THE ROLE OF THE SYSTEMIC INFLAMMATORY RESPONSE, THE JAK STAT PATHWAY AND THE MAPK PATHWAY IN THE PROGNOSIS OF RESECTABLE PANCREATIC CANCER

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ABSTRACT

Pancreatic cancer is a devastating disease with a five year survival of only 2-3%. Only 10-15% of patients have resectable disease at presentation and the only potential cure is major surgery with adjuvant chemotherapy. The outcomes of surgery are disappointing with a median survival of only15-17 months and operative mortality and morbidity figures of 5-10% and 40% respectively. This abysmal prognosis is likely due to the highly aggressive nature of the tumour, its resistance to adjuvant therapy, its late presentation and the likely presence of micro-metastases not detectable at staging or surgery.

A pre-operative systemic inflammatory response (as measured by CRP) is known to be associated with a poor prognosis in a number of cancers including pancreatic cancer. The reasons behind this poor prognosis are not yet known. The main driver of plasma CRP levels is the cytokine IL-6, known to be elevated in the plasma of patients with pancreatic cancer. This thesis hypothesises that upregulation of two IL-6-dependent pathways, the JAK STAT and MAPK pathways is responsible for the poor prognosis associated with an inflammatory response in pancreatic cancer. Both of these pathways are known to be involved in cellular growth, differentiation and apoptosis and when activated they may confer a growth or survival advantage to tumour cells. The aims of this thesis were to establish the prognostic role of a systemic inflammatory response in resectable pancreatic cancer in both a retrospective and prospective cohort and establish whether increased protein expression in either the JAK STAT or MAPK pathways is associated with a poor prognosis in the same retrospective cohort.

A retrospective database of 148 patients who had undergone Whipple resection for either pancreatic cancer (PC) or non-pancreatic peri-ampullary cancer (NPPC) was created with pre-operative CRP values and survival data. The author then created tissue micro-arrays (TMA's) with both tumour and normal pancreatic duct tissue from each of the 148 patients in the retrospective cohort and carried out immunohistochemistry on 12 antibodies known to be crucial in IL-6 signalling (6 in the JAK STAT and 6 in the MAPK pathways). Following staining the author scored each of the antibodies using the weighted histoscore to allow analysis of antigen expression. During the period of research the author also created a prospective database of 36 patients who underwent surgery for either PC or NPPC. Plasma was stored pre-operatively from each of the patients and this was later thawed and using an ELISA kit another research fellow (JL) was able to establish plasma levels of IL-6 in the prospective cohort.

On univariate analysis a raised pre-operative CRP was associated with poorer survival, 374 days versus 618 days (p=0.0001) in the retrospective PC group only. On multivariate analysis, only pre-operative CRP retained statistical significance amongst those factors shown to be significant on univariate analysis (P=0.009). In the prospective group, patients with low levels of IL-6 had a median survival of 799 days, against a median survival of 537 days in those with high plasma IL-6 levels (P=0.002) when all 36 patients were analysed together. On analysis of protein expression, no significant relationship between increased expression and poor survival was seen for any of the 12 proteins analysed.

The results from this thesis confirm that a pre-operative inflammatory response is associated with poor survival in patients with resectable pancreatic cancer. Raised

plasma levels of IL-6 are also associated with poorer survival in similar patients. However, the poor prognosis appears to be via a JAK STAT/ MAPK independent mechanism. Other possible explanations for this poor prognosis including the connection between inflammation and cachexia and other important inflammatory proteins such as NF-κB and SOCS are explored in the discussion of this thesis.

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AUTHOR'S DECLARATION

The work presented in this thesis was performed entirely by the author except as acknowledged. This thesis has not been previously submitted for a degree or diploma at this or any other institution.

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DEFINITIONS

APR	Acute Phase Response
BMI	Body Mass Index
CA 19-9	Carbohydrate Antigen 19-9
CEA	Carcinoembryonic Antigen
COX	Cyclo-oxygenase
CP	Chronic Pancreatitis
CRP	C-Reactive Protein
CT	Computed Tomography
DAB	Diaminobenzidine
ELISA	Enzyme Linked Immuno Sorbent Assay
ERCP	Endoscopic Retrograde Cholangio Pancreatography
ERK	Extra-cellular Regulated Kinase
ESR	Erythrocyte Sedimentation Rate
EUS	Endoscopic Ultrasound
FAMMM	Familial Atypical Multiple Mole Melanoma
FAP	Familial Adenomatous Polyposis
FISH	Fluorescent In-Situ Hybridisation
FNA	Fine Needle Aspiration
GPS	Glasgow Prognostic Score
HCC	Hepato-Cellular Carcinoma
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HP	Hereditary Pancreatitis
ICCC	Intra-Class Correlation Coefficient
IHC	Immunohistochemistry

IL-6	Interleukin-6
IL-6R	Interleukin-6 Receptor
IPMN	Intra-ductal Papillary Mucinous Neoplasia
JAK	Janus Kinase
LN	Lymph Node
LUS	Laparoscopic Ultrasound
MAPK	Mitogen Acivated Protein Kinase
MM	Multiple Myeloma
MRI	Magnetic Resonance Imaging
NF-κB	Nuclear Factor Kappa B
NO	Nitric Oxide
NPPC	Non-Pancreatic Peri-ampullary Cancer
NSAID	Non-Steroidal Anti-Inflammatory Drug
NSCLC	Non-Small Cell Lung Cancer
PanIN	Pancreatic Intra-epithelial Neoplasia
PC	Pancreatic Cancer
PD	Pancreatico-Duodenectomy
PDGF	Platelet Derived Growth Factor
PET	Positron Emission Tomography
PBMC	Peripheral Blood Mono-nuclear Cell
REE	Resting Energy Expenditure
RM	Resection Margin
RT-PCR	Reverse Transcription-Polymerase chain Reaction
Ser	Serine
SIRS	Systemic Inflammatory Response Syndrome
SOCS	Suppressor Of Cytokine Signalling

TAM	Tumour Associated Macrophage
TBS	Triphosphate Buffered Saline
TMA	Tissue Micro-Array
TNF	Tumour Necrosis Factor
Tyr	Tyrosine
US	Ultrasound
VEGF	Vascular Endothelial Growth Factor

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1.0 INTRODUCTION

1.1 Pancreatic Cancer

1.1.1 Epidemiology

In the year 2000 pancreatic cancer was the tenth most common cancer in the UK, accounting for 3% of the total cancer burden with nearly 7000 registered cases[1]. Over the last thirty years the incidence of pancreatic cancer in UK men appears to be falling, from just over 12 cases per 100,000 in 1971 to 10 in 2001. The rate in women appears to be static at around 7.5 cases per 100,000.

In 2002 pancreatic cancer was the sixth most common cause of cancer death, behind lung, bowel, breast, prostate and oesophageal cancer. It is a devastating disease with figures from 1996-1999 in the UK showing a 5 year survival of only 2% in men and 3% in women, the worst in both sexes.

The reason for this abysmal prognosis is the late presentation of pancreatic cancer, coupled with the highly aggressive nature of the tumour and its resistance to extant treatments. The only potential cure is radical surgery combined with chemotherapy which can only be performed for localised disease[2]. Figures from 2003 in the USA show that only 8% of patients presenting with pancreatic cancer had localised disease. This compared with 15%, 36%, 60%, 83% and 25% in lung, bowel, breast, prostate and oesophageal cancers respectively[3].

1.1.2 Pathology

1.1.2.1 Tumour Types

The terminology surrounding pancreatic cancer is often confused and it is important to establish it correctly early in this thesis. The term pancreatic cancer means a tumour arising from the pancreatic ductal tissue and from this point whenever the term pancreatic cancer is used it is referring only to tumours arising from pancreatic ducts. The term peri-ampullary cancer encompasses a number of different tumours that arise at, or near to the Ampulla of Vater. This term includes pancreatic cancer (cancer arising from the pancreatic ducts in the head of the pancreas only), Ampullary adenocarcinoma (arising from the Ampulla of Vater itself), distal Cholangiocarcinoma (adenocarcinoma arising from the distal bile duct adjacent to the Ampulla) and duodenal adenocarcinoma (tumour arising in the duodenum adjacent to the Ampulla). It is worth noting that the term peri-ampullary cancer does not include pancreatic cancer arising from the neck, body and tail of the gland, which account for approximately 30% of pancreatic cancers (the other 70% arising within the head of the gland)[4]. Another group of tumours that can also cause confusion are neuroendocrine tumours arising within the pancreas. These tumours are out with the scope of this thesis and are not referred to again.

Although the anatomical origins of these tumours are often only separated by millimetres it important to differentiate between them as their prevalence and prognoses differ significantly. Yeo et al looked at 242 patients who underwent pancreatico-duodenectomy for peri-ampullary cancer over a 22 year period[5]. Of the 242 patients identified 62% were pancreatic cancers, 19% were ampullary

adenocarcinomas, 12% were cholangiocarcinomas and 7% were duodenal tumours. Post-operative 5 year survival varied greatly with tumour type: duodenal adenocarcinomas 59%; ampullary adenocarcinomas 39%; cholangiocarcinomas 27%; pancreatic tumours 15%. The median survival times for the different tumours follows a similar pattern: duodenal tumour patients median survival was not reached at the time of publication; ampullary tumours 49 months; cholangiocarcinomas 22 months; pancreatic cancers 12 months.

The study by Yeo et al demonstrates the difference in outcome for patients with pancreatic cancer (PC) and non-pancreatic peri-ampullary cancer (NPPC). The factors associated with poor survival post-resection in this study were poorly differentiated tumours, tumour within resected lymph nodes and tumour at resection margins. All of these factors were shown to be associated more commonly with pancreatic cancers and may account for the extremely poor survival statistics for that sub-group of patients.

1.1.2.2 Pancreatic Cancer

Pancreatic adenocarcinoma is generally thought to arise from the pancreatic ductal cells, the first evidence for this being published by Cubilla and Fitzgerald in 1976. This seminal study showed an increased incidence of abnormal ductal structures in patients with pancreatic cancer[6]. Cubilla and Fitzgerald, examined 227 cases of pancreatic cancer and 100 autopsies of patients without pancreatic cancer as controls. In the patients with pancreatic cancer they found the incidence of ductal papillary hyperplasia to be 50%, 20% of these with marked atypia and 18% with carcinoma in-situ, neither features were found in control cases. It was noted that

there was a similar spatial distribution of these lesions to malignant tumours which is consistent with the theory that they are developing adenocarcinomas. These lesions are now named areas of pancreatic intra-epithelial neoplasia (PanIN).

Many invasive cancers have histologically well-defined precursor lesions, most notably carcinomas of the cervix, breast and colon. Screening programs aimed at discovering and treating these pre-clinical lesions in the cervix and breast have been in place for a number of years and studies have shown a reduction in mortality due to these screening programs[7]. These tumours are readily accessible for biopsy purposes, a significant advantage over the pancreas which is very difficult to sample and as a result, nearly all pancreatic specimens come either from patients who have undergone a pancreatic resection or autopsy specimens. These offer only a snapshot of the histological picture and it is virtually impossible to observe the natural history of these pre-cancerous lesions within the pancreas. A classification of these pre-cancerous PanIN lesions was published in 2001with three basic categories PanIN 1, 2 and 3 with PanIN 1 separated further into A and B[8].

The detection of pancreatic cancer at an early stage is the key to treating a disease which has almost always spread by the time the majority of patients present to their medical practitioner. Current imaging techniques are unable to detect areas of PanIN and the inaccessibility of the pancreas makes it impossible to biopsy as is done with the cervix looking for precursor lesions. We currently don't know what the risks of developing invasive cancer from these lesions are or the time scale involved, but with further research development of the correct tools, the

detection of PanIN lesions may offer a method of detecting very early and potentially curable pancreatic cancer.

Currently, only a small proportion of pancreatic cancers are resectable, the only potentially curative option being a pancreaticoduodenectomy and a number of large studies have looked specifically at the factors leading to a poor outcome following this resection. These include: poor tumour differentiation; large tumour diameter; positive lymph nodes; positive resection margins; surgery out with a specialist centre[9-13]. In 2005 a study was published that added another indicator of poor outcome to this list and forms the basis of this thesis, a raised pre-operative inflammatory response[14]. This will be discussed in detail later in this chapter.

1.1.2.3 Cholangiocarcinoma

Tumours of the bile duct or cholangiocarcinomas are uncommon lesions arising from the epithelial cells of the biliary tract. They can arise at any point along the intra or extra-hepatic biliary tree and have a peak incidence in the seventh decade of life. The exact cause of cholangiocarcinoma is unknown with most cases occurring sporadically, but there are a number of risk factors including primary sclerosing cholangitis, congenital biliary cysts, hepatolithiasis, biliary infection, dioxin exposure and dietary nitrosamines. A number of genetic defects have also been associated with cholangiocarcinoma including over-expression of the genes Bcl-2, K-ras, p53 and p16. All of these genetic alterations are also associated with pancreatic cancer and will be discussed in more detail later in section 1.1.3. The only potential cure comes following a pancreaticduodenectomy for those patients with localised disease.

1.1.2.4 Ampullary Adenocarcinoma

Cancers of the ampulla of Vater are the second commonest of the peri-ampullary tumours behind pancreatic cancers, accounting for approximately 20% of cases[15]. Ampullary tumours have a better prognosis and higher resectability rate than pancreatic cancers. A study by Talamini et al of 120 patients, all of whom had biopsy proven adenocarcinoma of the ampulla, showed a resectability rate of 88%, a median survival of 46 months and a five year survival of 38% in patients undergoing resections[15]. The reasons for this improved prognosis may include the advent of endoscopy, allowing visualisation of early changes in the area of the ampulla and the earlier onset of jaundice thought to be due to increased intra-luminal growth resulting in patients seeking more prompt medical attention. If they are resectable, ampullary tumours are removed by pancreaticoduodenectomy, but there has been a recent move to attempt local resection for very carefully selected localised ampullary tumours. With the aid of endoscopic ultrasound (EUS), benign lesions smaller than 3cm, small neuroendocrine tumours and T1 carcinomas have been treated with localised resections with mixed results [16-18].

1.1.2.5 Duodenal Adenocarcinomas

Of the four peri-ampullary tumours duodenal tumours are the rarest, accounting for less than 10%[5]. Cancers of the duodenum account for approximately 45% of all small intestinal cancers, with 20% of them appearing in the peri-ampullary region[19]. Patients with familial adenomatous polyposis have a 100 fold increased risk of developing duodenal adenocarcinoma, but the vast majority of cases are sporadic[20]. Duodenal adenocarcinoma is associated with a better prognosis than pancreatic cancer and a higher resectability rate. A study describing 40 years of experience with duodenal cancers by Ryder found a resectability rate of 63%, a

median survival overall of 22 months, and a 5year survival of 33%[19]. Including only those patients who underwent resection, a 5 year survival of 43% was achieved. This same study found the factors associated with decreased survival were large tumours, moderate and poor tumour differentiation and invasion of surrounding fat. These tumours as with the other peri-ampullary tumours when operable are again removed by pancreaticoduodenectomy.

1.1.3 Genes and the Development of Pancreatic Cancer

Pancreatic cancer, like many other malignancies appears to result from the accumulation of genetic abnormalities in key genes such as oncogenes and tumour suppressor genes, this is known as the multi-hit theory[21]. A number of key genetic abnormalities occurring with pancreatic cancer have been identified over recent years with the advent of modern genetic techniques. If the PanIN lesions described earlier are precursors to invasive adenocarcinoma then they should harbour some, but not all of the genetic alterations seen in invasive tumours and the number of these genetic abnormalities would be expected to increase with increasing severity of dysplasia[22].

1.1.3.1 K-Ras

K-ras is a proto-oncogene that encodes a protein involved in cell growth and differentiation[23]. K-ras is mutated in over 90% of pancreatic ductal tumours leading to its constitutive activation and a crucial role in signal transduction[24]. The fact that the vast majority of mutations are limited to one codon means it is relatively easy to detect and has led to a number of efforts to create a screening test for pancreatic cancer. Analysis of pancreatic juice[25], duodenal fluid[26] and stool samples[27] for K-ras mutations have been attempted, but none have yet been successful in producing a reliable screening test.

A number of papers over recent years have shown that PanIN lesions harbour mutations in the K-ras gene and that these mutations increase in frequency with increasing levels of atypia[28,29]. These mutations have been found in specimens

with minimal atypia and therefore K-ras mutations are believed to be an early genetic event in the development of pancreatic cancer.

1.1.3.2 HER-2/neu

HER-2/neu is a member of the epidermal growth factor receptor family and is over expressed in approximately 70% of pancreatic ductal carcinomas[30] . Day et al examined the expression of HER-2/neu in a series of ductal lesions and found there to be increasing over expression with increasing atypia in the pancreatic ducts[31]. HER-2/neu over expression is also an early event in the development of pancreatic cancer.

1.1.3.3 p16 (INK4A)

The p16 tumour suppressor gene is located on chromosome 9p and is inactivated in up to 95% of pancreatic cancers[32,33]. Inactivation of this gene leads to phosphorylation of a number of growth proteins and leads to loss of cellular control and unchecked proliferation[24]. Wilentz et al showed that 30% of flat ductal lesions, 55% of papillary without significant atypia and 71% of papillary ductal lesions with significant atypia had loss of expression of the p16 gene product[34]. Yamano et al found there was loss of heterozygosity at 9p in 13% of low grade ductal lesions compared with 90% of high grade lesions at the same location[35]. Although not exclusively, the loss of p16 expression occurs in higher grade ductal lesions and is felt to arise after HER-2/neu and K-ras abnormalities in the development of pancreatic cancer.

1.1.3.4 p53

The p53 gene is a tumour suppressor found on chromosome 17p and is crucial to the way a cell responds to stressful insults as it is essential for tumour growth suppression. The function of p53 is lost in around 50% of cancers secondary to mutation and the remaining cancers appear to have defective p53 signalling[36]. Under normal non-stressful conditions p53 is kept at very low levels, but when exposed to DNA damage, hypoxia or other insults, activated p53 induces growth arrest and/or apoptosis by the induction or repression of specific genes. The p53 gene is inactivated in 50-70% of infiltrating pancreatic cancers[37,38]. Maitra et al showed that the p53 gene was abnormal in 57% of PanIN 3 lesions[39]. Interestingly none of the earlier PanIN lesions exhibited p53 abnormalities suggesting that it is an abnormality that occurs late in the development of a pancreatic tumour.

1.1.3.5 DPCA4 (SMAD4)

The Deleted in Pancreatic Carcinoma 4 (DPCA4) gene is a tumour suppressor gene located on chromosome 18q and is a member of the transforming growth factor β (TGF β) family[39]. DPC4 is inactivated in approximately 55% of invasive pancreatic adenocarcinomas by a number of different mechanisms[40]. A study by Wilentz et al looked at DPC4 in 188 PanIN lesions and found expression to be normal in PanIN 1 and 2 but 31% of PanIN 3 lesions had loss of expression[41]. Maitra et al also found there to be inactivation of DPC4 in 28% of PanIN 3 lesions and completely intact in lesions with less atypia (PanIN 1 and 2)[39]. These studies both confirm that inactivation of DPC4 occurs late in the development of pancreatic cancer.

1.1.3.6 BRCA2

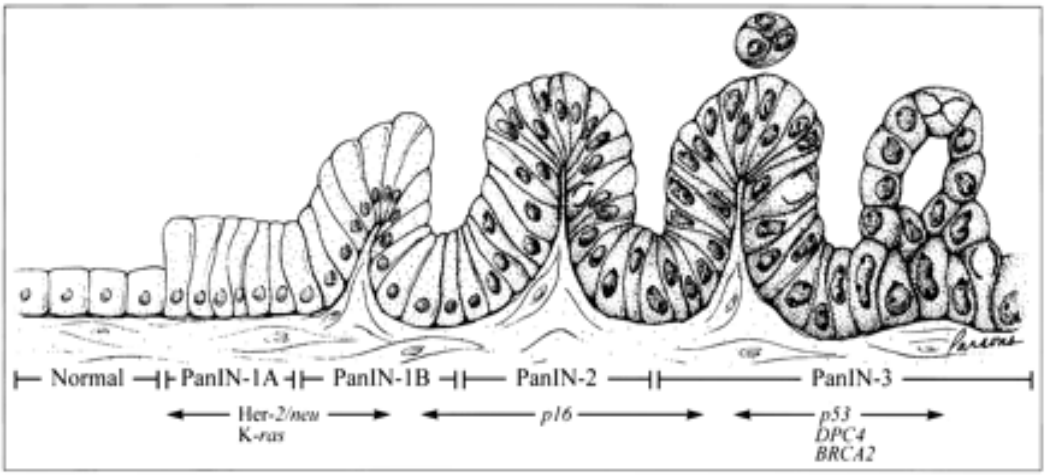
Those patients with inherited tumour suppression gene BRCA2 mutations are known to be at an increased risk of developing breast and ovarian tumours. The same patients are also at an increased risk of developing tumours in other areas such as the prostate, the gallbladder, the stomach, malignant melanomas and they have a relative risk of 3.51 (95% CI 1.87-6.58) of developing pancreatic cancer[42]. Goggins et al found Mutations in BRCA2 occurred late in the development of pancreatic cancer, although this was a very limited study of only 14 PanIN lesions with a single mutation[43]. It is possible that BRCA2 loss promotes the malignant progression of existing lesions in the pancreas. BRCA2 is necessary for maintaining genomic stability by regulating the DNA repair process. The fact that the mutation occurs late in the tumour development suggests that it requires other mutations such as p53 to be present to allow the DNA damage to be tolerated as BRCA2 loss in normal cells leads to lethal chromosomal abnormalities[44].

1.1.3.7 The Pancreatic Cancer Progression Model

Over the past 2-3 decades there have been huge advances in genetics, one of the key developments being the proof that cancer is a genetic disease[45]. Cancer is mostly caused by somatic mutations as opposed to germline mutations in other genetic diseases and generally requires an accumulation of mutations to develop. This theory is known as the multi-hit concept and is key to understanding the development of cancer[21]. The incidence of most common human cancers increases exponentially with age due to the multi-hit concept, where human beings collect genetic abnormalities until enough are present to cause invasive cancer[45].

Vogelstein et al published their progression model for colorectal cancers development from colonic adenomas in 1988[46]. A progression model for pancreatic cancer has more recently been published by Hruban et al (Figure 1.1) as a result of the improved understanding of genetic abnormalities involved in pancreatic cancer (discussed above) and the stage at which they appear from normal epithelium through the PanIN stages to invasive adenocarcinoma[47].

Figure 1.1: The pancreatic cancer progression model (Hruban et al[47])



The development of the progression model for pancreatic cancer paves the way for further research aimed at identifying lesions early before the inevitable spread has occurred. This research may involve the development of molecular genetic based screening tests looking at pancreatic or duodenal fluid[25,26].

1.1.4 Risk Factors For Pancreatic Cancer

As the vast majority of patients presenting with pancreatic cancer have incurable disease, a lot of research has been directed at disease prevention and the identification of risk factors involved with the development of pancreatic cancer. These risk factors can be divided into two categories, those that are modifiable and those that are not.

1.1.4.1 Non-modifiable Risk Factors

Pancreatic cancer is a disease of the elderly, its incidence increasing with age[48,49]. Over 80% of cases are diagnosed in the 60-80 year old age group, the main reason being the collection of genetic abnormalities discussed in section 1.1.3[50,51].
In the US it has been noted for a number of years that the rate of pancreatic cancer was higher for African Americans than Caucasians. A number of studies have examined the reasons for this, but the evidence appears to be conflicting, some studies claiming that any difference is due confounding environmental factors, others claiming differing genetic mutation rates and biomarker expression[52,53].

A relatively small proportion of pancreatic cancers (7-10%) appear to be due to an inherited disposition[49,54]. This "familial pancreatic cancer" appears to be distinct from a number of syndromes associated with an increased chance of developing pancreatic cancer discussed later. A study following 535 families on the National Familial Pancreas Tumour Registry found that in those families where there were three or more relatives affected, unaffected first-degree relatives were 57 times more likely to develop pancreatic cancer than the general population[55]. None of the families whose members developed pancreatic cancer had a history of

syndromes associated with aggregation of pancreatic cancer and this study appears to highlight a separate group of patients at a significantly higher risk of developing pancreatic cancer, but their genetic backgrounds are not yet understood.

Hereditary pancreatitis (HP) is an autosomal dominant disorder that accounts for 3-6% of all cases of pancreatitis and is caused by mutation in the cationic trypsinogen gene[56]. HP is associated with a recognised increase in the risk of developing pancreatic cancer. A longitudinal study followed 246 patients with a diagnosis of HP and compared the incidence of pancreatic cancer with that of the general population, showing a cumulative risk of developing pancreatic cancer of 40% by the age of 70, increasing to 75% with paternal transmission of HP [57].

The autosomal-dominant Peutz-Jeghers syndrome, which is caused by a LKB1/STK11 mutation and characterised by hamartomatous gastrointestinal polyps and mucocutaneous pigmentation is associated with an increased risk of developing pancreatic cancer compared with the general population[58].

Hereditary non-polyposis colorectal cancer (HNPCC) is caused by a mutations in the DNA mismatch repair genes and accounts for approximately 5% of new cases of colorectal cancer a year[4]. Lynch first noted the connection between HNPCC and pancreatic cancer and pancreatic cancer is now considered an integral lesion in the Lynch syndrome II, with patients being at a greater risk of developing pancreatic cancer when compared with the general population[59].

Familial adenomatous polyposis (FAP) is a dominantly inherited condition, the gene for which is carried on the long arm of chromosome 5. It is responsible for approximately 1% of colorectal cancers and is characterised by the formation of thousands of adenomas in the colon and rectum that left untreated will inevitably progress to colorectal cancer[4]. A study of 197 FAP pedigrees found a relative risk of 4.46 for pancreatic cancer in patients with the syndrome[60].

