3.7	0.008
0-110 ≥111	85 5	65 40	25	3.0	0.040
0-270 ≥271	86 4	65 25	40	4.8	0.011
0-430 ≥431	87 3	71 0	71	11.2	0.001
0-542 ≥543	88 2	70 0	70	7.9	0.006
0-1150 ≥1151	89 1	70 0	70	21.5	0.006

Table 4.4 Establishing the cut-off for CTCs in the 'training' set. Groups were split into below and above different CTC cut-offs in rows. 12 months PFS with survival difference between groups, hazard ratio and *P* value shown in rows.

To test for consistency, this CTC threshold was then tested with 85 subsequently enrolled patients, the 'validation set'. The distributions of patients above and below the cut-off level in the training and validation sets were compared with the use of Fisher's exact tests, and median PFS and median OS in the two sets compared with nonparametric *k*-sample test for equality of the medians. All *P*-values were two sided. Neither PFS nor OS were significantly different between the validation and training sets (log-rank PFS P=0.32, OS P=0.56) suggesting similar survival distributions. The distribution of patients with CTC levels above the cut-off of ≥ 1 CTC per 7.5ml blood did not differ between the training and validation sets (P=0.41). The cut-off level from the training set was confirmed as separating two significantly different prognostic groups in this validation set (Figure 4.3).

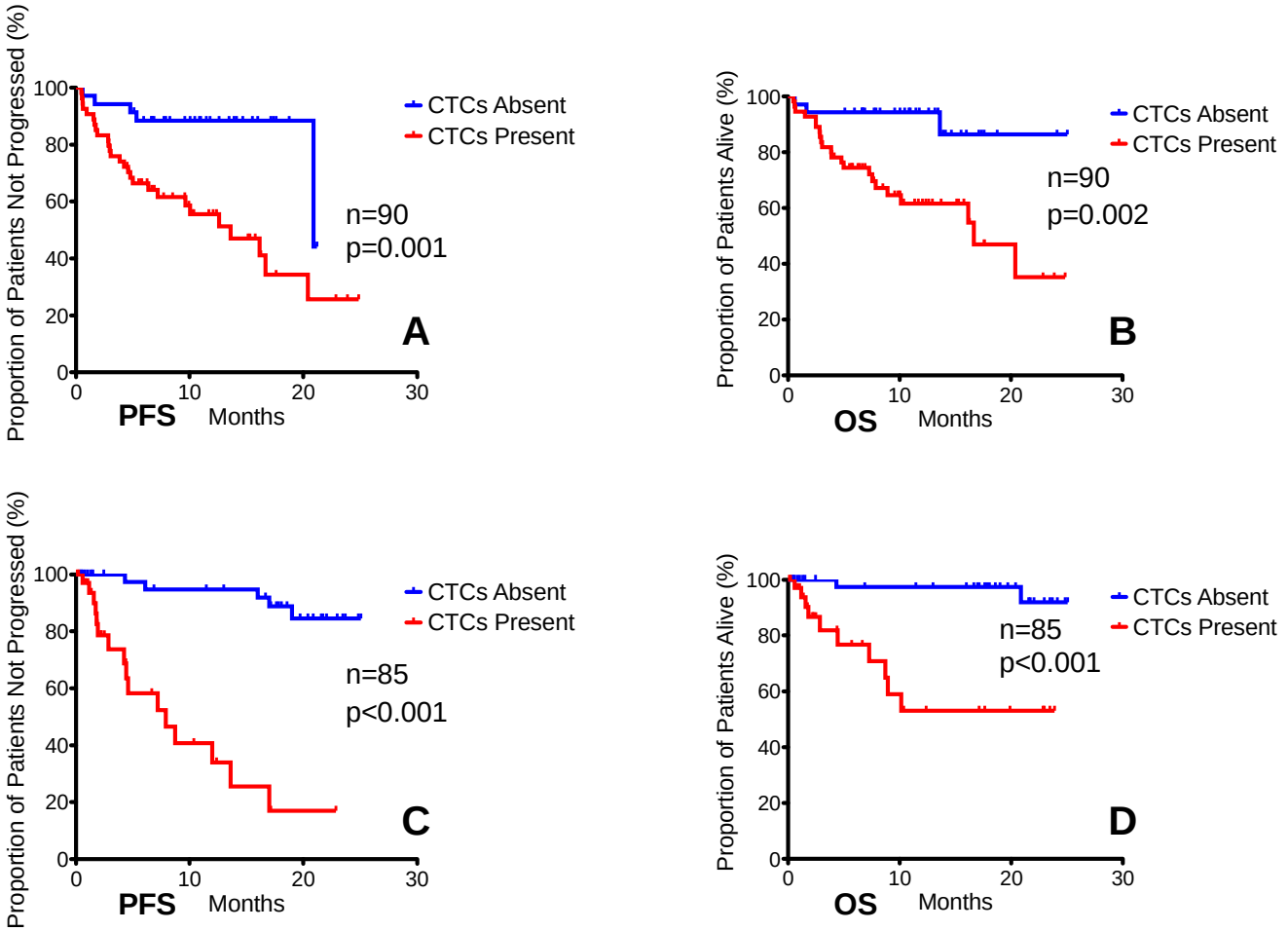


Figure 4.3 Survival curves for training set PFS (A), training set OS (B), validation set PFS(C), validation set OS(D), demonstrating the difference in survival between patients with CTCs present and those without CTCs i.e. a threshold of one or more CTCs

4.3.4. CTCs as Prognostic Markers al in the Combined Dataset

Using the combined dataset incorporating both the surveillance and treatment groups (n=175), Kaplan-Meier survival curves of PFS and OS are shown in Figure 4.4 with univariate and multivariate analyses of prognostic indicators shown in Table 4.5 and Table 4.6 respectively. Due to small numbers in some category groups, burden groups 25-50%, 50-75% and >75% were grouped together and PS 2, 3 and 4 were grouped together. The median follow-up was 12.6 months (range 5-28).

Patients with CTCs had significantly worse PFS and OS than those patients without CTCs. On univariate and multivariate analyses, presence of CTCs was a prognostic factor for worse PFS and OS. Although CgA was a prognostic factor for OS on univariate analyses, it was not significant on multivariate analyses and was not significant in terms of PFS. G3 (high grade) was also an independent poor prognostic indicator. A low burden (<25% liver involvement) was prognostic on univariate but not on multivariate analyses.

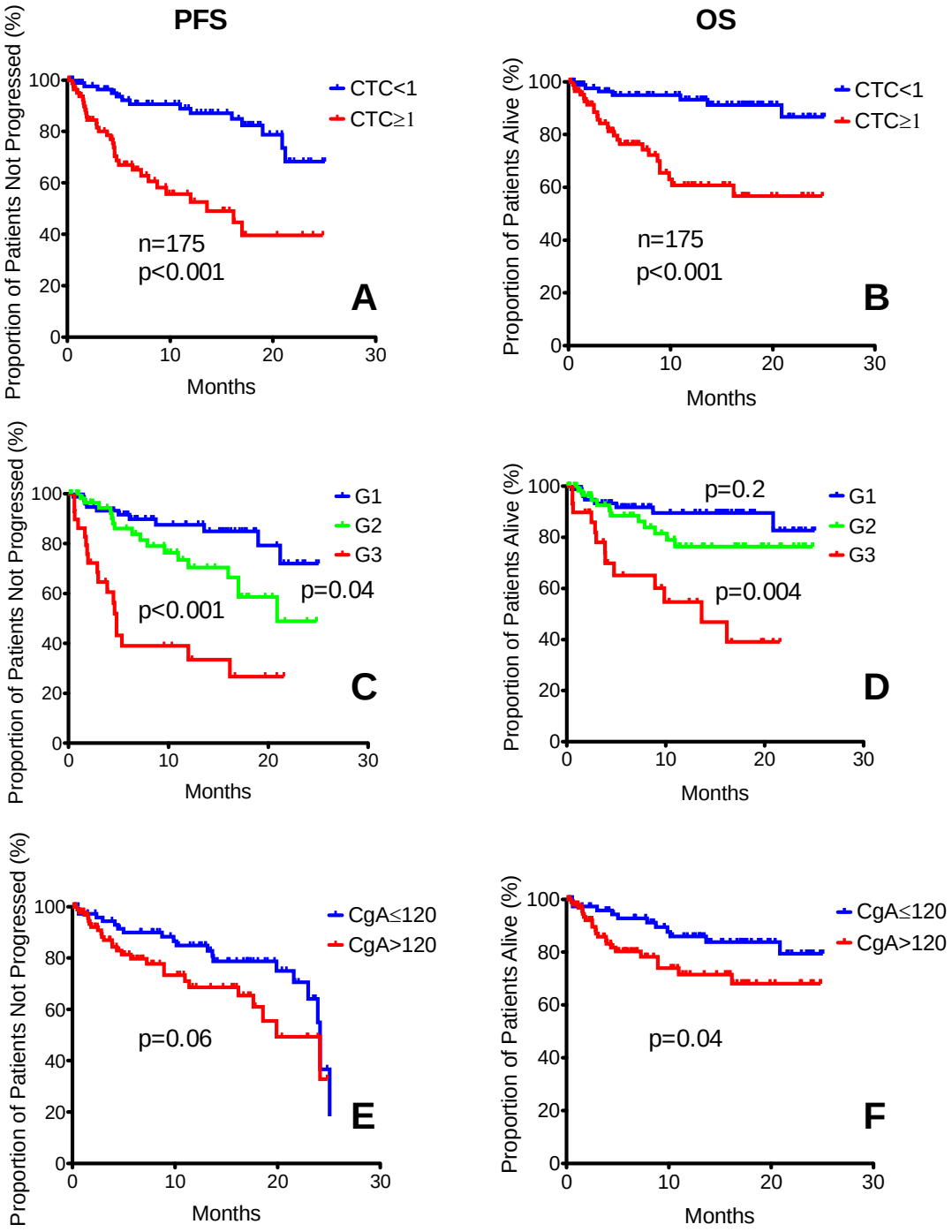


Figure 4.4 Survival curves demonstrating differences in PFS between groups according to (A) presence of CTCs, (C) Grade, (E) CgA; and differences in OS between groups according to (B) presence of CTCs, (D) Grade, (F) CgA (n=175)

Risk Factor	n	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
CTC					
Absence	89	1.0		1.0	
Presence	86	6.6 (3.2-13.6)	<0.001	8.0 (3.1-21)	<0.001
CgA					
CgA≤120	75	1.0		1.0	
CgA>120	100	1.8 (0.9-3.3)	0.057	2.5 (1.2-5.3)	0.018
Grade (Ki67)					
1	83	1.0		1.0	
2	63	2.4 (1.1-5)	0.025	1.6 (0.6-3.7)	0.330
3	29	6.4 (3.0-14.0)	<0.001	4.3 (1.8-10.2)	0.001
Burden					
<25%	83	1.0		1.0	
≥25%	92	2.5 (1.3-4.6)	0.004	3.6 (1.6-7.9)	0.002
PS					
0-1	167	1.0		1.0	
≥2	8	1.7 (0.5-5.4)	0.385	1.3 (0.8-5)	0.401
Age					
For every 10yrs	175	0.8 (0.6-1)	0.075	1.01 (0.8-1.4)	0.921

Table 4.5 Univariate analysis for prognostic indicators (n=175)

Risk Factor	n	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
CTC					
Absence	89	1.0		1.0	
Presence	86	3.3 (1.6-6.6)	0.001	3.7 (1.6-8.9)	0.003
CgA					
CgA≤120	75				
CgA>120	100	1.1 (0.5-2.2)	0.844	1.5 (0.6-3.7)	0.402
Grade (Ki67)					
1	83				
2	63	2.0 (0.9-4.2)	0.084	1.2 (0.5-3.1)	0.633
3	29	5.5 (2.4-12.3)	<0.001	3.4 (1.3-8.3)	0.008
Burden					
<25%	83				
≥25%	92	1.3 (0.6-2.6)	0.484	1.9 (0.8-4.6)	0.126
Age					
For every 10yrs	175	1.3 (1.1-2.1)	0.034	1.1 (0.8-1.4)	0.543

Table 4.6 Multivariate analyses for prognostic markers allowing for age (n=175)

4.3.5. CTCs as Prognostic Markers in Patients with Low (G1) and Intermediate (G2) Grade NETs

Patients with G1 and G2 tumours constitute a large subgroup of NETs yet the clinical behaviour within groups may vary significantly. There are no validated prognostic markers that can be applied within these groups and I therefore examined the prognostic value of CTCs within the G1 and G2 group combined and separately. Using the combined dataset of surveillance and treatment groups (n=175), a subgroup of patients who had either G1 or G2 grade NETs were analysed

There were 146 patients in this subgroup (28 progression and 19 death events). Survival curves of PFS and OS are shown in Figure 4.5, with univariate and multivariate Cox-proportional hazards regression shown in Table 4.7 and Table 4.8. Those with detectable CTCs had significantly worse outcome compared to those who had no CTCs. Presence of CTCs was an independent factor for worse PFS and OS whereas grade or CgA were not prognostic.

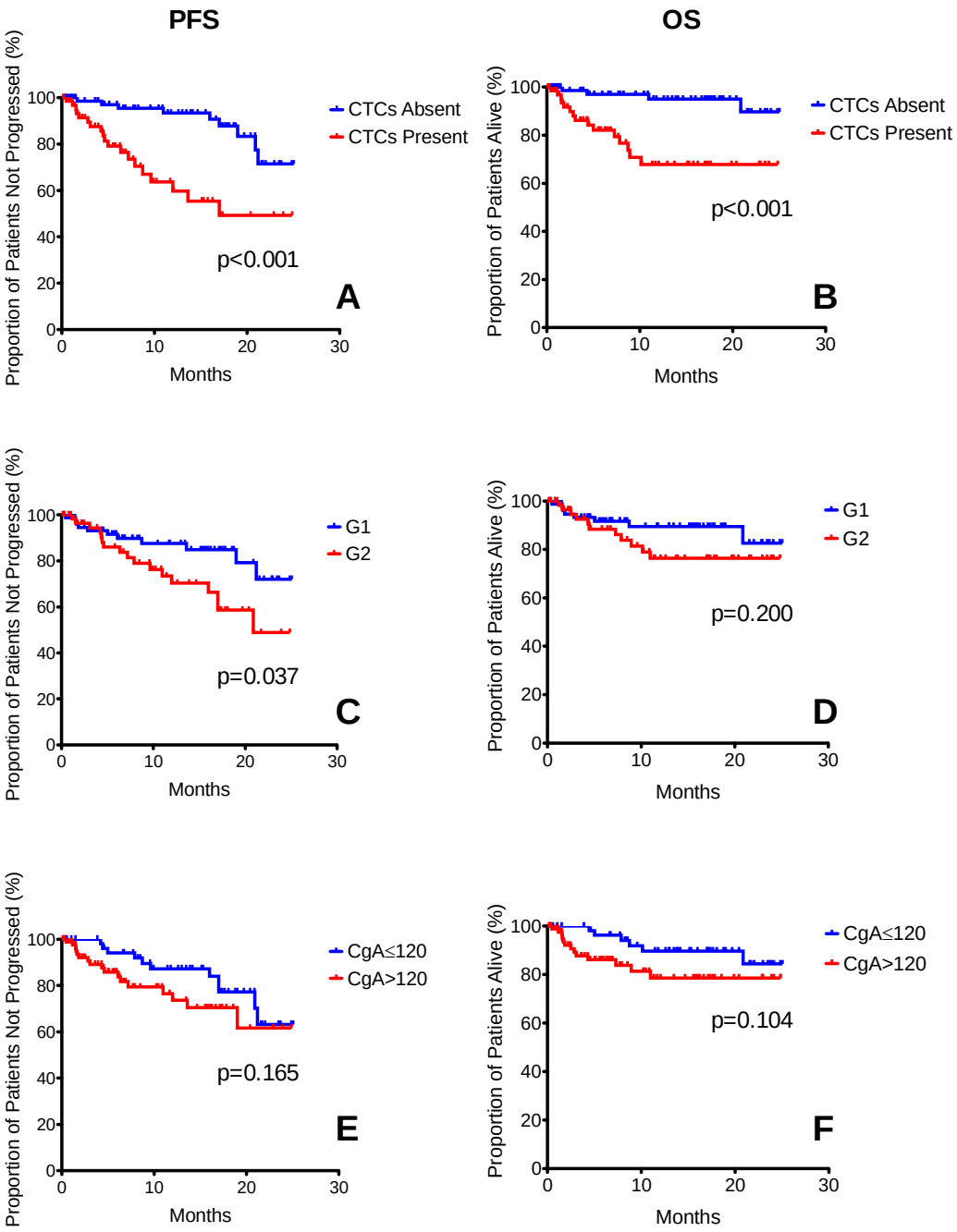


Figure 4.5 Survival curves demonstrating: differences in PFS between groups according to (A) presence of CTCs, (C) Grade (G1 or G2), (E) CgA; and differences in OS between groups according to (B) presence of CTCs, (D) Grade (G1 or G2), (F) CgA in a subgroup of G1 and G2 NETs

Risk Factor	n	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Baseline CTC					
Absence	79	1.0		1.0	
Presence	67	4.5 (2-10.1)	<0.001	6.0 (2.0-18.1)	0.002
Baseline CgA					
CgA≤120	58	1.0		1.0	
CgA>120	88	1.7 (0.8-3.7)	0.170	2.2 (0.8-5.8)	0.112
Burden					
<25%	75	1.0		1.0	
≥25%	71	1.7 (0.8-3.5)	0.169	2.2 (0.9-5.7)	0.090
PS					
0-1	139	1.0		1.0	
≥2	7	1.7 (0.4-7.1)	0.479	2.7 (0.6-11.6)	0.191
Grade					
1	83	1.0		1.0	
2	63	2.2 (1-4.7)	0.043	1.8 (0.7-4.5)	0.206
Age					
For every 10yrs	146	0.99 (0.7-1.4)	0.929	1.4 (0.9-2.0)	0.139

Table 4.7 Univariate analyses for prognostic factors in low and intermediate grade (G1 and G2) patients (n=146)

Risk Factor	n	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Baseline CTC					
Absence	79	1.0		1.0	
Presence	67	4.1 (1.6-9.4)	0.001	5.9 (2.0-18.1)	0.002
Grade					
1	83	1.0		Not significant	
2	63	1.7 (0.8-3.8)	0.160	in univariate	
Age					
For every 10 yrs	146	0.9 (0.7-1.3)	0.723	1.4 (0.9-2.1)	0.161

Table 4.8 Multivariate analyses for prognostic factors in low and intermediate grade (G1 and G2) patients (n=146)

4.3.6. CTCs as Prognostic Markers in Patients with Intermediate Grade (G2) NETs

Using the combined dataset from the surveillance and treatment groups (n=175), a subgroup of patients who had G2 grade NETs were analysed. This group was analysed as it is unclear whether this intermediate grade group should be treated aggressively or as more indolent tumours. There were 63 patients in this subgroup (17 progression events, 11 death events). Survival curves of PFS and OS are shown in Figure 4.6 with univariate and multivariate Cox-proportional hazards regression shown in Table 4.9 and Table 4.10. Those with detectable CTCs had significantly worse outcome compared to those who had no CTCs. Presence of CTCs was an independent factor for worse PFS where neither CgA nor burden was prognostic. When OS was evaluated, although presence of CTCs was prognostic on univariate analysis, it did not approach statistical significance as an independent prognostic factor on multivariate analyses.

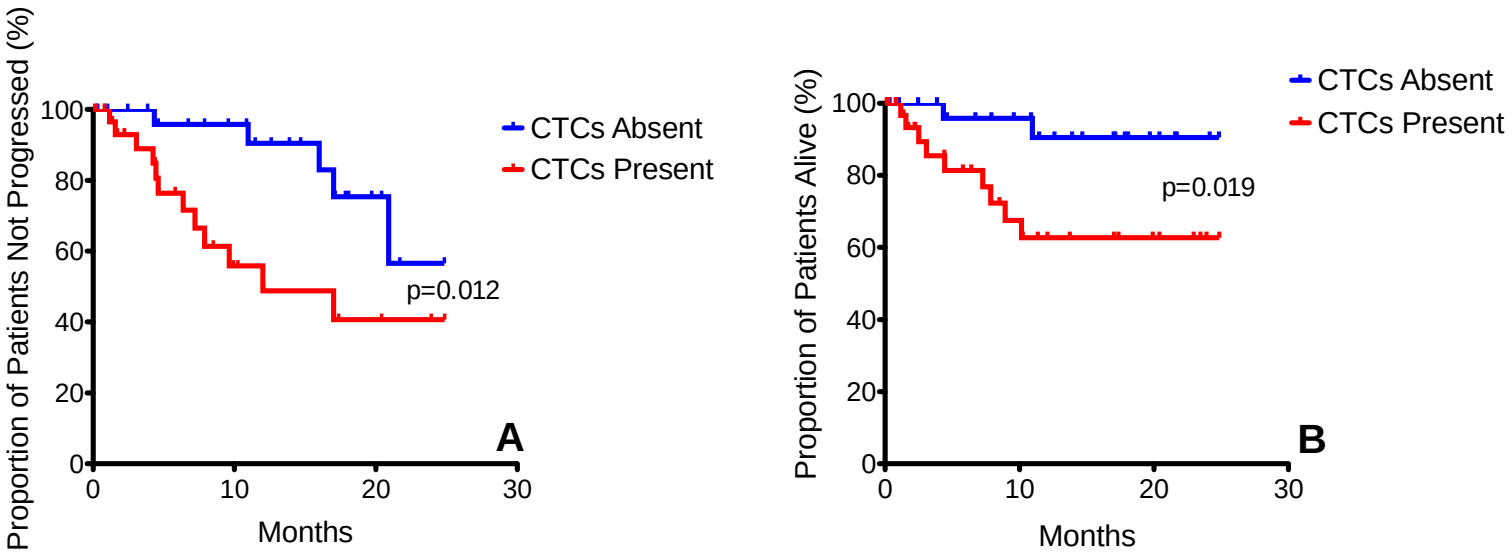


Figure 4.6 Kaplan-Meier survival curves of (A) PFS and (B) OS demonstrating differences between groups according to presence of CTCs in a subgroup with G2 NETs

Risk Factor	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Baseline CTC				
Absence (n-29)	**1.0**		**1.0**	
Presence (n=34)	**3.5 (1.2-10.1)**	**0.018**	**5.2 (1.1-24)**	**0.036**
Baseline CgA				
CgA≤120 (n=29)	1.0		1.0	
CgA>120 (n=34)	1.7 (0.6-4.4)	0.293	3.6 (0.9-13.6)	0.060
Burden				
<25% (n=31)	1.0		1.0	
≥25% (n=32)	0.9 (0.3-2.4)	0.890	1.7 (0.5-5.7)	0.419
PS				
0-1 (n=61)	1.0		1.0	
≥2 (n=2)	1.3 (0.2-10)	0.805	2.9 (0.4-22.9)	0.308
Age				
For every 10yrs	0.9 (0.6-1.4)	0.684	1.5 (0.9-2.6)	0.139

Table 4.9 Univariate analyses for prognostic indicators in intermediate grade (G2) patients (n=63)

Risk Factor	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Baseline CTC				
Absence (n=29)	1.0		1.0	
Presence (n=34)	3.7 (1.3-10.7)	0.016	4.2 (0.8-22)	0.091
Baseline CgA	Not significant			
CgA≤120 (n=29)	in univariate		1.0	
CgA>120 (n=34)			2.1 (0.5-8.8)	0.311
Age				
For every 10 years	0.9 (0.6-1.3)	0.472	1.6 (0.8-3.2)	0.161

Table 4.10 Multivariate analyses for prognostic factors in intermediate grade (G2) patients (n=63)

4.3.7. CTCs as Prognostic Markers in Patients with Low Grade (G1) NETs

Using the combined dataset from surveillance and treatment groups (n=175), a subgroup of patients who had G1 grade NETs were analysed. It was important to analyse this group as these are NETs with best survival, yet become more aggressive at some point during the disease process often many years after the specimen was taken that determines grade. There were 83 patients in this subgroup (11 PFS and 8 OS events). Survival curves of PFS and OS are shown in Figure 4.7 with univariate and multivariate Cox-proportional hazards regression shown in Table 4.11 and Table 4.12. Those with detectable CTCs had significantly worse outcome compared to those who had no CTCs. Presence of CTCs was an independent factor for worse PFS and OS whereas neither CgA nor burden was prognostic.