Familial atypical multiple-mole melanoma (FAMMM) is an autosomal dominantly inherited disorder characterised by multiple atypical nevi and multiple cutaneous malignant melanomas[59]. Patients with FAMMM who were p16 tumour suppressor gene positive were a subgroup at risk of developing pancreatic cancer and this subgroup is now termed FAMMM-PC (pancreatic cancer)[61,62].

A number of publications, many of them conflicting, have examined the role that diabetes mellitus has in pancreatic cancer[63-65]. A meta-analysis on the subject reveals that patients with diabetes do have an increased risk of developing pancreatic cancer[66]. This study identified 20 case-control and cohort studies between 1975 and 1994 that included cases with a duration of diabetes of at least 1 year prior to either pancreatic cancer diagnosis or death. Their results show an overall relative risk of 2.1 (95% CI 1.6-2.8) for developing pancreatic cancer in patients with diabetes. If all patients who had diabetes for less than 5 years prior to their diagnosis of cancer were excluded, the relative risk was still calculated to be 2.0 (95% CI 1.2-3.2). As pancreatic cancer is such an aggressive disease, with a high proportion of patients dead within a year of diagnosis, it is highly unlikely that the relationship between pancreatic cancer and diabetes mellitus is due to the former causing the latter. Interestingly, the only two studies in this meta-analysis

that made a distinction between insulin-dependent and non-insulin-dependent diabetes both found all cases of pancreatic cancer arose from the non-insulin-dependent group[65,67]. There are a number of possible explanations for this: the vast majority of patients developing pancreatic cancer are over 70 years of age and are therefore more likely to have non-insulin-dependent diabetes; the pancreas is exposed to low levels of exogenously administered insulin in insulin-dependent diabetes, but to high levels of insulin years before and after their diagnosis in non-insulin-dependent diabetes; the hyperinsulinaemia associated with non-insulin-dependent diabetes causes localised increases in blood flow and cell division and exposure to insulin has been reported to promote growth in one pancreatic cancer cell line[68,69].

A number of large studies have found an association between gastric surgery and pancreatic cancer. Caygill et al found there to be a four-fold risk of developing pancreatic cancer among 5010 post-gastric resection patients[70]. A review article found a statistically significant increased risk of developing pancreatic cancer after gastric resection for benign peptic disease in 4 out of 7 suitable papers[71]. This increased risk is postulated to be caused by hypoacidity leading to excess carcinogens such as N-nitroso compounds in gastric juice[23].

A number of other conditions are linked with an increased incidence of pancreatic cancer including Ataxia Telangiectasia and Pernicious Anaemia[72-74]. A persons height has also been postulated as a risk of developing pancreatic cancer, taller people having an association with the disease[75].

1.1.4.2 Modifiable Risk Factors

The most consistently reported modifiable risk factor associated with pancreatic cancer is cigarette smoking, which is named as a major risk factor in multiple review articles[48,76-78]. A prospective trial by Fuchs et al in the US looked at smoking as a risk factor for pancreatic cancer in healthcare professionals and included over 100,000 individuals followed up over a 12 year period[79]. They found the relative risk of developing pancreatic cancer to be 2.5 for current smokers and 1.6 for those people who had ever smoked, when compared with people who had never smoked. There was a dose dependent increase in risk between those who consumed less than 10 pack years and those who had consumed 26-50 pack years. The effect of cessation of smoking was also analysed and, the relative risk of former smokers decreasing precipitously and approaching that of never smokers within 10 years of giving up. Fuchs et al estimated that up to 25% of pancreatic cancers were attributable to cigarette smoking.

The role of alcohol in the aetiology of pancreatic cancer dates back to the 1960's in a retrospective review of chronic alcoholics by Burch et al[80]. A number of more recent studies have shown alcohol to be responsible for an increased risk of developing pancreatic cancer when consumed to excess[81-83].

A retrospective cohort study performed in Sweden recently looked at over 200,000 alcoholics admitted to hospital over a 30 year period[84]. All patients developing pancreatic cancer within a year of follow up were excluded to avoid selection bias. Alcoholics were shown have a modest 40% increased chance of developing pancreatic cancer when compared to the normal population (Standard Incidence Ratio of 1.4 [95% CI of 1.2-1.5]). The authors acknowledged that

information regarding smoking was deficient but using data regarding the rates of smoking among the different groups of patients in Sweden over the time period in question calculated that the increased risk of pancreatic cancer in those groups related to alcohol consumption could be entirely explained by the confounding of smoking.

Another large study published by a Canadian group looked again at the relationship between pancreatic cancer and alcohol consumption in 583 confirmed cases with 4813 controls[85]. The results showed that there were varying risks depending not only on the types and amounts of alcohol consumed, but also between the sexes. The fact that there are differing reports into the effects of alcohol on pancreatic cancer and so many confounding issues suggests that further research is needed including details into the specific types of alcohol consumed.

Although the connection between pancreatic cancer and alcohol consumption alone is not yet established, alcohol excess can lead to pancreatitis. Hereditary pancreatitis is associated with a significantly increased lifetime risk of developing pancreatic cancer, but are other forms of pancreatitis (chronic) associated with a similar risk? Two large studies have shown an increased risk of pancreatic cancer in patients with chronic pancreatitis varying between a relative risk of 2.04 and 18.5[86,87]. However, both are retrospective, relying on information gathered from patient registers and have differing definitions for the diagnosis of chronic pancreatitis. A prospective, single centre trial observing 373 consecutive patients with a definite diagnosis of chronic pancreatitis based on: pancreatic calcifications on imaging; moderate to marked pancreatic ductal lesions on ERCP (Cambridge criteria); typical histology on adequate surgical pancreatic specimens; shows a

connection between chronic pancreatitis and pancreatic cancer[88]. The results show a significantly increased risk of developing pancreatic cancer amongst these patients with a standardised incidence ration (SIR) of 26.7. However, this study contained only four cases of pancreatic cancers and no real conclusions about this relationship can be drawn from this or the previously discussed retrospective studies.

Early evidence indicated that coffee drinkers were associated with an increased relative risk of developing pancreatic cancer, but more recent studies have found no evidence for this[85,89-91].

The ingestion of fatty foods and foods high in cholesterol is associated with an increased risk of pancreatic cancer[76,92-95]. A high dietary intake of meat, particularly smoked meat is also associated with an increased risk of pancreatic cancer[92,95,96]. Other high risk foods for the disease include dairy products and diets including high amounts of carbohydrate, salt and sugar[92,95,97]. Food types which seem to have a protective role in other cancers also seem to be associated with lower rates of pancreatic cancer, these include fruit and vegetables and other high fibre foods[76,92,93,95].

A large cohort study involving over 150,000 healthcare professionals found obesity to be associated with an increased risk of pancreatic cancer with moderate physical activity having a protective role[75]. A body mass index (BMI) of 30kg/m^2 or greater was associated with a relative risk for developing pancreatic cancer of 1.72 (95% CI 1.19-2.48) when compared with those patients with a BMI of 23kg/m^2 or less. A relative risk of 0.45 (95% CI 0.29-0.70) was observed for those

undertaking moderate activity when compared to those in the lowest activity category.

1.1.5 The Presentation of Pancreatic Cancer

Pancreatic cancer is associated with a dismal prognosis, with only 5-15% of patients presenting with disease that is amenable to surgery, the only potential cure[98]. The idea that pancreatic cancer classically presents with painless jaundice does not always hold true and a number of other symptoms are commonly associated with the disease. When the literature is examined the commonest presenting symptoms are pain, jaundice, weight loss, anorexia, early satiety, nausea, vomiting, diarrhoea, xerostomia and are associated with signs such as jaundice, a palpable liver or gallbladder, ascites and thrombophlebitis.

One of the reasons the prognosis for pancreatic cancer is so poor is because the symptoms and signs that lead a patient to present to a medical practitioner are often only present once the tumour has reached an advanced stage. A review article by Brand describes a group of early vague symptoms that include non-specific abdominal pain, nausea, vomiting, sleeping difficulties, anorexia and general malaise[99]. These symptoms although troublesome may not lead a patient to their doctor and even if they do, may not prompt the necessary investigations to uncover an early pancreatic malignancy. The onset of jaundice which would usually lead to investigations of the biliary tract may be a late sign depending on the location of the growing tumour.

A study by Krech et al looked at the common symptoms experienced by patients with pancreatic cancer[100]. The commonest symptom described was abdominal pain with 82% of patients experiencing it, most commonly in the right upper quadrant. The other symptoms described by more than 50% of the patients were anorexia (64%), early satiety (62%), xerostomia (54%), sleep problems (54%),

and weight loss (51%). None of these symptoms are specific to pancreatic disease which again demonstrates the reasons for late diagnosis.

There are certain signs and symptoms that should prompt investigation, but are often associated with more advanced disease. Migratory thrombophlebitis (Trousseau's sign) has been reported in around 7% of patients with pancreatic cancer[101]. Diabetes Mellitus is present in around 15% of patients with pancreatic cancer and many more have glucose intolerance. New onset diabetes or a change in insulin requirements in a stable diabetic may be signs of a pancreatic malignancy. As mentioned earlier controversy exists around the role that pancreatitis plays in pancreatic cancer, whether it is just a sign of a developing tumour, or is itself a risk factor for the disease. Patients presenting with pancreatitis that have none of the normal risk factors (alcohol excess, gallstones) for the disease, should have further investigations to rule out a malignancy.

Jaundice, a sign that would lead to investigations of the biliary tract, can appear early or at an advanced stage depending on the location of the tumour. Kalser et al looked at the prognosis of 399 patients with pancreatic cancer by examining their clinical presentation[102]. The patients were grouped according to their disease stage, group 1 potentially curative, group 2 locally unresectable, and group 3 had metastatic disease. Group one had the smallest lesions and 90% had a pancreatic head tumour. In this group painless jaundice was the most frequent presentation (52%). Group 2 had 83% of their tumours located in the head of the pancreas with pain present in 80% of cases, jaundice in 62% and 48% having both. In group 3 lesions of the body and tail were 4 and 3 fold greater than in groups 1 and 2 respectively. Pain was present in 85% of cases and jaundice in only 31%. This

study suggests that those patients with potentially resectable disease do not tend to present with pain which is the commonest symptom in most other papers[100,103,104].

From the studies discussed above, pain appears to be associated with more advanced disease and is probably caused by invasion through the capsule and into the coeliac and superior mesenteric plexuses. We can conclude that the commonest symptom for patients presenting with pancreatic cancer is pain. These patients are likely to have more advanced disease and make up the vast majority of the 85-95% of patients who have inoperable disease. Those with tumours of the body and tail of the gland are likely to be in this group. Those patients who have tumours in close proximity to the bile duct (ie. head tumours) are much more likely to present with obstructive jaundice and undergo relevant investigations quickly. These are likely to be the patients with potentially resectable disease and are unfortunately in the minority.

1.1.6 The Diagnosis and Staging of Pancreatic Cancer

A patient presenting to either their GP or a hospital with any of the symptoms discussed above will need to undergo a number of different investigations before a diagnosis of pancreatic cancer can be made. Following a detailed history and examination it is likely the treating doctor will go on to request blood tests, the results of which are likely to lead to a number of radiological investigations. Only after the relevant investigations have been completed can the definitive diagnosis be made and the process of staging the tumour can begin.

1.1.6.1 Blood Tests

Unfortunately laboratory blood tests on patients with pancreatic cancer are generally non-specific. If jaundice is present the patient will likely have obstructive liver function tests (LFT's) with raised alkaline phosphatase, raised γ glutamyl transferase along with an increased bilirubin. If the transaminases are increased disproportionally, this may be a sign of extensive liver metastases. Along with the abnormal LFT's, the patients clotting may be affected due to the malabsorption of fat soluble vitamin K and hence decreased production of vitamin K dependent clotting factors. There may be other non-specific abnormalities such as a low albumin, anaemia and evidence of impaired glucose tolerance or frank diabetes.

There are a number of serological markers often used to help establish a diagnosis of pancreatic cancer. The most commonly used is Carbohydrate antigen 19-9 (CA19-9). The efficacy of this marker in detecting pancreatic cancer has been extensively studied, having a sensitivity of around 80% and a specificity of 60-

70%[103,105]. There are a number of drawbacks with this marker. First, increased concentrations are normally only found in tumours with a diameter of more than 3cm, excluding the vast majority of resectable tumours. Second, CA19-9 is not only raised in other GI tumours including the stomach, bile ducts and the colon, but in some benign conditions such as pancreatitis, hepatitis and cirrhosis[103]. However, when combined with radiological imaging such as CT, US or ERCP the diagnostic accuracy is improved and approaches 100%[106]. CA 19-9 has also been correlated with prognosis and tumour recurrence. Generally a higher CA 19-9 pre-operatively indicates a larger tumour and an increased chance of unresectability. When used post-operatively to assess adjuvant treatments, increasing levels of CA 19-9 generally indicate disease progression or recurrence, whereas stable or decreasing levels tend to indicate a stable tumour burden or absence of recurrence[107].

Other serological assays have been identified as potential markers for pancreatic cancer, these include carcinoembryonic antigen (CEA), carbohydrate antigen 50 (CA-50) and carbohydrate antigen 242 (CA-242). However, none of these have been found to be consistently superior to CA 19-9[108-110].

Currently none of the serological markers are able to detect an early pancreatic cancer and certainly none are suitable as a screening test which would lead to potentially improved survival figures due to earlier surgical treatment. The developments in molecular and genetic medicine are most likely to lead to the early detection of pancreatic cancer in the future, but at the moment serological tests are only used as adjuncts to radiological imaging.

1.1.6.2 Diagnostic Imaging

Over the last 20 years there have been huge advances in radiological imaging which have greatly enhanced the ability to diagnose and stage pancreatic cancer. During the process of diagnosing and then staging patients with pancreatic cancer the results of a number of radiological investigations are combined to provide adequate information on the tumour and enable accurate staging. There is currently no single modality that can accurately stage the disease in its own right.

The two aims of imaging in patients who have or may have either PC or NPPC tumours are:

1/. To confirm the diagnosis.

2/. To assess resectability.

The features that are generally accepted to preclude surgical resection are[111,112]:

1/. Metastatic spread to the liver or peritoneum.

2/. Vascular invasion/ encasement.

3/. Lymph node spread.

4/. Spread to contiguous structures such as the stomach, colon, spleen, or spine.

1.1.6.2.1 Computed Tomography (CT)

Abdominal CT scanning is currently the method of choice for the diagnosis and staging of pancreatic. Modern multi detector CT scanners (MDCT) mean faster scanning with thinner slices allowing multi-planar, three dimensional reconstruction. The use of water as oral contrast and timed IV contrast injections (venous, arterial, pancreatic) enable the visualisation of the course of blood vessels and bile ducts in and around the pancreas. CT has been shown to have excellent results in the diagnosis of pancreatic cancer with a positive predictive value of 92%, a sensitivity of 96%, a specificity of 81% and an accuracy of 88%[113,114].

CT is excellent for predicting unresectability of pancreatic tumours, helical CT having accuracies of non-resectability of between 93 and 96%[115,116]. However, of patients with tumours deemed to be resectable on pre-operative scanning, the accuracy of helical CT has been shown to be far less 72-91%[113,117]. The commonest reasons for the under staging of pancreatic tumours are small liver metastases, lymph node involvement, peritoneal spread and vascular invasion[113,118,119].

CT is an excellent modality for diagnosing PC's and NPPC's and for identifying non-resectable tumours but is not good at giving accurate information on borderline tumours or identifying small peritoneal or liver metastases. To try and identify these non-resectable tumours CT is used in conjunction with other radiological modalities described below.

Figure 1.2: MDCT reconstruction of pancreatic duct showing small pancreatic head cancer (arrow) and a dilated pancreatic duct immediately distal to the obstruction[120].

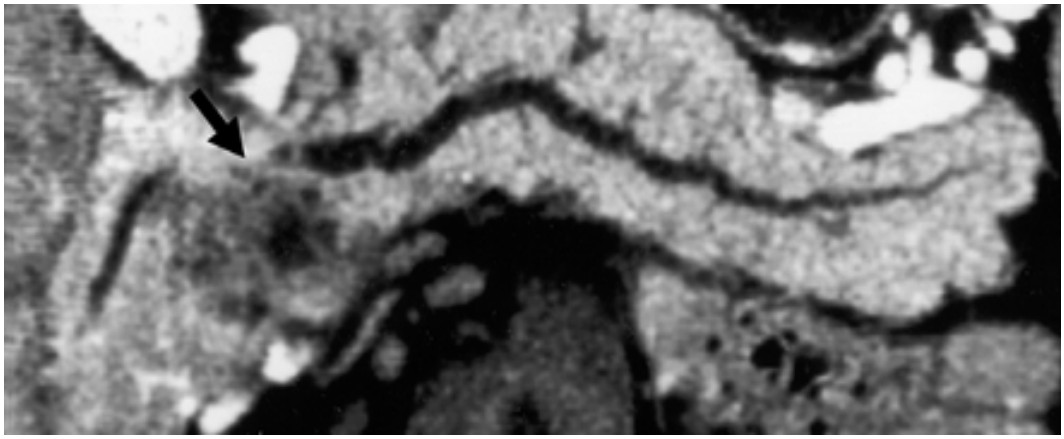
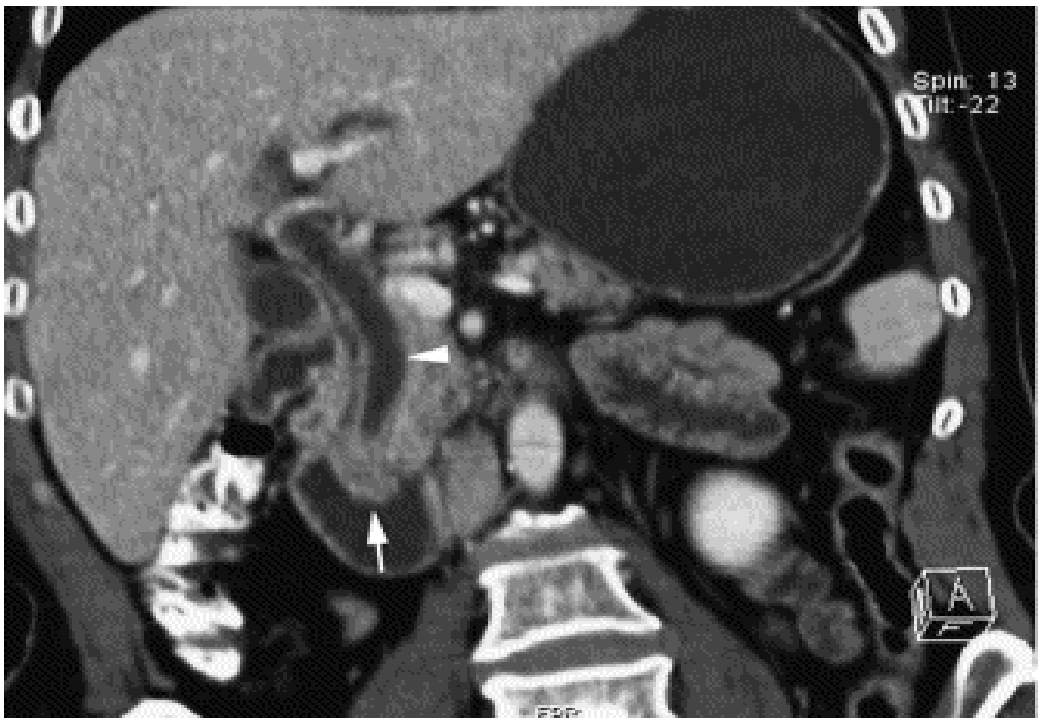


Figure 1.3: MDCT paracoronal reconstruction of dilated common bile duct (arrowhead) and a peri-ampullary tumour projecting into the duodenum (arrow)[111]



1.1.6.2.2 Endoscopic Ultrasonography (EUS)

Endoscopic ultrasonography (EUS) was developed in the 1980's to try and overcome the limitations of trans-abdominal US by eliminating interference from overlying bowel gas, improving resolution of the pancreas and surrounding structures and also enables tissue sampling [121].

Midwinter et al compared 48 patients with suspected pancreatic and ampullary cancers[122]. EUS demonstrated 33 of the 34 tumours compared to 26 with CT. EUS was particularly useful in demonstrating small tumours missed by CT.
Tierney et al found EUS to be more sensitive in the identification of vascular involvement in 51 patients with non-metastatic, peri-ampullary malignancies when compared to helical CT and concluded that EUS is particularly useful when CT findings were equivocal[123].

The added advantage of EUS is its ability to take tissue samples. The diagnostic ability of EUS guided fine needle aspiration (EUS-FNA) in diagnosing pancreatic malignancies has been examined in a number of studies[124-126]. Sensitivities of 91-94%, specificities of 67-100% and accuracies of 92% were achieved.

EUS cannot replace CT as it is unable to include the whole of the abdominal cavity in the staging process. However, EUS is of particular value as an adjunct to CT scanning, particularly where the diagnosis is not known, in small tumours and where resectability is uncertain after CT. EUS is also able to gain tissue diagnoses from pancreatic lesions or from lymph nodes if spread is being questioned.

1.1.6.2.3 Magnetic Resonance Imaging (MRI)

MRI has always had the advantage of not exposing the patient to ionising radiation and the ability of modern MRI scanners to perform pancreatography (MRCP) and angiography at one sitting has shown comparable accuracy in terms of the diagnosis and staging of pancreatic cancer[114,118].

With the current technology MRI is unlikely to replace CT as the Gold-standard investigation, but may have a role to play in those patients where CT is inconclusive or resectability is uncertain. Trials are awaited to see if the administration of secretin prior to MRI scanning and the resulting increased secretion of pancreatic juice improves results[112,127].

1.1.6.2.4 Ultrasound (US)

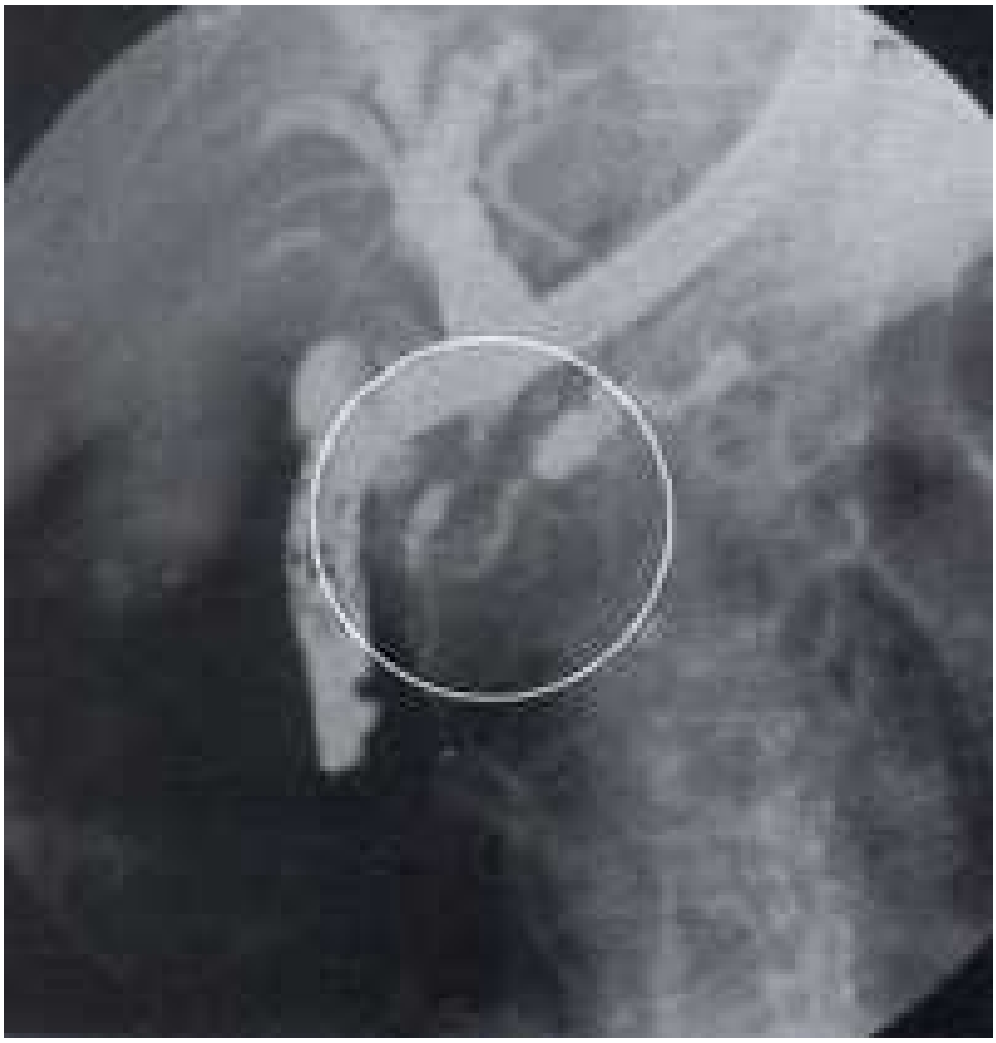
Abdominal US is often the initial screening investigation for patients with pancreatic cancer, particularly those suffering from jaundice as it is non-invasive, cheap and readily available. US can also identify gallstones, liver metastases, the level of biliary obstruction and the presence of abdominal ascites as well as the size, site and characteristics of the primary tumour[103]. Overlying bowel gas, abdominal ascites large amounts of fat and the operator can all significantly affect the accuracy of US, particularly when looking at the pancreas due to its retroperitoneal location. US will never be considered as a rival to CT, but with the advent of new technologies such as contrast harmonics it may have a role to play as an adjunct to CT looking for liver metastases for example, but trials of this new technology are awaited.

1.1.6.2.5 Endoscopic-retrograde-cholangiopancreatography (ERCP)

ERCP allows direct visualisation of the pancreatic duct, the ability to gain cytological information with brushings and the deployment of biliary stents where necessary. ERCP will never be the mainstay of staging and its role tends to be in those patients who present with jaundice and are found to have a dilated common bile duct (CBD). The finding of an irregular stricture in an otherwise normal pancreatic duct is a sign of a possible malignancy and a simultaneous stricture in the common bile duct, the 'double-duct sign' (Figure 1.4) is often considered a specific sign of ductal adenocarcinoma[128]. However, cancers arising in the pancreatic body or tail rarely present with a double duct sign and not all head cancers involve the common bile duct meaning this sign will not be present in a significant proportion of pancreatic cancers. Despite this, ERCP has been has been shown to predict pancreatic adenocarcinoma with a sensitivity of 70-92% and a specificity of 90-94%[129,130]. It should be remembered that ERCP is an invasive procedure with associated risks such as pancreatitis (moderate-severe 1.3%), cholangitis (0.87%), haemorrhage (0.76%) and perforation (0.58%)[131]. The relief of biliary obstruction in those patients with resectable disease by biliary stenting is controversial, a meta-analysis showing no statistical advantage and a higher post-op complication rate[132].

ERCP will continue to be used commonly in the investigation of obstructive jaundice, diagnosing the vast majority of pancreatic tumours that present in this way, however its main advantages are therapeutic in both operable and non-operable pancreatic and biliary malignancies.

Figure 1.4: Double-duct sign on ERCP



1.1.6.2.6 Positron Emission Tomography (PET)

Positron emission tomography scanning or PET scanning, is a newer imaging technique that works on the basis that an IV administered radio-labelled glucose analogue metabolism is increased in malignant cells when compared to normal tissue allowing them to be identified[121]. A number of studies have compared PET to CT scanning, but due to its current lack of availability PET will not replace CT in the near future. As with a number of imaging techniques it may add to the staging information of patients with pancreatic cancer, particularly in picking up small hepatic or peritoneal deposits.

Rose et al examined 70 patients who were being investigated for either primary (65) or recurrent (5) pancreatic cancer performing CT and PET[133]. PET scanning suggested changes in the management in 43% of patients after CT. In nine patients who underwent FDG-PET before and after neo-adjuvant chemotherapy, four had evidence of tumour regression, three showed stable tumours and two showed disease progression, none of these changes were identifiable on CT. This paper shows that FDG-PET not only has a role in the diagnosis and staging of pancreatic cancer, but may also be useful in evaluating treatment response and tumour progression.

The latest advances have combined CT (anatomical imaging) and PET (physiological imaging), with promising results. Heinrich et al looked at 59 patients with presumed resectable pancreatic cancer, comparing standard staging (chest x-ray, CT) and combined CT/PET[134]. The CT/PET demonstrated hepatic metastases in five patients and 2 patients with synchronous rectal tumours that

weren’t seen on standard staging. Management was changed in 16% of cases ($p<0.03$) as a result of CT/PET.

This new technology needs to be evaluated in large, prospective trials to see if these promising results hold true, but until PET scanners are more available it will not form part of standard staging for pancreatic cancer.

1.1.6.3 Surgical Staging

A proportion of patients who have been found to have potentially resectable tumours following a combination of the previously discussed imaging modalities, will in fact have metastatic disease discovered at the time of surgery. A large study in 768 patients undergoing laparotomy for peri-ampullary cancer found 33% had evidence of metastatic disease at laparotomy not seen on pre-operative staging[135]. Of this 33%, 68% were found to have metastatic deposits in the liver or peritoneum suggesting that a staging laparoscopy would have enabled surgeons to discover these small metastatic deposits thus avoiding a laparotomy and the morbidity and mortality it entailed (3.1% and 22% respectively in this study).

Conlon et al performed extended laparoscopy (with the use of several ports) on 108 patients felt to have resectable disease following radiological investigations and discovered 41 (36%) had metastatic deposits[136].

The addition of laparoscopic US (LUS) was examined in 90 patients with pancreatic cancer by Merchant and Conlon[137]. Of the initial 90, 17 were found to have non-resectable disease radiologically, laparoscopy went on to identify a further 41 (45%) patients with metastatic disease. Thirteen patients were deemed to have equivocal findings and underwent additional LUS, which deemed 8 non-resectable cases. Overall, only one patient deemed resectable actually had non-resectable disease, giving the combined technique a positive predictive index of 100% and a negative predictive index of 98%.

The largest study looked at 239 patients with peri-ampullary/pancreatic malignancy who underwent both CT and LUS for staging purposes[138]. Of the 239 patients, 190 were found by CT to have resectable disease, the addition of LUS identified 23 further patients who had metastatic spread. However, of the 167 patients identified by both CT and LUS as having resectable disease, 31 (16%) were found to have metastatic disease at laparotomy.

The role of laparoscopy with or without US in the staging of pancreatic cancer remains controversial. There appears to be substantial evidence in the literature supporting its use, however the largest study to date showed that LUS still missed 16% of the cases of metastatic spread. It can be argued that performing a laparotomy in the first instance allows the surgeon to perform a prophylactic bypass if a non-resectable tumour is found, but laparoscopic-gastro-jejunostomy is now a widely used and safe alternative and many patients will have a biliary stent in place[139]. The use of LUS in the staging of pancreatic cancer is not universal and performed at the discretion of individual investigators.

1.1.7 Treating Pancreatic Cancer

1.1.7.1 Background

Pancreatic cancer has one of the poorest outlooks of all malignancies, with surgical resection improving the outlook in the 10% of patients who are eligible for the procedure after staging[2,140]. Following surgery survival is poor and dependent on a number of factors, firstly the type of tumour, with pancreatic cancer having a median survival of 15-17 months, significantly worse than the other non-pancreatic peri-ampullary cancers (NPPC)[5,9].

When the reasons for treatment failure are examined, local spread is the major culprit with 72-86% of patients succumbing to recurrence in the pancreatic bed and 62-92% to hepatic recurrence[13,141]. These figures suggest microscopic spread present prior to surgery which is not visible either in pre-operative staging, or during the procedure itself and is an obvious target for adjuvant therapy.

1.1.7.2 Factors Associated with Poor Outcome

A number of factors have been shown to have a negative affect on survival on patients with pancreatic cancer on multivariate analysis. These factors are: the size of the tumour (larger tumours doing poorly); positive resection margin; lymph node invasion; poor differentiation; surgery performed out-with a specialist unit[9-13]. Table 1.1 below displays the factors associated with poor survival and the relevant publications.

Table 1.1: Factors associated with poor survival following Whipple operation for pancreatic cancer.

Author	Ref. Number	Patients	Tumour Size (>2cm)	+'ve RM	LN Invasion	Poor Diff	Surgery Out with Specialist Centre
Sohn TA	9	616	**X***	**X**	-	**X**	-
Yeo CJ	10	650	**X***	**X**	**X**	**X**	-
Lim JE	11	396	**X**	-	**X**	**X**	**X**
Birkmeyer JD	12	7229	-	-	-	-	**X**
Sperti C	13	78	**X**	**X**	**X**	**X**	-

* Size >3cm.

1.1.7.3 Surgical Treatment

Surgery for pancreatic cancer involves a major abdominal procedure with retro-peritoneal dissection and multiple anastamoses, resulting in significant morbidity and mortality. The technique of pancreaticoduodenectomy (PD) was first described by Kausch in 1912 but later popularized by Whipple whose name is now synonymous with the procedure[142]. Post-operative morbidity and mortality rates were exceptionally high, 60% and 25% respectively in early series in the 1960's. Now, the majority of Whipple operations are carried out in specialist centres, where mortality rates are reported as less than 5%, although morbidity still remains high at around 40%[10,143-146]. The most common causes of morbidity are often not life threatening, but amount to increased hospital stay and delayed onset of adjuvant therapy. These include delayed gastric emptying, pancreatic fistula and wound infection.

Despite the advances in surgical techniques, equipment and peri/post-operative care, survival rates in the 10% of pancreatic cancer patients who have disease amenable to surgery remain poor. Five-year survival for patients undergoing a potentially curative resection, vary greatly depending on the site of origin of the tumour, the best being duodenal (59%), followed by ampullary (39%), cholangiocarcinoma (27%) and finally ductal (15%) in a study of 242 patients[5].

The most common technique of a Whipple procedure consists of the en bloc removal of the distal segment (antrum) of the stomach, the first and second portions of the duodenum, the head of the pancreas, the common bile duct, and the gallbladder. Following removal of the specimen, continuity of the bowel is re-established along with a pancreaticojejunostomy and a hepaticojejunostomy.

More recently, the pylorus- preserving pancreatico-duodenectomy (Whipple) has been described. A large randomised trial of 214 patients comparing the two techniques was published by Seiler et al in 2005[147]. On analysis, the only significant differences between the two groups were operating time, blood loss and blood transfused, all being less in the pylorus-preserving group. There was no significant difference in mortality and morbidity (both medical and surgical which included delayed gastric emptying) and long-term follow up found no significant difference between the two groups in terms of overall survival, tumour recurrence or quality of life.

1.1.7.4 Adjuvant Treatment

A number of retrospective studies have suggested that adjuvant therapy may improve long-term survival, but until recently adjuvant therapy was not routinely used following surgery for pancreatic cancers and NPPC's [9,11,148].

A large, multi-centre randomised trial looking at surgery, combined chemo-radiation and chemotherapy alone was needed and The European Study Group for Pancreatic Cancer (ESPAC) was set up to do this[2]. The study used a two-by-two design where, following resection each patient was randomly assigned to receive chemoradiotherapy or chemotherapy, neither treatment, or both treatments, with 70 patients enrolled in to each group, yielding combined data for 140 patients in each group for the two main treatment comparisons (chemo-radiotherapy vs no chemo-radiotherapy and chemotherapy vs no chemotherapy). Patients who underwent a complete macroscopic resection of a histologically proven pancreatic ductal adenocarcinoma underwent randomisation.

Chemo-radiotherapy consisted of a 20-Gy dose to the tumour given in 10 daily fractions over a two week period plus IV bolus of fluorouracil on each of the first three days of radiotherapy and again after a break of two weeks. Chemotherapy consisted of an IV bolus of leucovorin, followed by an IV bolus of fluorouracil on each of five consecutive days every 28 days for six cycles. Combination therapy was the above regimes starting with chemo-radiotherapy followed by chemotherapy.

A total of 289 patients were enrolled from 53 hospitals all over Europe between February 1994 and June 2000. When the results were analysed for the two main areas of comparison, those patients who underwent chemo-radiation had a median survival of 15.9 months and estimated five year survival of 10% compared with 17.9 months and 20% for those who did not get chemo-radiation (p=0.05). Those that had chemotherapy had a median survival of 20.1 months and estimated five year survival of 21% compared to 15.5 months and 8% in those that didn't receive chemotherapy (p=0.009). When the four individual groups were examined their median survivals were 16.9 months in those that had observation alone, 13.9 months in those that had chemo-radiation, 21.6 months for those that got chemotherapy alone and 19.9 months in those that received both chemo-radiation and chemotherapy. The estimated five year survival for each of these groups was 11%, 7%, 29% and 13% respectively. The explanation given for the apparent detrimental effect of chemo-radiation on those that received chemotherapy was that the chemo-radiation delayed the onset of chemotherapy post-operatively. This study shows that the standard treatment for those patients with resectable pancreatic cancer should be curative surgery followed by adjuvant systemic chemotherapy.

1.1.8 Summary

In summary, pancreatic cancer is a devastating condition with only a very small proportion of patients with potentially curable disease. Current methods of staging still miss a significant number of patients who actually have non-resectable disease, although there have been improvements in radiological staging over the last couple of decades. The treatment hasn't changed significantly for half a century in those patients lucky enough to have resectable disease, but even after major surgery and more recently adjuvant chemotherapy, median survival is a mere 21 months[2]. There are a number of well established pathological factors that have a negative affect on survival including, larger tumours, positive resection margins, lymph node spread, and poorly differentiated tumours but these rely on the post-operative specimen[9-13]. Identifying the patients that do poorly after surgery and learning more about the genetic and molecular make-up of these patients may lead to new therapeutic options and improved survival.

In 2005 Jamieson et al published a paper looking at 65 patients who underwent resection for ductal adenocarcinoma of the head of the pancreas. On univariate analysis, sex ($p<0.05$), tumour size ($p<0.05$), vascular invasion ($p<0.001$), pre-operative CRP ($p<0.001$) and post-operative CRP ($p<0.001$) were significantly associated with survival. On multivariate analysis of the significant variables, tumour size, vascular invasion, pre and post-operative CRP retained independent significance. In fact the group of patients who did not have a raised pre-op CRP (<10mg/l) had a median survival of 18.2 months compared with only 8.3 months in those with a raised CRP ($p<0.001$). There was no significant difference in the stage, presence of positive resection margins, lymph node metastases or perineural invasion between the inflammatory and non-inflammatory groups.

This paper suggests that a pre-operative inflammatory response is significantly associated with a poor post-operative survival in patients with resectable pancreatic cancer. This paper forms the basis of this thesis and in the next section the relationship between inflammation and cancer is explored.

1.2 Inflammation and Cancer

1.2.1 Introduction

The link between inflammation and cancer has been recognised for many years, Virchow noting the presence of leukocytes in neoplastic tissue in 1863, suggesting that this reflected the origin of cancer at sites of chronic inflammation[149]. A number of cancers are known to have a relationship with chronic inflammation, including: squamous cell carcinoma development at the site of burned skin (Marjolins ulcer)[150]; Barretts metaplasia pre-disposing to oesophageal cancer[151]; the increased chance of colorectal cancer development in patients with inflammatory bowel disease (IBD)[152]; the development of mesothelioma following asbestos exposure[153]. As mentioned above hereditary pancreatitis[57] and more controversially chronic pancreatitis[88] are associated with an increased risk of developing pancreatic cancer. There are also a number of cancers associated with chronic infections leading to chronic inflammation which are thought to account for up to 15% of the worlds cancer burden[154], including: schistosomiasis and bladder cancer; liver fluke infection and cholangiocarcinoma; papillomavirus and cervical cancer; helicobacter pylori and gastric cancer; Hepatitis B and C and hepatocellular tumours. These examples demonstrate the link between inflammation and the development of malignancy, but what are the mechanisms behind this transformation?

1.2.2 Inflammation and the Pathogenesis of Cancer

Chronic inflammation may progress from acute inflammation if the initial cause is not removed, but can be chronic from the outset. Chronic inflammation is characterized by infiltration of damaged tissue by cells such as macrophages, lymphocytes and plasma cells that attempt repair of the damaged tissues[155]. Macrophages are the most important cells in the chronic inflammatory reaction due to their production of multiple inflammatory mediators and products. These include plasminogen activator, enzymes, plasma proteins, complement components, coagulation factors, reactive metabolites of oxygen, cytokines, chemokines, growth factors and nitric oxide. A number of these mediators and products are thought to be crucial in the transformation from inflamed tissue to cancer.

The pro-inflammatory cytokine tumour necrosis factor (TNF) is a key downstream promoter of inflammation and is known to regulate a cascade of cytokines, chemokines and inflammatory mediators[149]. The major source of TNF appears to be macrophages which have infiltrated the area of inflamed tissue. TNF's actions include both tissue destruction as well as recovery and whilst it can destroy a diseased cell at sites of inflammation it can also stimulate fibroblast growth. TNF can also be both pro and anti-angiogenic depending on the surrounding conditions and the amount present at a specific site. Chronically produced TNF, for example in areas of chronic inflammation, can act as a tumour promoter aiding the development of the necessary stroma tumours require for growth and spread. It may be that TNF is one of the ways in which inflammation acts as a tumour promoter. Evidence for this has been demonstrated using genetically engineered mice that lack the TNF gene. These mice were shown to be

resistant to skin carcinogenesis despite exposure to known carcinogens[156]. TNF has also been the target for a number of clinical trials involving inflammatory disorders such as rheumatoid arthritis and Crohns disease using licensed TNF antagonists such as inflixamab, with initial results showing symptomatic relief[157]. Anti-TNF drugs are licensed for cytokine/chemokine inhibition, reduced angiogenesis and prevention of leucocyte infiltration, all of which may be useful in a biological therapy for cancer. In fact there are a number of ongoing and planned studies involving anti-TNF therapies as single agents and in combination with other therapies in the treatment of cancer.

There are many similarities between tumour stroma and areas of inflammation and thus similarities between wound healing and tumour stroma formation. In a review article Dvorak described tumours as ‘wounds that do not heal’ and it may be that a persistent stimulus over a long period of time (chronic inflammation) disrupts the normal balance between key molecules in the inflammatory response leading to tumour promotion, with TNF being one of these molecules[158].

Another key molecule in the inflammatory pathway is the ubiquitously expressed transcription factor nuclear factor kappa B (NF-κB). NF-κB regulates the expression of various inflammatory, apoptotic and oncogenic genes[159]. Activation of NF-κB occurs by a number of stimuli including IL-1, protein kinase C, reactive oxygen species, viruses, but is primarily induced by TNF[160,161]. Once activated at sites of inflammation NF-κB causes increased transcription of 27 different genes encoding for cytokines, chemokines and receptors crucial for neutrophil adhesion and migration including IL-2, IL-2R, IL-6, IL-8, IL-12 and TNF[162]. These cytokines can in turn activate the NF-κB pathway establishing a positive

autoregulatory loop that sustains and amplifies the inflammatory response. NF-κB also induces nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX 2), two enzymes vital to the inflammatory response and may be responsible for the initiation of oncogenic changes in inflamed tissue.

NF-κB has been shown to exert important effects on cellular apoptosis and proliferation via the activation of anti-apoptotic genes including cellular inhibitors of apoptosis (c-IAP1, c-IAP2, and IXAP) the TNF receptor-associated factors (TRAF1 and TRAF2), the Bcl-2 homologue A1/Bfl-1, and IEX-IL[162]. In pancreatic cancer, TNF related apoptosis was shown to be inhibited by the co-activation of NF-κB which in turn activated the anti-apoptotic genes mentioned above blocking the activation of caspase-8, an initiator protease, involved at an early step in stimulating the apoptotic pathway[163].

Although more research is needed, NF-κB may well be the key molecule that connects inflammation and cancer. It is activated in inflammatory conditions going on to regulate many genes coding for key inflammatory proteins. This creates an autoregulatory loop that propagates and prolongs inflammation in the exposed tissues, as well as causing inhibition of apoptosis.

The generation of nitric oxide (NO) by the inflammatory cytokine induction of nitric oxide synthase (iNOS) is present in inflamed tissue and is thought to exert oncogenic effects via a number of mechanisms. These include direct DNA and protein damage, inhibition of apoptosis, mutation of DNA and cellular repair functions (e.g. p53) and promotion of angiogenesis[164]. As well as NO production

by macrophages, the iNOS gene is under transcriptional control of inflammatory cytokines which are in rich supply in inflamed tissue.

Another inducible enzyme that is active within inflamed and malignant tissues is cyclo-oxygenase-2 (COX-2)[155]. COX or prostaglandin H2 synthase is the key enzyme in the biosynthesis of prostaglandins mediating inflammation and other important physiological processes[165]. It has been shown to exist as two isoforms, COX-1 and COX-2. COX-1 is a housekeeping enzyme and expressed in nearly all tissues mediating physiological responses such as protection of the stomach, platelet aggregation and regulation of renal blood flow. COX-2 is expressed in cells that mediate inflammation such as macrophages and monocytes and is primarily responsible for the synthesis of prostaglandins involved in acute and chronic inflammatory states and hence may be involved in malignant change in inflamed tissues. Many malignancies produce more prostaglandins than normal tissue and this increased production comes from enhanced expression of COX-2. This increased expression is thought to be due to mutations activating signalling pathways such as the mitogen activated protein kinase (MAPK) pathway.

There are different mechanisms by which COX-2 is thought to enhance carcinogenesis each of which has evidence to support it. Accumulation of arachidonic acid favours apoptosis and its depletion favours cell survival therefore increased levels of COX-2 will decrease available arachidonic acid giving cells a survival advantage[166]. The second mechanism which is more widely accepted is that prostaglandins formed via COX-2 govern key processes involved in cell growth and differentiation such as the suppression of apoptosis and the

enhancement of angiogenesis, providing an optimal environment for tumour growth and spread.

Further evidence for the role of COX-2 in neoplastic progression comes from studies examining cancer risks amongst long-term users of non-steroidal anti-inflammatory drugs (NSAID's) which work by inhibiting COX. There is evidence that 15-20 years of NSAID ingestion can reduce the risk of sporadic colorectal cancer as well as reduce the adenoma load in patients with familial polyposis coli. Epidemiological evidence also suggests a protective role in stomach and oesophageal cancers with long term NSAID use[167].

The mechanisms by which areas of chronic inflammation undergo malignant change are far more complex but the above information connects both intrinsically. It is likely the change from inflammation to neoplasia is a very gradual process, with affected tissues being exposed to a range of mutagens over a long period of time causing a collection of mutations, in other words the multi-hit concept mentioned earlier when discussing the pancreatic cancer progression model. The role that inflammation and the inflammatory response has on established malignancies is explored in the next section.

1.2.3 Inflammation and the Progression of Cancer

A significant body of evidence exists showing that a systemic inflammatory response (as measured by C-reactive protein) is a predictor of poor outcome in a number of cancers including bladder[168], lymphoma[169], colorectal[170], renal[171], non-small cell lung cancer[172], and pancreatic cancer[14]. The mechanisms behind this poor prognosis are not fully understood. Does this systemic inflammatory represent the body attempting to mount a response to tumour load? Or, does this inflammatory response represent a pro-inflammatory tumour micro-environment with cytokines, chemokines and leucocytes[149,173].

The micro-environment in and around tumours is created by a number of interlinked mechanisms. It is characterised by the presence of host leucocytes including macrophages, T-cells and natural killer (NK) cells, with mast cells in smaller numbers[174]. Tumour associated macrophages (TAM) are a major component of almost all tumours and derive from circulating monocyte precursors attracted to tumours by chemokines[175]. TAM can, in the correct conditions produce growth and angiogenic factors (TNF, TGF, platelet derived growth factor and interleukin-6) creating an environment in favour of tumour growth and spread. Also activated in these conditions is NF-κB mentioned above, that itself induces the up-regulation of many pro-inflammatory protein genes, creating an auto-regulatory loop within and around the tumour.

Chemokines, mentioned above, play a central role in the recruitment of leucocytes to sites of inflammation and tumours and are induced by inflammatory cytokines. Their name is derived from their function, they are **chemo**tactic cyto**kines** and aid the migration of responsive cells. Most tumours produce the two major groups of

chemokines CXC and CC, with CXC chemokines active on neutrophils and lymphocytes and CC chemokines acting on monocytes, eosinophils, dendritic cells and lymphocytes[149]. Other than their function, CC and CXC chemokines differ by having an extra amino-acid separating the first two cysteine residues. Interleukin-8 (IL-8) is a chemokine of the CXC family and is known to be increased in pancreatic cancer. IL-8 is strongly inducible by hypoxia, another factor initiating an inflammatory response in tumours.

One of the major physiological differences between normal tissue and tumours is the presence of hypoxia in the latter. A hypoxic environment as found in tumours is known to induce a number of cytokines including TNF, IL-1, IL-6 and various chemokines such as IL-8[149]. Pancreatic tumours have been shown to have areas of significant hypoxia with normal tissue oxygen levels in surrounding unaffected pancreatic tissue, possibly explaining the reason for a localised inflammatory reaction at the site of a tumour[176]. The other reason for this rich pro-inflammatory environment at the sites of tumours is the fact that tumour cells themselves have been shown to produce a host of cytokines including IL-1, IL-6, TNF, IL-8, PDGF, TGF amongst others[177].

The pro-inflammatory environment within and surrounding tumours influences its survival, growth and spread via a number of different mechanisms. The action of reactive oxygen species such as NO has been mentioned above for their role in the pathogenesis of tumours. Evidence suggests that these compounds are induced at tumour sites by inflammatory cytokines such as IL-1 and TNF. Further more, in a study of cholangiocarcinomas the induction of reactive oxygen species was shown to be present in all tumours examined and was responsible for a 70% decrease in

DNA repair activity[178]. Further evidence linking the inflammatory response to DNA damage came when an inflammatory cytokine known as macrophage migration inhibitory factor (MIF) was identified as a negative regulator of p53 activity[179]. In some tumours there is evidence of p53 inhibition but without gene mutation meaning p53 regulation has been altered. MIF a known pro-inflammatory cytokine was shown to overcome p53 activity meaning the inflammatory response is responsible in potentially creating genomic instability and explains why for example, mutations created by reactive oxygen species may not be dealt with in the normal way at tumour sites.

To survive, proliferate and spread, tumours and their supporting stroma must grow and cytokines are crucial for tumour growth. To support the growing tumour the necessary vascular structures must develop and if there is an absence of new blood vessel formation then the tumour will reach equilibrium with the rate of apoptosis and not have the ability to grow and spread. One of the key molecules in angiogenesis is vascular endothelial growth factor (VEGF) which was first characterised in the 1980's as a vascular permeability factor[180]. It has subsequently been shown to be absolutely critical in tumour angiogenesis which is essential for tumour development and progression. More recently VEGF has been shown to have other characteristics crucial to tumour progression such as anti-apoptosis activity, lymphangiogenesis, and immunosuppression. VEGF is present in higher concentrations within tumours when compared with normal tissue and its levels are correlated with poor survival in breast cancer, ovarian cancer, non-small cell lung carcinoma, colorectal cancer and pancreatic cancer. VEGF expression is also correlated with tumour grade, depth of invasion, nodal mets, and TNM staging[180]. VEGF is a crucial part of tumour growth and its production

is known to be stimulated by the inflammatory infiltrate with TNF and IL-6 key players.

The micro-environment in and around tumours is clearly pro-inflammatory with a high concentration of cytokines, chemokines and leukocytes forming an environment where growth and angiogenesis can flourish. Key factors in this process include TNF, IL-6, IL-8, VEGF and NF-κB. There is also evidence of altered DNA repair that along with the abundance of growth and angiogenic factors appear to promote tumour growth and spread.