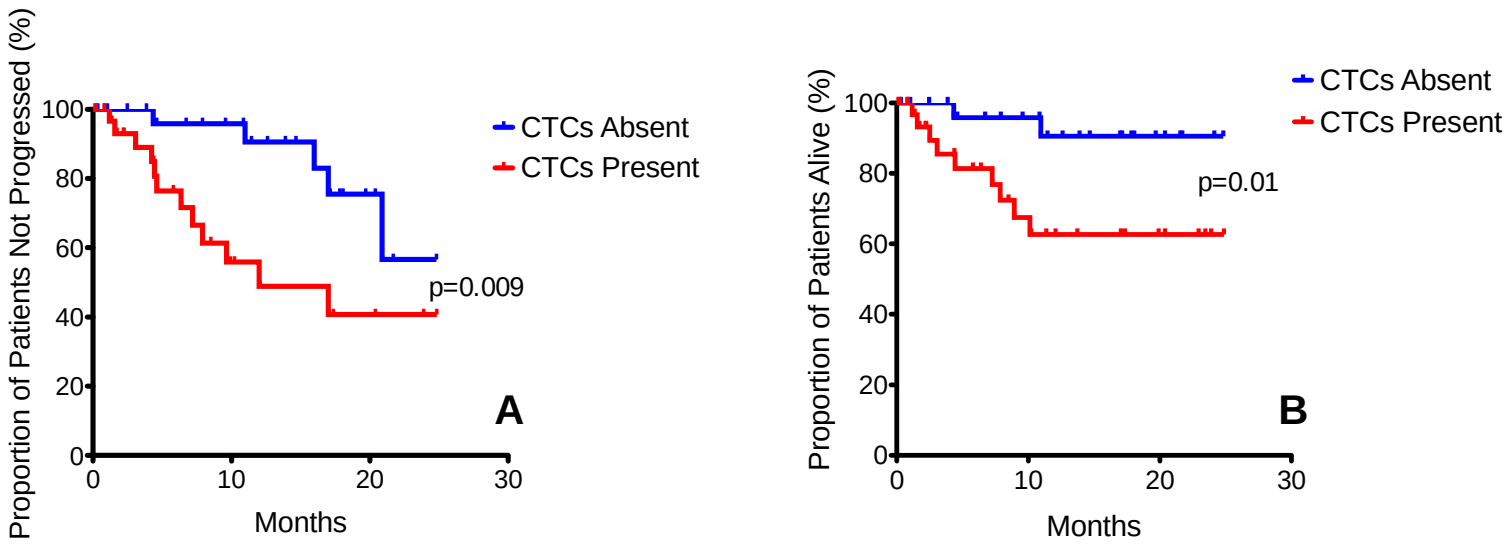


Figure 4.7 Kaplan-Meier survival curves of (A) PFS and (B) OS demonstrating differences between groups according to presence of CTCs in a subgroup with G1 NETs

Risk Factor	**n**	**PFS HR (95% CI)**	***P* - value**	**OS HR (95% CI)**	***P* - value**
Baseline CTC					
Absence	50	1.0		1.0	
Presence	33	5.0 (1.3-18.5)	0.017	7.2 (1.3-39.4)	0.023
Baseline CgA					
CgA≤120	29	1.0		1.0	
CgA>120	54	2.4 (0.6-9.4)	0.200	1.3 (0.3-5.6)	0.724
Burden					
<25%	44	1.0		1.0	
≥25%	39	2.8 (0.8-9.8)	0.098	2.6 (0.6-10.8)	0.197
PS					
0-1	78	1.0		1.0	
≥2	5	2.2 (0.3-18.0)	0.449	3.0 (0.4-25.0)	0.311
Age					
For every 10yrs	83	1.2 (0.7-2.0)	0.562	1.2 (0.7-2.2)	0.559

Table 4.11 Univariate analyses for prognostic factors in low grade (G1) patients (n=83)

Risk Factor	n	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Baseline CTC					
Absence	50	1.0		1.0	
Presence	33	4.9 (1.3-18.2)	0.019	7.0 (1.3-38.7)	0.025
Age					
For every 10 years	83	1.1 (0.6-1.9)	0.747	1.2 (0.6-2.1)	0.712

Table 4.12 Multivariate analyses for prognostic factors in low grade (G1) patients (n=83)

4.3.8. Background Characteristics of Patients Undergoing Treatment

Background characteristics of the patient sample (n=138) commencing treatment in the prospective study are shown in Table 4.13.

	Pancreatic n=31	Midgut n=81	Broncho-pulmonary n=12	Unknown Primary n=11	Hindgut n=3	Total n=138
Age, median years (range)	51.5 (23-72)	63 (34-85)	50.5 (30-77)	63 (31-78)	74 (43-75)	60 (23-85)
Sex, n (%)						
Male	20	47	4	4	1	76
Female	11	34	8	7	2	62
Grade						
Low	9	48	4	2	0	63
Intermediate	7	28	6	6	2	49
High	15	5	2	3	1	26
Burden of Liver metastases						
<25%						
25%≤50%	10	35	7	4	1	57
50%≤75%	13	30	3	4	0	50
>75%	3	11	2	2	1	19
	5	5	0	1	1	12
Duration of diagnosis, median months (range)	33 (1-145)	30 (1-149)	20 (9-116)	15 (1-67)	18 (5-22)	26 (1-149)
CgA (pmol/L), median (range)	56 (23-1000)	380 (26-1000)	129 (42-1000)	215 (51-1000)	64 (44-835)	264 (23-1000)
PS						
0	22	49	8	6	1	86
1	9	28	4	5	2	48
2	0	3	0	0	0	3
3	0	0	0	0	0	0
4	0	1	0	0	0	1
Naïve to non-surgical treatment	9	32	7	4	1	53
Previous treatments						
Resection of primary	15	43	5	0	1	64
SST	8	43	3	2	1	57
Chemotherapy	14	9	3	6	1	33
TAE	2	13	0	2	0	17
Radionuclides	10	4	0	1	0	15
Interferon	3	2	0	0	0	5
Liver resection	6	8	0	0	0	14
Number of previous non-surgical treatments						
0	9	32	7	4	1	53
1	16	29	4	4	2	55
2	3	13	1	2	0	19
3	3	6	0	1	0	10
4	0	1	0	0	0	1

Table 4.13 Background characteristics of sample commencing treatment

4.3.9. Distribution of CTCs in The Treatment Group

The number of CTCs detected across different primary NETs in the prospective study in patients undergoing treatment are shown in Table 4.14 (hindgut NETs are not shown).

	Pancreatic	Midgut	Bronchial	Unknown
n	31	81	12	11
CTC>0	15 (48%)	49 (60%)	6 (50%)	11 (100%)
Median CTC≥1	6	8	12.5	6
Mean CTCs	28	14.5	70.2	451.9
(±SEM)	15	7.2	48.2	343
95% CI	0-58	5-23	0-176	0-1217
Range of CTCs	0-430	0-294	0-542	0-3731

Table 4.14 Distribution of CTCs in prospective study across different primary NETs

4.3.10. Survival in Group Undergoing Treatment using Baseline CTC Count

Using the group commencing a new treatment (n=138), Kaplan-Meier survival curves of PFS and OS are shown Figure 4.8 with univariate and multivariate analyses of prognostic indicators shown in Table 4.15 and Table 4.16 respectively. Due to small numbers in some category groups, burden groups 25-50%, 50-75% and >75% were grouped together and PS 2, 3 and 4 were grouped together. The median follow-up was 9.7 months (range 5-29).

Patients with CTCs had significantly worse PFS and OS than those patients without CTCs. On univariate and multivariate analyses, presence of CTCs was a prognostic factor for worse PFS and OS. CgA at the same time-point was not a prognostic factor in any analyses. G3 (high grade) was also an independent poor prognostic indicator. Again, a low burden (<25% liver involvement) was prognostic on univariate but not on multivariate analyses.

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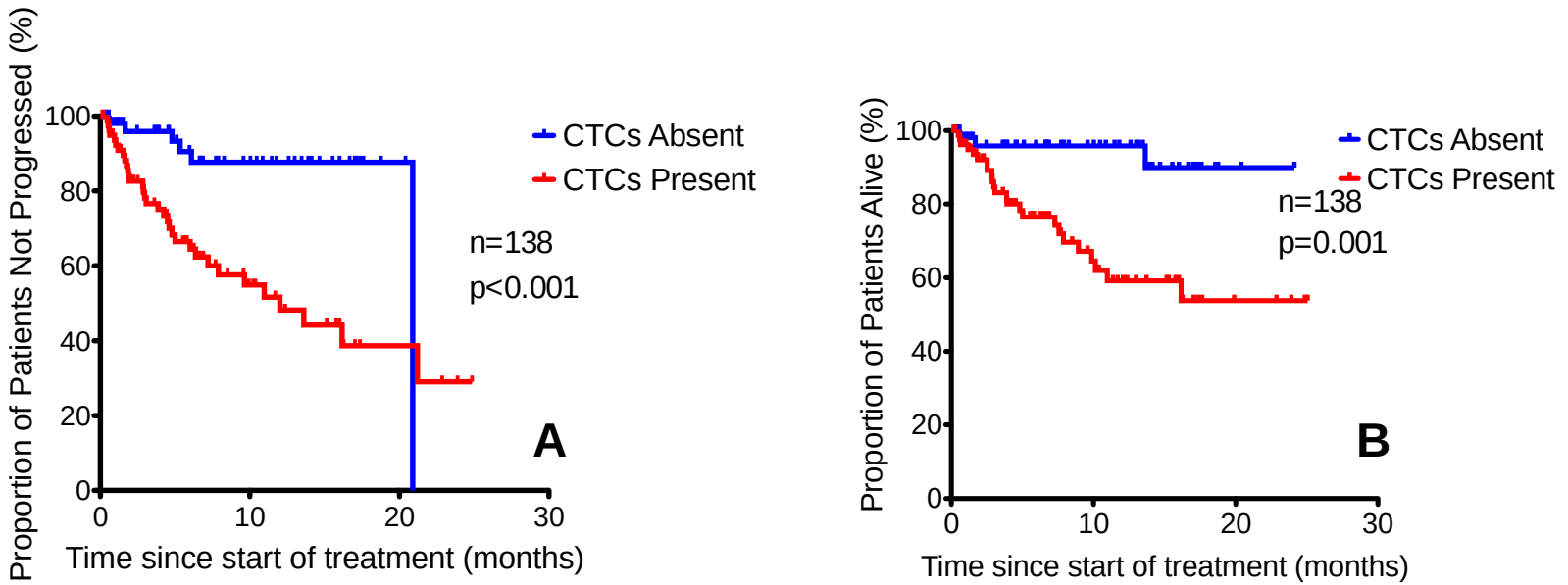


Figure 4.8 Kaplan-Meier survival curves of (A) PFS and (B) OS demonstrating differences between groups according to presence of CTCs at baseline in group of patients undergoing treatment

Risk Factor	n	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Baseline CTC					
Absence	56	1.0		1.0	
Presence	82	4.3 (1.8-10.3)	<0.001	6.1 (1.8-20.3)	<0.001
Baseline CgA					
CgA≤120	49	1.0		1.0	
CgA>120	89	1.53 (0.8-2.9)	0.190	2.14 (0.9-5.1)	0.070
Grade (Ki67)					
1	63	1.0		1.0	
2	49	1.3 (0.5-3.0)	0.590	1.5 (0.5-4.4)	0.439
3	26	4.7 (2.1-10.5)	<0.001	5.0 (1.8-13.2)	0.001
Burden					
<25%	57	1.0		1.0	
≥25%	81	2.5 (1.2-5.4)	0.015	4.6 (1.6-13.4)	0.005
PS					
0-1	134	1.0		1.0	
≥2	4	2.5 (0.6-10.5)	0.210	1.8 (0.2-13.3)	0.569
Age					
For every 10yrs	138	3.0 (1.1-1.7)	0.017	1.1 (0.8-1.4)	0.502

Table 4.15 Univariate analyses for prognostic factors in patient group undergoing treatment (n=138)

Risk Factor	n	PFS HR (95% CI)	*P* - value	OS HR (95% CI)	*P* - value
Baseline CTC					
Absence	56	1.0		1.0	
Presence	82	3.7 (1.5-8.9)	0.005	5.1 (1.5-17.3)	0.008
Baseline CgA					
CgA≤120	49	1.0		1.0	
CgA>120	89	1.7 (0.8-3.6)	0.189	1.6 (0.6-4.0)	0.294
Grade (Ki67)					
1	63	1.0		1.0	
2	49	2.0 (0.9-4.4)	0.078	1.3 (0.5-3.3)	0.544
3	26	5.5 (2.3-13.4)	<0.001	4.4 (1.8-10.9)	0.001
Burden					
<25%	57	1.0		1.0	
≥25%	81	1.4 (0.6-3)	0.449	2.7 (0.9-8.1)	0.069

Table 4.16 Multivariate analyses for prognostic indicators allowing for age in patient group undergoing treatment (n=138)

4.3.11. Changes in CTCs in Predicting Progression-free and Overall Survival

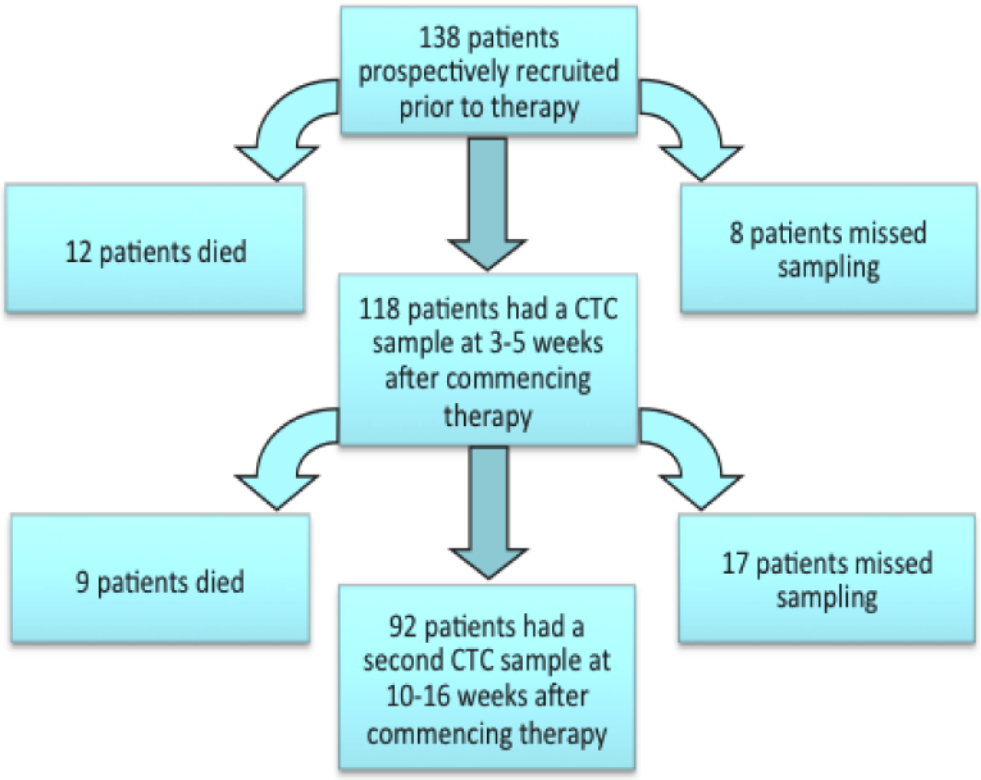
Having established the prognostic value of CTCs, I went on to investigate the significance of changes in CTCs with therapy. One-hundred and thirty-eight patients (138) who were about to commence a new treatment were recruited for the prospective study (Figure 4.9). Baseline blood samples for CTC enumeration were taken in all cases. Post-treatment samples were taken in 118 (86%) of cases at the first time-point (3-5 weeks, median 4.3 weeks) and in 92 (67%) at the second time-point (10-15 weeks, median 13.7 weeks). Reasons for missing post-treatment samples were death or inability for patient to either return to hospital (or send sample) at appropriate time-point. The median follow up was 9.7 months (range 5-29).

The prognostic significance of baseline CTC counts has already been discussed above. In order to analyse changes in CTC counts, each post-treatment time-point was considered separately (first and second post-treatment time-points). The percentage change from baseline CTC was divided into tertiles:

1) ≥65% reduction from baseline CTC
2) from <65% reduction to <33% increase from baseline CTC or no change (but with CTCs at baseline)
3) an increase ≥ 33% from baseline CTC

Patients who had zero CTCs before and zero CTCs after therapy were used as the reference group. Those with zero CTCs at baseline and >0 CTCs after treatment, i.e. an infinite increase, were included and then excluded from group 3 to investigate their effect on the analyses.

Using Cox-proportional hazards regression, the effect of changes in CTCs (using these four groupings) on PFS and OS were analysed (Table 4.17). Survival curves for these groups are shown in Figure 4.10.



??Figure 4.9 Flow diagram demonstrating numbers of patients undergoing sampling for CTCs before and after treatment

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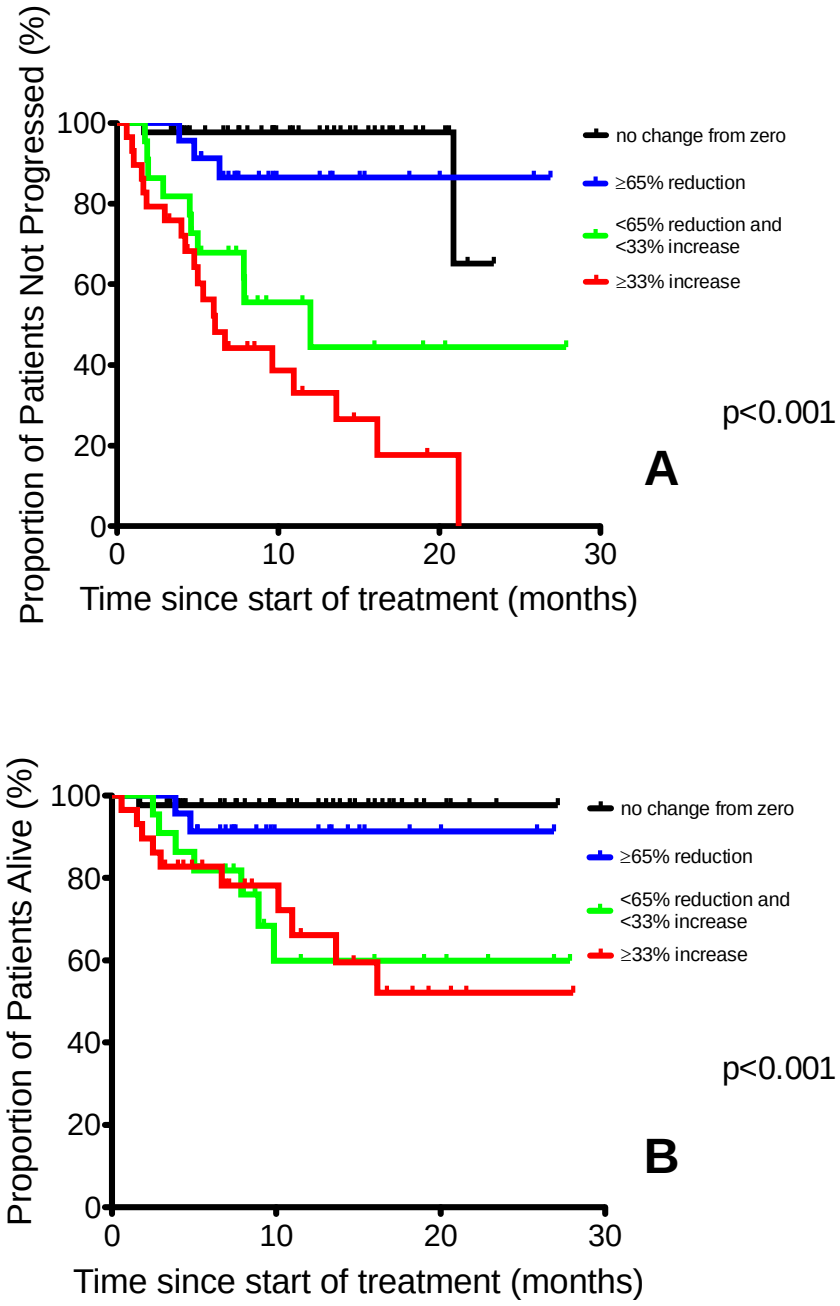

Figure 4.10 Survival curves estimating differences in (A) PFS and (B) OS across patients grouped according to changes in CTCs at time-point one (3-5 weeks) after treatment

Change from baseline (CTCs)	n	PFS HR(95% CI)	*P*-value	Events/n	OS HR (95% CI)	*P*-value	Events/n
No Change (zero CTCs)	43	1.00	<0.001	35/117	1.00	P<0.001	21/118
≥65% reduction	23	2.86 (0.47-17.1)			4.00 (0.36-44.2)		
<65% reduction or no change[1] or <33% increase	22	12.6 (2.76-57.8)			15.7 (1.92- 127.5)		
Increase[2] ≥33%	29	23.1 (5.37-99.0)			18.9 (2.4-146)		
[Increase[3]≥33%]	[22]	[21.7 (4.95- 95.1)]		[30/110]	[18.2 (2.27-146)]		[18/109]

Table 4.17 Effect on OS and PFS of changes in CTCs after treatment with groups comparing first post-treatment sample time-point to baseline CTC count

[1]But had CTCs at baseline

[2]Including patients who had zero CTCs at baseline and >0 CTCs after treatment

[3] Not including patients who had zero CTCs at baseline and >0 CTCs after treatment

According to Table 4.17, it appears that the groupings stratified according to CTC changes are associated with OS and PFS. In other words, the greatest increase in CTCs (≥33% increase) at time-point 1 after treatment had worse PFS and OS than those with a smaller increase or modest reduction (<65% reduction or <33% increase). Excluding those cases which had 0 CTCs before but CTCs of one or more after treatment, i.e. an infinite increase, made no significant impact and thus these cases were included in the group with ≥33% increase in CTCs.

The group with best outcome was those with zero CTCs before and after therapy, then followed by the group with ≥65% reduction. It might be argued since baseline CTC absence is associated with better survival from earlier data, that baseline CTC is a confounder. However, when looking at the characteristics of the different groupings, there was no obvious difference in median baseline CTCs.

The effect of baseline CTC (not just as a dichotomous variable) was further investigated. Splitting baseline CTCs into tertiles would result in one group of all zero CTCs and it would be impossible to adjust for these as they could only fall into two of the four 'change in CTC' groups in Table 4.17. To overcome this problem, both baseline and first post-treatment time-point CTC were divided into tertiles. Cox proportional hazards regression was used to evaluate the effect on survival of baseline and then separately first post-treatment time-point CTC. Then the effect of first post-treatment time-point CTC, *adjusted for the baseline CTC,* was analysed. This is shown in Table 4.18 which demonstrates that when adjusted for baseline CTC, the first post-treatment CTC is still predictive of PFS and OS. This is shown graphically in Figure 4.11 where the model appears to be a better predictor for OS than PFS.

When looking at post-treatment time-point 2, due to missing data and the number of cases which had already progressed or died by this time-point, it was not possible to categorise the changes in CTCs in tertiles as was possible with post-treatment time-point 1. However, it was possible to demonstrate a clear association between the absolute CTC count at the second timepoint and progression at that time-point (Table 4.19, $P<0.001$).

	n	OS HR (95% CI)	p-value	Events/n	PFS HR (95% CI)	p-value	Events/n
CTC Baseline							
0	55	1.00	0.002	28/138	1.00	<0.001	40/135
1-7	39	3.27(0.98, 10.89)			3.05 (1.21, 7.68)		
>7	44	5.83 (1.95, 17.45)			4.62 (1.95, 10.95)		
CTC Post treatment							
0	52	1.00	<0.001	21/118	1.00	<0.001	35/117
1-5	29	6.18 (1.20, 31.88)			10.48 (2.92, 37.67)		
>5	37	13.17 (2.99, 57.99)			16.60 (4.92,55.77)		
CTC Post treatment *							
0	52	1.00	0.005	21/118	1.00	<0.001	35/117
1-5	29	7.54 (1.25, 45.31)			15.16 (3.72, 61.75)		
>5	37	19.11 (2.95, 123.95)			42.07 (9.39, 194.25)		

Table 4.18 Effect of baseline and first post-treatment time-point CTC on OS and PFS *adjusted for baseline CTC count

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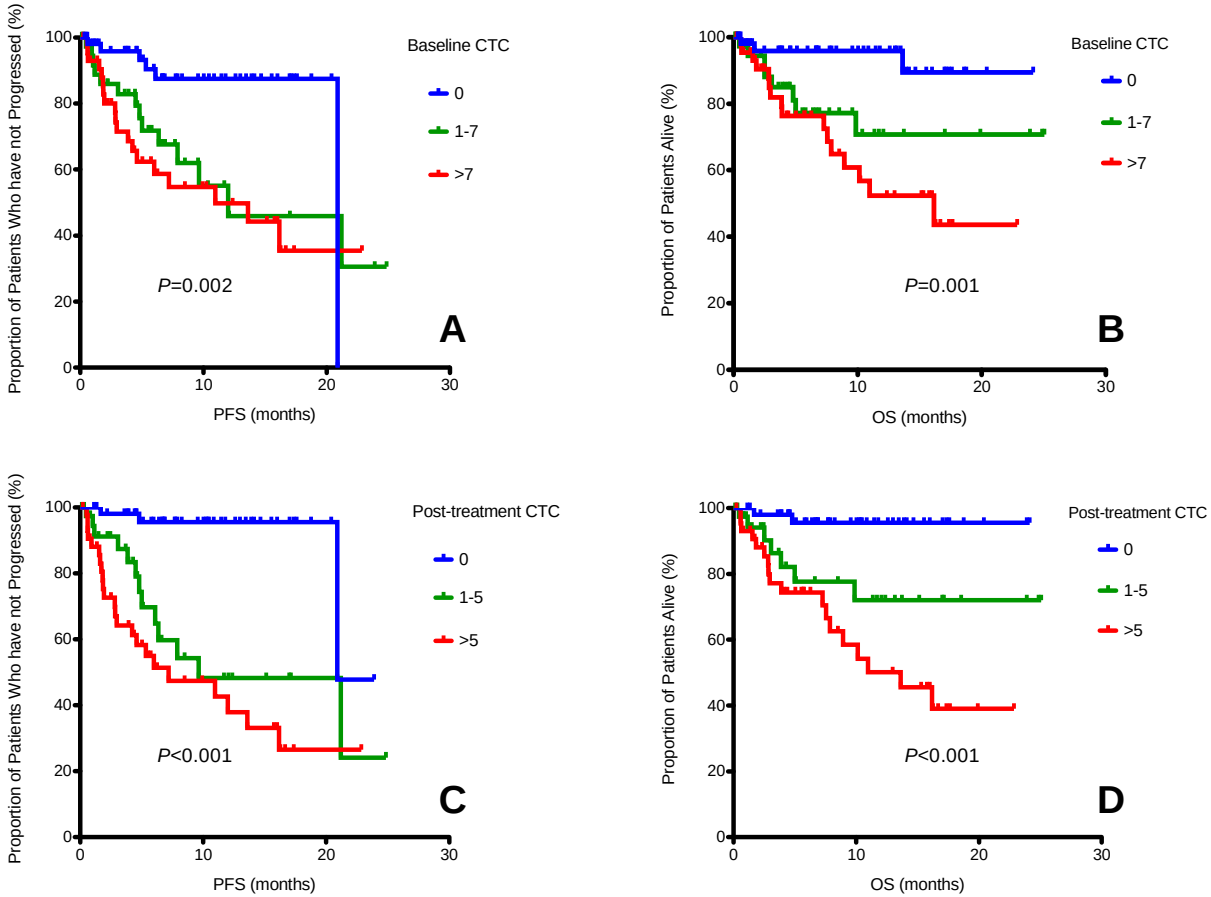

Figure 4.11 Kaplan-Meier Curves demonstrating (A) PFS and (B) OS stratified by baseline CTCs divided into tertiles; (C) PFS and (D) OS stratified by post-treatment CTCs divided into tertiles (as in table 4.18)

Status at time-point 2*	N (%)	Median CTC at time-point 2 (25^{th}, 75^{th} Centiles)
Have Progressed	21(22.8)	18 (4, 31)
Have not progressed	71(77.2)	0 (0, 2)

Table 4.19 Progression at time-point 2 (10-15 weeks) after treatment and progression

*Only patients who have CTC counts at time point 2 (Wilcoxon Mann-Whitney test p<0.001)

4.3.12. Changes in CTCs in Predicting Response to Treatment

Having established that changes in CTCs with therapy predict survival, I investigated the ability of these changes to predict response. The changes in CTCs, according to the groupings in Table 4.18 were analysed for an association with radiological response to treatment (Table 4.20). There was a significant association between an increase in CTCs after treatment and radiological progression (Fisher's exact P<0.001). Individual changes in cases' CTC levels, grouped by response, is depicted graphically in Figure 4.12 with a subgroup of patients who were treated for progressive disease depicted in Figure 4.13.