1.2.4 Markers of Inflammation

The most commonly used measurements of inflammation or the inflammatory response involve measuring acute phase proteins. The most widely used indicators are the erythrocyte sedimentation rate (ESR) and the C-reactive protein (CRP) mentioned above. The ESR measures the rate that erythrocytes fall through plasma and is largely influenced by the plasma concentration of fibrinogen (an acute phase protein). The ESR is therefore an indirect measurement of the acute phase response and is greatly influenced by the size, shape and quantity of erythrocytes as well as other plasma constituents leading to inaccuracies[181]. Although the ESR has been measured for over 70 years with supporting evidence, it has now been superseded by CRP. This is due to the ability to measure acute phase proteins (e.g. CRP and fibrinogen) directly, the inaccuracies mentioned above, the fact that the ESR rises with age and the inability of the ESR to change rapidly with a patient's condition.

CRP is a protein belonging to the pentraxin family, with five identical subunits forming a ring which conveys very high stability to the protein[182]. CRP was discovered in the 1930's and is so named because of its ability to react with the pneumococcal C-polysaccharide in the plasma of patients during the acute phase of pneumococcal pneumonia. CRP is synthesised predominantly in hepatocytes under the influence of cytokines. Although a number of cytokines are capable of inducing CRP, overwhelmingly the major inducer of CRP via hepatocytes is Interleukin-6 (IL-6)[183-186]. Other sites of CRP production include smooth muscle cells, macrophages, kidney tubular cells, neurons, and lymphocytes, however, these sites contribute only a small proportion of the CRP value and plasma CRP measurements reflect hepatocyte production.

CRP is able to bind to bacteria, fungi and parasites as well as damaged membranes in apoptotic or necrotic cells. Once bound to these molecules CRP facilitates their removal by phagocytes. This opsonization effect is in addition to CRP's ability to activate the classic complement pathway therefore aiding innate immunity.

CRP has been used to help differentiate inflammatory from non-inflammatory conditions for a number of years. Serial measurements are useful in managing inflammatory conditions (e.g. rheumatoid arthritis) as well as sepsis and reflect the patients response to treatment interventions as plasma levels change rapidly. More recently CRP measurements have been recommended for cardiovascular risk prediction. Studies in apparently healthy populations reported a relative risk of 2.3 for major adverse cardiovascular events at the highest quintile of CRP levels, but only 1.5 for the highest LDL cholesterol quintile, indicating that these events are more strongly associated with CRP than LDL cholesterol[187]. In a significant number of patients with normal LDL cholesterol levels, elevated CRP was shown to be associated with major adverse cardiovascular events.

As discussed earlier, an elevated CRP has also been shown to have prognostic value in a variety of tumours including bladder, colorectal, renal, non-small cell lung cancer, lymphoma and pancreatic cancer.

1.2.5 Inflammation and Pancreatic Cancer

The inflammatory response and its influence on survival in patients with unresectable pancreatic cancer has been explored by a number of authors. Engelken et al looked at a combined prospective and retrospective cohort of patients who were found to have non-resectable pancreatic cancer over a seven year period[188]. The authors looked at a variety of prognostic factors in 325 patients whose overall median survival was 5.7 months. In 51 patients they had CRP measurements available, although no information was given as to the timing of these CRP results. They found a normal CRP (<5mg/l) was associated with a three-fold increase in survival compared to those with a raised CRP (7.29 months versus 2.23 months, $p<0.001$). When they performed multivariate analysis on this subset of 51 patients, CRP was found to be the only statistically significant factor. It should be noted however that only 10 patients in this study had elevated CRP levels.

Ueno et al looked at 103 patients who had been treated with palliative systemic chemotherapy for metastatic pancreatic cancer[189]. The median survival overall was just 3.2 months and again CRP was found to significantly influence outcome. On multivariate analysis a raised CRP (>5mg/l) (along with performance status and CA19-9 levels) was an independent predictor of poor outcome ($p<0.0023$). In this study 17 patients had an elevated CRP which was taken prior to the start of chemotherapy in all patients. The authors also state that the patients with an elevated CRP had no other causes such as sepsis and they believed the inflammatory response was caused entirely by the malignancy.

Falconer et al looked at 102 patients with non-resectable pancreatic cancer[190]. A number of factors were found to negatively influence survival. On multivariate analysis increasing age, disease stage, elevated albumin and a raised CRP (>10mg/l) all retained statistical significance. Those patients with a normal CRP had a greater than three-fold survival advantage over those with a raised CRP (66 days versus 222 days respectively, p=0.001).

The only paper to look at patients with operative pancreatic cancer was mentioned in section 1.1.8 and published in 2005 by Jamieson et al[14]. This paper looked at 65 patients who underwent resection for ductal adenocarcinoma of the head of the pancreas and had pre and post (1 month) -operative CRP measurements taken. Of the 65 patients, 51% had a raised CRP (>10mg/l) pre-operatively. On univariate analysis, sex ($p<0.05$), tumour size ($p<0.05$), vascular invasion ($p<0.001$), pre-operative CRP ($p<0.001$) and post-operative CRP ($p<0.001$) were significantly associated with survival. On multivariate analysis of the significant variables, tumour size, vascular invasion, pre and post-operative CRP retained independent significance. In fact the group of patients who did not have a raised pre-op CRP had a median survival of 18.2 months compared with only 8.3 months in those with a raised CRP ($p<0.001$). There was no significant difference in the stage, presence of positive resection margins, lymph node metastases or perineural invasion between the inflammatory and non-inflammatory groups.

There is compelling evidence in pancreatic cancer patients that having a raised systemic inflammatory response as measured by CRP has a significant negative effect on patients survival in both resectable and non-resectable disease. The mechanisms behind this poor prognosis are not known. It is likely that a raised CRP is a by-product of another process which influences survival and as the main driver of plasma CRP, Interleukin-6 (IL-6) is likely to be involved.

1.2.6 Interleukin-6

Interleukin-6 (IL-6) belongs to a family of cytokines known as IL-6-type cytokines other members being IL-11, LIF (leukaemia inhibitory factor, OSM (oncostatin M), CNTF (ciliary neutrophic factor), CT-1 (cardiotrophin-1) and CLC (cardiotrophin-like cytokine). The IL-6-type cytokines are part of a larger group of cytokines characterised by their four-α-helix bundle structure, but are generally grouped together due to their similar and overlapping physiological actions as a consequence of their use of the common receptor subunit glycoprotein 130 (gp130), a signal transducer [191].

This family of cytokines are major players in a number of physiological processes including haematopoiesis, the developmental differentiation of lymphocytes, endocrine functions, bone formation, inflammation, acute phase and immune responses and target genes involved in differentiation, survival, apoptosis and proliferation[192,193]. Many cell types respond to IL-6, including, neural, lung, ovarian, endometrial, heart, renal, macrophages, monocytes, osteoblasts, mast cells, fibroblasts, leydig cell precursors, endothelial, hepatocytes and prostatic cells[193]. There are also many inducers of IL-6 including IL-1, TNF-α, NF-κB, prostaglandins, VEGF along with enterotoxins, growth factors, hypoxia, ionising radiation and oxidative stress[193]. With these multiple inducers and the many cell types sensitive to IL-6, the presence of IL-6 in tissues is not an abnormal occurrence.

The role of IL-6 in the immune and inflammatory responses to pathogens and tissue damage is essential in the survival of the host cells, but the protective effect cytokines have on these cells, by inhibiting apoptosis and promoting growth, may

also make them susceptible to neoplastic change. IL-6 has been implicated as an autocrine growth factor in a number of cancers including multiple myeloma, prostatic, bladder, renal cell, and cervical[194-197]. Elevated serum levels of IL-6 have also been found in a number of cancers including ovarian, renal cell, cholangiocarcinoma, lymphoma, lung and melanoma[198-203]. IL-6 has been shown in certain types of cancer to inhibit apoptosis. This was shown by Lauta in multiple myeloma and by Leu in oesophageal carcinoma[204,205].

When looking specifically at pancreatic cancer, IL-6 has been identified as an autocrine growth factor, serum levels of IL-6 have also been found to be elevated and it is thought to lead to activation of anti-apoptotic genes. Miyamoto et al examined the expression of IL-6 on pancreatic cell lines and the effect it had on ionising radiation[206]. They showed that in certain cell lines IL-6 inhibited apoptosis by causing up-regulation of Bcl-2 family proteins (known anti-apoptotic proteins) and concluded that IL-6 is likely produced in an autocrine and/or paracrine fashion by certain pancreatic cell lines. Barber et al found that serum levels of IL-6 were significantly raised in patients with advanced pancreatic cancer when compared to healthy subjects[207]. Okada et al also found serum IL-6 to be increased in patients with advanced pancreatic cancer, not only when compared to healthy controls but also patients with chronic pancreatitis[208]. They also found that higher levels of IL-6 corresponded with greater weight loss in the pancreatic cancer group. Ebrahimi et al examined the serum of 51 patients with pancreatic cancer and measured cytokine levels (using ELISA), comparing results with healthy controls[209]. Not only were IL-6 levels raised significantly when compared to controls, these higher levels were associated with poor performance

status, increased weight loss and decreased survival. On multivariate analysis raised serum IL-6 was shown to be an independent predictor of poor survival.

The actions of IL-6 are carried out via two intra-cellular pathways that transmit signals from the plasma membrane to the nucleus. These two pathways are the Janus Kinase (JAK) and signal transducers and activators of transcription (STAT) pathway and the mitogen-activated protein kinase (MAPK) pathway[192].

Both of these signalling pathways are known to be crucial in the development and progression of numerous types of cancer. There is also evidence that the two pathways far from being separate are connected with cross-talk between the two of them potentially being crucial in the progression of cancer. Both pathways are discussed below.

1.3 The JAK STAT Pathway

1.3.1 Introduction

The Janus kinase-signal transducer and activator of transcription (JAK STAT) pathway transmits information received from extra-cellular polypeptides (e.g. IL-6) via transmembrane receptors to target genes within the nucleus. These genes are responsible for a variety of biological functions including, the immune response, the acute phase reaction, inflammation, haematopoiesis and achieve this by regulating cell growth, differentiation, and survival[210]. The first stage in this complex process is the binding of IL-6 to its receptor.

1.3.1.1 IL-6 Receptor (IL-6R)

IL-6R is a member of the cytokine class I family of receptors which are defined by the presence of at least one cytokine binding module (CBM). The IL-6R can be divided into two sub-units, a non-signalling subunit (IL-6Rα) and a signalling subunit (gp130).

The first stage in the pathway involves the binding of IL-6 to the receptor subunit IL-6Rα. Although this is not involved in the intracellular signal transduction cascade, it is a crucial process as it is only after this step that the cytokine and α-receptor complex can efficiently recruit the signal-transducing subunit (gp-130). Although gp130 is ubiquitously expressed, the expression of the α-subunit is much more tightly regulated meaning the number of cells responding to IL-6 is restricted by expression of the non-signalling subunit[192].

The next stage in the pathway involves the recruitment of gp130 signalling molecules to the IL-6-receptor complex. The gp130 subunits have a similar structure to the IL-6Rα, but have a considerably larger cytoplasmic part due to their signal transducing properties. IL-6 (along with IL-11) differs from the other IL-6-type-cytokines as it induces gp130 homodimerisation (other members of the family cause heterodimerisation) when it binds to its receptor[211]. Once IL-6 binding has taken place gp130 homodimerisation occurs, followed by phosphorylation on serine, threonine and tyrosine residues[211]. The binding of IL-6 to its receptor leads to internalisation of the ligand. Internalisation has been shown to be entirely dependent on the signal transducing subunit (gp130) of the receptor and is modulated by the phosphorylation of the serine residue[212].

1.3.1.2 Janus Kinases (JAK's)

It wasn't until 1994 that it was discovered that IL-6-type cytokines utilised tyrosine kinases of the JAK family (and transcriptors of the STAT family) as mediators of signal transduction[213,214]. JAK's are intracellular tyrosine kinases, with mammalian cells expressing four types, Jaks1-3 and Tyk2. Jak1, 2 and Tyk2 are widely expressed but Jak3 expression is confined mainly to haemopoietic cells. JAK's all have a similar structure with a typical kinase domain and substitutions of single tyrosine residues within this domain have been shown to eliminate all tyrosine kinase activity.

Receptor dimerisation as described above brings the associated JAK's into close proximity with the receptors. JAK's bind to the membrane-proximal region of cytokine receptors and they transphosophorylate each other becoming activated[215]. Once activated, their kinase activity stimulated, JAK's go on to

phosphorylate tyrosine residues on the cytoplasmic portion of the receptors and thus create binding sites for STAT proteins[216].

IL-6 binding to its receptor has been shown to activate Jak1, Jak2 and Tyk2 but Jak1 has been shown to be dominant in IL-6 signal transduction. In fact Jak1 is essential for IL-6 signalling via the JAK-STAT pathway as Jak1 negative cells have absent signalling in response to IL-6[217,218].

1.3.1.3 Signal Transducers and Activators of Transcription (STAT's)

In human beings there are seven STAT genes, STAT 1-6 (including STAT5a and STAT5b). STAT genes are located in clusters on chromosomes and their expression is ubiquitous. STAT proteins are known to be activated by a wide variety of ligands, probably all known cytokines and large numbers of growth factors that exhibit tyrosine kinase activity, such as EGF and PDGF. In a given cell type more than one STAT protein can be activated by a given ligand, but the identity of the STAT activated depends on the state of cell differentiation and maturation. This builds a degree of variability into STAT activation and hence increases the panel of genes whose expression will be modulated in different cellular contexts[219].

As described above, ligand binding to the receptor leads to JAK binding and their close proximity to each other on the dimerised receptor subunit (gp130) leads to JAK transphosphorylation. Once phosphorylated, their kinase activity enabled, JAK's phosphorylate tyrosine residues on the cytoplasmic region of the receptor subunit, thus creating binding sites for STAT proteins. Once binding has taken

place the receptor-bound JAK's activate the STAT proteins by phosphorylating them at a single tyrosine residue[191].

Following phosphorylation at the receptor, the activated STAT proteins form stable homo and hetero-dimers in the cytoplasm. The choice of whether to bind with an active receptor or another STAT protein appears to be related to the state of phosphorylation of the STAT in question. Unphosphorylated STAT exhibits a high affinity for receptor docking sites, but activated or phosphorylated STAT have a much higher affinity to other STAT proteins, encouraging dimerisation following tyrosine phosphorylation[220]. This dimerisation of STAT proteins is essential for subsequent DNA binding[221]. Whether STATs form homo or hetero-dimers appears to be influenced by the original activating ligand, the receptor sub-unit and the individual STAT protein involved, adding further variability to the signalling process[191].

STAT activation and dimerisation takes place in the cytoplasm, but to exert their effect they need to enter the nucleus and bind to DNA. The mechanism by which the STAT dimers are transported to the nucleus remains unknown, but active transport is required as their size is greater than the limit of the nuclear pore[192]. Once in the nucleus the active dimers go on to bind with specific DNA sequences.

1.3.2 IL-6 Signalling and STAT3

There are seven known STAT proteins, as mentioned above, but not all are activated in response to IL-6. IL-6 potently activates STAT3 and to a lesser extent STAT1, but STAT3 is widely recognised as the key player in IL-6 signalling and the major activator of acute-phase gene transcription[191,193,222-225]. As described above, STAT3 undergoes tyrosine phosphorylation under the influence of Jak1 following its binding to the gp130 receptor subunit. This tyrosine phosphorylation is crucial for subsequent dimerisation and adequate to activate the protein, in STAT3 it takes place at tyrosine705 (tyr705)[191,219,226].

There is clear evidence that STAT3 can undergo phosphorylation at another site in addition to tyr705 and that it involves serine phosphorylation at serine727 (ser727)[191,205,215]. What induces this serine phosphorylation, its significance in cell signalling and the biological effects it has been the subject of some interest over recent years. It is thought that to completely activate STAT3 that serine phosphorylation also has to take place and that the MAPK pathway may well be involved in the process[227,228]. Further work is required to establish the precise role of serine phosphorylation in STAT3 proteins.

1.3.3 STAT3 and Cancer

Tumourogenesis is a multi-step process, with cells losing their normal ability to detect and repair DNA damage and adequately regulate cell cycle progression and apoptosis. There is mounting evidence that STAT proteins, and in particular STAT3 are heavily involved in cellular apoptosis, angiogenesis and differentiation via growth factor signalling and are therefore likely to play key roles in cancer development, growth and spread. STAT proteins, including STAT3 are persistently activated in non-malignant processes such as inflammation and their activation alone is not enough to lead to cellular transformation. However, with cells collecting crucial genetic mutations in the development of cancer, in the appropriate context, STAT3 is likely to be a crucial contributor to the cancerous phenotype.

Abnormal tyrosine kinase activity and increased levels of tyrosine phosphorylation are common in cancer cells and are usually caused by overexpression of growth factor receptors and their relevant ligands, IL-6 for example[229]. STAT3 has strong associations with many cancers including multiple myeloma, melanoma, lung, breast, renal, prostate, colonic, gastric, cervical, ovarian, hepatocellular, head and neck and pancreatic cancer[193]. The reason for the association between STAT3 and cancer appears to be the genes it targets once activated.

A gene profiling study by Croonquist et al using DNA microarrays determined the subset of genes activated by IL-6 signalling in multiple myeloma[230]. Out of 138 identified, 54% were found to be involved in cell cycle regulation. The genes involved in the cell cycle are activated by IL-6 and its subsequent activation of

STAT3, highlighting the importance of the JAK-STAT pathway in tumourogenesis.

A review article published in 2007 looked at the role of STAT3 and neoplastic cellular transformation and discussed a 12 gene STAT3 signature[231]. Using mouse fibroblasts, specific STAT3 target genes were identified, a number of which were known to be co-expressed by primary human tumours. Using this approach, the 12 gene STAT3 signature was identified. Closer analysis of these genes shows they have the ability to regulate almost all of the essential properties of a tumour cell. Part of this12 gene set are Egr-1, JunB and Cyclin D1, all genes involved in promotion of cell cycle progression. Three genes known to be prominent in the inhibition of apoptosis, Mcl-1, Bcl-2 and Bcl-x1 are also part of this 12 gene signature. The group also contains the genes Klf-4 and Bcl-6, both known to be involved in the inhibition of differentiation and the self-renewal of cells. STAT3 directly regulates matrix metallo-proteinases, enzymes which are known to be involved in tumour cell invasion and may suggest a role for STAT3 in the metastatic spread of a tumour. Also crucial to the growth and invasion of a tumour is its blood supply and one of the 12 signature genes is vascular endothelial growth factor (VEGF).

Levels of VEGF, a potent angiogenic growth factor in both benign and malignant tissue, have been shown in a number of trials to be related to IL-6 levels[232,233]. Cohen et al showed that IL-6 induces expression of VEGF, in fact the strength of this induction is comparable to that exhibited by hypoxia in tissues[234]. IL-6 exerts this effect via the JAK STAT pathway as activated STAT3 protein levels have been shown to correlate with the level of VEGF expression[180]. Abdelrahim et al

looked at the effect of NSAID's on certain transcription factors associated with vascular endothelial growth factor (VEGF) expression in mice[235]. Tolfenamic acid (an NSAID) was shown to cause degradation of Sp1, Sp3 and Sp4 (transcription factors involved in VEGF expression) and the resultant statistically significant decreases in VEGF mRNA, VEGF levels, tumour growth, tumour weight and hepatic metastasis.

More relevant to this thesis is the fact that VEGF, under the influence of IL-6 and hence STAT3 appears to have a key role in pancreatic cancer. In pancreatic cell lines it has been demonstrated that IL-6 is responsible for VEGF expression and the extent of VEGF expression appears to be related to the extent of IL-6 Receptor expression on cells[236,237]. Wei et al examined the relationship between STAT3 and VEGF in pancreatic cancer cell lines[238]. Immunohistochemical analysis demonstrated that STAT3 was constitutively activated in more than 80% of human pancreatic cell lines and this STAT3 activation correlated with expression levels of VEGF. Blocking STAT3 activation led to a significant suppression of VEGF expression and subsequent decrease in angiogenesis, tumour growth and metastasis in vivo. This study indicates that STAT3 regulates VEGF expression and is therefore involved in angiogenesis, tumour growth and metastasis in human pancreatic cancer.

In addition to the role of STAT3 in VEGF regulation there are other publications describing the relationship between the JAK STAT pathway, or more specifically STAT3 and pancreatic cancer. Greten et al examined the role of STAT3 in the regulation of anti-apoptotic genes in mice crossbred to develop ductal pancreatic cancer similar to the human form of the disease[239]. They demonstrated increased

transcription of Bcl-x, the anti-apoptotic gene described previously with STAT3 and subsequent down-regulation when it was blocked. This study also noted that the transcription factor NF-KappaB, first discussed in section 1.2.2, had a similar effect on Bcl-x and that down-regulation of the gene took place when either STAT3 or NF-KappaB was blocked, indicating that both may be required. If both transcription factors were blocked together programmed cell death was induced and the authors highlight this as a possible future avenue for therapeutic intervention. NF-KappaB is a key transcription factor in inflammation and carcinogenesis and will be discussed in greater detail later in this discussion.

Scholz et al examined the expression and activation of STAT3 in pancreatic cancer cell lines[240]. They were able to show that activated STAT3 is over-expressed in ductal cells but not in chronic pancreatitis cells. Subsequent inactivation of STAT3 led to decreased proliferation in vitro and tumour growth in vivo. The authors concluded that STAT3 promoted cellular proliferation by accelerating the G(1)/S-phase progression contributing to the malignant phenotype of pancreatic cancer.

Toyonaga et al examined the significance of JAK and STAT3 on the malignant potential of human pancreatic cancer cell lines[241]. Their studies revealed that STAT3 was constitutively activated in certain pancreatic cancer cell lines and went on to use a JAK inhibitor tyrphostinAG490. The JAK inhibitor led to a marked inhibition of STAT3 activation and also diminished expression of the genes cyclin D1, Bcl-x and VEGF, causing arrested growth in pancreatic cancer cells. The authors in this study again highlighted the JAK-STAT3 signalling pathway as a potential target for future treatment of pancreatic cancer.

A study using RNA-interference (RNAi) technology which works by silencing specific target genes was employed to look at the role that STAT3 plays in the invasion and metastasis of pancreatic cancer[242]. The authors used a metastatic pancreatic cancer cell line known to highly express STAT3 and using this RNAi technique, observed the effects of reduced STAT3 expression in both in-vitro and in-vivo conditions. They were able to demonstrate a significant suppression in the expression of matrix metallo-proteinases-2 (MMP2) and VEGF and a resulting inhibition of invasion and metastasis in-vitro and in-vivo. The authors make the conclusion that silencing the STAT3 gene may offer one way of controlling pancreatic cancer invasion and spread.

1.4 The Mitogen Activated Protein Kinase (MAPK) Pathway

1.4.1 Introduction

Mitogen-activated protein kinases (MAPK) belong to a larger family of serine-threonine kinases and form major cell-proliferation signalling pathways from the cell surface to the nucleus. There are three major subfamilies of MAPK: the extra-cellular-signal-regulated kinases (ERK-MAPK, Ras/Raf1/MEK/ERK); the c-jun N-terminal or stress activated protein kinases (JNK or SAPK); and MAPK14[243]. The ERK-MAPK pathway is one of the most important cascades in cell proliferation and will be the subject of further discussion in this report. Signals transmitted through Ras trigger ERK pathway signalling, which then activate Raf1, leading to a cascade involving MEK and ERK. The ERK-MAPK cascade is involved in the control of growth signals and cell survival.

1.4.1.1 Ras

There are more than 50 low molecular weight proteins in the Ras-related superfamily serving multiple functions in virtually all eukaryotic cells[244]. Ras-related proteins control various cellular signalling pathways including cell cycle entry and mitogenesis. The term Ras actually encompasses a group of proteins including H-Ras, K-Ras, N-Ras and R-Ras. Ras is a small GTP-binding protein which is modified with a hydrophobic lipid moiety allowing it to be anchored to the membrane. This feature allows the protein to be functional in the transduction of signals from extra-cellular ligands binding with membrane receptors. Multiple extra-cellular growth factors lead to Ras activation via membrane receptors. These

include EGF, TGFβ, HGF and importantly IL-6 via the gp-130 receptor subunit[245].

Activation of Ras protein occurs with the conversion of inactive GDP-bound Ras to the active GTP-bound Ras. The first stage of Ras activation is tyrosine phosphorylation of one of the Ras subunits termed Shc. Ras localisation on the interior of the plasma membrane leads to rapid conversion of Ras from its GDP bound (inactive) state to its GTP bound (active) state. K-Ras mutation is one of the commonest involved in human cancer and in particular pancreatic cancer where it is mutated in 90% of patients with the disease. The K-Ras oncoprotein differs from the normal protein by a single amino acid substitution at a critical position. This substitution leads to constitutive activation of the Ras protein (GTP bound) and subsequent activation of the downstream Raf/MEK/ERK pathway[244].

1.4.1.2 Raf-1

Raf-1 is the most extensively studied of the Raf proteins of which there are three forms, A-Raf, B-Raf and C-Raf (Raf-1)[246]. Raf-1 is a 72-76 kDa cytoplasmic protein with intrinsic serine-threonine kinase activity and is a direct effector of Ras. Once bound to active Ras at the cell membrane, Raf-1 proteins undergo activation via a complex series of events. Initially there is de-phosphorylation of an inhibitory site (serine 259) followed by the phosphorylation of a critical activating site (serine 338)[247] . Activated Raf-1 then goes on to interact with MEK further downstream in the pathway. In contrast to Ras proteins, Raf-1 mutations are extremely rare.

The MEK's are a family of protein serine/threonine kinases that have three isoforms in mammals- MEK-1a, MEK-1b and MEK-2[248]. Activation of MEK-1/2 involves phosphorylation of two serine residues at positions 217 and 221 by activated Raf-1 protein in the cytoplasm. Once activated MEK-1/2 goes on to activate MAPK again in the cytoplasm.

1.4.1.3 MAPK (ERK)

There are three members of this group of protein kinases ERK-1, ERK-2 and an alternative spliced form of MAPK. ERK-1 and ERK-2 are generally considered together as they are functionally very similar. The activation of these kinases requires phosphorylation of both threonine and tyrosine residues for full activation, and the main mechanism for this process is phosphorylation at both sites by activated MEK [248]. The phosphorylation at both sites has also been shown to be the main requirement for the translocation of ERK into the nucleus where it binds and regulates genes controlling cellular growth, proliferation, differentiation and apoptosis[249]

1.4.2 MAPK and Cancer

It has been suggested that to survive and proliferate cancer cells have to acquire a number of capabilities: independence of proliferation signals, evasion of apoptosis, insensitivity to anti-growth signals, unlimited replicative potential, the ability to invade and metastasise and to attract and sustain angiogenesis[250]. Abnormalities within the MAPK pathway can give rise to most of these properties which is why it is such a crucial pathway in the development and propagation of cancer. In all, 30% of all cancers contain a mutation to one of the Ras oncogenes. Table 1.2 demonstrates the frequency of these mutations in various cancers.

Table 1.2: Frequency of Ras mutation in human cancers[247].

Tumour type	**Percentage with Ras Mutation**
Pancreas	90%
Lung adenocarcinoma (non-small cell)	35%
Thyroid (follicular)	55%
Thyroid (papillary)	60%
Seminoma	45%
Melanoma	15%
Bladder	10%
Liver	30%
Kidney	10%
Myelodysplastic syndrome	40%
Acute myeloid leukaemia	30%

In the MAPK pathway it is the ligand-mediated activation of receptor tyrosine kinases that leads to the activation of Ras protein (GDP bound to GTP bound state) and subsequent recruitment of Raf-1 to the cell membrane followed by activation of the MAPK cascade. As previously stated, one of these ligands is IL-6 which leads to activation of the Ras protein via receptor tyrosine kinases, a fact crucial to this report. The majority of cancer-associated lesions that go on to cause constitutive activation of the MAPK pathway occur early in the cascade[247]. The large number of mutations centred around Ras-Raf suggest that this is the key stage in regulation of the pathway, particularly in pancreatic cancer with 90% of these tumours harbouring Ras mutations.

It is not surprising that the MAPK pathway has been the target for a number of potential cancer therapies. Sorafenib targets Raf kinases and was found to be well tolerated but with little anti-tumour activity in malignant melanomas, the intended target[251]. A number of MEK inhibitors have been trialled, firstly CI-1040 was found to be well tolerated but with little anti-tumour activity, a second MEK 1/2 is now involved in trials. In fact there have now been around 25 small molecule inhibitors aimed at the MAPK pathway that have reached early to late stage clinical trials in many different tumour types[252]. This fact alone signifies the importance of the MAPK pathway in cancer research.

As discussed above, IL-6 is known to be an important ligand involved in the activation of receptor tyrosine kinases and subsequent activation of Ras protein[253]. IL-6 has been shown not only to be increased in the plasma of patients with pancreatic cancer but is produced in an autocrine manner by pancreatic cancer cells[206-208]. These two particular facts not only emphasise the potential importance

of the MAPK pathway in pancreatic cancer, but also link it with the JAK-STAT pathway.

A number of studies in the published literature have shown the MAPK pathway to influence proliferation, mitosis and apoptosis in pancreatic tumours and cell lines. Tong et al showed that activation of the MAPK pathway was crucial for tumour proliferation in pancreatic cell lines[254]. Gysin et al looked at the role of the MAPK pathway on pancreatic cancer cell line proliferation by treating cell lines with a MEK inhibitor[255]. They were able to demonstrate that this inhibition led to a cessation of cellular proliferation and cell cycle arrest in all eight cell lines tested suggesting that the MAPK pathway may have a crucial proliferative role in pancreatic cancer cells.

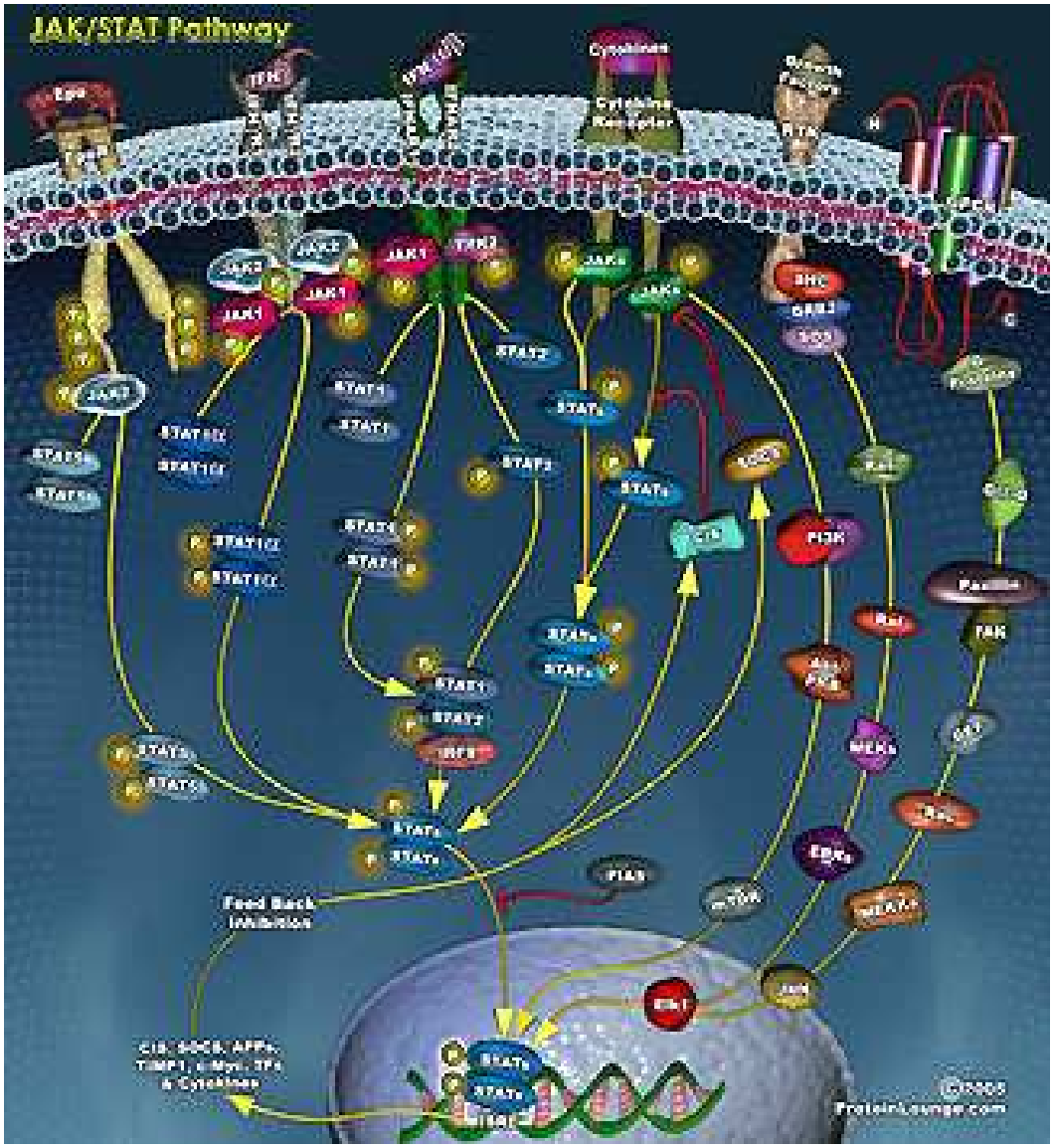
AURKA is a gene encoding a kinase crucial in the control of cellular mitosis. Using a known MAPK inhibitor and mapping the subsequently downregulated genes, Furukawa et al showed that AURKA is a downstream target of the MAPK pathway. Its overexpression in pancreatic cancer is induced by increased expression of the MAPK pathway, most likely via its constitutive activation secondary to KRas mutation[256].

Boucher et al looked at pancreatic cancer cell lines and looked at the role that ERK 1/2 had in cell survival[257]. By using a MEK inhibitor and studying the resulting changes in expression in genes known to have anti-apoptotic effects. They were able to demonstrate that activation of the MAPK pathway protects pancreatic cancer cells from apoptosis by regulating crucial genes involved in apoptosis such as Bcl-2, Bcl-x and Mcl-1.

The transcription factor NF-κB is of great importance in the inflammatory response, but increasingly it is being touted as a key player in carcinogenesis. A number of publications have shown that K-Ras may be one of the factors that is responsible for the activation of NF-κB and this will be explored later in this thesis.

Figure 1.5: Diagramatic Representation of JAK STAT and MAPK pathways

(http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/PathFinder.html)



1.5 Hypothesis and Aims

The link between inflammation and cancer is now well established. When looking specifically at pancreatic cancer, an inflammatory response, as measured by CRP is associated with a poor outcome in patients with both operable and non-operable disease. As the main driver of plasma CRP, IL-6 is the likely cause of this inflammatory response, but is IL-6 responsible for this poor prognosis, and if so how does it exert its effect? The main intra-cellular pathway associated with IL-6 signalling is the JAK-STAT pathway described above and it is known to induce cell growth, de-differentiation and inhibit apoptosis. IL-6 is a known activating ligand in the MAPK pathway also described above. The MAPK pathway is also known to be involved in the regulation of cellular growth, proliferation, differentiation and apoptosis and this thesis aims to explore the relationship between these two intracellular pathways and survival in patients with pancreatic cancer.

1.5.1 Hypothesis

The presence of a systemic inflammatory response is associated with a particularly poor prognosis in pancreatic cancer. Pro-inflammatory cytokines, in particular IL-6, are known to induce CRP production and also may influence tumour growth and differentiation through well-described cellular pathways. Upregulation of IL-6 dependent pathways in tumour cells may explain the poor prognosis associated with a systemic inflammatory response.

1.5.2 Aims

The ultimate aim of this thesis is to disprove the above hypothesis. Below are the ways in which this hypothesis will be tested:

1/. Establish pancreatic cancer patient database with basic demographics, pathological and survival data.

2/. Confirm the connection between a raised CRP and survival in pancreatic cancer in a larger cohort and establish if there is a relationship between CRP and non-pancreatic peri-ampullary cancers.

3/. Simultaneously create a prospective database and establish the connection between CRP and survival in a prospective cohort.

4/. Create tissue micro-arrays using tissue from the pancreatic cancer database to enable the efficient examination of relevant antibodies using standard immunohistochemical techniques.

5/. Using immunohistochemistry, stain with antibodies from the JAK-STAT and MAPK pathways and establish whether poor survival in these patients with pancreatic cancer is associated with upregulation of these pathways.

2.0 MATERIALS & METHODS

2.1 Patients

2.1.1 Retrospective Patients

The author (SD) gained ethical approval from the trust Ethics committee and met the approval of the local Research & Development department. The author identified all patients who had undergone resection for pancreatic cancer by interrogating the pathology department database at Glasgow Royal Infirmary with the assistance of the mortician (AM). A search looking for the words ‘whipple’ or ‘pancreaticoduodenectomy’ in all pathology reports was carried out by the author who went on to carefully examine each individual report. Only patients who had undergone a pancreatic resection for pancreatic cancer, cholangiocarcinoma, ampullary adenocarcinoma, duodenal adenocarcinoma and tumours termed by the pathologist as peri-ampullary adenocarcinoma were included. This left 148 patients, 90 pancreatic cancers (PC) and 58 non-pancreatic peri-ampullary tumours (NPPC) made up of 35 ampullary adenocarcinomas, 15 distal cholangiocarcinomas, 3 duodenal adenocarcinomas and 5 that were described as peri-ampullary adenocarcinomas.

With the pathology data, an anonymised database was created by the author for the 148 patients using their unique pathology numbers as identifiers. Basic patient demographics, pre-operative CRP values and survival data were retrieved to complete the database. All CRP values in this thesis were analysed in the biochemistry department of Glasgow Royal Infirmary. A fluorescence polarisation immunoassay was performed using an Abbott TDX analyser and Abbott reagents (Abbott Labs, Abbott Park, Il, USA).

2.1.2 Prospective Patients

The author (SD) gained ethical approval from the trust Ethics committee and met the approval of the local Research & Development department. All patients undergoing a Whipple resection for either pancreatic cancer (PC) or non-pancreatic peri-ampullary cancer at Glasgow Royal Infirmary whom gave written consent had blood taken pre-operatively by the author (SD) between 30/10/03 to 05/05/05 (collection has continued by JL after this date as the author left the department).

Blood was anonymously labelled and stored in the department of surgery at -20^{o}C. An anonymous prospective database was then created containing basic patient demographics, pathology data and survival data. The blood samples for the first 36 patients were analysed by an additional research fellow in the department of surgery at Glasgow Royal Infirmary (JL). Circulating concentrations of IL-6 were measured by JL using a commercially available enzyme-linked immuno-sorbent assay (ELISA) (R&D Systems Europe Ltd).

2.2 Creating Tissue Micro-Array's (TMA's)

Prior to undertaking the slide retrieval process the author underwent a period of gastro-intestinal histology revision, with emphasis on pancreatic and hepato-biliary histology under the guidance of a consultant pathologist (KO) within the pathology department of Glasgow Royal Infirmary. This revision included recognition of normal tissue but also on the recognition of pancreatic tumours and degrees of tumour differentiation.

2.2.1 Slide Retrieval, Identification of Tumour and Slide Marking

After the identification of the 148 patients to be included in this study, the slide retrieval process began. As stated in section 2.1, the pathology numbers were used as unique identifiers and the author collected every haematoxylin and eosin stained slide from the pathology archive that corresponded to the pancreatic resection for each of the 148 patients. This resulted in approximately 40 slides for each of the 148 patients. Following the period of histology revision, the author (SD) looked at every H&E slide collected for the 148 patients under a microscope, marking areas of tumour and areas of normal pancreatic duct. Due to the heterogenous nature of pancreatic cancer, the author marked multiple areas of each tumour aiming to include areas of differing differentiation in addition to normal pancreatic ducts. A consultant pathologist (KO) checked a proportion of the marked slides, agreeing with the selected tumour and normal duct marking.

2.2.2 Planning the TMA's

A precise plan of the TMA's was created by the author. A number of factors were taken into account when planning the TMA's: the number and size of cores (3 tumour and 2 normal ductal cores of 0.6mm for each of the 148 patients); control tissues; the ability to orientate around the TMA's; the minimum safe distance between each core; the size of the recipient wax block.

Three cores of tumour tissue and two cores of normal ductal tissue were planned for each of the 148 patients making a total of 740 cores. Two cores for each of nine control tissues (breast, testis, pancreatic ducts, pancreatic acinar tissue, pancreatic islets, bile duct, duodenum, liver and prostate) on every TMA were also planned for. The minimum distance between cores is accepted at 0.8mm but the author planned for a minimum of 1mm apart with regular larger gaps to enable easy core identification during analysis. A plan was then created using a spreadsheet with all these factors taken into account. Three TMA's were necessary, each being asymmetrical and individually identifiable (Figures 2.1-2.3).

At this stage a practice TMA was also planned containing 40 cores. This would be used for the optimisation of antibodies, avoiding waste of the full TMA's.

Figure 2.1: Plan of TMA

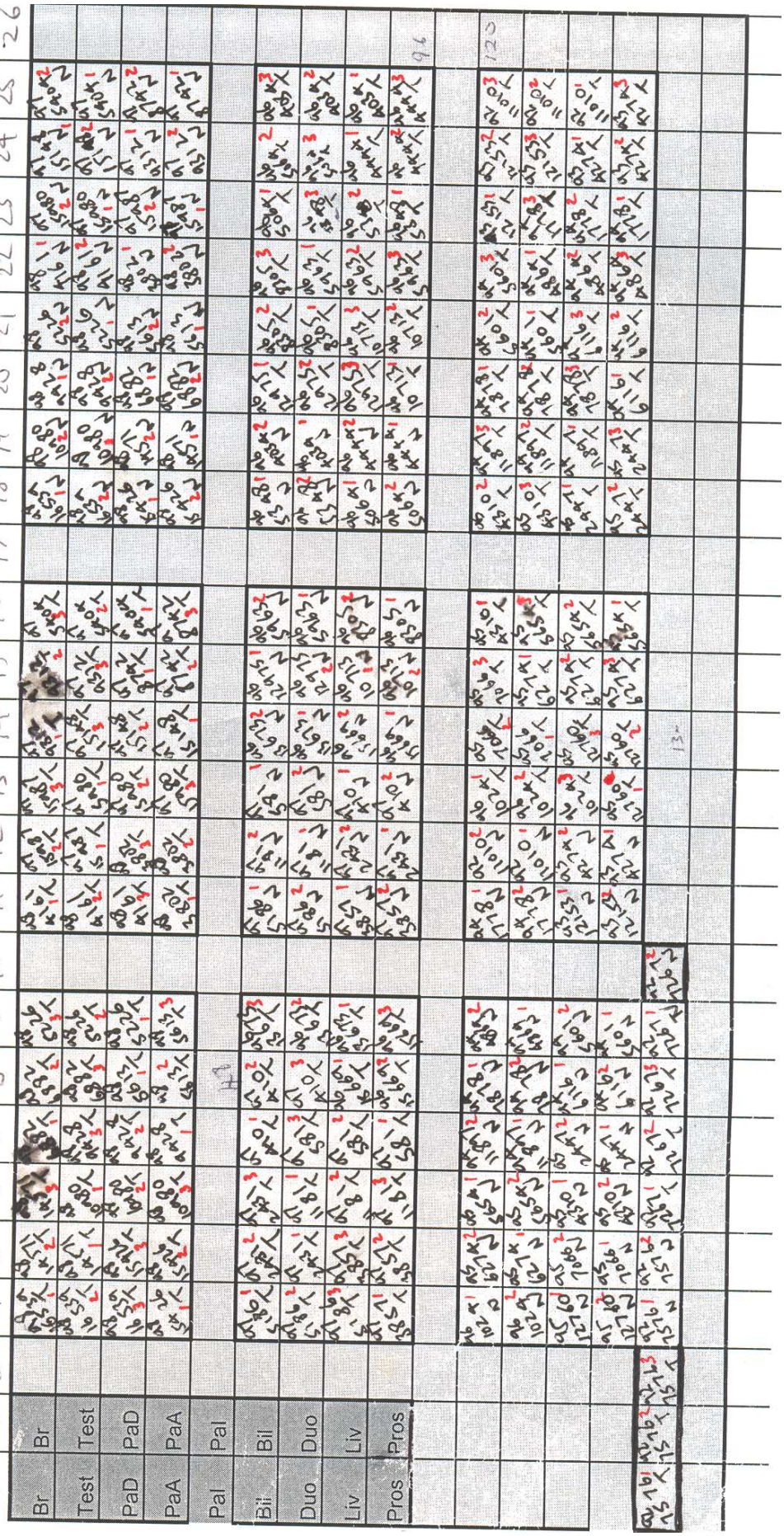
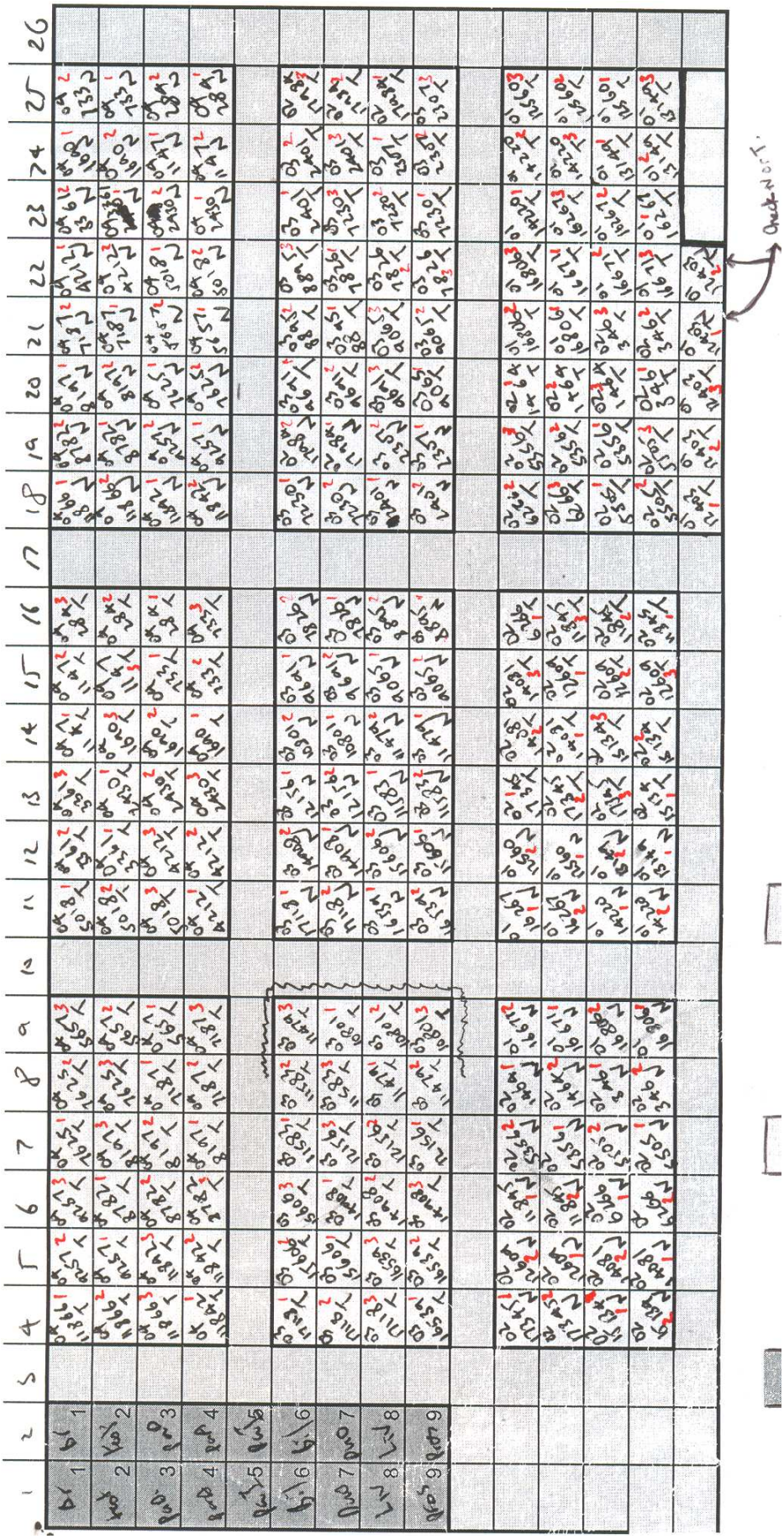


Figure 2.2: Plan of TMA 2

Figure 2.3: Plan of TMA 3



2.2.3 Making the TMA's

Following marking of the relevant H&E slides, the author collected all the corresponding paraffin embedded tissue blocks from the pathology archive of Glasgow Royal Infirmary. All the formalin fixed paraffin embedded tissue blocks paired with their corresponding pre-marked H & E slides were transferred to the pathology department of The Western Infirmary, Glasgow. The author then underwent a period of training at the Western Infirmary into the manufacturing of TMA's under the guidance of a scientist (AK) within the department with years of experience in making TMA's. A manual tissue arrayer (Beecher Instruments© Micro-array technology, figure 2.4) was used to create a number of practice lines of tissue cores. The quality of these practice runs were assessed by AK. The author then went onto create the formal practice pancreatic TMA. Following this the three full pancreatic TMA's were manufactured by the author.

The following procedure was followed for the manufacturing of the TMA's (shown in figure 2.5).

1/. Blank wax block is fixed in position.

2/. A core of wax from a pre-planned position is removed.

3/. The coring mechanism is flipped on the arrayer.

4/. A core of tissue (identified by the pre-marked H&E slide) is removed from the donor tissue block. The tissue core is slightly larger than the space to allow a tight fit.

5/. The coring mechanism allows this process to occur without moving the wax block and the tissue core is placed precisely in the space on the recipient block.

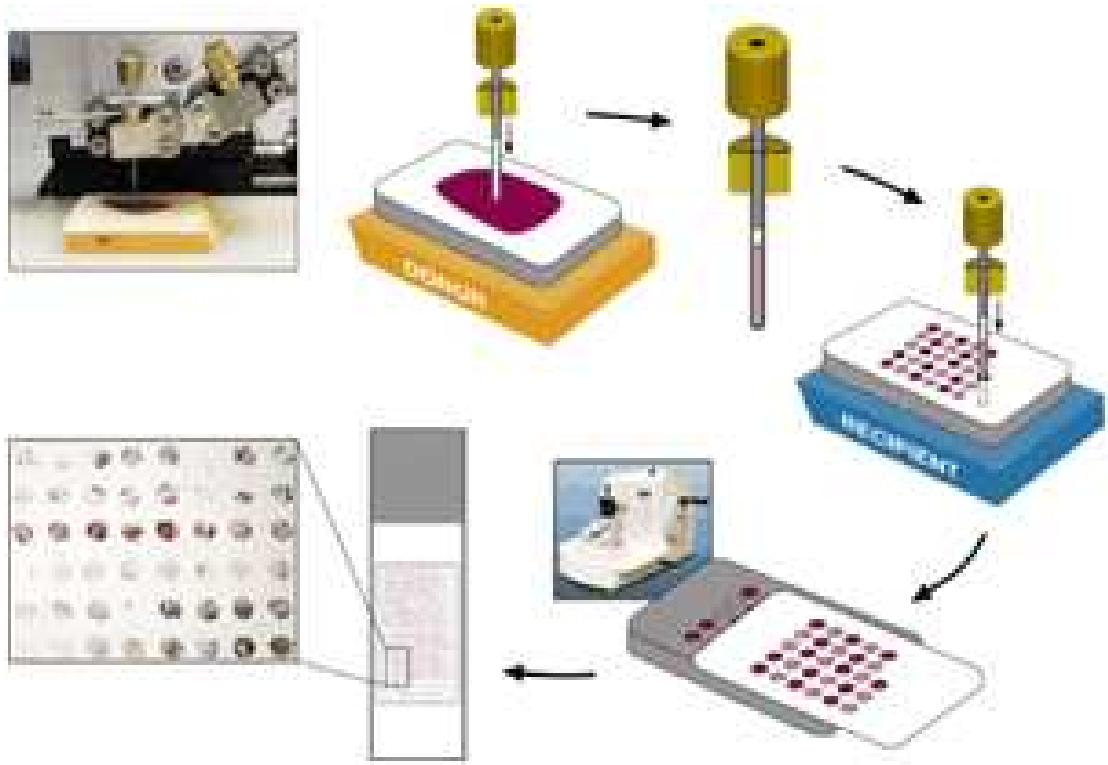
6/. Using the micrometer the coring mechanism is moved for extraction of the next wax core and the process is repeated until the TMA is completed.