Response	0-0 CTCs	≥65% reduction	<65% reduction to ≤33% increase	≥33% increase
Disease progression	1	2	10	16
Disease stabilisation*	35	18	8	10

Table 4.20 Association between changes in CTCs from baseline and radiological response to treatment (Fisher's exact p-value <0.001) *stable disease or partial response

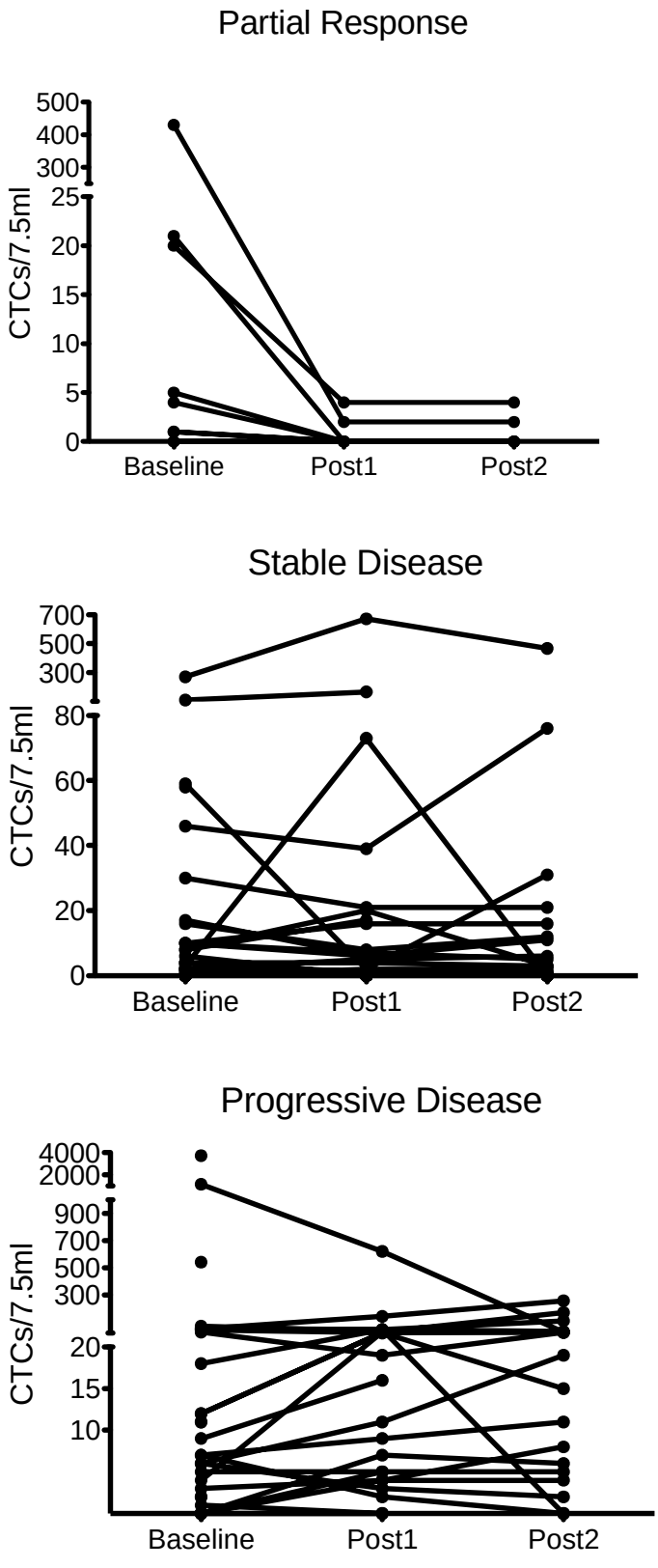

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Figure 4.12 Graphs displaying CTC changes from baseline grouped by radiological response to treatment (partial response, stable disease, progressive disease). Single dots represent cases where post-treatment samples were not taken due to death.

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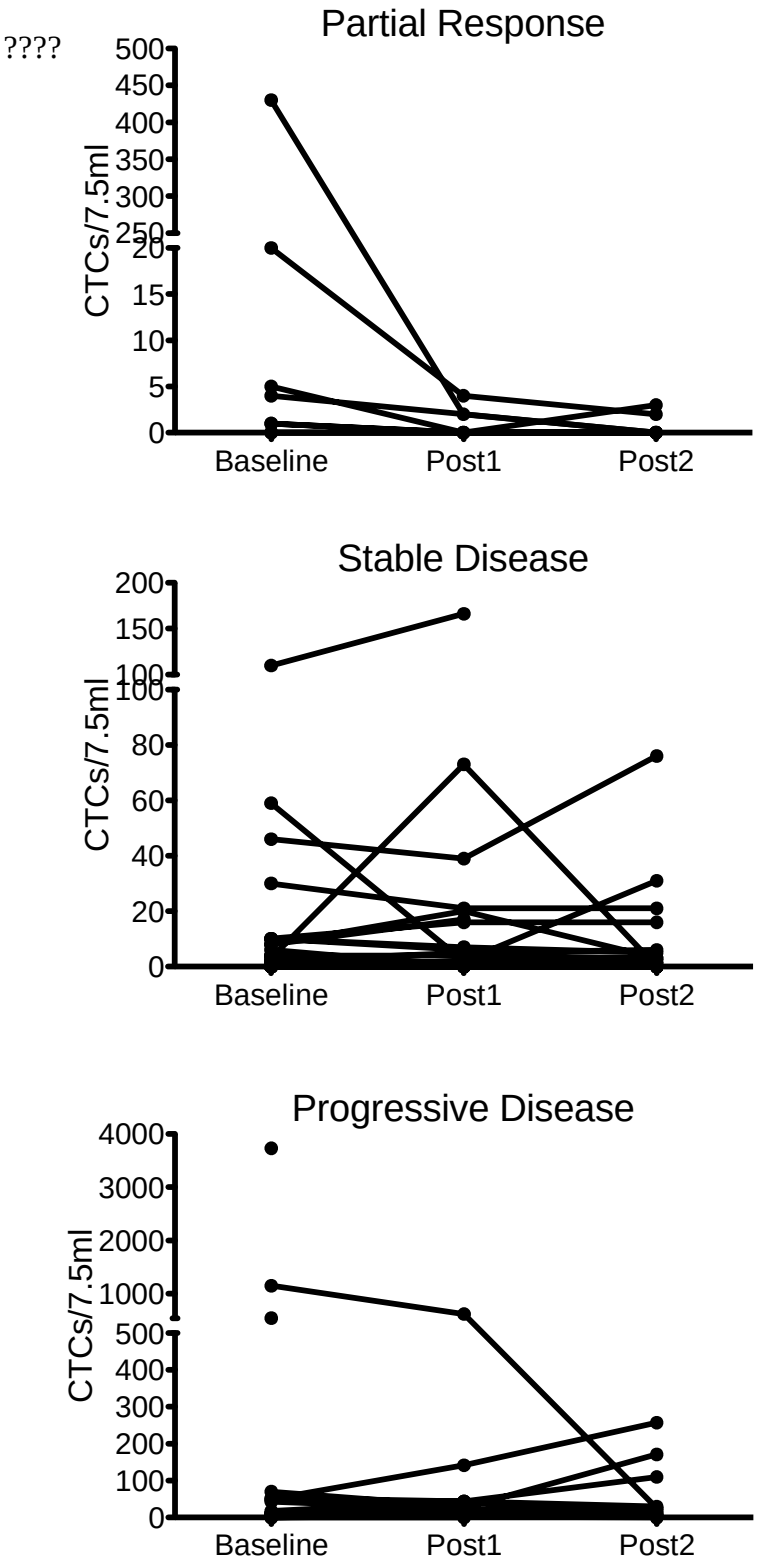

Figure 4.13 Graphs demonstrating CTC changes before and after therapy grouped by responses to treatment in a subgroup who had progressive disease prior to treatment. Single dots represent cases where post-treatment samples were not taken due to death.

4.3.13. Differences in CTC Changes Across Types of Treatment

Changes in CTC levels grouped according to type of therapy is depicted in Figure 4.14.

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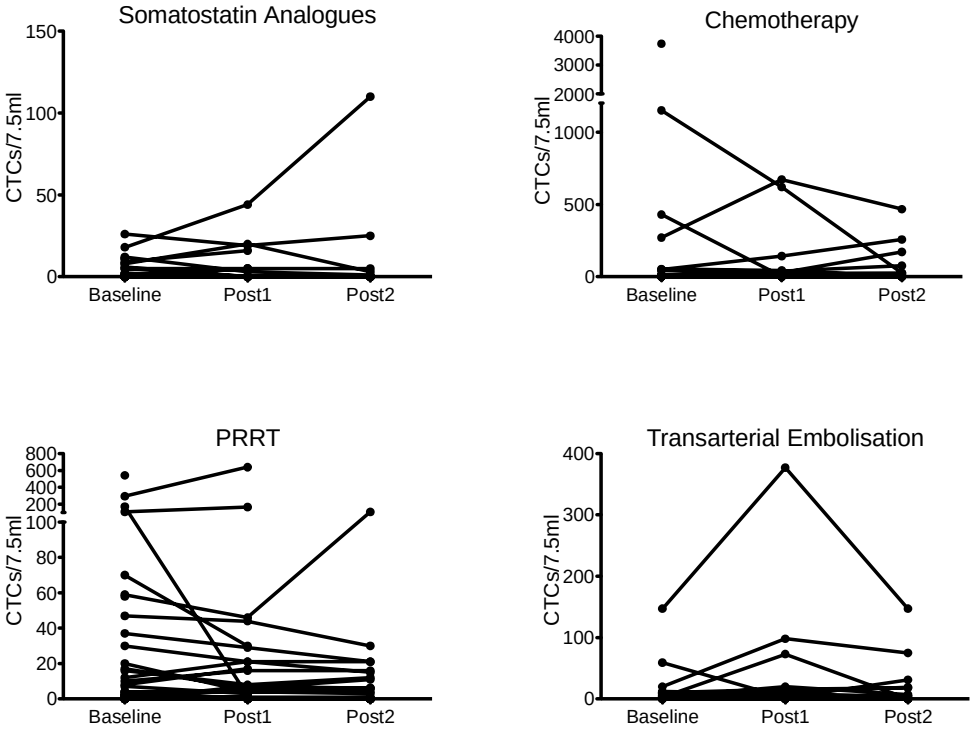

Figure 4.14 Graphs displaying CTC changes before and after therapy grouped by treatment type

4.3.14. Changes in Chromogranin A with Treatment

Having looked at the dynamic nature of CTCs with treatment in predicting outcome, I investigated changes in the existing marker CgA with 107 cases having CgA evaluated at the first time-point following treatment. Interestingly, as shown earlier, baseline CgA was not an independent prognostic marker. CgA at time-point 1 (3-5 weeks) after treatment was analysed similarly to the CTC analysis. Changes in CgA were divided into tertiles which are shown in Table 4.21, together with median baseline and post-treatment CgA levels. Using Cox-proportional hazards regression, the effect on PFS and OS of these groups was investigated (Table 4.22). Changes in CgA after treatment were not predictive of outcome. Figure 4.15 shows survival curves demonstrating that changes in CgA do not predict outcome. In fact, although not significant, the group with greatest increase in CgA had a slightly better outcome which is contradictory. Interestingly, baseline CgA was higher in the group with greatest CgA reduction which may account for the paradoxical hazard ratios in univariate analyses. These changes in CgA were not predictive of radiological response to therapy (Table 4.23, P=0.645).

Percentage change in CgA (pmol/L)	N	Median CgA Baseline (pmol/L)	Median CgA at Time point 1 (pmol/L)
>27% reduction	36	395	172
≤27% reduction or <12% increase	36	212	200
≥12% increase	35	107	372

Table 4.21 Changes in CgA from baseline to first time-point after treatment (3-5 weeks) divided into tertile groups shown with median CgA levels

Percentage change in CgA (pmol/L)	PFS HR (95% CI)	p-value	Events/n	OS HR (95% CI)	p-value	Events/n
>27% reduction	1.00	0.54	31/104	1.00	0.42	21/107
≤27% reduction or <12% increase	0.95 (0.41-2.21)			0.50 (0.17-1.48)		
≥12% increase	0.63 (0.25-1.58)			0.64 (0.24-1.74)		

Table 4.22 Effect of changes in CgA after treatment on PFS and OS

Response	**>27% reduction**	≤27% reduction or <12% increase	≥12% increase
Disease progression	2	13	7
Disease stabilisation*	3	34	23

Table 4.23 Association between radiological response to therapy and percentage changes of post-treatment CgA at time-point 1 (3-5 weeks) compared to baseline CgA (Fisher's exact p-value = 0.645) *stable disease or partial response

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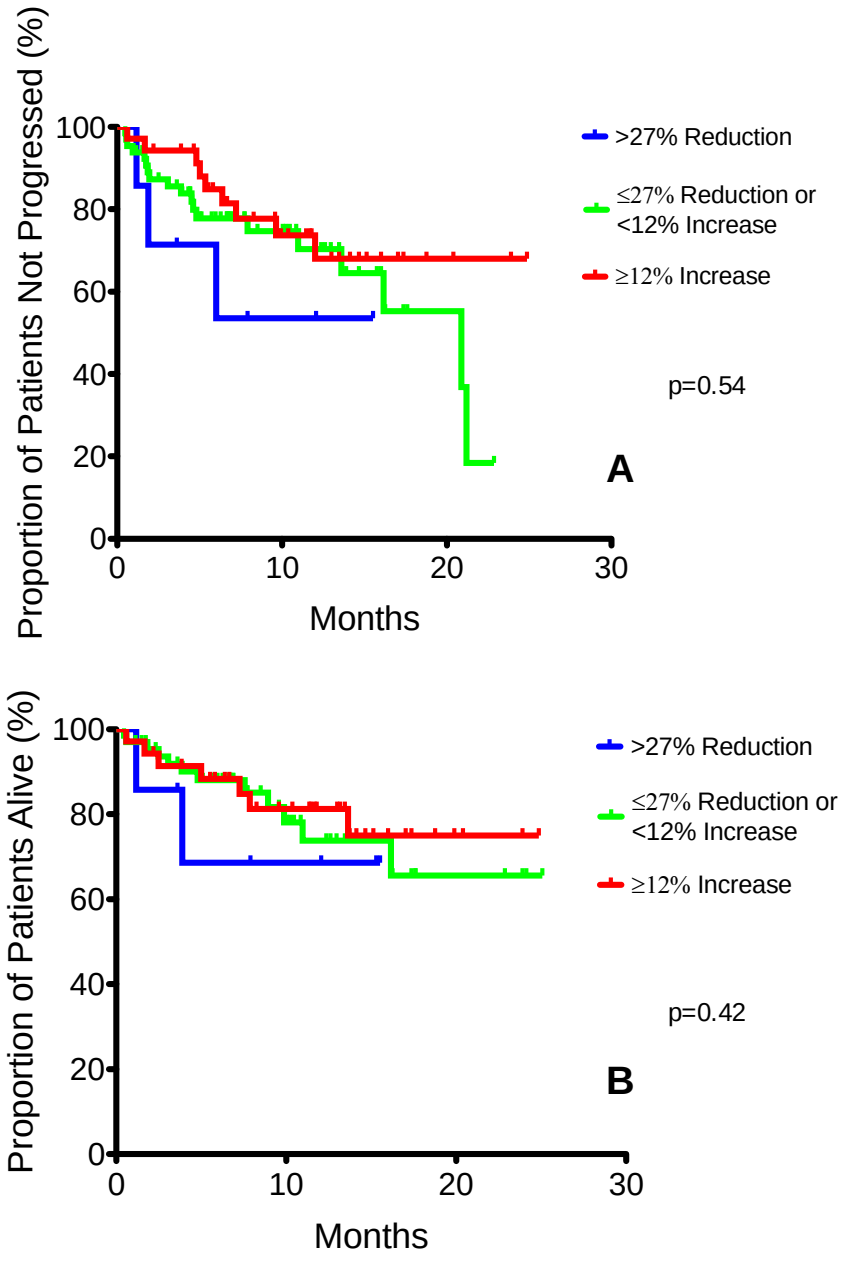

Figure 4.15 Survival curves estimating differences in (A) PFS and (B) OS across patients grouped according to changes in CgA at time-point one (3-5 weeks) after treatment

4.4. Discussion

The results from this study indicate that the number of CTCs detected is a useful predictor of progression-free and overall survival in metastatic NETs. More importantly, in patients with metastatic NETs undergoing therapy, the level of CTCs at first follow-up at a median of 4.3 weeks are predictors of eventual response, PFS and OS.

Using data from the initial pilot dataset comprising patients undergoing surveillance or treatment (n=63), it appears that the absence of CTCs in metastatic NETs is associated with stable disease as defined by RECIST, whereas the presence of CTCs is associated with progressive disease. I recognize limitations in this analysis whereby tumours were classified as progressive using retrospective comparisons with previous scans in heterogeneous groups. However, the median interval between scans and the distribution of histological grades was similar between patients who had tumour progression and those with no tumour progression. The pilot study was hypothesis-generating and the findings have been confirmed in the prospective study.

This relationship between CTCs and progression allowed us to investigate CTCs prospectively as a prognostic and predictive biomarker.

Using a training set, I evaluated the prognostic value of stepwise CTC cut-offs and confirmed a cut-off of 1 CTC or more as carrying a worse prognosis in a validation set. This cut-off is lower than thresholds in breast (5)[204], colorectal (3)[206] and prostate (5)[205] but is clearly the threshold at which groups are split evenly with the greatest survival difference, identified by log-rank testing and Cox regression. Since CTC images are analysed by an operator, human error may classify cases incorrectly as the difference between 0 and 1 CTCs is small. However, this also applies to other thresholds in other cancers, and is limited here by the use of two independent blinded operators, and operator consistency has previously been demonstrated in large studies[153].

Of note, the proportion of 138 patients who had CTCs in the prospective study were higher than in the first CTC chapter (pancreatic NETs 48% Vs 36%, midgut NETs 60

Vs 51%). This is probably due to a higher proportion of patients with progressive disease (as they were all undergoing treatment) which I have shown is associated with CTCs.

Baseline CTC count was found to be a prognostic factor in terms of PFS and OS on univariate and multivariate analysis. The only other significant factor on multivariate analysis was grade. However, only having a high grade NET was prognostic (not G2 vs G1); but G3 tumours are uncommon (29/175 patients) and are already known to confer a worse survival. Although higher CgA was associated with worse OS on univariate analysis in the large dataset, it was not prognostic on multivariate (at either 60 or 120 pmol/L thresholds) in any dataset. A higher burden was a significant predictor on univariate but not in the multivariate model. Thus when adjusted for other factors, CTC count was the only clinically useful prognostic indicator.

For poorly differentiated or G3 tumours it is clear that the clinical course is aggressive and immediate treatment is usually warranted. However, for G1 and G2 tumours, the clinical course may be uncertain at diagnosis and during follow-up. It was therefore important to understand if CTCs were prognostic in the G1 and G2 subgroup. For this analysis 29 G3 tumours were excluded from the total cohort of 175. The presence of CTCs was prognostic and had clearly separable survival curves compared to CgA and grade (Figure 4.4). Once again, the presence of CTCs was the only significant predictor of PFS and OS (HR 4.1 and 5.9 respectively) when adjusted for other factors. This is important as the presence of CTCs appears to be a better prognostic marker than grading. This could be clinically useful where those with CTCs could be treated earlier and more aggressively than those without CTCs as they have a worse survival. These results were similar when the subgroup of G1 NETs were analysed and did approach significance in G2 NETs. However, these are subgroup analyses with fewer events and must be interpreted with caution.

Some categorical variables analysed with regression, including performance status and metastatic burden, were grouped together due to small numbers in certain categories. Although continuous information was lost, this led to more evenly distributed groups and robust analysis.

Importantly, the percentage change in CTCs 3 to 5 weeks after commencement of treatment compared to baseline appears to predict PFS, OS and response to therapy. The latter is best shown graphically (Figure 4.10) especially in those treated for progressive disease (Figure 4.12). The best outcome occurred in those with 0 CTCs before and 0

CTCs after therapy and the worst outcome with an ≥33% increase. Those with detectable CTCs after treatment but with none pre-treatment had similar outcome to the ≥33% increase group and were thus incorporated into this group in further analysis. These findings were confirmed when data were analysed by an alternative method which may be more clinically useful. When baseline and post-treatment CTCs were grouped into tertiles, these groups were predictive of PFS and OS. Hazard ratios for post-treatment CTCs were higher than those of baseline CTCs indicating that the post-treatment sample divided into tertiles could be used clinically to assess outcome. If post-treatment CTCs were 0, this confers a good outcome compared to 1-5 CTCs, which confers a better outcome than >5CTCs post-treatment.

My methods are alternative methods of analysing changes in CTCs which tackles the problems with studies in other cancers where small changes across the CTC threshold are significant which is prone to error e.g. where a change in 1 CTC could confer a change in group[204].

Interestingly, those with a ≥65% reduction in CTCs over baseline adopted a PFS, OS and response almost similar to that of the reference group (0 before, 0 after treatment) implying two groups with similar outcomes. This included 15 patients who had undetectable CTCs at this time-point, having had detectable CTCs prior to commencing treatment. This may be a clinically useful finding to help early identification of patients who are likely to respond to treatment.

The change in CTCs after treatment gives additional information than solely using baseline CTC count. In other words, changing the CTC count by treating the tumour can change the outcome. Even if high at baseline, if the CTC count can be reduced by ≥65% with treatment, the survival can be improved. Given that this change in outcome and the response to therapy can be predicted at 3-5 weeks into treatment, it may give the early opportunity to stop therapies which may be toxic and expensive or to continue them if beneficial rather than awaiting imaging several months later.

There was no significant difference in clinical factors (age, gender, burden, CgA, baseline CTC) between the groups stratified according to percentage change in CTCs that could account for the difference in survival. Although grade could possibly be a confounder, there was no clear incremental association and on multivariate analysis, it was only G3 NETs (in addition to CTCs) that were a significant factor, which is an expected finding.

It was impossible to adjust these groupings (according to CTC change after therapy) for baseline CTCs (as a continuous variable) but our alternate method demonstrated that post-treatment CTC counts were still predictive when adjusted for the baseline CTC (when divided into tertiles). The change in CTC after treatment at 3 to 5 weeks was still predictive of PFS in the multivariate model but only approaching significance with OS. However, with four categories in this variable, three other variables in the model, and few events in each group, confidence intervals were wide. Thus given the median follow-up of 10 months, a robust multivariate analysis might require longer follow-up when there have been more progression events and deaths.

Importantly, in addition to CgA not having the same prognostic value as CTCs at baseline, changes in CgA were not predictive of response to therapy, nor of PFS or OS. The group with greatest reduction in CgA had a higher baseline CgA which may account for the paradoxical hazard ratios curiously implying worse survival with larger reductions in CgA. Although caution must be taken in interpreting this, it is clear from the data and the survival curves, that CgA was not a independent prognostic or predictive biomarker.

Unfortunately when time-point 2 (10 to 15 weeks after commencing treatment) was analysed, there were only 92 cases and it was not appropriate to split into tertiles due to missing data and the number of cases that had already progressed or died by this time-point. Although there was an association between the absolute CTC count at this time-point and progressive disease, it may be that this is not an optimal time-point.

There are limitations to my study. I have analysed CTCs as a dichotomous variable i.e. absence and presence. Such simplicity may cause some intrinsic problems, notably loss of information and power, increased possibility of false-positive results, and impossibility of detecting non-linear relationships between the variable and outcome which may mean information is lost. However, this is a threshold which I have validated with a training and validation set and is a method which has been used in other studies[154, 155, 205]. Although I have shown variation between different treatments in individual changes in CTCs graphically, further studies are required to look at the effect of different treatments on CTCs in a homogenous population.

I have also analysed two overlapping populations in this chapter, one from an early chapter and one group about to commence therapy. Although the latter was less heterogeneous, both were recruited prospectively. Regardless of this, due to heterogeneity in grade, treatments undertaken, time since diagnosis, further studies

would be useful in more homogeneous populations but given the rarity of NETs, this may be difficult.

The subgroup survival analysis of G1 and G2 NETs as well as individual NETs could provide clinically useful information. It is often these grades where questions exist with regards to optimum treatment and optimum timing of treatment. If CTCs predict a prognostically worse phenotype, this may mean more aggressive treatment is required earlier. However, given that this is subgroup analysis with smaller numbers of events, caution must be taken.

In addition to the problems with analysing time-point 2 post-treatment (10-15 weeks) and missing data, limitations exist in timing of sampling. The post-treatment time-point 1 (3-5 weeks) was chosen as it is a clinically useful time for review, assessing toxicity, and is similar to other CTC studies in other cancers. However, in order to find the optimal time-point for predictive marker, studies are required at smaller intervals and also may be different for different therapies.

Lastly, I acknowledge that CTCs were evaluated in a heterogeneous group with different primary NET types and heterogeneity in terms of previous and subsequent treatments undertaken. I would recommend that these findings are validated in prospective trials of defined treatments in defined tumour groups with longer follow up.

Current methods of monitoring for progression and response to treatment in NETs include radiological imaging. This may be confounded by inter-observer variability and the fibrotic reaction often seen in NETs.[259, 260] This dependency on serial imaging is costly and exposes patients to radiation considering the varied survival with NETs compared with other tumours. Given the varied survival with NETs compared with other tumors, this dependency on serial imaging is costly and exposes patients to radiation. In NETs, CTCs may be of prognostic value in discriminating progressing from stable tumors, which may assist stratification for aggressive therapy at time of diagnosis. Given the recent focus on the delayed response seen in NETs with chemotherapy and radionuclides,[54, 232], CTCs may offer predictive information early during therapy and may be useful in monitoring response to therapy without repeated exposure to radiation.

This study met REMARK criteria for biomarker evaluation and from this study, I conclude that CTCs are clinically prognostic and predictive biomarker in metastatic NETs.

Chapter 5. Circulating cell-free DNA (cfDNA) in NETs

5.1. Introduction

Nucleic acids were identified in human plasma in both healthy subjects and patients with various diseases five years prior to Watson and Crick elucidating the double-helical structure of DNA[261]. It was not until the 1960's that interest was revived in serum or plasma DNA in diseases including systemic lupus erythematosis (SLE)[262]. Hereafter, increasingly sensitive assays were developed leading to detection of DNA in serum or plasma of healthy individuals at levels between 10 and 30ng/mL[263]. Cell-free DNA (cfDNA) is defined as extracellular DNA occurring in blood[264].

5.1.1. Origins of cfDNA

Theories of the origin of tumour-related cfDNA in the circulation include: apoptosis or necrosis of tumour (or of circulating tumour cells), or active release of DNA into the circulation from a tumour.

In healthy subjects, it is assumed that cfDNA originates from lymphocytes and other nucleated cells[265, 266] but It is not known why cancer patients have higher quantities of plasma cfDNA. The origins of plasma cfDNA in the initial studies were thought to be from tumour cells or from activated lymphocytes. Since DNA originating from cancer cells has decreased strand stability when carcinogens are added, strand separation was found to occur at lower temperatures and *in vitro* DNA synthesis was increased compared with DNA from healthy subjects using electrophoresis[267]. Detection of loss of heterozygosity (LOH) in plasma cfDNA also suggests mutant cfDNA is the predominant subtype of cfDNA[156, 267]. This suggested that a significant fraction of cfDNA in plasma originated from the tumour rather than from lymphocytes.

cfDNA found in plasma of cancer patients is likely to originate from necrotic or apoptotic tumour cells. Apoptotic or necrotic cells result in small fragments of 70 to 200 base pairs and large fragments of 21 kilobases[268]. The theory of tumour necrosis is supported by high amounts of cfDNA found in plasma with large tumours or with advanced diseases with metastases[267, 269, 270, 271]. However, radiation therapy reduced plasma DNA levels by up to 90% which would be inconsistent with this[156]

as one would expect a shower of cfDNA if necrosis played an important role in the production of cfDNA.

More recently, there has been increasing evidence to support apoptosis as the origin of cfDNA. Plasma and serum derived cfDNA often has several bands on electrophoresis similar to patterns shown by apoptotic cells[267, 270, 272]. An argument against this, however, is that apoptosis is a mechanism lost by proliferating cancer cells.

The theory of circulating tumour cells as sources of cfDNA is supported by a study that found an association between CTCs and quantity of cfDNA[273]. Certainly, a small proportion of cfDNA comes from lysis of fragile cancer cells that become detached from the tumour and enter the bloodstream[274]. However, if cfDNA is due to lysis of circulating cancer cells, there needs to be many more CTCs detected than is found in studies to date.

Another possibility is that tumour actively releases DNA into the circulation in a similar process to lymphocytes after phytohaemaglutinin activation *in vitro*[275] [276]. This has also been demonstrated in mice after a mitogenic effect of bacterial lipopolysaccharide[277] and also from human leukaemic cell lines[278] and again resulted in a ladder pattern.

In summary, the origins of cfDNA remains disputed.

5.1.2. Structure of cfDNA

Few studies have studied the form of soluble cfDNA in the circulation. In an early study in a number of cancers, plasma cfDNA was found in double-stranded fragments and, using a ^{32}P labeled human DNA probe, was identified as human in origin[270]. Another early study utilised transmission electron microscopy (TEM) and demonstrated the bulk of cfDNA in normal individuals averaged 1.2-1.6 μm in length with a few at 0.1 μm and the longest 20 μm[279].

More recent studies using nick-translation of the extracted DNA and autoradiographic evaluation after electrophoresis, concluded that at least some of the cfDNA exists in association with nucleosomes[280, 281, 282]. A nucleosome is a histone octamer core wrapped twice by a 185-200base pair-long DNA strand.

Electrophoresis bands of plasma cfDNA in patients in one study were stronger and larger than controls, with a greater aggregate density of oligonucleosomal bands in one study in pancreatic cancer, but this may have resulted from cfDNA nicking in these

samples[282]. Consistent with other studies, a higher concentration of cfDNA was found in cancer patients compared to controls. Using electrophoresis, this study demonstrated the minimum cfDNA length of ~145bp (49nm) approximating to the length of a DNA strand wrapped around a single histone octamer[282]. Low molecular weight bands predominated.

Higher molecular weight cfDNA found in plasma is known to originate from apoptosis[283]. The finding that short oligonucleosomal strands comprise the majority of plasma cfDNA suggests that degradation of genomic DNA has occurred intracellularly during apoptosis, and not after the DNA was released into the plasma. Under physiological conditions, nucleosomes are packed into apoptotic particles and engulfed by macrophages[284]. Rapidly proliferating cancer cells or chemotherapy treatment leads to saturation of macrophage engulfment process and increases the number of nucleosomes in the circulation[285]. Increased levels of these circulating nucleosomes have been associated with breast cancer progression[286].

Digestion of DNA during apoptosis is caused by endonucleases breaking the DNA strands randomly between nucleosomes[287, 288, 289, 290], to date these endonucleases have not been identified in plasma[291]. Animal studies have demonstrated that nucleic acids are cleared from the blood by the liver and kidneys with a half-life varying from 15 minutes to several hours[292, 293, 294]. When compared with human fetal cfDNA, which can be detected in maternal circulation, a shorter half-life of 4 to 30 minutes was found[295].

Although RNA is detectable in plasma RNA, the focus of this chapter was on cfDNA and thus circulating RNA will not be discussed[296, 297].

5.1.3. Quantification of cfDNA

Using a radioimmunoassay, Leon *et al.* discovered high levels of circulating DNA in various cancer patients compared to non-malignant diseases (180ng/mL versus 13ng/mL)[156]. No correlation between detected cfDNA quantity and size of tumour was found, however higher quantities were found in those with metastases compared to localized disease. Quantity of cfDNA detected varies depending on the cancer type. For example, 90% of 65 patients with pancreatic carcinoma had serum DNA levels of >100 mg/mL compared to only 40% of 85 patients with colorectal carcinoma using the same assay[269]. It must be remembered however that cfDNA is also found in other

conditions such as SLE[298], viral hepatitis[281], following surgery and with pulmonary embolism[299].

The range of cfDNA in plasma or serum varies between 0 and >1000ng/mL of blood[298] in cancer patients compared to between 0 and 100ng/mL in healthy controls[293]. The quantity or concentration of cfDNA varies according to the method of extraction and differential sample handling as demonstrated in a multi-centre prospective trial[300].

Plasma cfDNA appears to inversely correlate with outcome and tends to fall with effective treatment[156, 267]. Several studies in lung cancer have also found a correlation between response to therapy and a decrease in plasma cfDNA levels. An increase in plasma cfDNA was associated with progression after chemotherapy[157, 301]. Following surgery, levels of cfDNA and nucleosomes can decrease to levels of healthy individuals and when remains high, may indicate residual disease[302, 303, 304]. This suggests quantification of cfDNA may be useful as a prognostic and predictive biomarker. However, there are also other studies suggesting no correlation[305].

cfDNA includes coding and non-coding genomic DNA which can be used to examine loss of heterozygosity (LOH), mutations, polymorphisms, methylation and repeated elements.

5.1.4. Repeated Elements Throughout the Genome in cfDNA

Non-coding repetitive sequences, once thought to be ‘junk DNA’, such as *ALU*, a short interspersed nucleic element (SINE), and long interspersed nucleic elements such as *LINE1* are known to be hypomethylated in cancer cells compared to normal cells[306]. Their importance have been demonstrated in DNA repair, transcription, genomic stability and involvement in epigenetic phenomena[307, 308]. Although these assays are in their infancy when applied to cfDNA, *ALU*, *LINES* and *SINES* (200-400bp) can be found in serum or plasma with potential prognostic and diagnostic purposes in a variety of cancers[309] [310, 311]. For example, using PCR, the integrity of *ALU* sequences in blood is sensitive for early stage breast cancer including micrometastases[312].

5.1.5. The Use of Tumour-specific Mutations as Biomarkers in cfDNA

Cancer is characterized by multiple somatic genetic and epigenetic alterations that could potentially be used as molecular markers for detecting tumour-specific DNA in different bodily fluids. The development of the polymerase chain reaction (PCR) aided research into the nature of circulating DNA with mutations in the primary tumour also being detected in plasma DNA. For example, Anker *et al*. identified *KRAS* mutations in 7 of 14 colorectal primary tumours with Identical mutations found in the plasma of 6 of these 7 patients[313]. These results have since been confirmed in other studies in colorectal cancer demonstrating the same mutations in the tumours as blood specimens[314, 315]. Clinically relevant mutations in *BRAF*, epidermal growth factor receptor (*EGFR*) and adenomatous polyposis coli (*APC*) have been studied in cfDNA in colorectal, lung cancer and melanomas[158, 316, 317, 318].

Pancreatic adenocarcinoma ls an ideal cancer to detect common mutations in plasma since 90% of pancreatic adenocarcinomas have mutations in the *KRAS* gene[319]. Identical *KRAS* mutations have been found in tumour and plasma using restriction fragment length polymorphism (RFLP)-PCR followed by DNA sequencing[320, 321]. It might be expected that specificity would be low since *KRAS* mutations have been found in tissue from chronic pancreatitis[322]. However, *KRAS* mutations were not found in these patients making raising its possibility as a diagnostic test for pancreatic adenocarcinoma[323].

The presence of a *KRAS* mutation is an highly specific negative predictor of response to EGFR tyrosine-kinase inhibition e.g. Cetuximab. Kimura *et al.* obtained tissue and serum samples for *EGFR* mutation status in patients with lung cancer and found a strong correlation between presence of *KRAS* mutation in serum and response, in addition to a better PFS[158]. Interestingly, in a similar study, Moran *et al*. demonstrated a complete response in 20% of patients with both tissue and serum *KRAS* mutations and in 4% of patients with only a mutation in tissue[324]. Using digital PCR to quantify common *KRAS* mutations, Yung *et al.* demonstrated that plasma levels of mutant *KRAS* correlated with clinical response to therapy and a reduction in levels was observed in all patients who had a partial or complete response[325]. *KRAS* mutations were also found to be associated with worse overall survival in mucinous ovarian cancer[326].

New approaches such as cfDNA sequencing have been studied recently. The *BRAF* mutation, V600E, found in >70% of metastatic melanomas, can be detected using a

quantitative real-time clamp PCR assay with higher quantities found in more advanced disease[318]. This approach can also be used to monitor response to therapy, which would be beneficial when assessing efficacy of anti-BRAF drugs in development[327].

Mutations in the *p53* tumour suppressor gene found in ovarian[328], head and neck[305] and colorectal cancers[329] have also been determined and analysed in cfDNA.

Disadvantages include low assay sensitivity and specificity and in addition, the low frequency of tumour specific somatic mutations which occur in neuroendocrine tumours[298] [21]. Furthermore, there is no complete concordance between alterations found in the primary tumour and cfDNA in any cancer[330, 331, 332].

5.1.6. Epigenetics

Hypermethylation of tumour suppressor promoters in tumour tissue is an important epigenetic mechanism for tumour suppressor gene inactivation.

Aberrant DNA methylation can also be identified and utilised as a biomarker in the serum of cancer patients. Methylation-specific PCR can determine changes in promoter hypermethylation of the tumour suppressor gene p16, the DNA repair gene O6-methylgunaine-DNA-methyltransferase (*MGMT*), and the detoxification gene glutathione S-transferase P1 (*GSTP1*). When promoter methylation analysis of *GSTP1* was performed on patients with non-small-cell lung cancer, aberrant methylation was found in 15 of 22 patients, 11 of which had abnormal methylation in the matched serum sample[159].

Bastian *et al.* demonstrated that hypermethylation of *MDR1* was observed in serum samples of 17% of metastatic prostate cancers[333]. When correlation with clinical factors was studied, promoter hypermethylation of *GSTP1* in serum was detected in 28% of patients with metastatic prostate cancer compared with only 12% with localized prostate cancer[334]. Chuang *et al.* also found hypermethylated *GSTP1* promoter in 11 of 36 plasma samples from patients with prostate cancer but did not report a correlation with diagnostic or prognostic variables[335]. Other bodily fluids can also be used. Promoter hypermethylation of *GSTP1* in urinary derived DNA has been used to distinguish prostate cancer from benign prostatic disease[336].

Ponomaryova *et al.* found methylation of the gene *RARbeta2* in cfDNA was associated with disease progression in non-small cell lung cancer[337].

The association between circulating tumour cells and methylated DNA has been explored. Using methylation specific PCR on serum of patients with metastatic prostate cancer, circulating tumour cells (CTCs) were detected in 36 of 39 patients with tumour-related methylated DNA but only in 11 of 37 without[338]. Promoter methylation was studied in *APC*, *GSTP1*, prostaglandin-endoperoxide synthetase 2 (*PTGS2*), *MDR1*, and Ras association domain family 1 isoform A (*RASSF1a*). Hypermethylation of only one of this panel of loci conferred a shorter survival. In another study, patients with melanoma who had CTCs and methylated *RASSF1a* and *RARB* in blood had a poorer response to chemotherapy and a shorter PFS and OS[339]. Van der Auwera *et al.* also demonstrated a correlation between promoter methylation of *APC, RASSF1a* and oestrogen receptor 1 (*ESR1*) in cfDNA and CTCs in breast cancer[340]. This suggests that CTCs are a potential source of cfDNA due to association of cell-free, methylated *APC*, *RASSF1a* molecules with CTCs.

Methylation of *RASSF1a* and *PITX2* in plasma DNA has also recently been found to be prognostic of PFS and OS in breast cancer[341].

5.1.7. Microsatellite alterations and Loss of Heterozygosity

Microsatellites are short repetitive and highly polymorphic DNA sequences. Microsatellite alterations defined as the presence of allelic imbalance (AI) or loss of heterozygosity (LOH) and valuable as clonal markers for cancer[342]. These microsatellite alterations have also been detected (and matched) in the plasma of patients in head and neck cancers[271], lung cancer[343, 344] and renal cancer[345]. However, there are limitations using these markers in plasma and serum as LOH or microsatellite alterations may be masked by normal DNA from lymphocytes.

5.1.8. Assays, Methodology and Technical Aspects

There are several factors which affect cfDNA yield from patient to patient such as whether plasma or serum is used, time between blood collection and centrifugation, and storage temperature can have influence on yield[346]. Levels of cfDNA were unchanged in EDTA stabilized blood stored at room temperature for 8 hours or at 4°C for 24 hours[347]. However, another study demonstrated a 3- to 5-fold increase in serum DNA upon storage at room temperature without the addition of additives[348].

Although not comprehensively investigated, storage of frozen serum and plasma samples may affect yield. Intact mRNA was detected in samples after 2 years of storage at -70°C and *KRAS* mutations in plasma DNA after 6 years of storage at -70°C [315,

349]. Studies on foetal DNA in serum found a decline in cfDNA by a factor of 0.66 genome equivalents/mL per month for storage at -20°C[350]. Additional freeze-thaw cycles of plasma but not of extracted DNA affects fragmentation of cfDNA[351].

Both plasma and serum have been studied as compartments for cfDNA but there is no definitive answer on which is better. Yield appears to be higher from serum most likely due to clotting and release of DNA from destroyed white blood cells[352, 353].

There are a number of different methods for DNA extraction from blood. Most studies use commercial kits, utilizing silica-gel membrane technology by selective binding and stepwise elution, but there is no accepted standard[354]. Generally, quantities of cfDNA are low and of poor quality irrespective of extraction technique. Use of commercial kits over crude techniques have made extraction steps more reliable but some DNA is lost as columns do not bind small DNA molecules (less than 150 base pairs) efficiently[355, 356].

The methods used in studies to quantify cfDNA include fluorometric methods and spectrophotometry[357], electron microscopy[279], and more recently, real-time quantitative PCR. A flurorescence-based (PicoGreen staining) and real-time PCR (SybrGreen and Taqman format) were compared to quantify cfDNA in patients with non-small cell lung cancer in one study[358]. Higher quantities of cfDNA was detected by the fluorescence-based method and this is considered to be sensitive method to a detection of 1 ng/mL.

Studies conflict on the fluctuation of levels of cfDNA in plasma. A variation of 2.2-fold was demonstrated of foetal cfDNA in maternal circulation over three days[359] whereas no fluctuation was found in three days in patients with colorectal cancer[360].

5.1.9. Theory of Genometastases

cfDNA in plasma may partake in tumorigenesis which is supported by a study where plasma of colorectal cancer patients induced the oncogenic transformation of susceptible cultured cells[361]. This followed on from experiments where cfDNA from tumour-bearing rats was taken up by cells and incorporated into the genome[362]. More specifically, tumour-specific methylated DNA fragments can penetrate into cells more efficiently and have a higher transformation potential than unmethylated counterparts[363]. However, this is an area which has not been extensively investigated.

5.1.10. Circulating Nucleic Acids in NETs

Although circulating messenger RNA (mRNA) in patients' plasma has been described in NETs as a means for distinguishing metastatic versus local disease[140], the field of circulating nucleic acids is not an area that has been thoroughly investigated in NETs. RNA is more unstable than DNA as RNA is readily degraded by RNases during cellular degeneration[296, 297] and by RNases in blood plasma[364, 365]. Hence I focused on DNA in these experiments since the applications on cfDNA mentioned above i.e. promoter methylation, amplification of repeated elements and mutational analyais could then be possibly applied in patients with NETs. To date, there is no study exploring cfDNA in NETs. Therefore the aims of these experiments were:

- to identify cfDNA in the blood of patients with NETs
- to compare cfDNA extracted between matched plasma and serum samples from patients in NETs
- to explore the relationship between cfDNA and CTCs in patients with NETs

5.2. Materials and Methods

5.2.1. Patient Recruitment

Eighty-eight patients were recruited between December 2009 and June 2011 for blood sampling. All eligible participants had histologically proven NET, metastatic disease measurable by RECIST criteria. They were categorised according to the site of primary NET: midgut, pancreas, bronchopulmonary, hindgut or of unknown primary. Additionally 20 healthy volunteers were recruited for blood sampling. Volunteers were excluded if they had a current or past cancer, active infection, recent surgery within 1 month, current inflammatory disease or chronic disease (apart from hypertension). This study was approved by the Local Ethics Committee (Ref 09/H0704/44) and all patients and volunteers provided written informed consent.

5.2.2. Blood Sample Preparation

For serum preparation, blood samples were drawn into a 10ml plain red blood collection tube (Becton Dickinson, Oxford, UK). Samples were left to clot for approximately15 minutes and then centrifuged within two hours at 1,500 X g for 10 minutes at 4^{o}C. Serum was separated and aliquoted into 0.5 mL Cryobank vials (Nunc, Denmark). Vials

containing serum were immediately transferred to a -80 °C freezer and stored until required.

For plasma preparation, blood samples were drawn into a 4ml EDTA blood collection tube (Becton Dickinson, Ox, UK). Samples were centrifuged within 2 hours at 1,500 X g for 10 minutes at 4^{o}C. 2mls plasma were separated carefully and aliquoted into Cryobank vials. Cell pellets were discarded. Vials containing plasma were immediately transferred to a -80 °C freezer and stored until required.

5.2.3. DNA Extraction

All samples and reagents were equilibrated to room temperature. 20µL of proteinase K (Qiagen, Germany) was pipetted into the bottom of a 1.5mL microcentrifuge tube. 200µL of the plasma (or serum) sample, or PBS (control) was added to the tube. 200µL of lysis buffer (Qiagen) was added to the mixture and mixed by pulse-vortexing for 15 seconds. The microcentrifuge tube was incubated at 56°C for 10 minutes. The microcentrifuge tube was briefly centrifuged to remove drops from inside the lid.

200µL of 100% ethanol was added to the sample and mixed by pulse-vortexing for 15 seconds. The tube was once again centrifuged briefly to remove drops from the lid.

The mixture from the above steps were carefully transferred to a QIAamp Mini spin column (Qiagen) and this was centrifuged at 6000 X *g* for 1 minute. After centrifugation, the spin column was transferred to a clean 2ml collection tube. The tube containing the filtrate was discarded.

500µL of wash buffer (Qiagen) was added to the spin column. After centrifugation at 6000 X *g* for 1 minute, the spin column was transferred to a clean 2ml collection tube. The tube containing the filtrate was discarded.

500µL of wash buffer was added to the spin column. After centrifugation at 20,000 X *g* for 3 minutes, the spin column was transferred to a clean 2ml collection tube and the tube containing the filtrate discarded. This was centrifuged further at 20,000 X *g* for 1 minute and the spin column transferred to a clean 1.5ml microcentrifuge tube. The filtrate was discarded.

For the elution step, 200µL distilled water was added to the spin column and incubated at room temperature for 3 minutes. After centrifugation at 6000 X *g* for 1 minute, the spin column was discarded and the extracted DNA processed for further experiments or stored at 4°C until ready for use.

5.2.4. DNA Separation, Sizing and Quantification

For the determination of cfDNA fragment length and quanitification, high sensitivity DNA kits (Agilent Technologies, CA, USA) were used containing microfluidic chips. This is useful for low concentrations of dsDNA (50pg/u μl) from 50-7000 base pairs (bp).

Chip-based capillary electrophoresis was performed on the Agilent 2100 Bioanalyzer (Agilent Technologies) in combination with a high sensitivity DNA kit. A validated High Sensitivity DNA assay was available within the Agilent 2100 expert software. The on-chip electrophoresis was performed according to the High Sensitivity DNA Kit guide as per manufacturer's protocol.

Briefly, to prepare the gel-dye mix, high sensitivity DNA dye concentrate and high sensitivity DNA gel matrix were equilibrated to room temperature for 30 minutes. The dye concentrate was vortexed, briefly centrifuged and then transferred to the vial containing gel matrix. After vortexing, the gel-dye mixture was transferred to the top receptacle of a spin filter. This was centrifuged at 2240 X g for 10 minutes at room temperature.

After equilibrating to room temperature, 9μL of gel-dye mix was loaded, using a chip-priming station, into a designated well in a 16-well high sensitivity DNA microfluidic chip. Further 3 X 9μL of gel-dye mix were dispensed into 3 designated wells. 5μL of high sensitivity DNA marker was dispensed into the 12 sample/ladder wells. 1μL of high sensitivity DNA ladder was added to the designated ladder well. The chip was vortexed carefully for 60 seconds on the IKA vortex mixer (Agilent Technologies) before analysis on the 2100 Bioanalyzer.

5.2.5. CTC Isolation and Enumeration

The process of CTC isolation by immunomagnetic separation and subsequent enumeration has been described previously in Chapter 3.

The total number of CTC events, i.e. including those not classified as CTCs, were also recorded.

5.2.6. Comparison of DNA Yield from Serum and Plasma

To compare DNA extracted from plasma and serum, plasma and serum from patients were collected. 44 pairs of samples (plasma and serum) from a subset of 44 patients underwent DNA extraction and quantification as above. Results were compared. Serum

from the 20 healthy volunteers were also processed for DNA extraction and quantification and results compared to patient cases.

5.2.7. Analytical/Laboratory Reliability

5.2.7.1. Extraction Replicates

To test for reproducibility of the DNA extraction technique, 20 samples underwent DNA extraction in duplicate. 400μL of plasma from the same aliquot was divided into 200μL fractions and both underwent DNA extraction as above. Results were compared.

5.2.7.2. DNA Quantification Replicates

To test for reproducibility of the DNA quantification assay using capillary electrophoresis on DNA chips, 23 extracted DNA samples underwent DNA quantification in duplicate. 2μL of extracted DNA was divided into 1μL fractions and each were processed separately on the DNA chip as above.

5.2.7.3. Aliquot storage Replicates

To test for reproducibility after storage at different durations at -80°C, 5 plasma samples underwent DNA extraction and quantification in duplicate. These samples were processed, as above, within 1 month of storage and then repeated after 6 months of storage at -80°C.

5.2.8. Intra-subject Reliability

To test for intra-subject variability, 17 patients had at least 2 blood samples for plasma DNA extraction at different time-points. The repeat sample was taken within a range of 4 to 12 weeks. Patients were included if no new or change in therapy was planned.

Five of these patients had more than 2 samples taken at serial time-points with no intervening treatment change.

5.2.9. Statistical Analysis

When considering the presence or absence of cfDNA i.e. a dichotomous variable, prevalence-adjusted bias-adjusted κ (PABAK) was used to evaluate reliability of DNA extraction replicates, DNA quantification replicates, storage replicates and intra-subject replicates as well as validity when comparing to CTCs. This was used since some values were 0, rendering Cohen's κ unreliable. When cfDNA was considered as a

continuous variable, a natural log transformation resulted in a normal distribution, thus intraclass correlation coefficients (ICC) were calculated to evaluate reliability of DNA extraction replicates, DNA quantification replicates, storage replicates and intra-subject replicates. Kruskal-Wallis test was used to assess intra-subject variability of DNA assays at serial timepoints.

Chi squared and student *t* tests were estimated to assess differences between healthy controls and patients with regards to gender and age distributions respectively. Student *t* test was estimated to assess difference in cfDNA concentrations between healthy controls and patients (Chi squared when cfDNA considered as a dichotomous variable i.e. present or absent).

Correlations between cfDNA and CTCs, between serum and plasma DNA concentrations were assessed using Spearman's rank test (Chi squared when presence of cfDNA/CTC considered as a dichotomous variable).