7/. Only after each core is inserted into the TMA is the pathology number of that core added to the formal TMA map which is used to identify cores for later scoring.

Figure 2.4: Beecher Instruments Manual Tissue Arrayer

(www.beecherinstruments.com)

Figure 2.5: Technique for creating TMA (www.tissue-array.org)



2.2.4 Preparation of Tissue Sections

The manufacturing of the TMA's was done entirely by the author (SD). The sectioning of the TMA's was performed by an MLSO with extensive TMA sectioning experience using the following protocol. Before sectioning of the TMA's could take place they were heated to 37^{o} for 10 minutes to allow the wax from the block to bind with the wax in the tissue core to avoid shearing of the cores during sectioning. Prior to sectioning, tissue blocks were cooled to $-10^{o}C$ (Tissue Tec© cooler). Three micrometer thick sections were then cut from the blocks using a Leica© RM 2135 microtome. These sections were then floated onto a waterbath and heated to $45\ ^{o}C$ allowing any tissue folds to flatten out. Following heating the sections were applied to aminopropyltriethoxysilane-treated (sialinised) slides (BDH 1.0mm thick, clear glass). These slides were sialinised to create greater adherence with the tissue using the following protocol:

1. Slides placed in acetone for 5 minutes, followed by 2% silane (v/v 8ml in 400ml).

2. Slides then washed in running tap water for 5 minutes, before being bathed in tap water for a further 20 minutes before being dried overnight in a fume hood.

Slides were then ready for immunohistochemical staining.

2.3 Antibodies and Antibody Specificity

2.3.1 Antibodies

Twelve antibodies were used in this study, six from the JAK-STAT pathway and six from the MAPK pathway. They were:

1. Interleukin-6 Receptor (IL-6R)- (C20, SC-661, Santa Cruz)
2. Jak-1- (#3332, Cell Signalling Technology, US)
3. Phosphorylated.Jak-1- (New England Biolabs, UK)
4. STAT-3- (#9132, Cell Signalling Technology, US)
5. Phosphorylated.STAT-3$^{Tyr\ 705}$- (#9131, Cell Signalling Technology, US)
6. Phosphorylated.STAT-3$^{Ser\ 727}$- (#9134, Cell Signalling Technology, US)
7. K-Ras- (Sigma R3400 Clone 234-4.2)
8. Raf-1- (E-10, Santa Cruz)
9. Phosphorylated.Raf-1$^{Ser\ 259}$- (#9421, Cell Signalling Technology, US)
10. Phosphorylated.Raf-1$^{Ser\ 338}$- (#05-538, Upstate)
11. MAPK (p42/44)- (#9012, Cell Signalling Technology, US)
12. Phosphorylated.MAPK (p42/44)- (#9101, Cell Signalling Technology, US).

2.3.2 Antibody Specificity

All antibodies except Phospho.JAK-1 were available in the laboratory of the University Department of Surgery, Glasgow Royal Infirmary. All eleven of the available antibodies had been used successfully within the same laboratory, the JAK-STAT proteins on prostatic tissue and the MAPK proteins on prostatic and breast tissue. The specificities of these eleven antibodies had all been established by Western Blotting within the same laboratory by previous students. The JAK-STAT antibodies (IL-6R, JAK-1, STAT3, Phospho.STAT3 Tyr705,Phospho.STAT3 Ser727) had Western Blotting performed by an MSc student (LT), demonstrating specificity in all five. The six MAPK antibodies (K-Ras, RAF-1, Phospho.RAF-1 Ser259, Phospho.RAF-1 Ser338, MAPK, Phospho.MAPK) underwent Western Blotting by a PhD student (LM) and again all six had their specificity confirmed.

2.3.3 Western Blotting for Phospho.JAK-1

The antibody Phospho.JAK-1 was not available nor had it been previously used within the laboratory. The author contacted Cell Signalling Technology who were able to supply a free sample of Phospho.JAK-1 antibody, but did state that this had not been used for immunohistochemistry before. Despite repeated attempts using a standard Western Blotting technique used successfully in the laboratory for other antibodies, the author was unable to demonstrate specificity for this antibody. After consultation with the senior scientist (JE) within the department, it was decided to perform immunohistochemical staining with the Phospho.JAK-1 antibody sample.

2.4 Immunohistochemistry

Protein expression was determined using immunohistochemistry (IHC). This is a staining technique which relies on the use of specific antibodies raised against epitopes of the protein antigens to be analysed. In this study a standard indirect immunoperoxidase technique was used to stain tissue sections. Slides contained 3µm sections of tissue, cut from paraffin embedded tissue-blocks and required de-waxing in xylene and progressive rehydration through graded alcohol solutions in order to remove any remaining paraffin. Some cells contain endogenous peroxidase enzymes which can interfere with the use of peroxidase-labelled antibodies later on during the procedure. Treatment with hydrogen peroxide (3%) is therefore required to destroy this pre-existing peroxidase within the tissue.

The next stage is an antigen-retrieval process which is required to counteract the effects of formaldehyde fixatives. These fixatives react with basic amino-acids to form cross-links masking antigen from the antigen. The cross-links can be removed using a variety of methods (incubating slides in heated trypsinised solution or citrate buffer solution) depending on the particular antigen in question. Non-specific binding of antibody to protein can take place resulting in background staining. This makes subsequent scoring difficult as differentiating between specific and background staining is impossible. A blocking agent is therefore required and takes the form of serum, which is usually derived from horse.

The addition of the antibody, which may be monoclonal or polyclonal is next. Polyclonal antibodies are by definition derived from several different clones of

plasma cells upon inoculation of protein, meaning they will react to different epitopes on the same antigen and can be used at low concentrations. This also means that they may also contain non-specific antibodies, leading to background staining. This effect can be minimised by titrating the most appropriate concentration of antibody. Monoclonal antibodies are produced from a single clone of B-cells and react with a specific epitope on the antigen and consequently leading to less background staining.

Primary antibodies are incubated for varying lengths of time, ranging from 1 hour at room temperature to overnight at 4^0C dependent on the antibody used. Following this initial incubation period the addition of a secondary antibody is required as part of an indirect method of immunostaining. This takes place in two parts, initially using a biotinylated secondary antibody which links to a peroxide enzyme-streptavidin conjugate. Streptavidin has a low potential for non-specific binding which results in more sensitive staining. Endogenous biotin may however result in non-specific binding of secondary antibody which may be reduced by the addition of a biotin block.

The next stage is staining which is achieved by the addition of 3,3-diamino-benzidine tetrahydrochloride (3,3-DBT). In the presence of peroxidase at the site of the antigen DBT polymerises to form a visible brown reaction product. Following this, the nuclei are counterstained blue with haematoxylin and Scotts tap water substitute. The final stage involves dehydration of the sections in graded alcohol solutions before clearing in xylene. Finally DPX resin-coated coverslips are added and the slides are ready for scoring.

All immunohistochemistry was performed by the author using a number of protocols. All protocols involved a number of similar steps which are described below. The individual variations between antibodies are summarised in Table 2.1.

2.4.1 De-waxing and Rehydration

1. Xylene (Fisher Laboratories, UK) for 5 mins x2
2. 100% alcohol (Fisher Laboratories, UK) for 5 mins
3. 90% alcohol for 2 mins
4. 70% alcohol for 2 mins
5. Rinse in tap water

2.4.2 Antigen Retrieval

Formalin fixation causes cross-linking within the sections, causing alteration of the tertiary structure of the protein and may therefore mask the antigenic site in the protein that the antibody has been raised to. Antigen retrieval is necessary to break these bonds and unmask the antigen and involves a process with enough energy to break the disulphide bonds without destroying the intrinsic protein structure. In this study the author used two methods depending on the antibody used:

Microwave under pressure:

1. Add 0.55g TRIS (VWR International LTD, UK) and 0.37g EDTA (BDH Laboratory Supplies, UK) to 1L of distilled water.
2. Microwave under pressure for 5 mins
3. Allow slides to cool for 20 mins
4. Rinse in tap water

Citrate buffer incubation:

1. Incubate in Citrate buffer solution (Epitope Retrieval Solution x10, K-5205, Dakocytomation, Denmark) diluted in distilled water (72:8) at 96^0C in a calibrated water bath for 20 mins
2. Allow slides to cool for 20 mins
3. Rinse in tap water

2.4.3 Block of Endogenous Peroxidase Activity

Many human tissues contain endogenous peroxidase. All twelve antibodies underwent treatment hydrogen peroxidase to irreversibly inactivate endogenous peroxidase. This was done as described below, either before or after antigen retrieval depending on the individual antibody protocol:

1. H_2O_2 (VWR International LTD, UK) 0.3% 400mls for 10 mins

2. Rinse in tap water

2.4.4 Block of Non-specific Background Staining

Non-specific background staining can cause problems during analysis and requires blocking. In the majority of cases this was done using 1.5% v/v normal horse serum (S-2000, Vector Laboratories Inc. Burlingame CA 94010) in tri-phosphate buffered saline (TBS). This was done using the following protocol:

- Block with 15uL horse serum (Vector, US) per 1ml of TBS for 20 mins, then blot.

In addition to horse serum some antibodies required further blocking using Avidin and Biotin block as described below:

1. Add Avidin block (Vector Laboratories, US) for 15 mins
2. Wash in TBS for 5 mins
3. Add Biotin block (Vector Laboratories, US) for 15 mins
4. Wash in TBS for 5 mins

A number of antibody protocols did not use horse serum and instead background, non-specific staining was blocked using the below protocol:

- Incubate with ‘Power Block’ – 1xCasein (diluted in TBS) for 10 minutes, then blot.

2.4.5 Incubation with Primary Antibody

The primary antibody was then added to the sections. For each antibody a number of different concentrations were tried on the practice TMA. The optimum concentration was chosen as the one that gave the least amount of background staining with clear tumour staining. All antibodies were diluted using DAKO antibody dilutent (S-0809, Dakocytomation, Denmark) and incubated overnight at either room temperature or 4^0C in a moisture chamber. See Table 1 for concentrations of each antibody.

2.4.6 Visualisation Method

Once the antibody has bound to its target protein, a visualisation method is needed to identify the location and intensity of the antibody. In the twelve antibodies used in this study three different methods were used depending on the individual protocols, each being described below.

Following each of the three visualisation methods described below 3,3-diaminobenzidine (DAB) solution is added. DAB binds to the peroxidase domain leaving an insoluble brown stain at the site of cleaving. The addition of DAB is common to all twelve antibodies using the protocol described below and follows each of methods 1, 2 and 3.

Method 1:

1. Add secondary antibody (K-0690, Dakocytomation, Denmark). Leave for 15 minutes
2. Wash in TBS for 5 mins x2
3. Add Biotinylated Streptavadin-HRP complex (K-0690, Dakocytomation, Denmark). Leave for 15 mins
4. Wash in TBS for 5 mins

Method 2:

1. Add Envision block (Dakocytomation, Denmark) for 30 minutes
2. Wash in TBS for 5 mins

Method 3:

1. Incubate slides with ‘SuperBlock Reagent’ (HK518-06K, BioGenex) for 20 mins
2. Wash in TBS for 5 mins x2
3. Incubate slides with ‘Poly-HRP Reagent’ (HK519-06K, BioGenex) for 30 mins
4. Wash in TBS for 5 mins x2

DAB protocol (follows each of the above methods 1-3):

1. DAB (SK-4100, Vector Laboratories, Burlingame, CA 94010, US) Kit contains separate H_2O_2, DAB reagent and buffer (pH 7.5) - 2 drops buffer: 4 drops DAB: 2 drops $H_2O_{2\ in}$ 5mls distilled water.
2. Leave for 2-10 minutes until brown staining evident.
3. Rinse in tap water for 10 minutes.

2.4.7 Counterstaining

Counterstaining is essential to stain areas on the section that do not contain the antibody so that all cells and stroma on the section can be visualised. All twelve antibodies underwent the following protocol:

1. Stain in haematoxylin (Thermoshandon) for 90secs
2. Rinse in tap water
3. Add to Scotts Tap water (Magnesium Sulphate 7-hydrate and Na, H+ Carbonate , BDH Laboratory Supplies, UK) until blue staining
4. Rinse in tap water

2.4.8 Dehydration and Mounting

Following staining the slides undergo dehydration through increasing concentrations of alcohol and then xylene before being mounted on a coverslip to preserve the tissue and allow examination under a microscope. All twelve antibodies underwent the following protocol:

1. 1 min 70% alcohol
2. 1 min 90% alcohol
3. 1 min 100% alcohol x2
4. 1 min xylene x2
5. Mount in DPX (a mixture of distyrene (a polystyrene), tricresyl phosphate (a plasticizer) and xylene). DPX has the same refractive index of glass and enables visualisation via light microscopy.

The above protocols were based on work done on prostate tissue for the JAK-STAT pathway and breast tissue for the MAPK pathway. They were developed for pancreatic tissue using the practice TMA described earlier until adequate quality of staining was achieved. The new protocol was then used upon the three pancreatic TMA's. The variables in IHC for each antibody are summarised in Table 2.1.

Table 2.1: Variations in IHC for each of the twelve antibodies.

Antibody	Antibody dilution	Antigen retrieval method	Blocking of peroxidase activity (before/after antigen retrieval)	Blocking of non-specific background staining	Incubation with primary antibody	Visualisation method
IL-6R	1:500	Citrate buffer	Before	Horse serum	4^0C	2^0 antibody & biotin
Jak-1	1:200	Microwave	After	Horse serum (+avid & biotin)	Room temp	2^0 antibody & biotin
pJak-1	1:25	Microwave	After	Horse serum	Room temp	Envision
STAT-3	1:100	Citrate buffer	Before	Horse serum	4^0C	2^0 antibody & biotin
pSTAT-3$^{Tyr\ 705}$	1:50	Microwave	After	Horse serum	Room temp	2^0 antibody & biotin
pSTAT-3$^{Ser\ 727}$	1:50	Microwave	After	Horse serum	Room temp	2^0 antibody & biotin
K-Ras	1:10	Microwave	After	Power block	4^0C	Super-block & Poly-HRP
Raf-1	1:80	Citrate Buffer	After	Horse serum	4^0C	Super-block & Poly-HRP
pRaf-1^{Ser259}	1:10	Citrate buffer	After	Power block	4^0C	Envision
pRaf-1^{Ser338}	1:250	Microwave	After	Power block	4^0C	Envision
MAPK	1:200	Citrate buffer	After	Horse serum	4^0C	Envision
pMAPK	1:50	Microwave	After	Horse serum	Room temp	Envision

2.5 Scoring

Scoring was performed using the weighted histoscore. When scoring each core/slide, the investigator multiplies the proportion of cells staining by the intensity of that staining to give on overall histoscore out of 300 for each part of the cell, the membrane, the cytoplasm and the nucleus. The proportion of cells staining is given as a percentage and the intensity is based upon a scale of 0-3, where 0 is no staining, 1 is weak staining, 2 is moderate staining and 3 is strong staining. The total score out of 300 is achieved by adding the following: (0 x percentage not staining) + (1 x percentage weakly staining) + (2 x percentage moderately staining) + (3 x percentage strongly staining). For example, a section with 50% of tumour staining with an intensity of 1; 25% staining with intensity of 2 and 25% staining with an intensity of 3 would have a total histoscore of:

(50x1) + (25x2) + (25x3) = 175

All 12 of the antibodies were double scored (a 10% sample of each antibody) by the senior scientist (JE) within the department of surgery with years of experience and scoring consistency was confirmed using the Intra-class correlation coefficient (ICCC). If there was a difference of 50 or greater for any score then the two observers looked at the core again and came to an agreement about the appropriate score.

2.6 Statistical Analysis

All statistical analysis in this thesis was performed using SPSS 11&15 (SPSS Inc, Chicago, Illinois) by the author (SD) with advice from a statistician (WA) in the University Department of Surgery, Glasgow Royal Infirmary The statistics in Section 3.4.6 (Further Statistical Analysis) were performed entirely by a statistician (JP) within the Beatson Oncology Centre, Gartnavel General Hospital, Glasgow, using his own statistical package and are reproduced in this thesis with his kind permission.

In section 3.1.2 (Patient Demographics) median age and range was calculated by analysing the basic patient demographics and using descriptive statistics followed by frequencies. Section 3.1.3 (Clinical and Pathological Prognostic Factors) involved the analysis of a number of factors against survival. The database contained survival data for all of the 148 patients and survival analysis was performed using Kaplan Meier curves and the log-rank test for significance. The event in the analysis was death and for each of the variables the patients were split into groups before the survival analysis was performed: pancreatic cancer and NPPC (Section 3.1.3.1); tumours >2cm and <2cm (Section 3.1.3.2); N0 and N1 tumours (Section 3.1.3.3); tumour differentiation (Section 3.1.3.4); R0 and R1 tumours (Section 3.1.3.5).

Section 3.2 dealt with CRP and for the statistics the tumours were again separated into Pancreatic cancer and NPPC. Before survival analysis, again with Kaplan Meier curves and the log-rank test, the patients were divided into two groups depending whether their pre-operative CRP was raised or not. A CRP of

>10mg/ml was taken as a raised CRP, again analysing against survival with death being the event. Following univariate analysis of all clinical and pathological prognostic factors, all statistically significant factors were entered into a Cox's regression analysis, again with length of survival and death as the event to establish significant factors following multivariate analysis.

The author scored all of the antibody staining and 10% of this scoring was repeated independently by the senior scientist within the laboratory (JE) to check scoring consistency. This consistency was analysed using the ICCC via scale and then reliability analysis.

The frequency histograms plotted in section 3.4.4 (Protein Expression) were produced by analysis and descriptive statistics to give a visual representation of the distribution of scoring for each antibody in each cellular compartment and are displayed in full in Appendix 1 and Appendix 2 (for the JAK-STAT and MAPK pathways respectively).

These frequency histograms demonstrate skewing to the right in the vast majority of cases in both the JAK-STAT and MAPK pathways and after consultation with the unit statistician (WA) it was decided that the optimum cut-off between what was considered low and high scoring should be the upper quartile. This was used to again plot Kaplan Meier curves with the log-rank test to assess significance for each of the twelve antibodies in each cellular compartment.

3.0 RESULTS

3.1 Patients

3.1.1 The Retrospective Database

Ethical approval was achieved from the trust Ethics committee and met the approval of the local Research & Development department. Identification of all patients who had undergone resection for pancreatic cancer was achieved using the pathology department database at Glasgow Royal Infirmary under the guidance of Mr Alan Moffat (mortician, Glasgow Royal Infirmary). The start date for this search was June 1992, when the pathology department database was created. A search looking for the terms ‘whipple’ or ‘pancreaticoduodenectomy’ in all pathology reports was carried out. All of the identified reports were then examined on a computer, those patients with Whipples disease biopsy results being excluded, leaving only those who had undergone a Whipple operation or pancreaticoduodenectomy. Each report was then carefully examined and only patients who had resection for either pancreatic cancer (PC) or non-pancreatic peri-ampullary cancer (NPPC) were included (neuro-endocrine tumours were excluded). This left 148 patients, 90 PC and 58 NPPC. The NPPC group was made up of 35 ampullary adenocarcinomas, 15 distal cholangiocarcinomas, 3 duodenal adenocarcinomas and 5 that were described as peri-ampullary adenocarcinomas on the pathology report.

Patient demographics were entered onto an anonymised database using pathology and hospital numbers as identifiers along with their pathological details. Further information including pre-operative CRP and survival data was gained from hospital records and added to the database.

3.1.2 Patient Demographics

Between June 1992 and July 2004, 148 patients underwent a Whipple procedure for malignant disease and were included in this study. During the first 6 years (June 1992- June 1998) of this period only 23 resections were performed by a single surgeon. The increase in resections over the latter six years (July 1998- July 2004) reflects the centralisation of pancreatic services in the West of Scotland, with three surgeons performing resections.

Overall median age was 63.5 years (range 38-77), in the PC group 63.5 years (range 38-77) and in the NPPC group median age was 64 years (range 42-77) (Table 3.1).

Table 3.1: Basic Patient Demographics

Variable	**Overall group (N=148)**	**PC group (N=90)**	**NPPC group(N=58)**
Median Age	63.5 years (38-77)	63.5 years (38-77)	64 years (42-77)
Male: Female	77:71	45:45	32:26
Median Survival	477 days (7-3851)	374 days (8-2873)	618 days (7-3851)
Alive: Dead	27:121	11:79	16:32

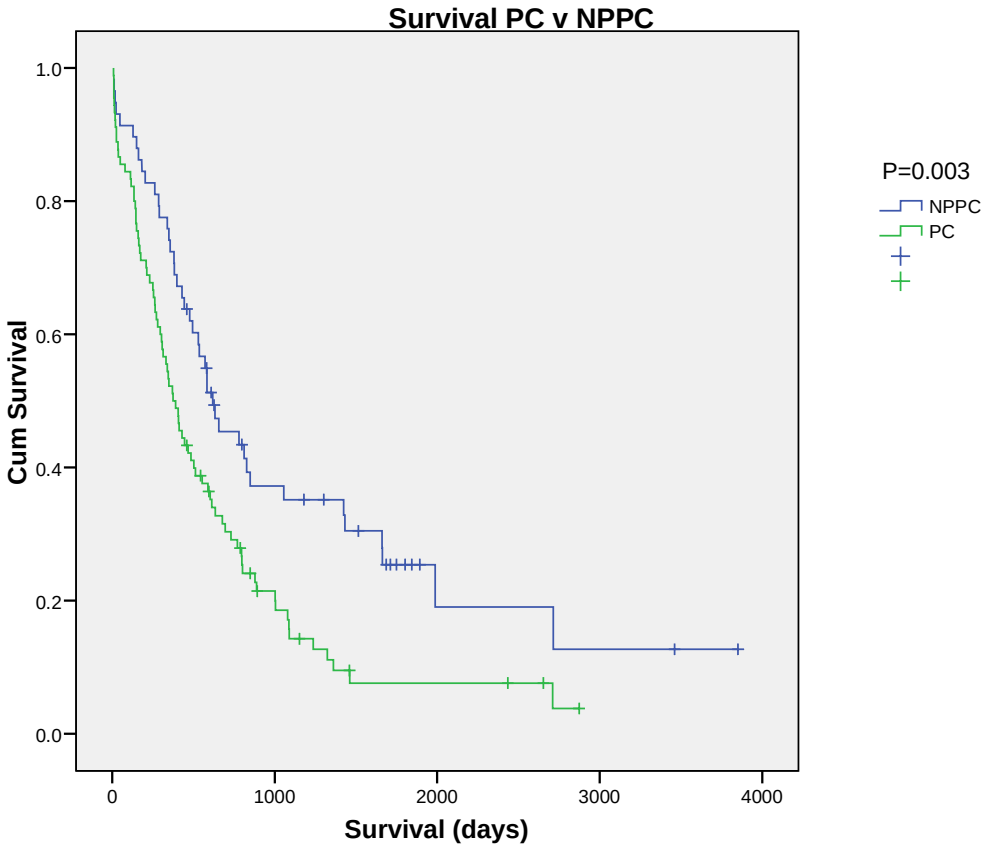
3.1.3 Clinical and Pathological Prognostic Factors

There are a number of clinical factors that are known to influence survival in resectable pancreatic cancers (see introduction). These include: histological tumour type; tumour size; lymph node status; tumour differentiation; resection margin status. As this thesis discusses CRP as a prognostic factor, the prognostic indicators above are discussed individually below.

3.1.3.1 Histological Tumour Type

The histological origin of the tumour is known to be crucial to post-operative survival, PC patients doing poorly when compared to NPPC[5]. In this cohort overall median survival was 477 days (range 7-3851) with 27 patients alive at the end of the study period. When patients were divided into PC and NPPC groups, using Kaplan Meier curves and the log rank test for statistical significance the median survival times were 374 days (range 8-2873) and 618 days (range 7-3851) respectively (p=0.003) (Figure 3.1).

Figure 3.1: Kaplan Meier Curve of PC versus NPPC post-op survival.