Kaplan-Meier estimates of survival were based on the presence or absence of cfDNA. For the purposes of survival analyses, progression-free survival (PFS) or overall survival (OS) was defined as the time between the date when the baseline blood sample was taken and the date of radiological progression, death due to neuroendocrine cancer or last follow-up visit. Survival curves were compared using log-rank testing.

5.3. Results

5.3.1. Background Characteristics of Patient Population

The background characteristics of the 88 patients recruited for plasma cfDNA evaluation are shown in Table 5.1.

	All NETs	Pancreatic	Midgut	Broncho-pulmonary	Unknown Primary	Hindgut
	n=88	n=12	n=65	n=4	n=4	n=3
Age, years mean ± SD	61 ± 13	55 ± 15	61 ± 12	80 ± 0.7	61 ± 16	64 ± 18
Sex						
Male	47	7	36	1	2	1
Female	40	5	29	3	2	2
Grade						
Low	48	2	45	1	0	0
Intermediate	27	4	18	3	1	2
High	12	6	2	0	3	1
Burden of Liver metastases						
≤25%	49	4	39	2	3	1
25%≤50%	22	3	18	2	0	0
50%≤75%	10	2	6	0	1	1
>75%	6	3	2	0	0	1
Duration of diagnosis, median months (range)	29 (1-150)	24 (1-146)	44 (2-150)	73 (58-87)	11 (1-30)	15 (5-22)
PS						
0	58	8	45	2	2	1
1	25	4	17	2	1	2
2	3	0	3	0	0	0
3	1	0	0	0	1	0
4	0	0	0	0	0	0
Previous treatments						
Resection of primary	38	7	29	1	0	1
SST	54	6	45	1	1	1
Chemotherapy	12	6	4	0	1	1
TAE	14	2	11	0	1	0
Radionuclides	12	2	10	0	0	0
Interferon	4	0	3	0	1	0
Liver resection	5	2	3	0	0	0

Table 5.1 Background characteristics of patient sample group undergoing plasma DNA evaluation

5.3.2. Healthy Controls Vs Patient Samples

There was no significant difference observed in the proportion of males vs females in healthy controls compared to patients (χ^2=0.332 *P*=0.565) (Table 5.2) or age between the control and patient groups (t=1.4 *P*=0.169).

In patients where plasma cfDNA was detected, this was found to have a fragment size of 150 base pairs with additional fragment sizes at 300, 450 and 600 base pairs in several plasma samples where total cfDNA was high (and serum samples as explained later) (Figure 5.1). Only one of the control samples had a small quantity of DNA at 150 base pairs.

Data of healthy controls compared to patients are shown in Figure 5.2 and Table 5.3. There was a significant higher quantity of plasma DNA in patients compared to healthy controls (t=2.69 *P*=0.009).

Gender	***Healthy Control***	***Patient***	**Total**
Male	9	47	56
Female	11	41	52
Total	20	88	108

Table 5.2 Distribution of gender across healthy control and patient groups

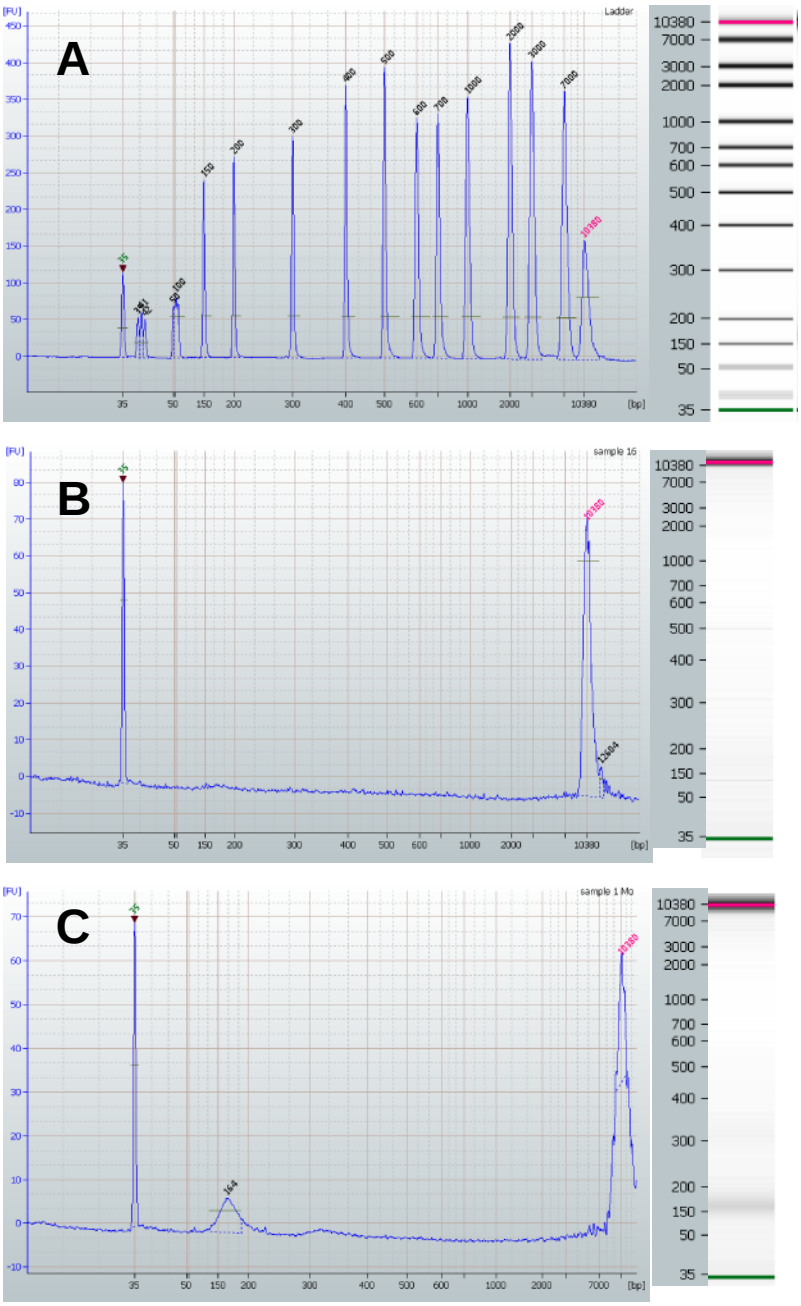

Figure 5.1 Examples of electropherograms (EPG), demonstrating cfDNA with fragment size in base pairs (bp) along the x-axis and cfDNA concentration along the y-axis. Corresponding traditional digital electrophoresis gels are displayed to the right of each EPG. Each EPG displays small (lower) and large (upper) marker DNA at 35bp and 10380bp respectively. (A) demonstration of typical calibration 'ladder' markers run with each batch of samples. (B) an example of sample with no cfDNA detected (apart from upper and lower markers) which was also the finding in the majority of healthy controls. (C) an example of a sample with detectable fragment at 150bp

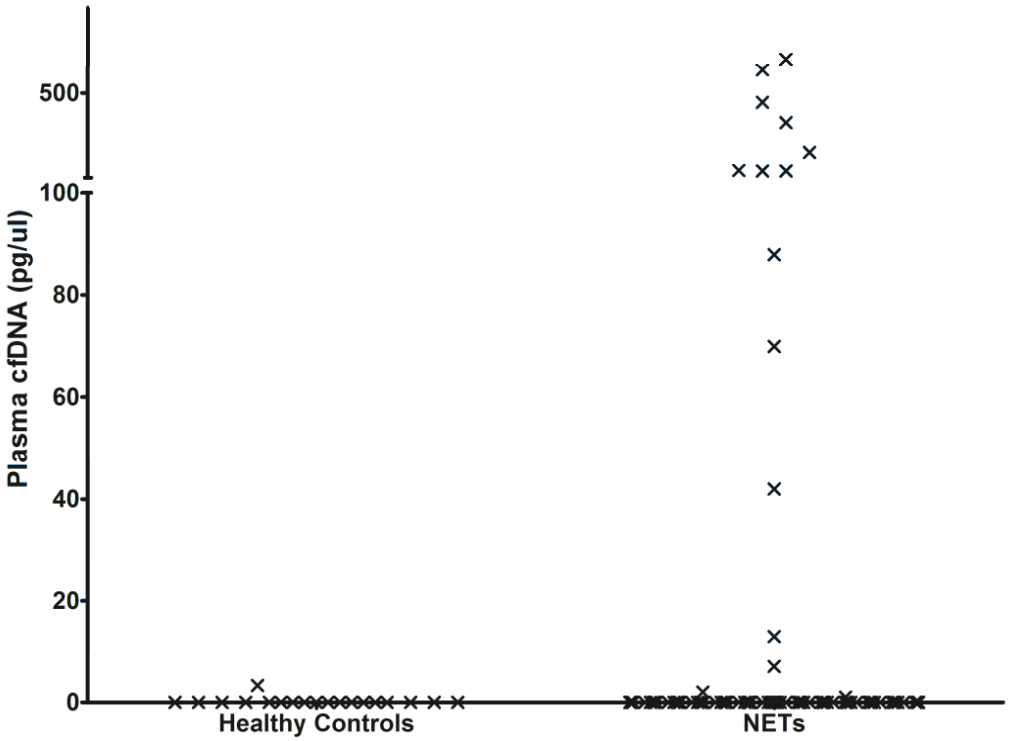

Figure 5.2 Plasma cfDNA in controls Vs NET patients

	Control (n=20)	**Patients (n=88)**
No. cases with detectable cfDNA (%)	1 (5)	22 (25)
Median (pg/uL)	0	0
Range (pg/uL)	0-3.3	0-660
Mean ± SD (pg/uL)	0.17 ± 0.76	34.1 ± 117
95% CI (pg/uL)	0-0.5	9-59
Median of cfDNA>0 (pg/uL)	3.3	110
Mean of cfDNA>0 (pg/uL)	3.3	183 ± 220

Table 5.3 Plasma cell-free DNA in healthy controls and patient groups

5.3.3. Analytical/Laboratory Reliability

5.3.3.1. DNA Extraction Reliability

	Detectable cfDNA	No cfDNA	Total
Detectable cfDNA	19	0	19
No cfDNA	0	1	1
Total	19	1	20

Table 5.4 Reliability of assay assessed on extraction replicates

100% of samples that had cfDNA present at the 150 base pair fragment, had cfDNA present in the extraction replicate (Table 5.4). The Prevalence-adjusted Bias-adjusted kappa (PABAK) coefficient was estimated at 1.0 indicating a perfect agreement. When quantity of cfDNA of was considered (Figure 5.3), the Intra-class coefficient (ICC) was estimated at 0.762 indicating excellent reproducibility.

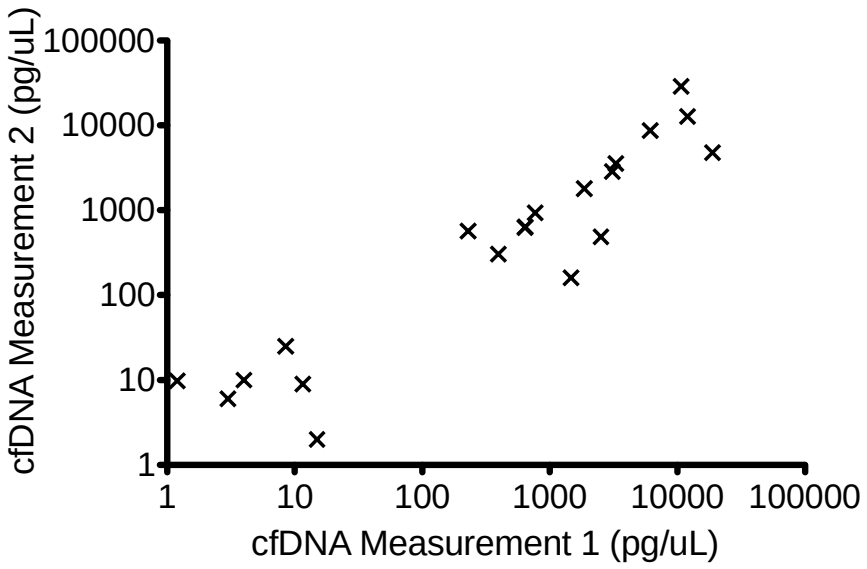

Figure 5.3 Scatter plot of reproducibility between cfDNA extraction replicates

5.3.3.2. Reliability of DNA Quantification

	Detectable cfDNA	No cfDNA	Total
Detectable cfDNA	13	0	13
No cfDNA	0	10	10
Total	13	10	23

Table 5.5 Reliability of assay assessed on chip replicates

100% of samples processed who had detectable cfDNA at 150 base pairs had detectable cfDNA on the chip replicate (Table 5.5). All those without detectable cfDNA had no cfDNA on the chip replicate. The PABAK coefficient was 1.0 indicating perfect agreement. When concentrations of cfDNA were compared, the ICC was 0.993 indicating excellent reproducibility (Figure 5.4).

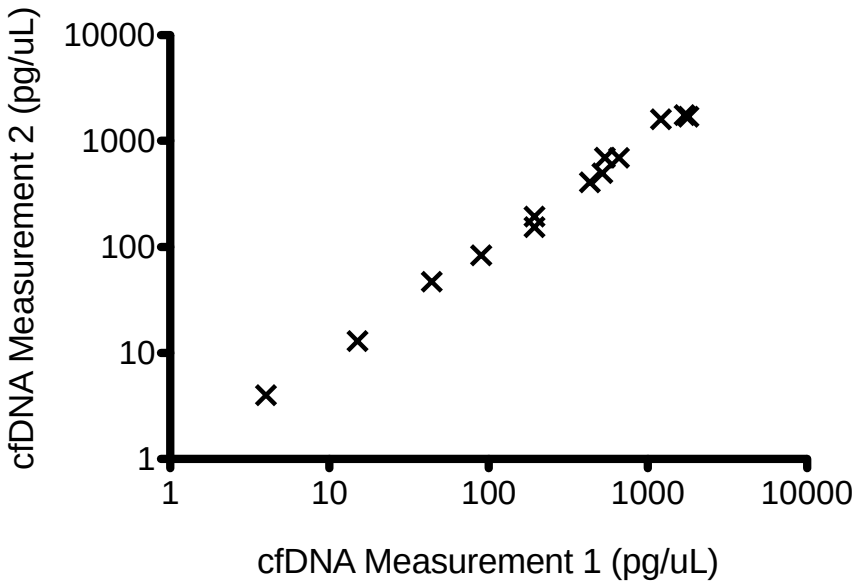

Figure 5.4 Scatterplot of reproducibility between cfDNA samples replicated on the microfluidic chip (chip replicates)

5.3.3.3. Reliability of Assay and Storage Duration

	Detectable cfDNA	No cfDNA	Total
Detectable cfDNA	2	0	2
No cfDNA	0	3	3
Total	2	3	5

Table 5.6 Reliability of assay based on storage replicates

Storage replicates are shown in Table 5.6. The PABAK coefficient for storage replicates was 1.0 indicating perfect agreement. When quantity of cfDNA was taken into consideration, the ICC was 0.76 indicating excellent reproducibility.

5.3.4. Intra-subject Reliability

	Detectable cfDNA	No cfDNA	Total
Detectable cfDNA	6	1	7
No cfDNA	1	9	10
Total	7	10	17

Table 5.7 Intra-subject reliability

The PABAK coefficient for intra-subject variability was 0.76 indicating substantial agreement (Table 5.7). When concentrations of cfDNA were compared, the ICC was estimated at 0.77 indicating excellent reproducibility (Figure 5.5).

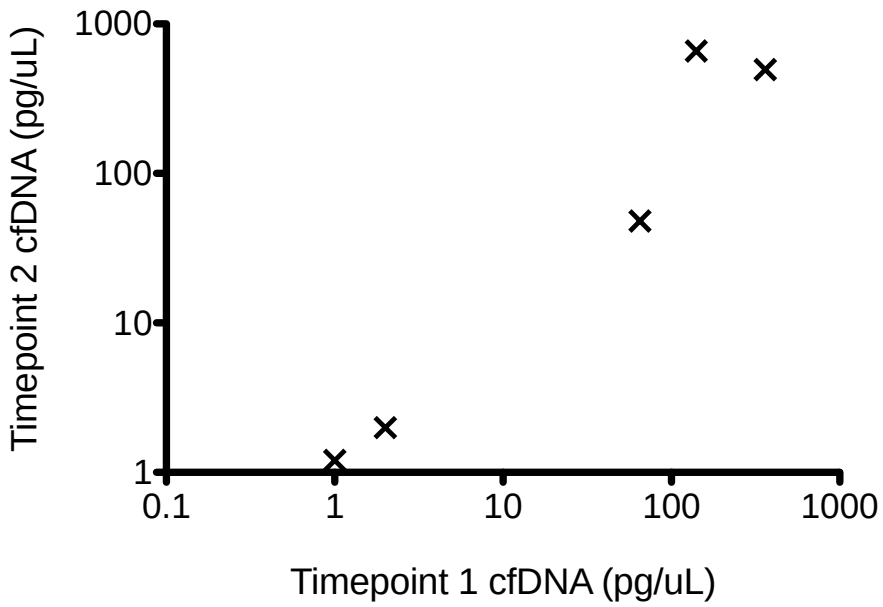

Figure 5.5 Scatterplot of intra-subject variability

Five patients had more than two samples taken and underwent no change in therapy. Samples were taken at 3-5 weekly intervals for 10-16 weeks. Three patients had detectable plasma cfDNA and continued to do so at later time-points. The other two patients did not have detectable cfDNA and continued not to have any cfDNA on further time-points. When cfDNA concentrations were assessed, there was no significant difference across time-points (Kruskal-Wallis=0.31 p=0.99). Serial changes are shown in Figure 5.6.

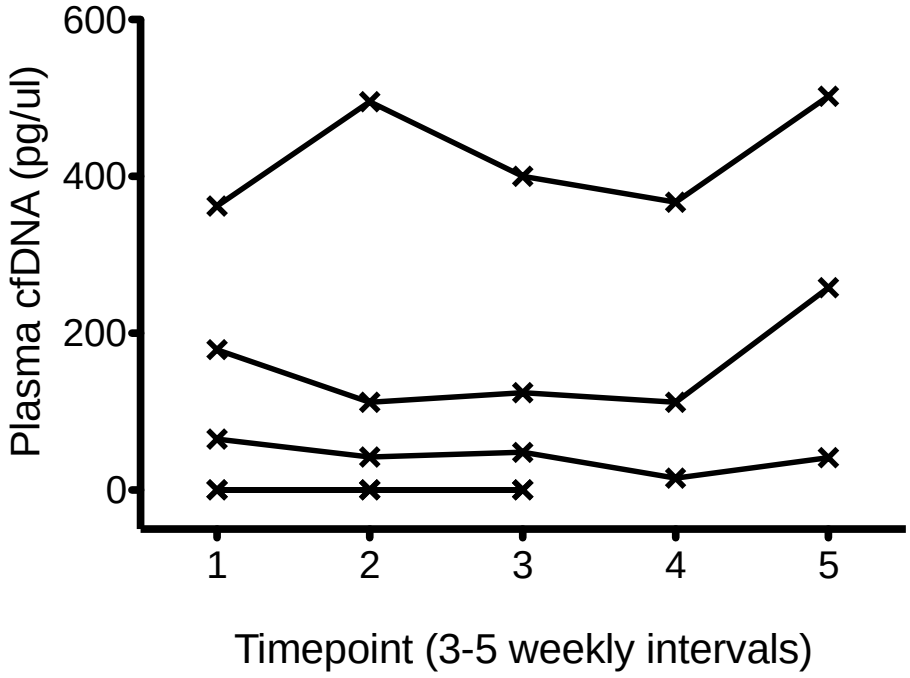

Figure 5.6 Graph demonstrating changes in cfDNA concentrations over serial samples. Each line represents a single patient except the line at y=0 which represents two cases. Sampling occurred at 3-5 weekly intervals.

5.3.5. cfDNA and Circulating Tumour Cells

75 of the patients who underwent evaluation for cfDNA also had samples taken for CTC enumeration. There was a significant association between presence of CTCs and presence of cfDNA (X^2=11.6 *P*=0.001) (Table 5.8). When cfDNA was detected, (19/22) 86% of patients had CTCs.

cfDNA levels were higher in those with CTCs present compared to patients without CTCs (Mann-Whitney *P*=0.001). This is shown in Figure 5.7 and Figure 5.9. The quantity of cfDNA positively correlated with number of CTCs (r-0.45 *P*<0.001) but not with the total number of CTC events (r=0.21 *P*=0.08). Interestingly, in a handful of cases with very high CTCs counts, fragments of 300 base pairs were detected in addition to the 150 base pair fragments (Figure 5.9). If the presence of cfDNA was used as a marker for presence of CTCs, the PABAK coefficient was 0.31, indicating only fair concordance.

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	cfDNA detected	*No cfDNA*	**Total**
CTC ≥1	19	23	42
CTC =0	3	30	33
Total	22	53	75

Table 5.8 Presence of CTCs and detection of cfDNA

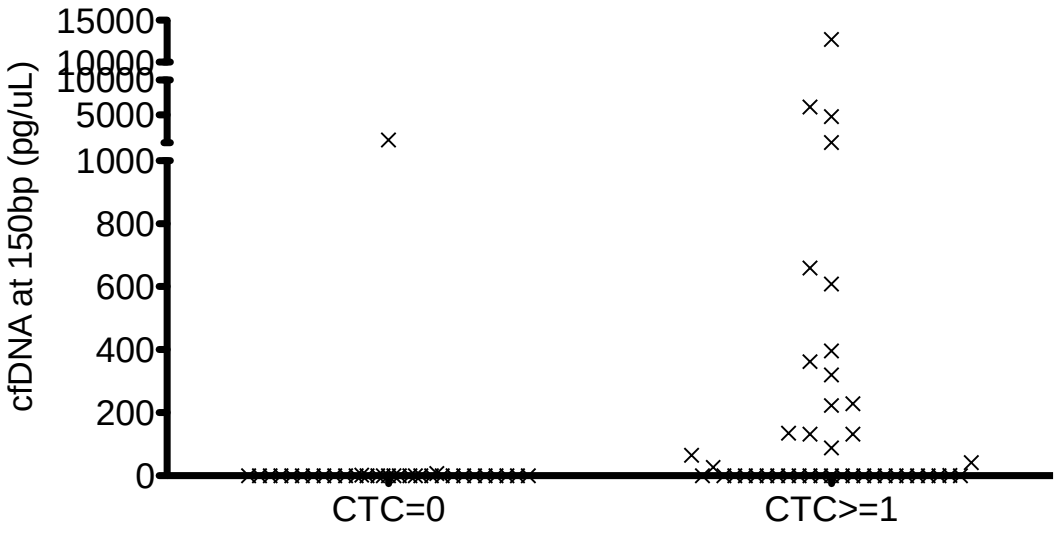

Figure 5.7 Quantity of cfDNA in patients without and with CTCs

5.3.6. Comparison of DNA Yield from Serum and Plasma

Compared to plasma of the 20 healthy controls where negligible cfDNA was detected, cfDNA was detected at multiple fragment lengths in serum of controls but no fragment of 150 base pairs was demonstrated (Figure 5.8).

44 patients with NETs had serum and plasma samples from which cfDNA was extracted and analysed. Compared to plasma where cfDNA was mainly present at 150 base pair fragments, serum contained a greater number of larger size fragments (Figure 5.9). However, when there was cfDNA detected in plasma at 150 base pairs, invariably (94%) there was cfDNA detected in serum at 150 base pairs (Table 5.9). When there was no cfDNA at 150 base pairs in plasma, despite a number larger size fragments in serum, 78% did not have any cfDNA at 150 base pairs. Thus there was an association between presence serum and plasma cfDNA (Fisher's exact = 21.5 $P<0.001$).

cfDNA was detected at higher quantities in serum of patient cases compared to controls (Mann-Whitney $P<0.001$) (Figure 5.10).

Figure 5.11 demonstrates levels of cfDNA at 150 base pairs in plasma compared to the same patient's serum. The concentration of cfDNA at 150 base pair in serum was higher than the concentration of the same fragment size in plasma (Wilcoxon $P<0.001$).

DNA at 150bp	**Serum Yes**	**Serum No**	
Plasma Yes	16	1	17
Plasma No	6	21	27
	22	22	44

Table 5.9 Presence of 150 base pair fragment of cfDNA when extracted from plasma and serum

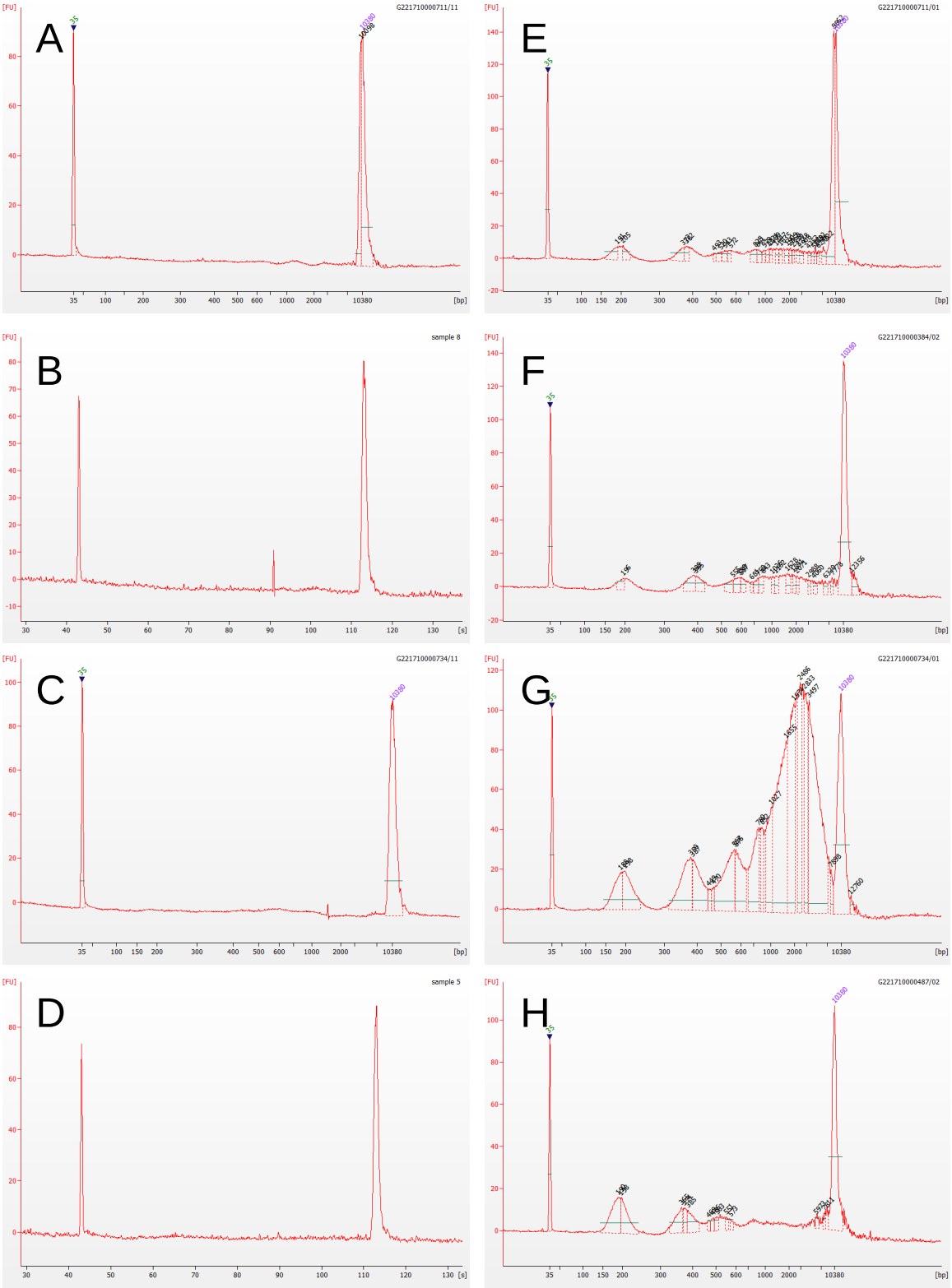

Figure 5.8 Electropherograms from control cases demonstrating difference in DNA fragments obtained in plasma (A, B, C, D) and corresponding serum (E, F, G, H respectively). There was no demonstrable fragment (at 150 base pairs, or otherwise, apart from the upper and lower assay markers) in the plasma of control cases. Multiple fragments of cfDNA in a ladder pattern were found in corresponding serum but no 150 base pair fragment was found.

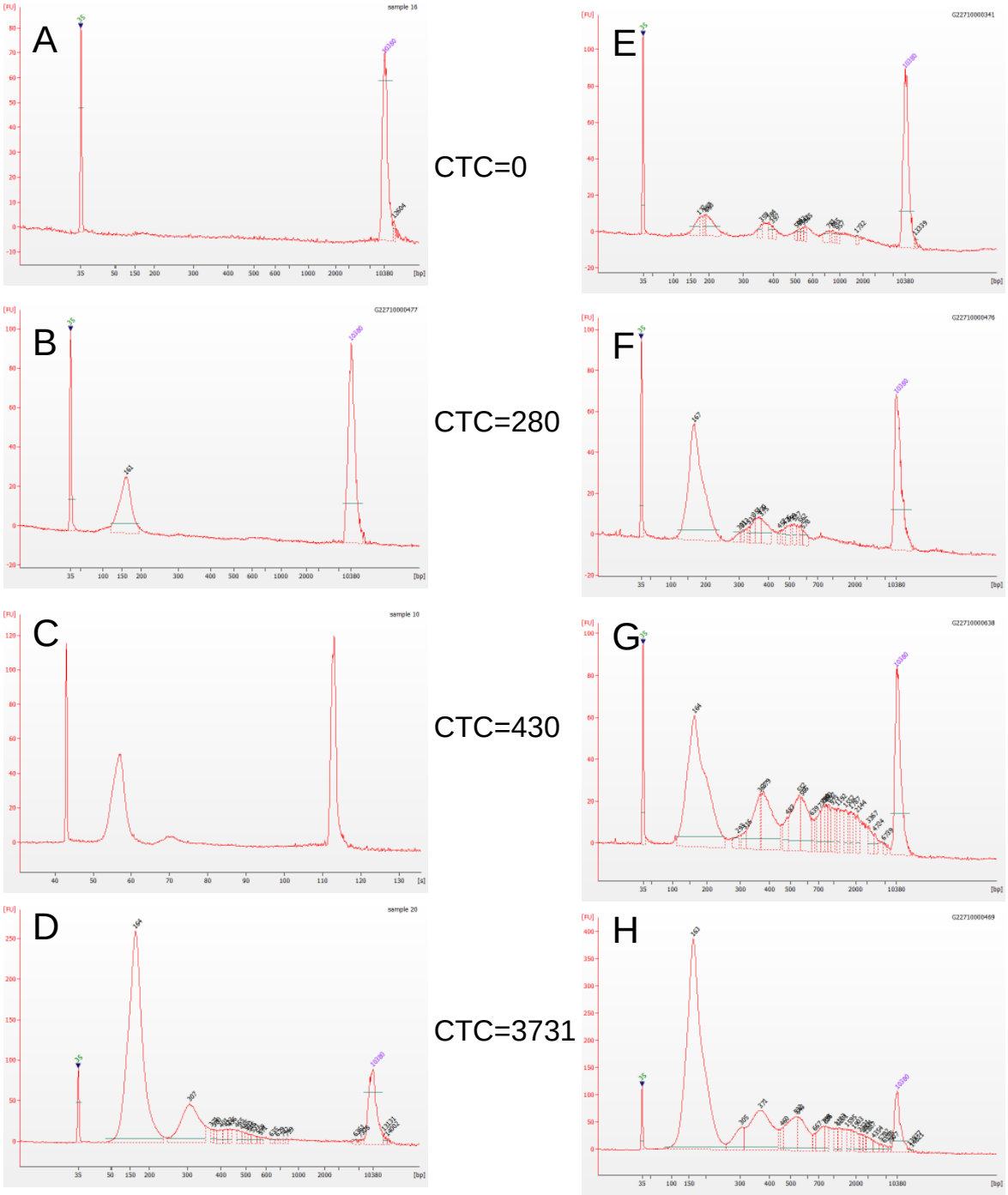

Figure 5.9 Electropherograms from cases of NETs demonstrating difference between cfDNA detected in plasma (A, B, C, D) and corresponding serum (E, F, G, H respectively). CTC count is displayed for each case. Large numbers of CTCs were associated with a fragment of 300 base pairs in addition to 150 base pair fragments (C, D). A ladder pattern with multiple fragment lengths were found in serum compared to corresponding plasma.

Figure 5.10 Levels of serum cfDNA at 150 base pairs in healthy controls and NET patients (horizontal line representing median)

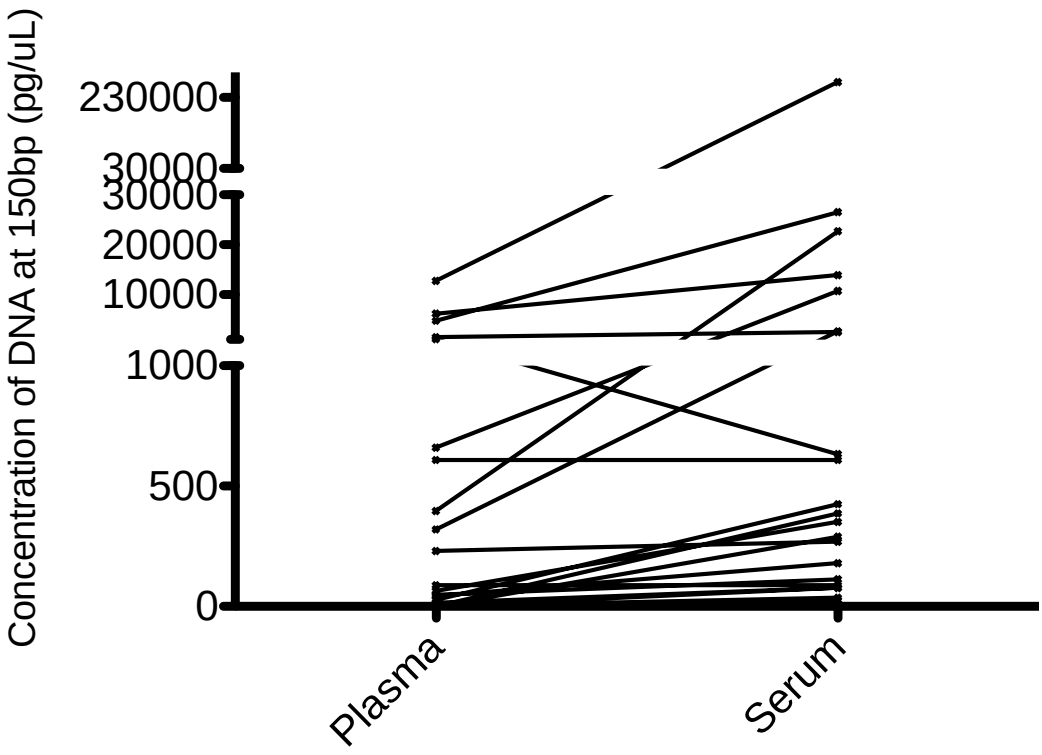

Figure 5.11 Quantities of cfDNA in plasma and serum (lines represent increase/decrease when serum analysed)

5.3.7. cfDNA and Survival

Outcomes were available in 87 of patients who had plasma cfDNA evaluated, median follow up 21 months. PFS was significantly worse in those with detectable cfDNA (log-rank $P<0.001$). Median PFS was 12 months in those with detectable cfDNA and not reached in those without cfDNA. OS appeared to be worse in those with detectable cfDNA but did not reach statistical significance ($P=0.162$). Kaplan-Meier survival curves are shown in Figure 5.12 and Figure 5.13.

Patients were grouped into those with no cfDNA and no CTCs (group A); either detectable cfDNA or CTCs (group B); or both detectable cfDNA and CTCs (group C). Both PFS and OS appeared to be worse for group C than group B than group A but only reached statistical significance for group C Versus group A. Kaplan-Meier survival curves are shown in Figure 5.14 and Figure 5.15.

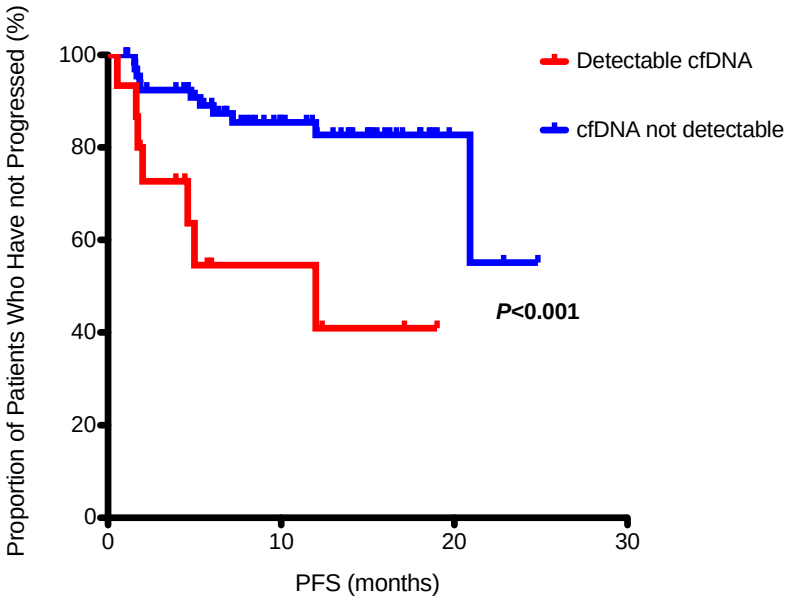

Figure 5.12 Progression-free survival of those with and without detectable cfDNA

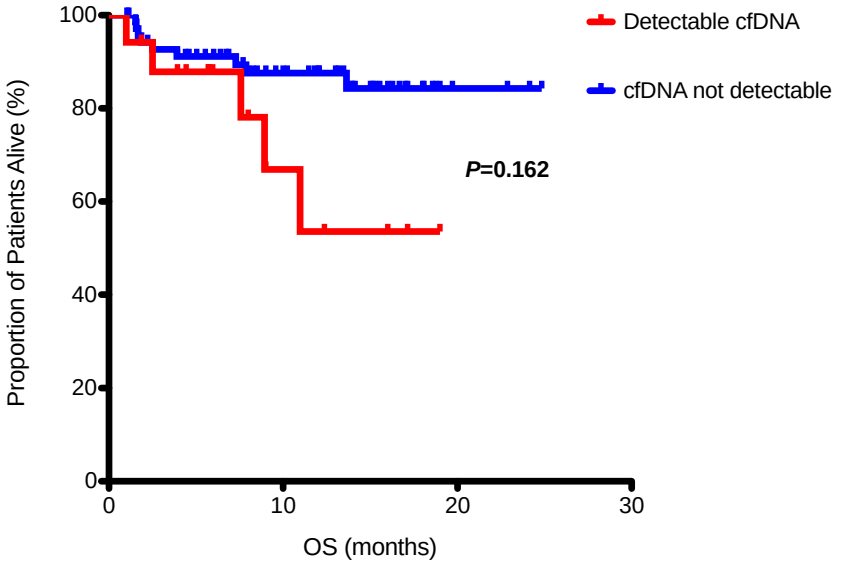

Figure 5.13 Overall survival of those with and without detectable cfDNA

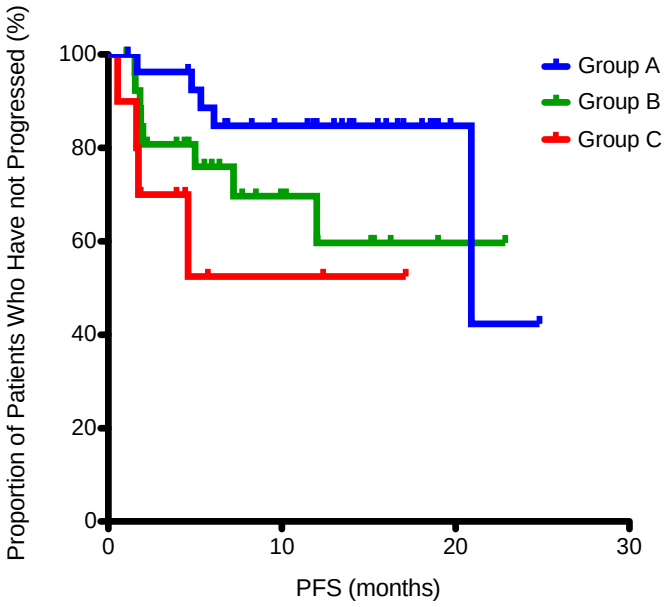

Figure 5.14 Progression-free survival of those with no cfDNA and no CTCs (group A); either detectable cfDNA or CTCs (group B); or both detectable cfDNA and CTCs (group C). Logrank A Vs B *P*=0.145; B Vs C *P*=0.177; A Vs C *P*=0.004

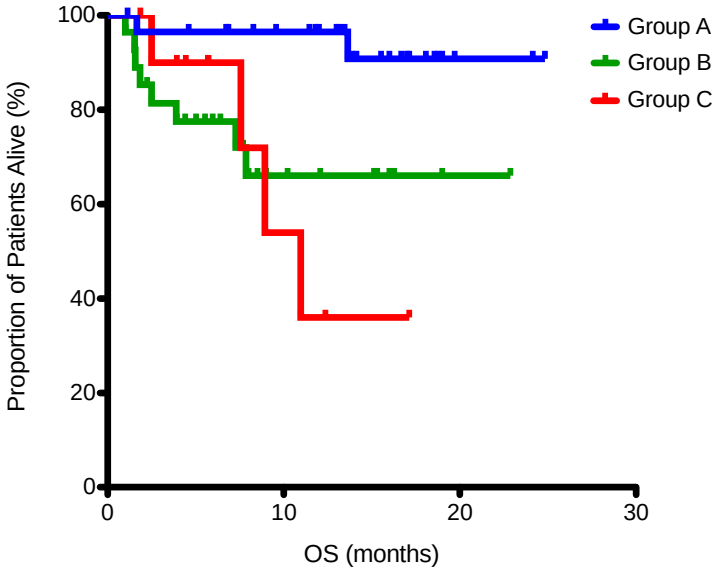

Figure 5.15 Overall survival of those with no cfDNA and no CTCs (group A); either detectable cfDNA or CTCs (group B); or both detectable cfDNA and CTCs (group C). Logrank A Vs B *P*=0.022; B Vs C *P*=0.614; A Vs C *P*=0.001

5.4. Discussion

To date, circulating free-DNA (cfDNA) has not been identified or investigated in patients with NETs yet with these experiments, I have demonstrated that cfDNA is detectable in patients with NETs in both plasma and serum and correlates with numbers of CTCs.

I have shown that when cfDNA is present in plasma of patients with NETs, it is predominantly at a fragment length of 150 base pairs (bp) which is a small fragment length previously found released from necrotic or apoptotic cells[268]. This is approximately the length of single strand of DNA wrapped around a single histone octamer, a length measured by electron microscopy when investigating plasma oligonucleosomes in a study in pancreatic cancer[282].

In certain cases, there were fragments of cfDNA found at multiples of 150bp e.g. 150, 300, 450, 600. These were present in plasma of cases with extreme high counts of CTCs (over approximately 100 per 7.5ml blood). This 'ladder' pattern was also demonstrated in serum. An explanation for higher counts of CTCs in blood being associated with a 'ladder' pattern of cfDNA theory may be that circulating nucleases that break down cfDNA are saturated in this situation resulting in release of excess larger fragments in multiples of 150bp. Why serum has multiple fragment lengths cannot be explained but may be due to the clotting process, which occurs when collecting serum, causing shearing of DNA, perhaps from leucocytes.

However, in a recent study, using qPCR with primers targeting fragments <100bp in colorectal cancer, a significant proportion of cfDNA was found in shorter lengths[366]. This study was the first to suggest that the size distribution profile of cfDNA fragments can be used to distinguish healthy from cancer patients using grouping by <100bp, 150-400bp and >400bp fragments, the latter two groups which I have demonstrated in our sample. However, our methodology may not be as sensitive to detect smaller fragments <100bp. This group showed that fragmentation increased with tumour size and cfDNA concentration. They also demonstrated that non-tumoral cfDNA is less fragmented than cfDNA from tumours.

In our study, although one out of twenty healthy volunteers had a small quantity of cfDNA, patients had much higher concentrations of cfDNA with a greater proportion (25%) having cfDNA present. This is consistent with previous studies demonstrating

greater quantities of cfDNA in patients with other solid cancers when compared to healthy controls.

Quantities of cfDNA in healthy controls ranged from 10 to 30 ng/mL in some of the early studies in the 1970s with radioimmunoassays[156, 263] which are considerably higher than found in our healthy subjects (range 0-3.3 pg/uL equivalent to ng/mL). However these previous studies utilised serum and did not focus on one particular fragment length as I have done. More recent studies utilizing qPCR, spectrometry or fluorometric methods on plasma have found similar concentrations to early studies with a range from 7 to 63 ng/mL[267, 352, 367, 368, 369].

Quantities of cfDNA in patients with other cancers in previous studies range from 41 to 709 ng/mL[157, 267, 305, 368, 369, 370, 371, 372, 373] which is similar to the cfDNA range of 0-660 ng/mL found in our study. When considering specific gastrointestinal cancers in previous experiments, concentrations in metastatic colorectal cancer ranged from 101 to 709 ng/mL[372, 374] and 568 ng/mL in pancreatic adenocarcinoma[375]. Thus I have demonstrated concentrations of cfDNA in NET patients similar to those in other cancers.

However, only 25% of our patient samples had detectable plasma cfDNA despite all having metastatic disease, which is less frequent than the majority of previous studies. A possible explanation is our NET population mainly consisting of performance status 0 or 1 (94%) and few having high-grade tumours (14%). Furthermore, previous studies vary significantly in reported frequencies of detectable cfDNA. In a study of head and neck cancers, 35% had measurable plasma cfDNA[305] using QIamp blood kit (QIagen) for DNA extraction and fluorometry for quantification. Another study in lung cancer found cfDNA in 53% of patients[301] using the same extraction method as our experiments but quantifying with qPCR. Yet, again with the same techniques, all 20 patients in one study with oesophageal cancer had detectable cfDNA[371]. Although direct comparisons are difficult due to heterogeneous populations, the frequency of detectable cfDNA I demonstrated was lower in NETs using this methodology. A possible explanation is the quantification method I employed i.e. microfluidic chips, which have not been reported extensively in the literature for plasma, albeit using the ‘high-sensitivity’ chip (Agilent). The extraction method is unlikely to be a plausible explanation since the Qiamp Blood Kit or Blood Mini Kit (which I use) have been employed by the majority of studies. Other explanations are the delay in centrifugation

after sample collection or the differential storage durations at -80°C resulting in a lower yield over a longer time period as has been shown with foetal cfDNA[350].

Interestingly, the presence of fragments of 150bp in serum was associated with fragments of the same length in plasma. Despite healthy serum producing fragments of multiple lengths, there was no healthy case with a 150bp fragment. The results demonstrating higher levels of this fragment in serum than in plasma and the fact that in some cases, this fragment length was present in serum and not in plasma, could indicate that quantification of cfDNA is more sensitive in serum than plasma. This is consistent with previous studies demonstrating higher yields in serum but this could be at the expense of purity since DNA is released from destroyed leucocytes[352, 353]. Our study and previous studies have focused on total concentration of cfDNA or of fragments of 150 base pairs or longer.

I have demonstrated an association between the presence of cfDNA and presence of CTCs in patients with NETs as has been demonstrated in previous studies in breast and prostate cancer (including methylated cfDNA)[273, 340]. However, not all patients with cfDNA had CTCs and not all patients with CTCs had presence of cfDNA. Certainly, I have shown that concentrations of cfDNA are higher in those with CTCs than those without. If CTCs are validated as being a prognostic marker elsewhere in this thesis, the presence of cfDNA is still not sensitive enough as a surrogate marker for CTCs since only fair concordance was seen between cfDNA and CTCs. This may support the theory of cfDNA originating from CTCs but as mentioned, a few patients without CTCs did have cfDNA. I also looked at the total number of 'CTC events' produced by the CellSearch™ system which includes events that appear (on the semi-automated microscope images) to consist of nuclear material not defined as clear CTCs. These did not correlate with cfDNA and thus I do not have sufficient evidence that this nuclear material relates to cfDNA.

Our preliminary survival data suggests that presence of cfDNA conferred a worse PFS and although not statistically significant, a suggestion of poorer OS. However, this is limited by the few events of progression and death. When combining presence of cfDNA with presence of CTCs, the presence of both conferred a worse PFS and OS than if both were absent. This needs to validated with a longer follow-up period in order to analyse more events but this preliminary data suggests that cfDNA could be combined with CTCs in a prognostic model.

Reliability of an assay is defined as the degree to which the results obtained by a measurement can be replicated and is interchangeable with the terms repeatability and reproducibility. When considering cfDNA as a dichotomous variable i.e. presence of absence, I demonstrate perfect reliability when duplicating DNA extractions from samples, duplicating cfDNA quantification on the microfluidic chips and when repeating measurement on samples stored at -80°C for 6 months.

When considering cfDNA as a continuous variable i.e. cfDNA concentration, although not perfect, reliability was excellent when duplicating measurements on microfluidic chips. There was more variability when replicating DNA extractions from samples and when repeating measurements after storage at -80°C for 6 months but even then, reproducibility was classed as excellent. One limitation of this validation is the few samples without cfDNA in the extraction replicate subgroup.

When looking at intra-subject reliability at two sample points, there was excellent reliability for both presence of cfDNA and as a continuous variable. However, two (of 17) samples had cfDNA at one time-point and none at the other time-point. This may be due to true appearance or disappearance of cfDNA but may also be explained by a sample not being representative of the whole circulation or slight differences in analytical methods on that day. The five patients who had more than two sample time-points confirmed excellent reliability over a longer period but I do not have enough evidence to discuss changes in cfDNA over the natural course of disease without any interventions. I demonstrate good intra-subject reliability but ideally further studies are required to validate quantification of cfDNA with more homogenous, larger samples and stricter time intervals although this would be a difficult study to conduct in this patient group.

Even if presence or quantification of cfDNA is not perfect as a biomarker, the consequence of its detection in NET patients could be significant. Specific mutations in midgut and pancreatic NETs have not been extensively established as in other cancers. However, as recently discovered in sporadic pancreatic NETs, *MEN1* and *DAXX*/*ATRX* mutations[21] could be studied in cfDNA and since associated with a better prognosis, these mutations in cfDNA could be developed as a biomarker. A disadvantage is the heterogeneity of mutations in these genes. Similarly, deletion of *p16/MTS1* in pancreatic NETs could be explored[22]. Important epigenetic alterations implicated in some NETs are suggested by *DAXX/ATRX* mutations, hypermethylation of *RASSF1* in bronchial NETs[376] and *p16* methylation (associated with poor outcome in foregut/midgut

NETs)[377]. Thus, methylation specific PCR could be utilised to detect these epigenetic alterations in cfDNA in the development of future biomarkers.

Overall, presence of cfDNA as a dichotomous variable is highly reproducible with slightly less reliability when measuring concentration of cfDNA. Concentrations of cfDNA are higher in patients with NETs than in healthy controls and higher in serum than in plasma with the predominant fragment length 150bp. However, in serum, and in plasma in patients with high CTC counts, a ‘ladder’ pattern is demonstrated. Yields are lower than in other studies which may be due to methodology differences but these results produce a platform for biomarker development studies utilising cfDNA.

Chapter 6. Circulating Endothelial Cells

6.1. Introduction

Circulating endothelial cells (CECs) were first described almost 40 years ago with techniques including vital light microscopy, Giemsa staining and separation density centrifugation[378, 379], and were identified in various conditions such as smoking, acute myocardial infarction[379]. These older studies identified CECs by morphological criteria only, but current methods isolate CECs using immunomagnetic isolation or fluorescence-activated cells sorting (FACS) based on a defined immunophenotype.

CECs are thought to represent mature endothelial cells that have been shed into the circulation from the vascular cell lining due to vascular damage. In healthy individuals, the endothelial layer is continuously renewed at a low replication rate of 0-1% per day[380]. It is therefore no surprise that in healthy adults, detection of CECs is a rare event with a frequency of about 0-12/ml blood, or a representation of 0.01 to 0.0001% of mononuclear cells. The rarity of these cells creates a challenge for developing detection assays that are sensitive and specific[381, 382, 383]. The size of CECs spans a wide spectrum ranging from multi-nucleated cell conglomerates to single cells and endothelial microparticles but a consensus paper suggests they are greater than 10μm[384].