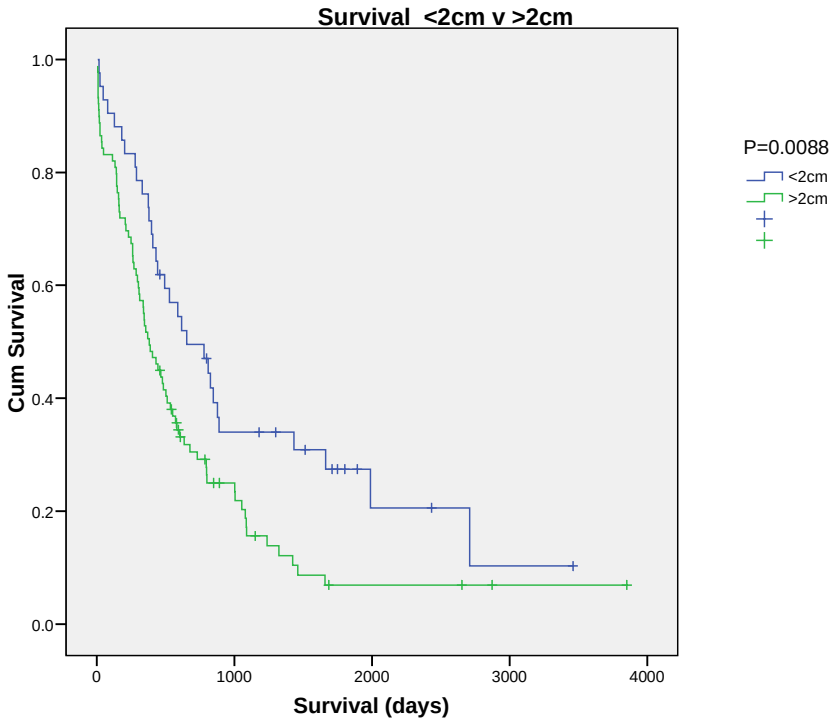
3.1.3.2 Tumour Size

In the current TNM classification of pancreatic cancers, tumours are separated into those less than and those greater than 2cm to differentiate between T1 and T2 tumours respectively. Tumour size is, as mentioned above another known prognostic factor, with larger tumours associated with poorer survival[11]. In the analysis of tumour size against survival, 2cm (maximum diameter) was used as the cut off before analysis using Kaplan Meier curves and the log rank test for statistical significance. Analysis revealed a significant difference in survival when the group was analysed as a whole, but not when separated in PC and NPPC groups (Table 3.2).

Table 3.2: Survival analysis of tumour size.

Tumour Type	Size	Median Survival (days)	Range	P-Value
OVERALL	T1	655	17-3460	**0.0088**
	T2	381	7-3851	
PC	T1	430	79-2710	**0.3469**
	T2	345	8-2873	
NPPC	T1	817	17-3460	**0.2045**
	T2	477	7-3851	

Figure 3.2: Kaplan Meier curve of tumour size and survival in overall group.



3.1.3.3 Lymph Node Status

The presence of tumour within lymph nodes following resection is associated with a negative impact on survival[13]. In the current TNM classification N0 and N1 are used to described tumours without and tumours with regional lymph node invasion respectively. Using lymph node status from post-operative resection specimens, Kaplan Meier curves and the log rank test for statistical significance were used to establish if lymph node positivity had an impact on survival in this patient cohort.

Following analysis, N1 disease was associated with significantly poorer survival when compared with N0 disease when all patients were analysed together and when separated into PC and NPPC patients (Table 3.3 & figures 3.3-3.5). In the PC group the Kaplan Meier curve (figure 3.4) illustrates the poorer survival with N1 disease but the median survival is actually longer than N0 patients. This is due to half of the patients in both groups dying in the first year, where the two curves are close. After the first year there is separation of the two curves as a number of N0 patients are long-term survivors. In the NPPC group, N1 disease is associated with significantly poorer survival ($P<0.0001$) (Figure 3.5).

Table 3.3: Survival Analysis of Lymph Node Status

Tumour Type	Nodal Status	Median Survival (days)	Range	P-Value
OVERALL	N0	1055	7-3851	**<0.0001**
	N1	411	8-2713	
PC	N0	304	9-2873	**0.04**
	N1	389	8-1461	
NPPC	N0	1659	7-3851	**<0.0001**
	N1	494	12-2713	

Figure 3.3: Kaplan Meier Curve of lymph node status and post-op survival in all patients.

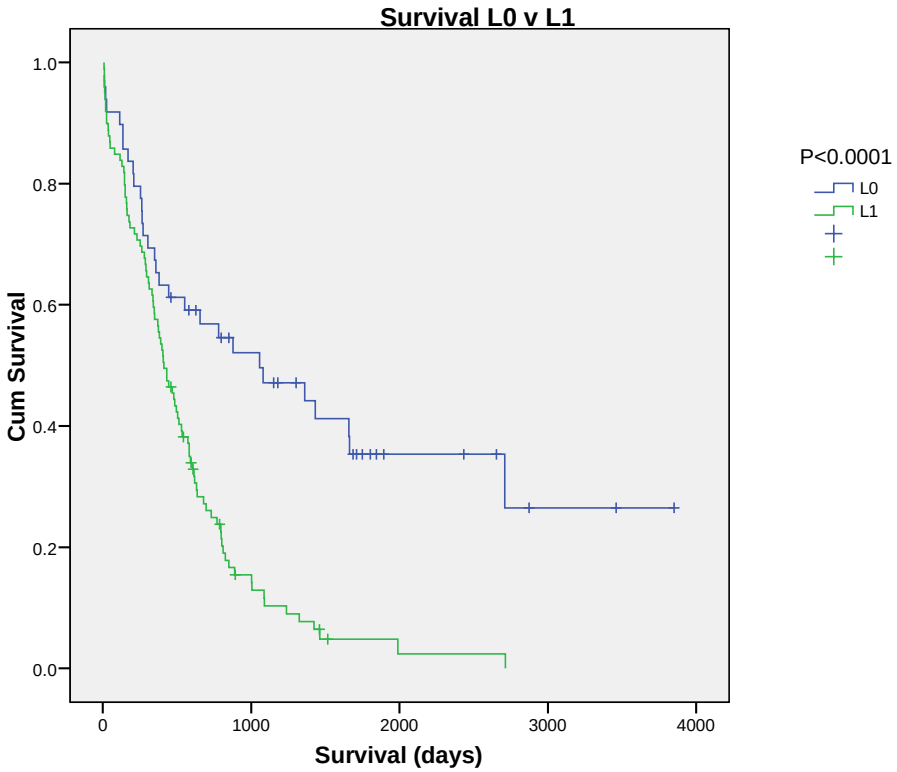


Figure 3.4: Kaplan Meier Curve of lymph node status and post-op survival in PC patients.

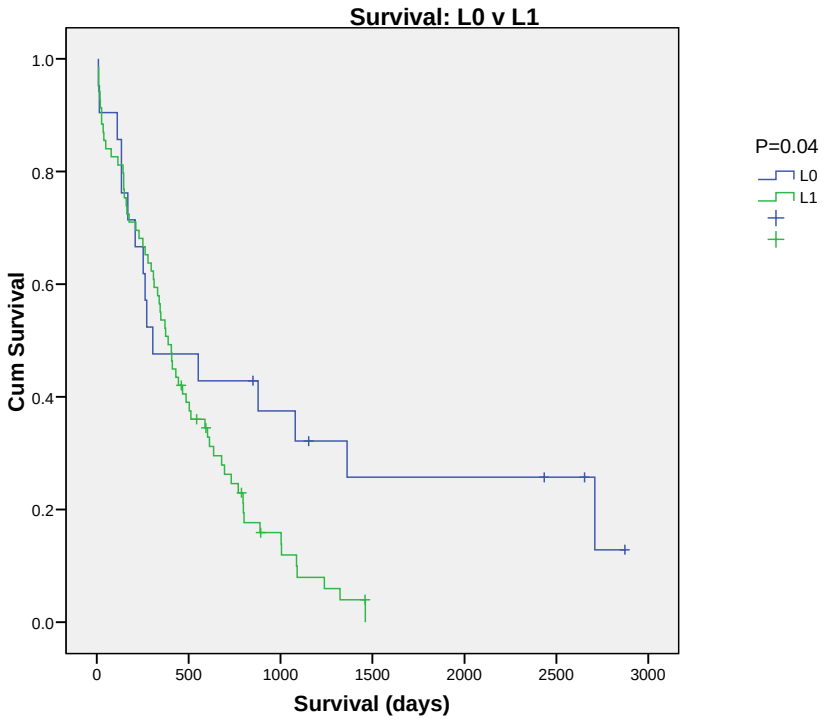
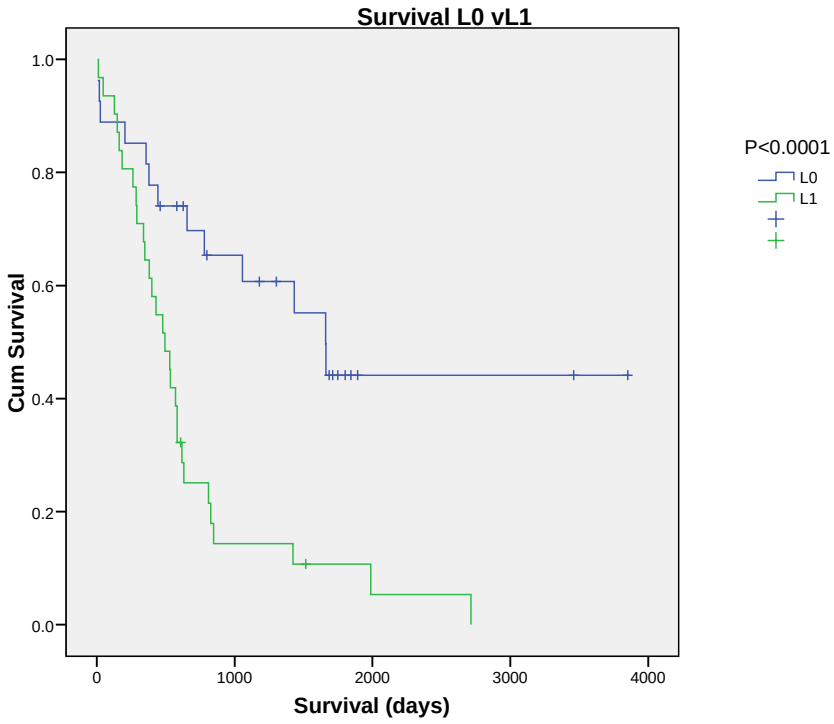


Figure 3.5: Kaplan Meier Curve of lymph node status and post-op survival in NPPC patients.



3.1.3.4 Tumour Differentiation

The differentiation of tumours significantly affects outcome after curative resection for pancreatic tumours, poorly differentiated tumours having a significantly poorer outcome compared to well or moderately differentiated tumours[9]. Patients were divided into PC and NPPC groups before analysis using Kaplan Meier curves and the log rank test for statistical significance. When all patients were analysed together, patients with poorly differentiated tumours survived a shorter period (p=0.028) (figure 3.6). In the PC group, tumour differentiation was a significant prognostic factor, patients with a poorly differentiated tumour surviving for a shorter period (p=0.0091) (figure 3.7). There was no significant difference in the NPPC group (p=0.34).

Table 3.4: Survival analysis of tumour differentiation

Tumour Type	Differentiation	Median Survival (days)	Range	P-value
OVERALL	Well	589	8-2710	**0.0281**
	Moderate	552	10-3851	
	Poor	290	7-2434	
PC	Well	589	8-2710	**0.0091**
	Moderate	467	10-2653	
	Poor	208	10-2434	
NPPC	Well	1301	356-1988	**0.3409**
	Moderate	618	17-3851	
	Poor	443	7-1515	

Figure 3.6: Kaplan Meier curve of tumour differentiation and post-op survival in the overall group (p=0.0281).

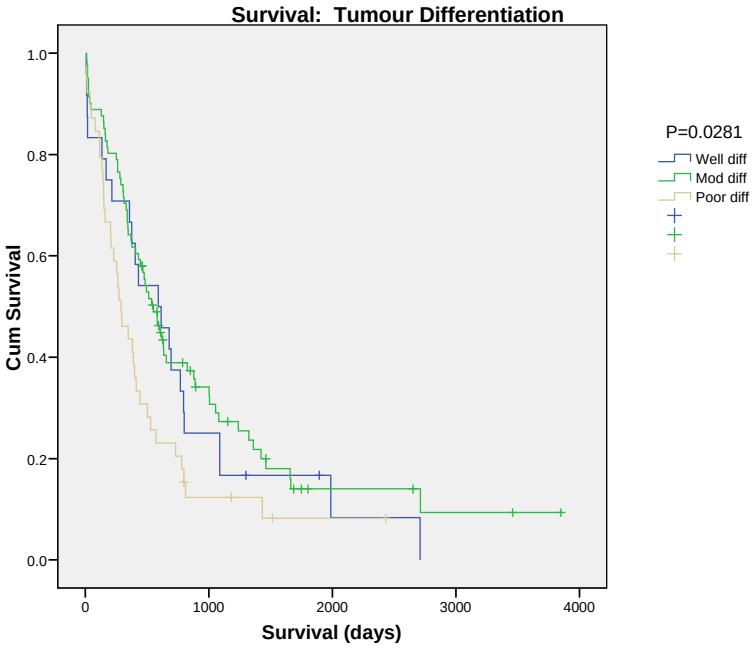
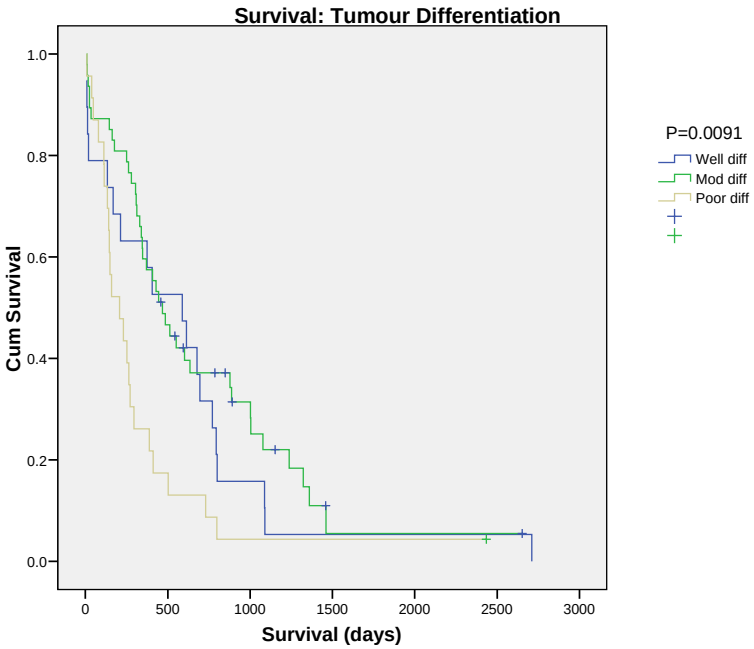


Figure 3.7: Kaplan Meier curve of tumour differentiation and post-op survival in the PC group (p=0.0091).



3.1.3.5 Resection Margin Status

The presence of tumour at the resection margins following potentially curative resection for pancreatic cancer causes a significant reduction in post-operative survival[9]. As with other clinical parameters, patients were split in to PC and NPPC groups prior to analysis using Kaplan Meier curves and the log rank test for statistical significance.

Following analysis a positive resection margin is significantly associated with poor survival when all patients are analysed together (figure 3.8), but appears to have no significant affect on survival in PC patients in this cohort (Table 3.5). In the NPPC group, positive resection margins were associated with significantly shorter survival than patients with a R0 resection (Figure 3.9) ($p<0.001$).

Table 3.5: Survival Analysis of Resection Margin Status

Tumour Type	Resec.Margin	Median Survival (days)	Range	P-Value
OVERALL	R0	655	7-3851	**<0.0001**
	R1	370	10-1460	
PC	R0	589	8-2873	**0.16**
	R1	345	10-2710	
NPPC	R0	779	7-3851	**0.001**
	R1	379	47-826	

Figure 3.8: Kaplan Meier Curve of resection margin status and post-op survival in all patients.

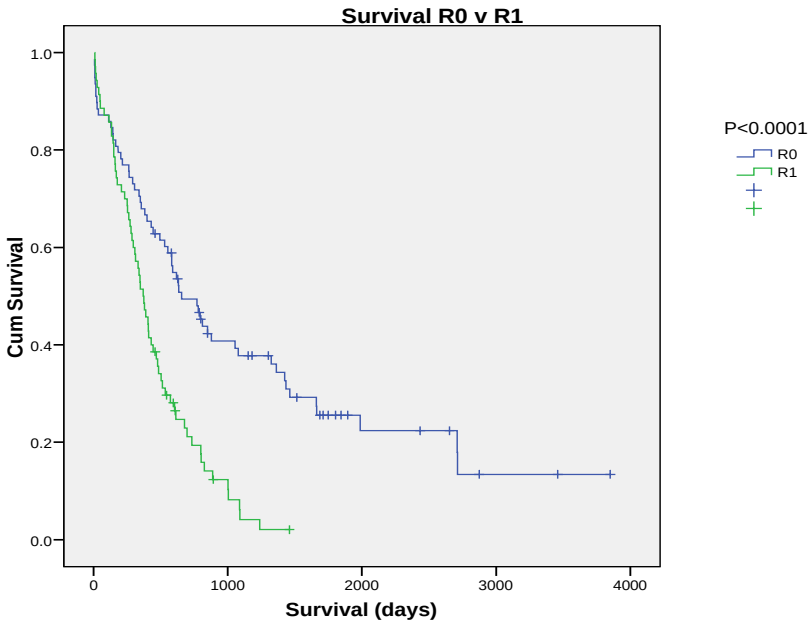
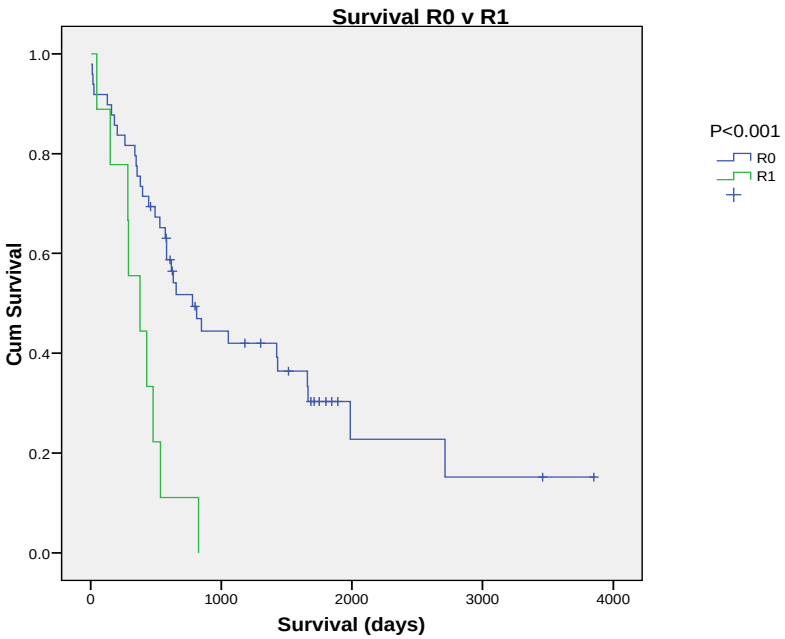


Figure 3.9: Kaplan Meier Curve of resection margin status and post-op survival in NPPC patients.



3.2 C-reactive Protein

3.2.1 Retrospective CRP

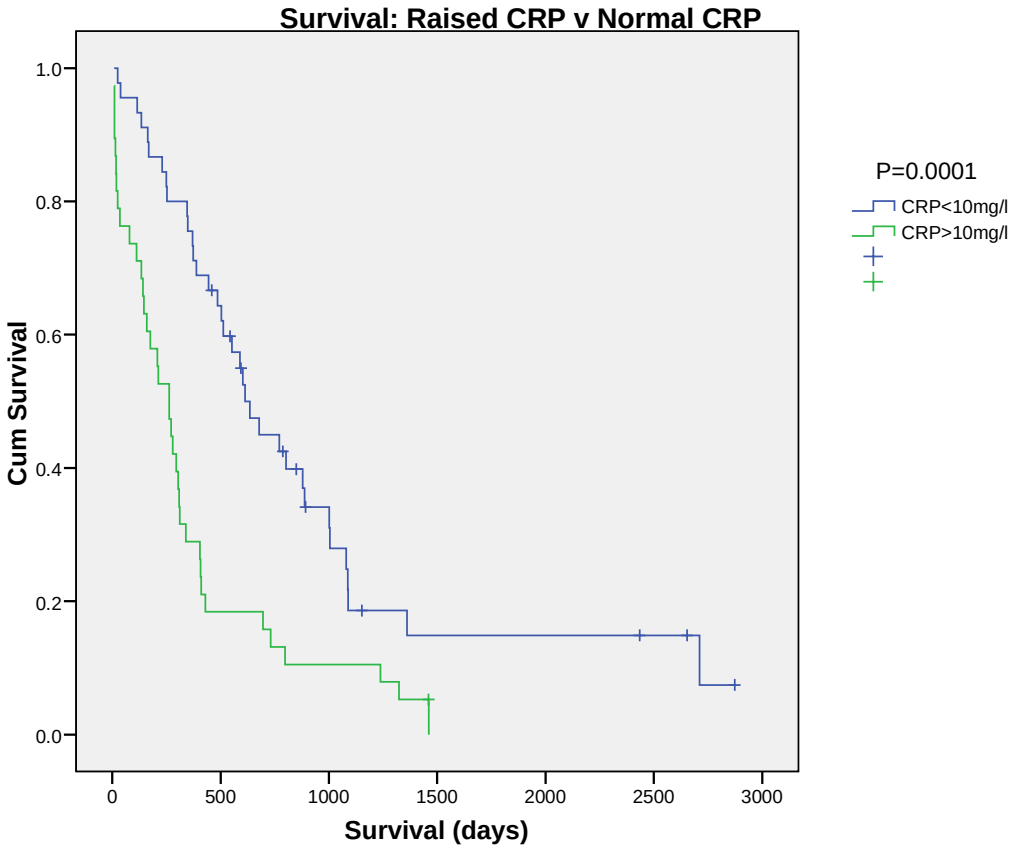
Overall 118 of the 148 patients had pre-operative CRP measurements available, 83 of these were in the PC group and 35 in the NPPC group. Of the 83 PC patients with pre-op CRP available 38 had a raised CRP level (>10mg/l). In the NPPC group15 out of the 35 CRP values available were raised (Table 3.6).

Table 3.6: Available CRP measurements/ proportion of patients with raised CRP.

Group	Pre-op CRP Available	Pre-op CRP >10mg/l
Overall (No-148)	118/148	53/118
PC (No-90)	83/90	38/83
NPPC (No-58)	35/58	15/35

The two groups (PC and NPPC) were separated and survival analysis using Kaplan Meier curves and the log rank test for statistical significance was performed using a CRP of 10mg/l as the cut off between normal and raised levels. An elevated pre-operative CRP was associated with poor survival (p=0.0001) (Figure 3.10). Those patients in the PC group with an elevated pre-operative CRP (>10mg/l) had a median survival of 262 days compared with 594 days in those with a normal CRP. In the NPPC group, pre-operative CRP was not significantly associated with survival (p=0.225).

Figure 3.10: Kaplan Meier curve of pre-op CRP levels and post-op survival in the PC group (p=0.0001).



3.2.1.1 Multivariate Analysis

All clinical variables found to be statistically significant on univariate analysis in the PC group (p<0.05) (pre-operative CRP, tumour differentiation, nodal status) were entered into Cox's regression analysis. Following analysis **only pre-operative CRP retained statistical significance (p=0.009)**.

3.2.2 Prospective Cohort

Ethical approval was granted by the trust Ethics committee meeting the approval of the local Research & Development department for a prospective study. All patients undergoing a Whipple resection for either pancreatic cancer (PC) or non-pancreatic peri-ampullary cancer at Glasgow Royal Infirmary whom gave written consent had blood taken pre-operatively by the author (SD) between 30/10/03 to 05/05/05 (collection has continued by JL after this date as the author left the department).

The blood was then anonymously labelled and stored in the department of surgery at -20^{o}C. An anonymous prospective database was then created containing basic patient demographics, pathology data and survival data. The blood samples for the first 36 patients were analysed by an additional research fellow in the department of surgery at Glasgow Royal Infirmary (JL).

3.2.2.1 Patient Demographics

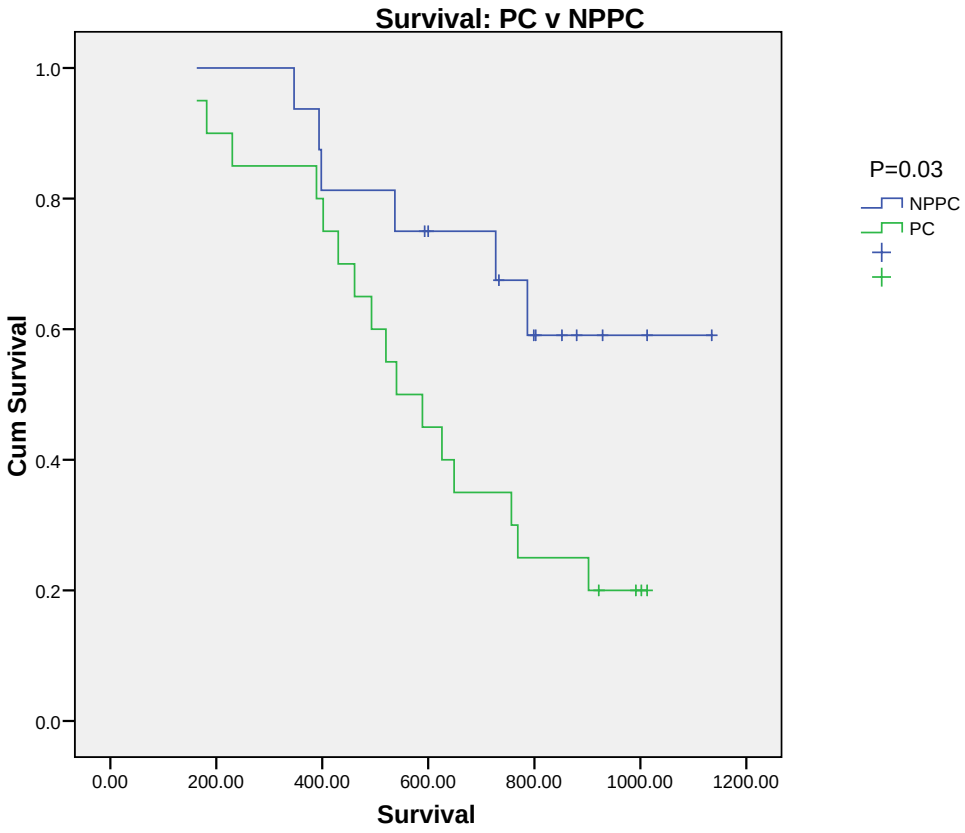
Between 30/10/03 and 05/05/05, 36 patients underwent a Whipple procedure for malignant disease and were included in an anonymous, prospective database. Pre-operative blood samples were collected by the author (SD) and analysed by another research fellow in the department of surgery (JL). The resections were performed by three consultant surgeons.