Immunomagnetic separation isolates endothelial cells from whole blood with paramagnetic particles which have been coated with anti-endothelial antibodies, most commonly CD146[385, 386]. CD146 (also known as melanoma cell adhesion molecule) is involved in cytoskeleton formation and signalling, and is present on endothelial cells, activated T-lymphocytes, mesenchymal stem cells and some malignant cells[387, 388]. Using immunomagnetic separation, whole blood is incubated with antibody-coated magnetic particles. Then, target cells with bound anti-endothelial antibody and magnetic particles are recovered with a magnetic field. CECs can then be enumerated with fluorescent antibodies to other EC markers. To distinguish activated T-lymphocytes from CECs, co-staining with CD45 or CD3 may be of use. CD133 or CD34 may identify endothelial progenitor cells (EPCs) and CD105 can identify activated CECs in cancer patients although agreement of phenotypic differentiation is lacking[389, 390]. The semi-automated immunomagnetic Cellsearch™ platform, designed to detect CTCs, can also be used for CEC detection utilising expression of CD105 (endoglin) to identify

malignant CECs (Figure 6.1). The system characterises CD146+CD105+CD45-DAPI+ cells as CECs, and this approach has been validated by morphology and global gene expression using this platform[391, 392].

CECs have been found to be raised in a variety of conditions including those with widespread vascular damage, infection, vasculitis, and myocardial infarction[161, 381].

Since angiogenesis is crucial for tumour growth and metastasis, there has been interest in CECs associated with cancer and they have been reported to be increased in a number of human malignancies[391, 393, 394]. A number of anti-cancer agents target tumour vasculature including monoclonal antibodies to VEGF and small molecule tyrosine kinase inhibitors . Circulating biomarkers including VEGF, s-VEGFR-2, placenta growth factor, soluble Tie2, E-selectin, and vascular endothelial cadherin have been examined as surrogates of response but none have been clinically validated[395, 396]. Given the importance of targeting tumour vasculature, CECs may be a potential tool to assess drug effect on tumour vasculature.

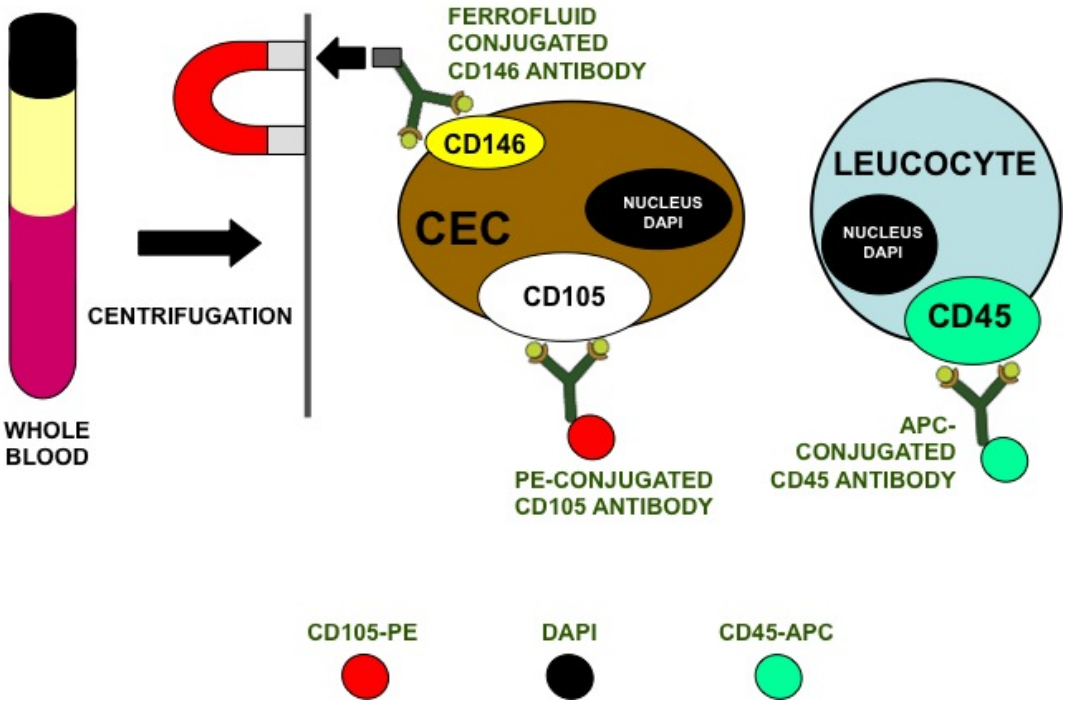

Figure 6.1 Schematic representation of immunomagnetic separation and immunoflurorescent staining employed by the CellSearch platform to enrich CECs from peripheral blood; CEC Circulating Endothelial Cell; CD146 (MelCAM, Melanooma Cell Adhesion Molecule); CD105-PE CD105(Endoglin)-Phycoerythrin; CD-45APC CD-45 Allophyocyan DAPI 4', 6-diamidino-2-phenylindole

Neuroendocrine tumours often produce hypervascular metastases with elevated levels of pro-angiogenic factors, such as VEGF[397] and angiopoeitin-2[134]. Consequently there had been interest in anti-angiogenic therapies for NETs and both Bevacizumab and sorafenib have been investigated in small studies[398] [399] [400]. However recently a large, multicentre, randomised, placebo-controlled trial reported sunitinib improved PFS, OS and response in well differentiated pancreatic NETs[61]. Sunitinib is a multi-targeted tyrosine kinase inhibitor with activity against VEGFR-1, VEGFR-2, VEGFR-3, KIT (stem cell factor [SCF] receptor), platelet derived growth factor receptor (PDGFR-a), and PDGRF-b[401]. With reports that anti-angiogenic therapy may elicit tumour adaptation with heightened invasiveness and increased metastatic potential in animal models of pancreatic NETs and glioblastoma, real-time biomarkers which can reflect the dynamic nature of angiogenesis would be valuable[402].

In earlier chapters, I have demonstrated the utility of CTCs as a biomarker to predict progression and response to therapy in patients with NETs. Similarly, there is a need for clinically effective biomarkers to determine optimal dosing, monitoring response and selecting or stratifying patients most likely to benefit from anti-angiogenic therapy. Given the lack of any data on CECs in NETs, I went onto conduct a pilot study exploring numbers of CECs compared to healthy controls and the relationship with clinicopathological factors.

6.2. Materials and Methods

6.2.1. Patient Recruitment

Patients (n=55) were recruited between July and December 2009 from the Royal Free Hospital. All eligible participants had histologically proven NET and metastatic disease measurable by RECIST criteria. They were categorised according to the site of primary NET: midgut, pancreas, bronchopulmonary, hindgut or of unknown primary. This study was approved by the Local Ethics Committee (Ref 09/H0704/44) and all patients provided written informed consent.

Patients that had undergone chemotherapy, interferon, receptor-targeted radiolabeled therapy, or embolisation within the previous 2 months were excluded.

Data were collected on primary site, duration of diagnoses, any previous treatment received, WHO performance status and whether the primary tumour had been resected. Grade of tumour according to Ki-67 proliferation index was recorded according to ENETs guidelines[23, 24]. Radiological burden was assessed by quantification of hepatic tumour load from 4 to 6 slices of a CT/MRI scan with the most amount of disease by a semi-quantitative approach. Hepatic tumour burden was categorised as 25% or less, more than 25% but 50% or less, more than 50% but 75% or less, or more than 75%.

6.2.2. Healthy Volunteer Recruitment

Healthy volunteers (n=23) were recruited for blood sampling. Volunteers were excluded if they had a current or past cancer, active infection, recent surgery within 1 month, current inflammatory disease or chronic disease (apart from hypertension). All healthy volunteers provided written informed consent.

6.2.3. Circulating Endothelial Cells (CEC) Isolation

Blood samples (4ml) from patients and volunteers were drawn into CellSave tubes (Veridex LLC) containing EDTA and a cellular preservative. Samples were maintained at room temperature and processed within 96 hours using the Cellsearch™ (Veridex LLC) platform for the isolation and enumeration of CECs. The platform consists of a semi-automated system that enriches the sample for cells expressing CD146 by immunomagnetic separation. The system incubates samples with ferrofluids coated with CD146 and labels the cells with the fluorescent nucleic acid DAPI. Fluorescently-

labelled monoclonal antibodies specific for leukocytes (CD45–allophycocyan) and activated endothelial cells in cancer (PE-CD105/endoglin) were used to distinguish CECs from leukocytes.

Briefly, 4mL of blood were mixed with 10mL of buffer, centrifuged at 800 X g for 10 minutes and then placed on the AutoPrep component of the platform. The instrument then added ferrofluids after aspirating the plasma and buffer layer. After incubation and subsequent magnetic separation, unbound cells and remaining plasma were aspirated. The staining reagents were added together with a permeabilisation agent to fluorescently label the immunomagentically labelled cells. After incubation, excess staining reagents were aspirated and magnetic separation repeated. In the final step, cells were resuspended in the MagNest Cell Presentation device (Veridex LLC) which consists of a chamber and two magnets that orient the cells for analysis.

6.2.4. CEC Analysis

The identification and enumeration of CECs on the display unit were performed with the use of the CellSearch™ Analyzer II, a semi-automated fluorescence-based microscopy system that permits computer-generated reconstruction of cellular images in the MagNest Cell Presentation device. All evaluations were performed without knowledge of the clinical status of the patients by 2 independent operators (M.K and T.T.). Out of the total events detected by the platform, CECs were defined as nucleated cells (DAPI+) lacking CD45 and expressing CD105 (Figure 6.2). Any discordant results were reviewed together to reach agreement. The total number of CEC events was also recorded i.e. including all CD146 positive events that the machine recorded despite not meeting the criteria for CEC identification. The total number of CEC events thus included nuclear material (DAPI+) with or without associated CD45, leucocytes and nuclear material with associated CD105.

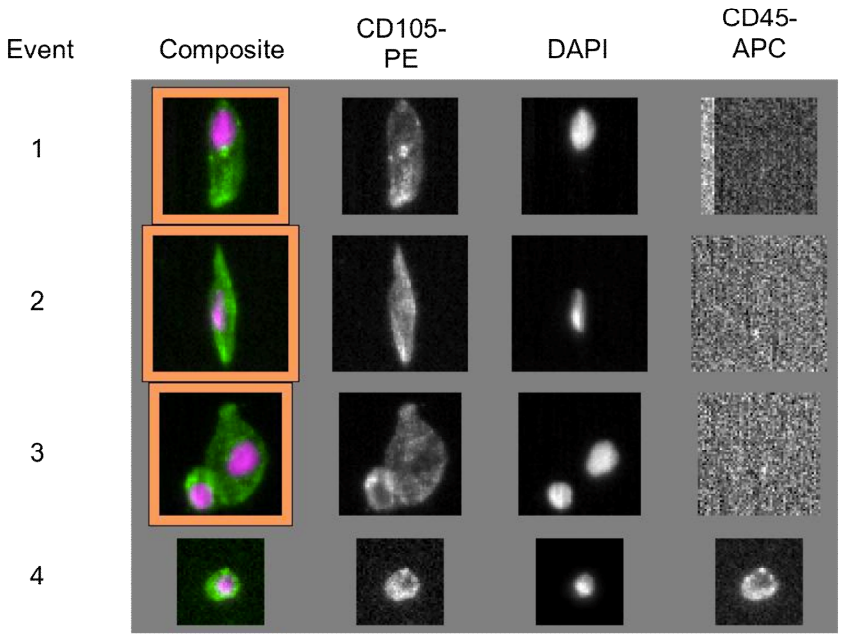

Figure 6.2 Image reconstruction from the CellSearch™ Analyzer with identification of CECs. Each horizontal 'event' is a possible cell or cells focused on by the automated microscope; there are 4 events depicted here. The columns refer to channels where the same event is imaged through different filters to view staining patterns: from right to left, CD45 (APC filter), DAPI (for nuclear staining), CD105 (PE filter) and a composite of all 3 stains. Event number 1 demonstrates a CEC - a nucleus within a CD105 skeleton with absence of CD45 expression. Event 4 represents a leucocyte rather than a CEC as the cell stains for CD45. Events 2 and 3 demonstrate further CECs.

6.2.5. Interobserver Reliability

Since the identification of CECs has not been extensively validated compared to CTCs, the interobserver reliability of enumerating CECs was assessed by comparing the two observers CEC counts.

6.2.6. CTC Isolation and Enumeration

Blood collected in Cellsave tubes (Veridex, LLC) was used for Circulating Tumour Cell (CTC) isolation and enumeration according to methods detailed in a previous chapter.

6.2.7. Statistical Analysis

Differences in gender and age distributions between healthy control and patient samples were assessed by Chi-squared and student t tests. Differences in number of CECs across groups were assessed using Mann-Whitney and Kruskal-Wallis tests. Spearman's rank assessed correlation of CTCs with CECs.

Interobserver reliability was assessed by intraclass correlation coefficient (ICC) and Bland-Altman plots. Kaplan-Meier estimates of survival were based on groups below and above a CEC threshold identified from baseline data. For the purposes of survival analyses, progression-free survival (PFS) or overall survival (OS) was defined as the time between the date when the baseline blood sample was taken and the date of radiological progression, death due to neuroendocrine cancer or last follow-up visit. Survival curves were compared using log-rank testing.

6.3. Results

6.3.1. Healthy Controls

The gender distribution across healthy controls and NET patients is shown in Table 6.1. There was no significant difference in proportion of males or females between controls and patients (χ^2=0.98 p=0.323).

There was also no significant difference in age between healthy controls (mean 53.6, standard deviation 17) and NET patients (mean 58.2 standard deviation 13) (t test, *P*=0.234).

Gender	Healthy Control	Patient	Total
Male	11 (48%)	22 (40%)	33
Female	12 (52%)	33 (60%)	45
Total	23 (100%)	55 (100%)	78

Table 6.1 Gender distribution across healthy controls and NET patients

6.3.2. Background Characteristics of NET Patients

The background characteristics of the NET patient group for CEC evaluation are shown in Table 6.2.

	Pancreatic	Midgut	Broncho-pulmonary	Unknown Primary	All NETs
	n=15	n=27	n=9	n=4	n=55
Age, years mean ± SD	61.5 ± 11.6	62.6 ± 11.4	46.6 ± 10.3	48.3 ± 16.8	58.2 ± 13
Sex					
Male	9	8	5	0	22
Female	6	19	4	4	33
Grade					
Low	9	15	3	1	28
Intermediate	3	10	4	3	20
High	3	2	2	0	7
Burden of Liver metastases					
None	3	0	3	0	6
≤25%	6	8	4	2	20
25%≤50%	5	14	1	2	22
50%≤75%	1	4	1	0	6
>75%	0	1	0	0	1
Duration of diagnosis, median months (range)	53.2 (1-164)	59.6 (1-274)	49.6 (9-283)	35.2 (12-86)	30.3 (1-283)
Performance Status					
0	12	15	4	3	34
1	3	9	5	1	18
2	0	3	0	0	3
3	0	0	0	0	0
4	0	0	0	0	0
Previous treatments					
Resection of primary	2	11	3	0	16
SST	1	11	1	1	14
Chemotherapy	3	2	2	3	10
TAE	1	4	0	1	5
Radionuclides	2	6	1	0	9
Interferon	0	0	0	0	0
Liver resection	1	2	0	0	3

Table 6.2 Background characteristics of patients undergoing CEC evaluation

6.3.3. Circulating Endothelial Cells (CECs) in Patients and Controls

Although there appeared to be a higher number of CECs in NET patients than in healthy controls, there was not strong enough statistical evidence to support this (Mann-Whitney, P=0.056) (Table 6.3). However, the distribution of CECs varied much less in the healthy controls compared to NET patients (standard deviation 12 Vs 148 CECs/4ml respectively). This can be seen in Figure 6.3 with the F-test supporting this (F=25.1 P<0.001). Looking at the distribution, it appears that healthy controls did not have greater than 45 CECs/4ml blood but NET patients did. 31% of NET patients had greater than 45 CECs/4ml and 27% had greater than 50 CECs/4ml. The majority of CECs were irregular or spindle-shaped. However, a minority were small and round (Figure 6.4).

	Controls	**Patients**
n	23	55
CECs		
Mean ± SD	27 ± 12	66 ±148
95% CI	12-82	26-106
Median, range	30, 5-44	23, 0-985
Interquartile range	15	38
No. ≥ 45	0/23 (0%)	17/55 (31%)
No. ≥ 50	0/23 (0%)	15/55 (27%)
Total CEC Events		
Median	349	432
Mean ± SD	504 ± 388	534 ± 752
95% CI	336-672	331-738

Table 6.3 Statistics of CECs and total CEC events in healthy controls and NET Patients

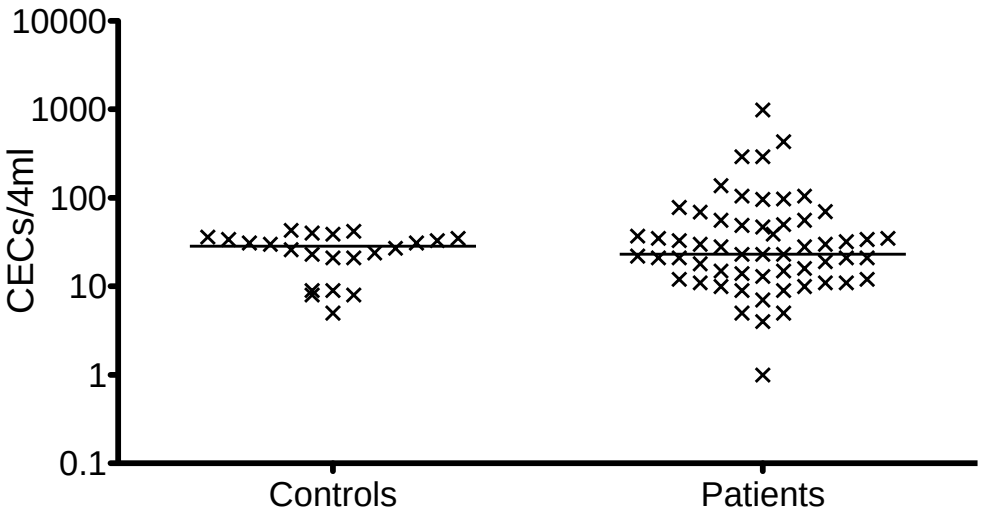

Figure 6.3 Number of CECs in healthy controls and NET patients (horizontal line representing median)

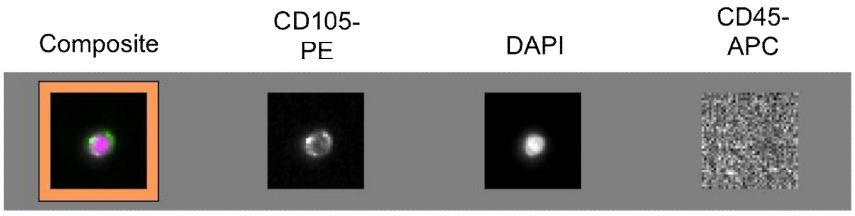

Figure 6.4 Image reconstruction from the CellSearch™ Analyzer with identification of one CEC which appears small and round compared to the 'normal' phenotype seen in

Figure 6.2

6.3.4. Total CEC Events

The total number of CEC events, including those that did not meet the definition criteria as stated in the methods, was recorded. There was no significant difference in these total events between healthy controls and NET patients (Mann-Whitney *P*=0.926) (Table 6.3) (Figure 6.5). Thus the total number of CD146 events detected by the platform did not appear to have any clinical relevance.

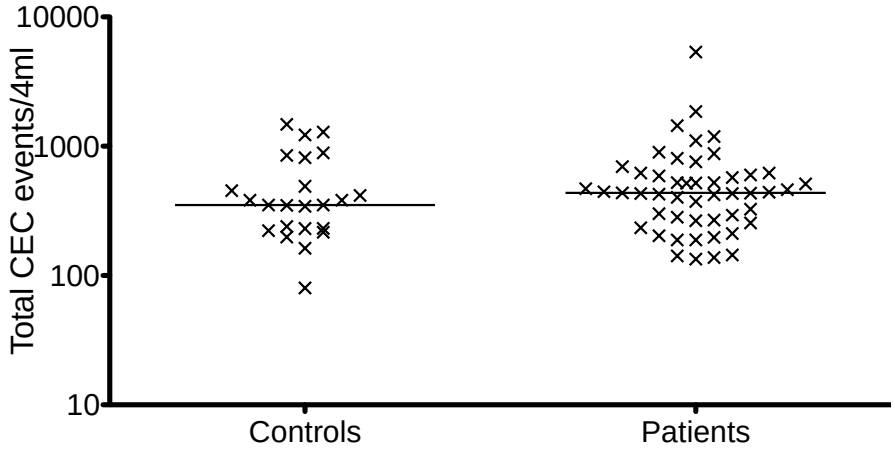

Figure 6.5 Total CEC events in healthy controls and NET patients

6.3.5. CECs Across Different Primary Tumours

The number of CECs across different primary NET types is shown in Figure 6.6. Using Kruskal-Wallis test for variance, the number of CECs was not different between tumour types (*P*=0.051). Although statistical significance was almost reached, the number of CECs was not dependent on tumour primary.

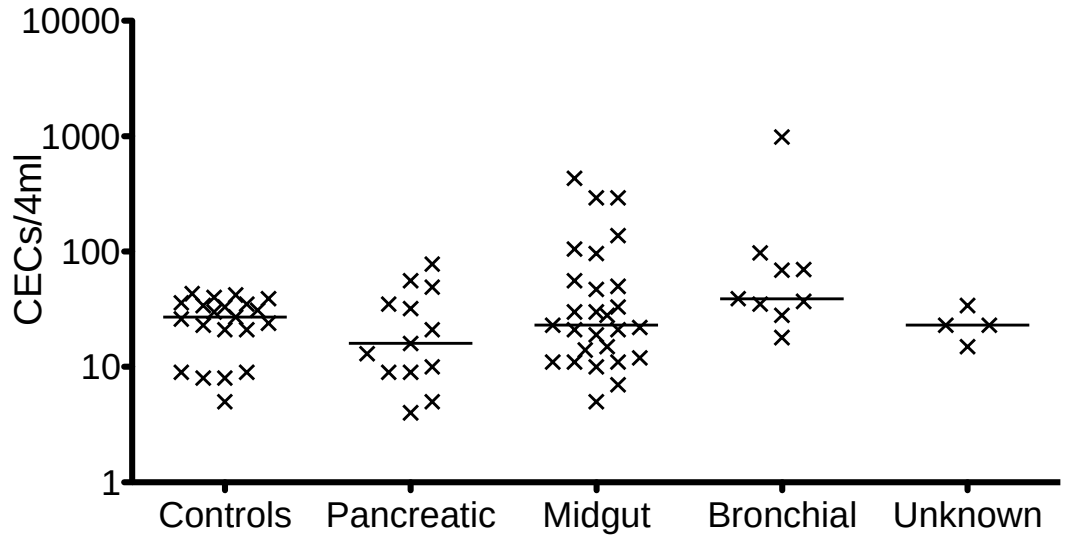

Figure 6.6 Number of CECs according to primary NET

6.3.6. Relationship with CTCs

A scatterplot demonstrating relationship between CECs and CTCs is shown in Figure 6.7. There was no significant correlation between CECs and CTCs (Spearman's ρ=0.029 *P*=0.855). When dichotomised, there was no association with presence of CTCs and number of CECs (*P*=0.398). There was also no correlation between total CEC events and CTCs (Spearman's ρ=0.6 *P*=0.208).

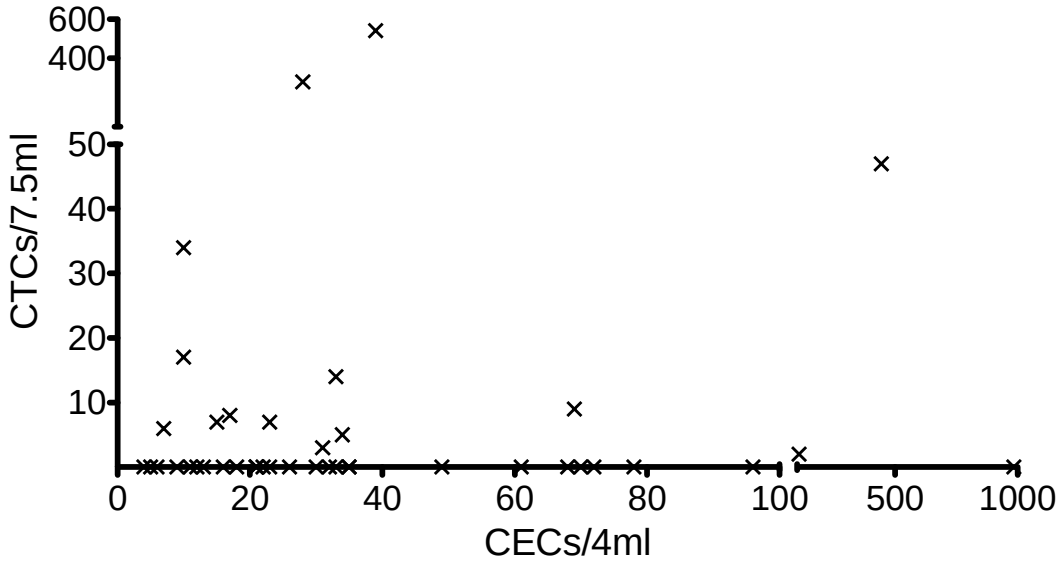

Figure 6.7 Relationship between CECs and CTCs

6.3.7. Relationship of CECs with Clinical Parameters

Liver metastases burden groups 25-50%, 50-75% and >75% were combined due to small numbers so that the group with <25% was compared with that ≥25%. Grades 2 and 3 were also combined for the same reason and so too were performance statuses 2, 3 and 4. There was no association between tumour burden and CECs (Mann-Whitney *P*=0.278) (Figure 6.8). nor between grade of tumour and CECs (Mann-Whitney *P*=0.079) (Figure 6.9). There was also no association between performance status – PS 0-1 vs PS 2-4 – and CECs (Mann-Whitney *P*=0.444).

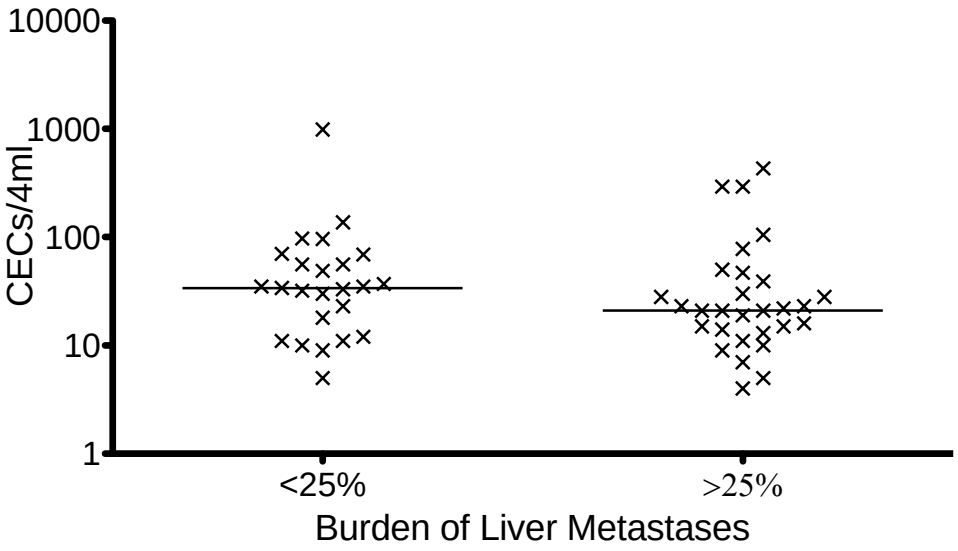

Figure 6.8 Relationship between burden of liver metastases and number of CECs

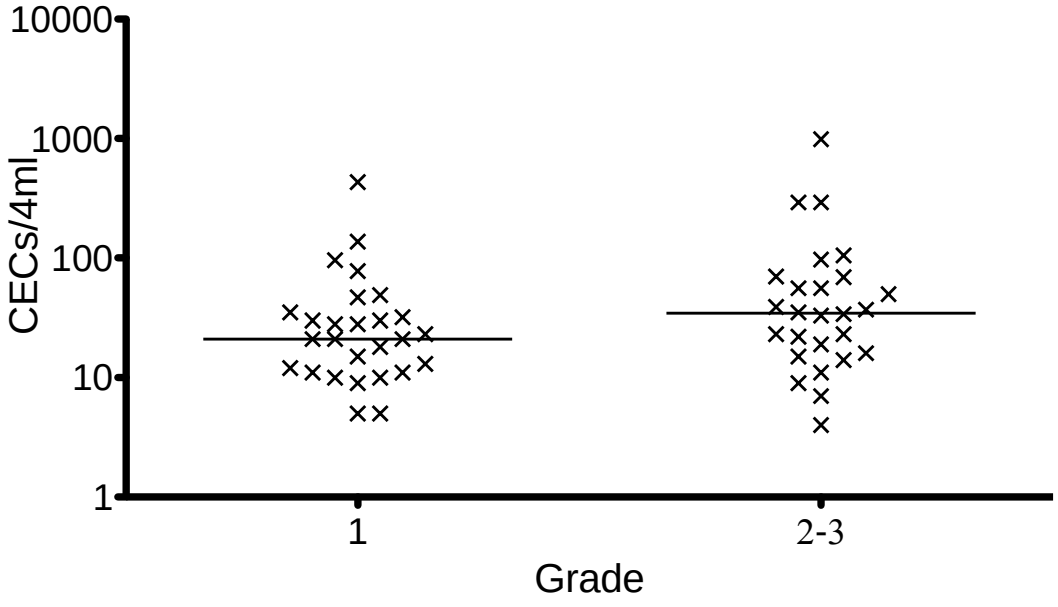

Figure 6.9 Relationship between grade of NET and number of CEcs

6.3.8. Interobserver Reliability

There was excellent correlation between the CECs counts assessed by observer A and observer B (r=0.99 *P*<0.001) (Figure 6.10).

A better method of assessing interobserver agreement is the Bland-Altman plot shown in Figure 6.11. The interobserver difference was generally low. It appeared to be larger on higher counts. The 95% limits of agreement were estimated from -11 to 8 CECs. The intraclass correlation coefficient was 0.999 (*P*<0.001) indicating excellent agreement.

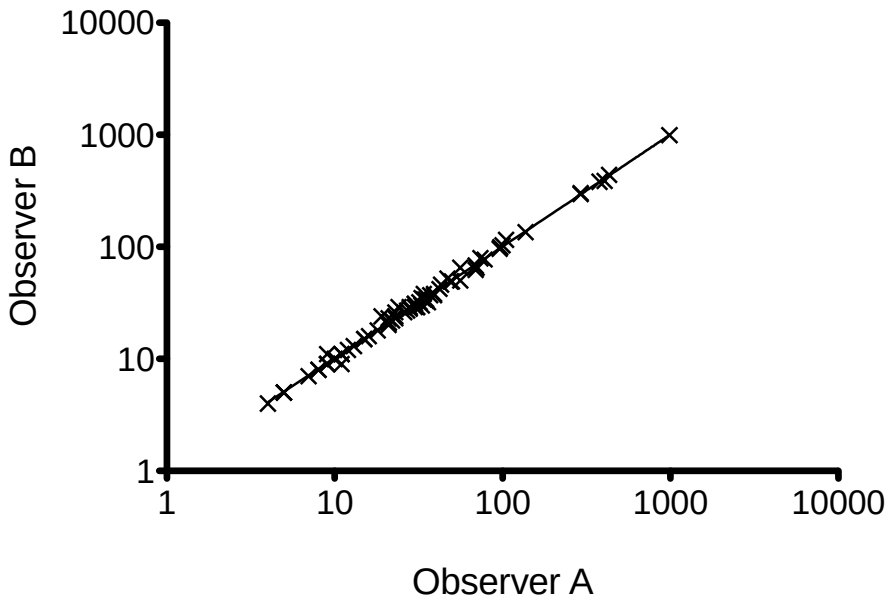

Figure 6.10 Association between observer A and observer B CEC counts

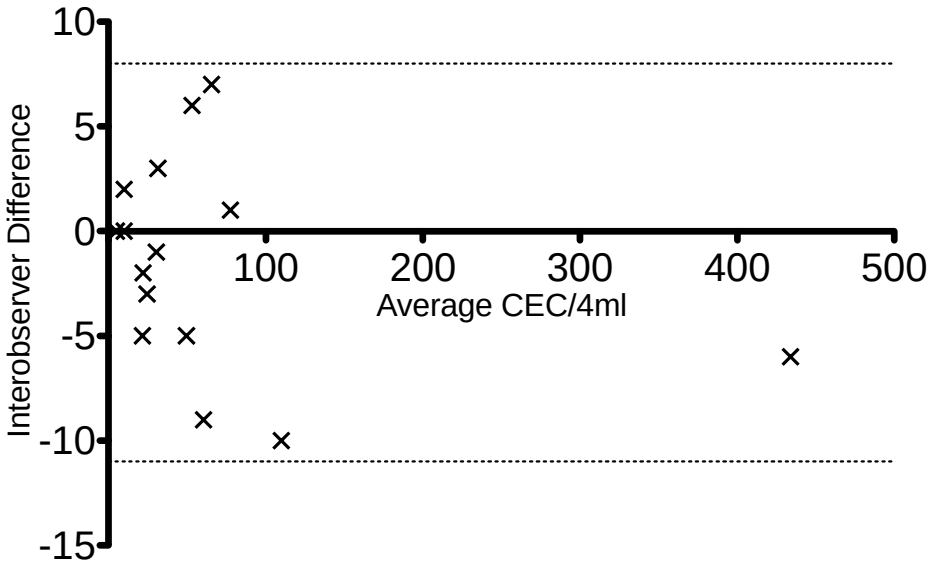

Figure 6.11 Bland-Altman plot of the average CEC count between observers plotted against the interobserver difference (dashed lines represent 95% limits of agreement)

6.3.9. CECs and Survival

Patients were grouped in to those above and below the threshold of 45 CECs/4ml blood defined above. Kaplan-Meier survival curves demonstrating PFS and OS according to these groups are shown in Figure 6.12. Median follow-up was 20 months (range 5-25). Although it appears that patients with CEC counts over 45 had a worse PFS and OS, this did not reach statistical significance (*P*=0.300, *P*=0.148 respectively).

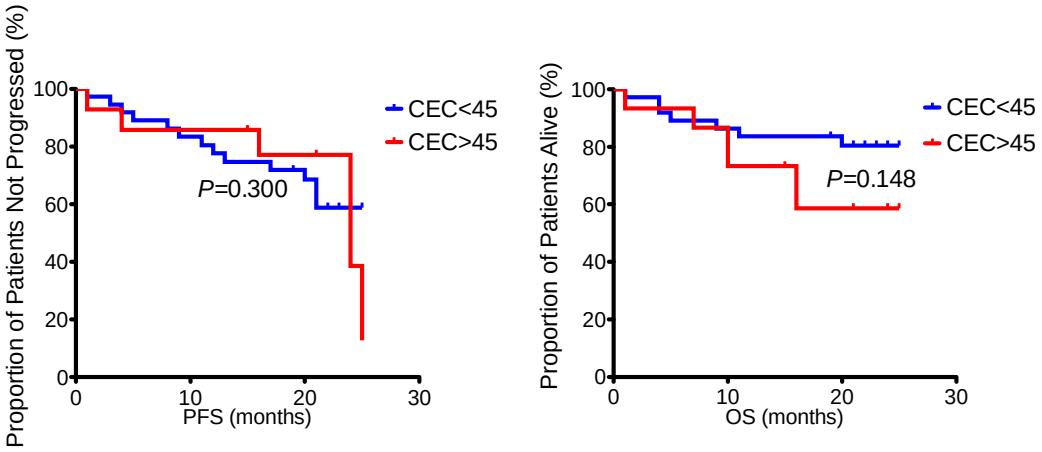

Figure 6.12 Survival curves of patients grouped by below and above the threshold 45 CECs/4ml blood defined from earlier data

6.4. Discussion

My data failed to show that CECs were increased compared to healthy controls which is in contradiction to other studies. CECs have been found to be increased in a number of human malignancies including head and neck, prostate, colorectal, breast, renal cell, gastric and oesophageal cancers[391, 393, 394, 403]. For example, in breast cancer and lymphoma, CECs, measured by flow cytometry, are increased 5-fold and correlate with plasma vascular endothelial growth factor (VEGF)[404].

CECs have also been found to be raised in conditions with vascular damage such as rickettsial infection, sickle cell anaemia or vasculitis[381]. Elevated CEC numbers have been detected in vascular conditions including acute coronary syndrome[161], cardiac failure[405], ischaemic stroke[406], pulmonary hypertension[407]. Additionally, pathogens such as cytomegalovirus (CMV) have been identified in CECs[408]. In patients with renal transplantation, highest CEC numbers were seen in those with acute vascular rejection[409]. In order to limit this effect of non-cancer pathology in our pathology, healthy controls were recruited with exclusion of chronic or vascular conditions such as these. However I acknowledge that co-existing vascular pathology in the patient group may have had an effect on CEC numbers.

Our numbers of CECs in healthy controls are comparable to other studies using the CellSearch platform™. We demonstrate that CECs ranged from 0 to 45 CECs per 4ml blood. When expressed differently, this is 0-11 cells/ml, similar to the 1-20 cells/ml found in one study using the same platform[391]. Immunomagnetic capture and density centrifugation techniques tend to show values of 1-20 cells/ml but numbers yielded by flow cytometry are greater (up to 1000-fold increase)[404, 410, 411]. The reasons for this variation are unknown but are probably methodological (choices of cell surface markers or technical gating) and a consensus of CEC definition is required which is being addressed by a European collaborative group.

My data did not show elevated CEC numbers in cases which may be particular to NETs compared to other cancers. However, a false negative result may have arisen given the relatively small numbers in the control sample or the heterogeneous nature of the NET sample with inevitable varying levels of tumour vascularity. Another reason for conflicting with previous studies is the non-standard methods of CEC isolation and identification throughout the literature. However, this was limited by using a semi-automated platform with generally accepted antibodies. I also addressed this limitation

by using two independent observers to identify CECs from events obtained by the automated microscope based on staining pattern and intact cells, regardless of morphology. The interobserver reliability was excellent with, expected, higher variability at lower CEC counts.

However, when looking at the spread of the data, numbers of CECs were widely spread with a standard deviation of 148 compared to 12 of healthy controls with standard deviation. This is statistically significant when using the F-test and an interesting finding. The range of CECs in healthy controls was narrow with a cut-off identified of 45 CECs/4ml which suggests a reference range may be possible but requires further validation in larger studies. Although levels of CECs were not higher in NETs, this variation suggests that CECs may reflect underlying tumour biology, perhaps a reflection of angiogenesis. Varying CEC levels add support to the vast heterogeneity of NETs, even within metastatic NETs, as suggested by published and unpublished genetic studies [21]

Considering the variation in CECs in NETs, there was no difference in levels across different primary tumours. However, I acknowledge the small numbers and limitations of this subgroup analysis. There was also no relationship with grade, performance status or tumour burden. This may be due to an insufficient sample size to detect these. The latter contradicts one study which looked at CECs isolated by the CellSearch platform[412]. This group found the number of CECs was found to correlate with tumour size and also revealed a dose-dependent decrease with anti-angiogenic agents, bevacizumab and cedirinab[412]. This group also characterised apoptosis in CECs with the addition of the terminal deoxynucleotodyl transferase dUTP nick end labelling (TUNEL) assay on the CellSearch™ platform and found that round CECs were apoptotic or deactivated CECs. There was a 3-fold induction in CEC apoptosis and inhibition of VEGFR2 in CTCs with the anti-angiogenic agents at 24-hours with no change in number of CTCs.

Similarly, in my study, there was no relationship between CECs and CTCs which suggest that CECs may not reflect the metastatic potential and radiological progression that CTCs represent. Interestingly, in a recent study, NET tissue expression of endoglin (CD105) was shown to be increased compared to normal tissue and associated with tumour burden and presence of neuroendocrine metastases[413]. CD105 is a co-receptor for TGF-β1 which is a multifunctional cytokine involved in numerous physiological and pathological processes. Due to its principal expression on endothelial cells of newly

formed blood vessels, studies suggest that CD105 is a specific marker of neovascularistaion in cancer[414, 415]. The expression of CD146 in NETs is unknown but given the similarity between melanoma (which strongly express CD146) and NETs, it may be that some of the CECs identified, especially the unusually shaped small and round CECs, were actually neuroendocrine CTCs, bearing a similar morphology too. Further studies investigating these markers in NETs are required.

I also looked at the total number of events (which were CD146+) obtained by the automated microscope on the platform. This added no further information as there was no relationship with CTCs or elevation in NETs. This is not surprising as contaminating leukocytes would be included into this group.

When using the cut-off of 45 CECs/4ml identified about, those NET patients with CECs above this cut-off appeared to have worse overall survival but this did not reach statistical significance. Baseline CEC may be prognostic with low CEC levels associated with better outcome in several studies in colorectal and breast cancer[416, 417, 418, 419]. However, other studies using the Cellsearch™ platform suggested baseline CECs were not prognostic in breast and prostate cancer[393, 420, 421]. With a longer follow up period and larger homogenous sample, this potential as a prognostic marker could be explored in a future study in NETs.

The clinical significance of CECs in cancer is poorly understood and it is unsure whether they are markers of altered vascular integrity or contributors to the neoplastic process. However, there is limited data on their use as biomarkers. There are a limited number of studies studying the change of CEC numbers in response to treatment and survival. In patients with prostate cancer treated with docetaxel-based regimens, an early increase of CECs was associated with worse overall survival especially when combined with CTC numbers. Another supportive study at the same time suggested an increase in CECs at seven days predicts survival and response in cancer patients treated with various chemotherapeutics[422]. A number of studies have studied outcome after anti-angiogenic therapy with increase of CEC numbers associated with clinical benefit, specifically in renal cancer[423], gastrointestinal stromal tumour (GIST)[424], and breast cancer[420]. Other studies have noted an increase in mature CECs in response to anti-angiogenic agents[425]. However, in contrast, other studies showed an increase in CECs was associated with worse clinical outcome in colorectal cancer and glioblastoma[426, 427]. Several studies have shown correlation between changes in CECs and objective response to treatment such as RECIST[419, 428, 429]. These

discrepancies may be accounted for by differences in timing of CEC detection, inclusion of apoptotic CECs, different assays and different treatments. Other uses of CECs include as biomarkers for optimal biological drug dosage[430] and identification of new novel tumour-associated endothelial markers for potential therapeutic targets[431].

My study is hypothesis-generating but this is the first systematic evaluation of CECs in NETs. We have focussed on patients with advanced, metastatic disease to make the group more homogenous. Although 3 patients in a small study had ‘carcinoid’, the sample was very small and grouped with large numbers of other cancers without any details of primary site[403]. Although not elevated in significant numbers compared to controls, there is considerable variability in CEC numbers in patients with NETs which need to be explored. Initially, validation studies similar to that conducted with CTCs need to be performed, looking at intra-sample and temporal variation of CECs in order to calculate a coefficient of variation. The significance of CECs in NETs needs to be explored by studying relationships with circulating level of angiogenic factors including VEGF, VEGFR, angiopoeitin; with vascularity on imaging; and with microvessl density on histopathological samples. Apoptotic markers on CECs may be useful, once validated, to identify response to anti-angiogenic agents. Finally, the use of CECs as a prognostic and predictive biomarker needs to be explored in large prospective studies in homogenous groups, especially in patients undergoing treatment with sunitinib.

Chapter 7. Summary and Future Directions

NETs are heterogeneous malignancies with diverse biology ranging from indolent to highly aggressive cancers. With the varied survival and increasing number of treatment options available, there is a requirement for biomarkers to predict prognosis and to predict outcome with therapy.

The aim of this thesis was to look at the current state of biomarkers in NETs and to explore new biomarkers in the circulation of patients with NETs: CTCs, CECs, and cfDNA. In chapter 1, I concluded that despite plasma CgA and urinary 5-HIAA being accepted as established blood biomarkers, there are a lack of prospective studies systematically investigating their prognostic and predictive value.

In chapter 2, I investigated the established histopathological biomarker, Ki-67 proliferation index, which forms part of a number of international NET clinical guidelines. ENETS propose a three-tiered grading system separate from their TNM staging classification. This stipulates that either Ki-67 or mitotic count on NET tissue can be used to assign grade but there is no evidence to suggest that these indices are equivalent in their prognostic value. In a series of 131 metastatic pancreatic and 136 metastatic midgut NETs, I demonstrated a discordance of 44% and 38% respectively when assigning grade using Ki-67 or mitotic count. This may have important implications if one index is used over the other, since treatment decisions are often based on grade. On univariate and multivariate analyses, grade according to Ki-67, but not mitotic index, was able to determine three prognostically different groups in both pancreatic and midgut NETs. The prognostic value of Ki67 was not improved if tertiles were used as cut-offs, but was improved when the low-grade threshold was raised to a Ki-67 of 5% (from 3%). Since Ki-67 index and mitotic count are both measures of proliferation, and I have demonstrated that Ki67 index is a superior prognostic marker, the additional value of mitotic index is questioned. I conclude that the international guidelines in NETs need to be changed so grade is assigned using Ki-67 alone and not mitotic count.

In chapter 3, EpCAM expression was investigated in a series of 74 NET tissue samples. All midgut and pancreatic NETs demonstrated strong EpCAM expression, which implies that NETs could be epithelial in origin, adding evidence against NETs

originating from the neural crest. With EpCAM directed therapy being investigated in other cancers, this also opens up this targeting area to NETs.

NET EpCAM expression allowed me to investigate whether it would be possible to isolate CTCs from blood of patients with NETs, using the semi-automated Cellsearch™ platform, which uses an EpCAM-based immunomagnetic separation technique. In 175 patients prospectively recruited with metastatic NETs, CTCs were clearly identifiable in blood. Although there is no 'gold standard' technique for CTC isolation, the Cellsearch™ platform has been extensively validated, approved by the FDA, and hence I did not recruit healthy controls. One or more CTCs were present (in 7.5ml blood) in 51% of midgut and 36% of pancreatic NETs with 42% of cases having 2 or more CTCs. The frequency and levels of CTCs in metastatic NETs was similar to that in metastatic breast and prostate cancers and may pave the way for more studies investigating CTCs in NETs.

Having proven the existence of CTCs in NETs, in chapter 4, I demonstrated that the presence of one or more CTCs was associated with progressive disease (defined by RECIST 1.1) in a pilot dataset of 63 patients with metastatic NETs. Consequently, I went on to investigate CTCs as prognostic and predictive biomarkers by prospectively recruiting 138 patients with metastatic NETs who were about to commence a new treatment. This is one of the largest prospective biomarker studies in this tumour type. Using a validation and training set, I identified a cut-off of one CTC as the optimal threshold. A baseline of one or more CTCs was a poor prognostic factor in terms of PFS and OS.

In the prospective study in chapter 4, grade according to Ki-67 was also a prognostic factor confirming retrospective findings from chapter 2. However, on multivariate analysis, only the G3 stratum was a significant factor, which constitutes a very small proportion of NETs. Importantly, when studying a large subgroup of G1 and G2 tumours where decisions on when and how to treat are difficult, CTCs were of more prognostic value than grade.

Importantly, a change in CTCs at 3 to 5 weeks after commencing therapy was predictive of response to treatment, PFS and OS. Using CTCs as an early marker of response may provide an opportunity to stop or change treatment sooner in those that are not benefiting. However, the validity of making treatment decisions based on CTCs needs

to be investigated in prospective studies, in more homogenous groups in terms of tumour type and treatment. Such trials are currently being conducted in breast cancer.

Given the varied survival of patients with NETs, grade may be based on a small sample of tumour mass taken many years ago and may not reflect current tumour biology. Therefore, grade as a 'biomarker' has limitations and biomarkers that can be repeated in a relatively non-invasive manner, such as from blood, may better reflect current tumour biology and can be used dynamically. With this in mind, although grade at the time of diagnosis provides some prognostic information, CTCs have a dynamic advantage over histological grade as they can be repeated at various time-points throughout what can be a variable time course. Baseline CTCs, especially in G1 and G2 NETs, could be used be used to stratify those patients who should have more aggressive or earlier therapy. However, prospective studies utilising baseline CTCs in making treatment decisions are required.

Notably, in chapter 4, CTCs were also of more prognostic value than baseline CgA, a generally accepted biomarker. Using CgA to monitor response to therapy was also not of value. This suggests the established biomarker, CgA, when subjected to a prospective study, does not provide as much as much information as consensus suggests.

Having characterised CTCs as neuroendocrine in origin using additional markers, synaptophysin and CD56, further protein, genetic, and epigenetic investigation of CTCs may also facilitate the understanding of the process of metastases and tumour biology of NETs, reflecting the term 'liquid biopsy' given to CTCs. However, due to their infrequency, sensitive single cell technology is required and other isolation techniques which increase the CTC yield may be better suited for this purpose than the Cellsearch™ platform. Given the long survival of some patients with NETs, changes in CTCs could also be used as a surrogate outcome in order to facilitate more rapid drug development.

Another potential circulating biomarker, cfDNA, was evaluated in chapter 5. For the first time, I demonstrated cfDNA in plasma and serum in a series 88 patients with NETs. Interestingly, the predominant fragment length was 150 base pairs approximately the length of DNA associated with oligonucleosomes, which are found in apoptotic cells. In certain cases, especially those with large quantities of cfDNA, a 'ladder' of

cfDNA was observed, indicating fragmentation, the significance of which is uncertain but could include circulating nucleases or shearing during processing.

Consistent with other studies, I demonstrated a higher concentration of cfDNA in patients with cancer (0-660 ng/mL) than in healthy controls (0-3.3ng/mL). However, only 25% of the patient samples had detectable cfDNA which may be explained by indolence of the tumour type or sample processing. I demonstrated that there is an association between cfDNA and CTCs and that the presence of cfDNA conferred a worse survival but not statistically significant. Given that 75% of cases did not have detectable cfDNA and the requirement statistically to dichotomise cfDNA as a variable, cfDNA may not be as useful as CTCs as a prognostic or predictive biomarker. However, large prospective studies are required. If the yield of cfDNA is increased with more sensitive and reliable technology, by analysing the primary tumour from the patient it may be possible to identify genetic mutations or epigenetic signatures of NETs in cfDNA. This may provide a 'tailored' biomarker, repeatable throughout the course of the patient's disease course, offering personalised therapy.

In chapter 5, I demonstrated the presence of CECs in a series of 55 patients with NETs using the same platform for CTC isolation and enumeration. Although not significantly elevated in NETs compared to healthy controls, there was significantly increased variation in numbers and a wider range of CECs in NETs. No definite conclusions can be derived from this but it suggests that CECs can vary in NETs and may reflect some underlying angiogenic process. Although no correlation with CTCs were found, given that CD105 is expressed in NETs, it may be that some of these CECs are actually CTCs. I did not demonstrate any correlation between CECs and tumour burden or survival. However, further studies are required, investigating intra-sample and temporal variation, relationship with circulating and histological markers of angiogenesis, and changes with anti-angiogenic therapy. CECs may offer a different facet in the circulating biomarker field to CTCs given recent anti-angiogenic therapies studied in NETs.

The population studied all had metastatic disease since this makes up the majority of clinical practice and gives a less heterogeneous group. However, these biomarkers should be studied in patients with localised disease undergoing therapy, resection or surveillance in order to compare their concentrations/frequencies to levels demonstrated in this thesis.

In conclusion, the incidence and prevalence of NETs is increasing. Given the increasing number of treatment options available and prolonged survival, it is unclear what treatments to offer, in which patients, and in which order. Grade according to Ki-67, but not mitotic count, certainly offers some prognostic information at the time of diagnosis. However, my research suggests that circulating biomarkers, specifically CTCs, offer additional and better prognostic and predictive information and provide the opportunity for sequential monitoring.

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