Overall median age was 64.5 years (range 41-76), in the PC group 66 years (range 41-76) and in the NPPC group median age was 59 years (range 42-76) (Table 3.7). The patients were split into PC and NPPC groups and survival analysis using Kaplan Meier curves and the log rank test for statistical significance was performed (Figure 3.11). Despite the small numbers in this prospective cohort, a statistically significant difference in survival is seen between the two groups. Patients with PC had a median survival of 564.5 days (range 163-1013) and those with NPPC 760 days (range 347-1135) (P=0.03).

Table 3.7: Basic Patient Demographics

Variable	**Overall group (N=36)**	**PC group (N=20)**	**NPPC group(N=16)**
Median Age	64.5 years (41-76)	66 years (41-76)	59 years (42-76)
Male: Female	19:17	11:9	8:8
Median Survival	637.5 days (163-1135)	564.5 days (163-1013)	760 days (347-1135)
Alive: Dead	14:22	4:16	10:6

Figure 3.11: Kaplan Meier curve of PC and NPPC and survival in prospective group.



3.2.2.2 Prospective CRP

All of the 36 patients had pre-operative CRP measurements available. A CRP of >10mg/l was used as the cut-off for an inflammatory response. Of the 36 patients 8 had a raised CRP level (>10mg/l). In the PC group 5 out of the 20 CRP values available were raised compared with 3 out of 16 in the NPPC group (Table 3.8).

Table 3.8 : Available CRP measurements/ proportion of patients with raised CRP.

Group	Pre-op CRP >6mg/l
Overall (No-36)	8/36
PC (No-20)	5/20
NPPC (No-16)	3/16

All 36 patients were analysed together before separating into two groups (PC and NPPC) and survival analysis using Kaplan Meier curves and the log rank test for statistical significance was performed. No significant difference in survival was seen between patients with and without an inflammatory response when all 36 were analysed together or when patients were separated into PC and NPPC groups (Table-3.9).

Table 3.9: CRP levels and survival in prospective group

Tumour Type	CRP Level	Median Survival (days)	Range (days)	P-Value
Overall	<10mg/L	727	163-1135	**0.739**
	>10mg/L	493	394-922	
PC Group	<10mg/L	589	163-1013	**0.970**
	>10mg/L	493	402-922	
NPPC Group	<10mg/L	787	347-1135	**0.917**
	>10mg/L	593	394-803	

3.2.2.3 Interleukin-6 and Survival

Using the prospectively collected blood samples, plasma levels of IL-6 were obtained by JL using a commercially available ELISA kit (R & D Systems Europe Ltd). All 36 patients were analysed together before separating into two groups (PC and NPPC) and survival analysis using Kaplan Meier curves and the log rank test for statistical significance was performed. Previous papers using an identical ELISA kit used an IL-6 level of 4pg/ml as the cut off between normal and raised levels and this value was employed by the author (Table 3.10)[258].

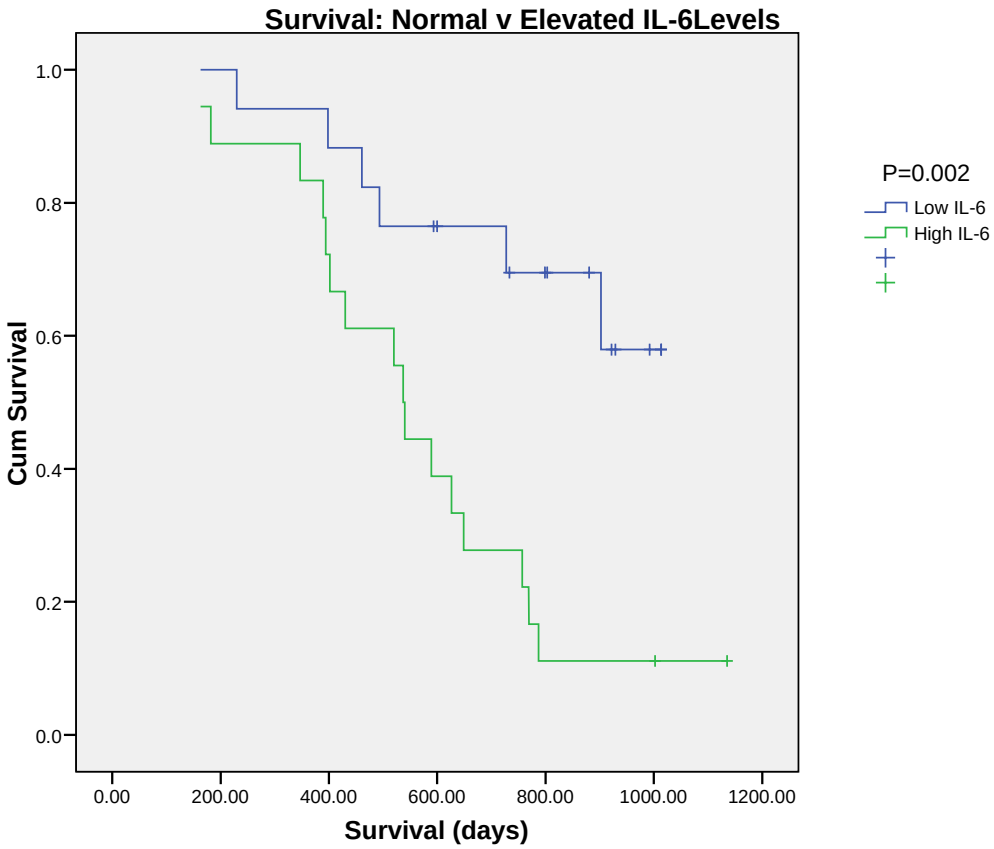
When all of the 36 patients were analysed together there was a statistically significant difference in survival between patients with low levels of plasma IL-6 and those with higher levels (Figure 3.12). Median survival in patients with low levels of IL-6 was 799 days (range 230-1013) compared with 537 days (range 163-1135) in those with higher levels (P=0.002).

When patients are separated into PC and NPPC groups there was no significant difference in survival in the PC group. In this group, median survival in those with lower levels of IL-6 was 902 days (range 230-1013) compared with 540 days (range 163-1002) in those with higher IL-6 levels (P=0.104). In the NPPC group there was a significant difference in survival. Those patients with low IL-6 levels had a median survival of were 766 days (range 398-1013) and 537 days (range 347-1135) in those without and those with an inflammatory response respectively (P=0.027). As with the prospective CRP results, the numbers in this IL-6 analysis are extremely small, with only 5 patients in the NPPC group actually having high levels of IL-6. These results represent the initial trends as data collection continues.

Table 3.10: IL-6 levels and survival in prospective group

Tumour Type	IL-6 Level	Median Survival (days)	Range (days)	P-Value
Overall	Low	799	230-1013	**0.002**
	High	540	163-1135	
PC Group	Low	902	230-1013	**0.104**
	High	540	163-1002	
NPPC Group	Low	766	398-1013	**0.027**
	High	537	347-1135	

Figure 3.12: Kaplan Meier curve of Normal v Elevated IL-6 and Survival in Overall Prospective Group.



3.2.2.4 Correlation Between CRP and IL-6 Levels

If the hypothesis stated in the introductory chapter to this thesis is correct then there should be a correlation between the plasma levels of IL-6 and CRP as the main driver of plasma CRP is IL-6. To analyse possible correlations, Spearmans test for two data sets with non-linear distribution was used (Figure 3.13). This revealed a statistically significant relationship between plasma CRP and plasma IL-6 (P=0.031).

Figure 3.13: Analysis of correlation between plasma IL-6 and CRP.

Correlations

			CRP	IL-6
Spearman's rho	CRP	Correlation Coefficient	1.000	.360(*)
		Sig. (2-tailed)	.	.031
		N	36	36
	il6elisa	Correlation Coefficient	.360(*)	1.000
		Sig. (2-tailed)	.031	.
		N	36	36

* Correlation is significant at the 0.05 level (2-tailed).

3.3 Tissue Micro-Arrays (TMA's)

3.3.1 Planning the TMA's

Before embarking on the creation of TMA's a period of planning took place. One of the key factors when planning the TMA's was the distance between cores. The accepted minimum between cores is 0.8mm, below that and the wax blocks are likely to disintegrate during coring. For the TMA's in this thesis 1mm gaps between cores were chosen. To gain representation of pancreatic tumours, 3 cores of tumour tissue were taken for each patient along with 2 cores of normal pancreatic ductal tissue, a total of 5 cores for each patient. For the 148 patients this meant a total of 740 cores. A number of control tissues were also required. A literature review was carried out by the author to select control tissues that have previously been shown to give positive immunohistochemical staining in the relevant antibodies. Table 3.11 below shows each of the antibodies, tissues they have previously been shown to stain and the relevant references. In addition to pancreatic ducts, acinar tissue, islet tissue, bile duct tissue and duodenal tissue, breast, testis, liver and prostate tissue were also included on the control panel.

Table 3.11: Selected tissue for positive controls.

ANTIBODY	TISSUE	REFERENCES
IL-6R	Prostate, Liver	Tam[259], Rose[260]
JAK1	Prostate	Tam[259]
Phospho.JAK1	Prostate, Breast	Tam[259], Yeh[261]
STAT3	Prostate, Liver, Breast	Tam[259], Levy[262]
Phospho.STAT3 Tyr705	Prostate, Liver	Tam[259], Yang[263]
Phospho.STAT3 Ser727	Prostate, Breast	Tam[259], Yeh[264]
KRas	Breast, Prostate, Testis	McGlynn[265], Mukhergee[266], Traynor[267], Yanagihara[268]
RAF-1	Breast, Prostate	McGlynn[265], Mukhergee[266]
Phospho.RAF-1 Ser259	Breast, Prostate	McGlynn[265], Mukhergee[266]
Phospho.RAF-1 Ser338	Breast, Prostate	McGlynn[265], Mukhergee[266]
MAPK	Breast, Prostate	McGlynn[265], Mukhergee[266]
Phospho.MAPK	Breast, Prostate	McGlynn[265], Mukhergee[266]

Using standard sized wax recipient blocks, it was calculated that 3 TMA's would be required for the 740 cores in addition to 18 control cores on each TMA (2 cores of each control tissue). The other crucial factor when planning TMA's is orientation. Each of the three TMA's had to be unique to allow identification and to make analysis easy, each TMA was asymmetrical. In addition to the 1mm gap between cores, larger gaps were made after 6, 6 and 8 cores in the horizontal plane and every 4 cores in the vertical plane creating 9 sections of cores on each TMA in addition to the panel of controls. This made orientation and subsequent scoring significantly easier. The construction of the TMA's is described in chapter 2.

For the purpose of antibody optimisation, a practice TMA was created with a total of 40 cores. The cores were made up of a selection of tumour and normal tissue to avoid using a significant number of slides from the actual TMA's.

3.3.2 TMA Quality

During the manufacturing of TMA's there is some inevitable loss of cores. This can occur at a number of stages and is the reason why a number of cores are taken for each patient. If the donor tissue block is thin then a number of cores will be required to fill the recipient block, often referred to as double or triple stacking. This inevitably creates gaps in the TMA which will manifest themselves as lost cores on subsequent sections. During sectioning it is also generally accepted that some cores will be lost, those at the peripheries being more vulnerable. Another potential problem that can arise with TMA's occurs before manufacturing has taken place in the selection of tissue. If the original tissue selection is incorrect then a good quality TMA will simply show cores of unwanted tissue that should be excluded at the scoring stage.

During the scoring of the 12 antibodies analysed in this thesis a record was kept of the number of lost or damaged cores and the number of cores of inappropriate tissue that had been stained for both tumour and normal pancreatic tissue.

3.3.2.1 JAK STAT Lost cores

There were a total of 740 cores that underwent staining for 6 antibodies (excluding control cores) making a total of 4440 cores that underwent scoring for the JAK-STAT pathway. Of those 4440, 2664 were tumour and the remaining 1776 were normal ductal tissue. The numbers of lost and incorrect cores are displayed below (Table 3.12).

Table 3.12: Numbers of cores excluded from JAK-STAT analysis.

	Total Cores	Lost/Damaged	% Lost	Incorrect Tissue	% Incorrect
Overall	4440	74	1.7	812	18.3
Tumour	2664	32	1.2	174	6.5
Normal	1776	42	2.36	638	35.9

3.3.2.2 MAPK Lost Cores

As in the JAK-STAT analysis there were a total of 4440 cores with 2664 tumour and 1776 normal ductal cores. The table below (Table 3.13) displays the breakdown of lost cores for the MAPK pathway.

Table 3.13: Numbers of cores excluded from MAPK analysis.

	Total Cores	Lost/Damaged	% Lost	Incorrect Tissue	% Incorrect
Overall	4440	108	2.4	1512	34.1
Tumour	2664	75	2.8	576	21.6
Normal	1776	33	1.9	936	52.7

3.4 The JAK STAT Pathway

3.4.1 Antibody Selection

For a detailed literature review of the JAK-STAT pathway see section 1.3, a brief summary follows. The cytokine Interleukin-6 (IL-6) binds to its cell surface receptor IL-6R. Ligand binding leads to dimerisation of the gp130 receptor subunit, followed by receptor phosphorylation[211]. This phosphorylation causes internalisation of the ligand-receptor complex allowing cytoplasmic parts of the receptor to bind cytoplasmic JAK proteins.

Following receptor phosphorylisation JAK proteins bind to the receptor and auto-phosphorylate causing JAK activation[269]. A number of JAK proteins can be activated by IL-6, but JAK-1 is dominant in IL-6 signal transduction[270]. The receptor-bound active JAK-1 proteins then cause phosphorylisation of tyrosine residues on the cytoplasmic region of the IL-6R which creates binding sites for STAT proteins. Although a number of STAT proteins can be activated in this process, STAT3 is the dominant protein in IL-6 signalling[191].

Once bound to the IL-6R complex STAT3 proteins are phosphorylated by the active JAK-1 proteins and these active STAT3 proteins form dimers in the cytoplasm, before transportation to the nucleus by unknown mechanisms to bind with DNA.

The most suitable antibodies (IL-6R, JAK-1, Phospho.JAK-1, STAT3, Pospho.STAT3 Tyr705, Phospho.STAT3 Ser727) for testing the stated hypothesis were chosen (Table 3.7). Five of these antibodies (all but Phopsho.JAK-1) were

already available for use, their specificity having been established within the laboratory as described in section 2.3.2. Each of these five antibodies had all been successfully used in immunohistochemistry with prostate tissue in the same laboratory. Phospho.JAK-1 antibody had not been previously used within this laboratory nor had it been approved for use in immunohistochemistry. A suitable product was identified (Phospho.JAK-1, New England Biolabs), but despite repeated attempts antibody specificity could not be established (described in section 2.3.3). It was decided that despite the lack of evidence of specificity, that optimisation of Phospho.JAK-1 should still take place.

Table 3.14: Antibodies Used in Analysis of JAK STAT Pathway

Antibody	Manufacturing Company
Il-6R	Santa Cruz
JAK-1	Cell Signalling
Phospho.JAK-1 Tyr1022/1023	New England Biolabs
STAT3	Cell Signalling
Phospho.STAT3 Tyr705	Cell Signalling
Phospho.STAT3 Ser727	Cell Signalling

3.4.2 Optimisation of Antibodies

As stated in section 3.4.1, five of the antibodies had been previously used for immunohistochemistry on prostate tissue within The University Department of Surgery Laboratory, Glasgow Royal Infirmary. The IHC protocols used for prostate tissue were taken as the starting point for IHC protocols by the author of this thesis (SD) and used on the pancreatic TMA's with permission of author LT. Using a practice TMA described earlier in this thesis, key variables, primary antibody dilution, blocking agents and epitope retrieval method were altered until optimal staining was achieved. Once optimal staining was achieved on the practice TMA, the protocol was repeated on the actual TMA's.

With every IHC run, a practice TMA slide was included that underwent the same protocol but without incubation with the primary antibody as a negative control. Positive control cores were already incorporated onto the TMA's as described earlier. The quality of the staining for each antibody was assessed by the author (SD) and confirmed by the senior scientist within the laboratory (JE). Representative immunohistochemical staining patterns for each of the antibodies are shown in figure 3.14.

3.4.2.1 IL-6R

The IL-6R rabbit polyclonal antibody (Santa Cruz) was used on the practice TMA initially with a protocol successfully used on prostate tissue. This protocol used citrate buffer and an antibody dilution of 1:500 and optimal staining was achieved on the first run with no staining on the negative control. This was successfully repeated on the actual TMA's. Staining was expected in both the cytoplasm and the membrane cellular compartments according to the product datasheet, but in this study staining was seen in all 3 cellular compartments.

3.4.2.2 JAK-1

The rabbit polyclonal JAK-1 antibody (Cell Signalling) was used on the practice TMA using a previously successful IHC protocol for prostate tissue. This antibody detects total JAK-1 staining. Antigen retrieval was with EDTA buffer and an initial antibody dilution of 1:200 was used. The initial staining run was successful and was repeated exactly on the TMA's with a negative control. Optimal staining was achieved. Staining was seen in the cytoplasm and to a lesser degree at the membrane.

3.4.2.3 Phospho.JAK-1 Tyr 1022/1023

As stated earlier the, rabbit polyclonal P.JAK-1 antibody (Cell Signalling) was obtained but had not been used for immunohistochemistry in the past. This antibody only detects levels of JAK1 when it is phosphorylated at Tyrosine residues 1022 and 1023. As this antibody had not been worked up in the past, the successful staining protocol for JAK-1 was used as a starting point with EDTA buffer and dilution of 1:200. This resulted in very weak staining and the protocol

was repeated with dilutions of 1:100, 1:50 and 1:25. Despite increasing concentrations of primary antibody, staining was extremely weak. Following discussion with the senior scientist within the laboratory (JE) the blocking agent was changed from Avidin and Biotin block (Vector Laboratories) to Envision block (Dakocytomation, Denmark). The protocol was otherwise unchanged and dilutions of 1:50 and 1:25 were performed on the practice TMA's, again with negative controls. Optimal staining was achieved with 1:25 and this was repeated successfully on the pancreatic TMA's. As P.JAK-1 had not been used for IHC the datasheet did not contain any information on expected staining, but staining was seen in all three cellular compartments on the pancreatic TMA's.

3.4.2.4 STAT3

The starting point for staining was a previously used protocol used successfully on prostate tissue with the same antibody. This STAT3 antibody detects total levels of STAT3 protein. This protocol used a citrate buffer and initial dilution of 1:100 and was followed successfully on the practice pancreatic TMA. This was then repeated on the 3 TMA's and optimal staining was achieved without staining of the negative control. The antibody datasheet showed staining in the cytoplasm and nucleus, however on the pancreatic TMA's staining was seen in the cytoplasm and at the cell membrane but not in the nucleus.

3.4.2.5 Phospho.STAT3 Tyr705

The IHC protocol previously used successfully on prostate tissue was used on the practice pancreatic TMA with EDTA buffer and a 1:50 dilution of the rabbit polyclonal antibody P.STAT3 Tyr705. This antibody only detects levels of STAT3 when it is phosphorylated at tyrosine 705 residue. This protocol resulted in excellent staining and was repeated successfully on the full pancreatic TMA's without any staining on the negative controls. Staining was seen in the cytoplasm and the nucleus.

3.4.2.6 Phospho.STAT3 Ser727

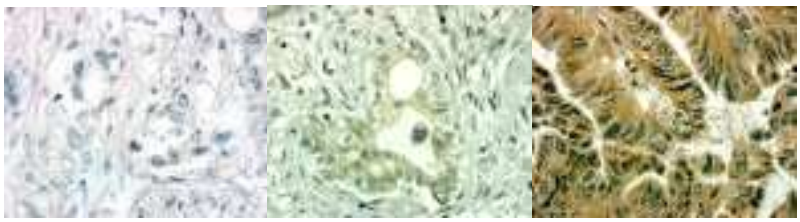
The same protocol used successfully on P.STAT3 Tyr705 was used on this rabbit polyclonal antibody which detects levels of STAT3 only when phosphorylated at Serine 727. Good staining was achieved on the practice run and repeated on the full pancreatic TMA's with excellent results and no staining on the negative control. Staining was seen in both the cytoplasm and the nucleus.

Figure 3.14: Representative Immunohistochemical Staining of JAK-STAT Proteins (0=no staining, 1=pale staining, 2=moderate staining, 3=strong staining)

IL-6R Staining

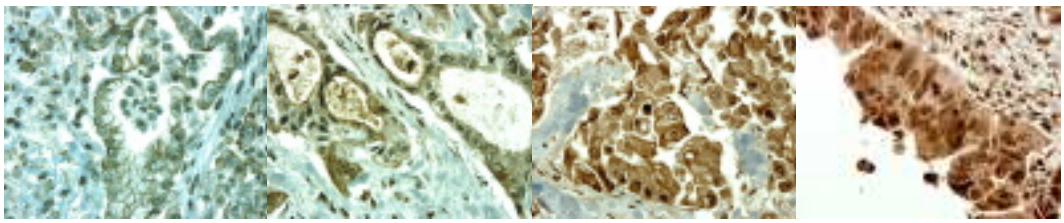
0 1 2

JAK-1 Staining



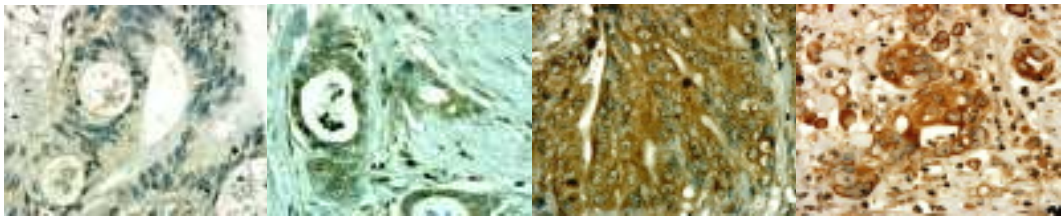
0 1 2

Phospho.JAK-1 Staining



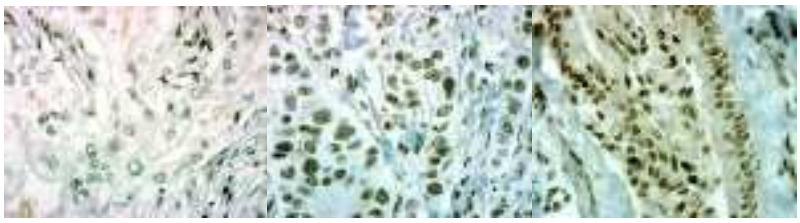
0 1 2 3

STAT3 Staining



0 1 2 3

Phospho.STAT3 Tyr705 Staining



0 1 2

Phospho.STAT3 Ser727 Staining

0 1 2

3.4.3 Variation in Scoring

The weighted histoscore (discussed in section 2.5) was used for all of the immunohistochemical staining. As discussed earlier, all scoring was performed by the author of this thesis (SD). The senior scientist within The University Department of Surgery, Glasgow Royal Infirmary (JE) independently double-scored 10% of the cores. The variation in observer scoring was calculated by the interclass correlation coefficient (ICCC). An ICCC of 0.7 or greater is considered excellent with an ICCC of 1 indicating identical scores. Table 3.15 below shows the individual ICCC scores for each of the antibodies.

Table 3.15: ICCC scores for JAK-STAT IHC scoring.

Antibody	**ICCC score (>0.7 excellent).**
IL-6R	0.7284
JAK-1	0.8757
P.JAK-1	0.9377
STAT3	0.8390
P.STAT3 Tyr705	0.9325
P.STAT3 Ser727	0.7059

3.4.4 Protein Expression

For the six JAK-STAT proteins examined in this study, expression was seen in all three cellular compartments in IL-6R, and Phospho.JAK 1. Cytoplasmic and membrane expression only was seen for JAK1 and non-activated STAT3. Both active forms of STAT 3 (Phospho.STAT3 Tyr705 and Phospho.STAT3 Ser727) had protein expression observed in the cytoplasmic and nuclear compartments only. The distribution of scoring for each of the antibodies in each of the cellular compartments was then examined. The distribution of scoring was demonstrated by plotting frequency histograms (SPSS 15.0, Chicago). These histograms are shown in full in Appendix 1.

When these histograms are analysed the majority of these graphs demonstrate skewing to the right. Before further statistical analysis took place the continuous data needed to be dichotomised, splitting the data in to high and low staining before survival analysis could take place. Following statistical advice the upper quartile was chosen as the cut-off between high and low scoring. Table 3.16 shows the median and inter-quartile ranges of each antibody in each cellular compartment and hence the cut-off values used for subsequent survival analysis.

Table 3.16: Median histoscores and interquartile range

Protein		Median Histoscore	Interquartile range (Lower Quartile to Upper Quartile)
IL-6R	Cyto	16.67	5.42-30.00
	Mem	6.67	0.0-20.00
	Nuc	6.67	0.0-20.00
JAK 1	Cyto	53.33	26.67-83.33
	Mem	0.00	0.0-16.67
	Nuc	-	-
P.JAK 1	Cyto	83.33	56.67-100.00
	Mem	60.00	30.00-86.67
	Nuc	120.00	83.33-145.00
STAT 3	Cyto	93.33	56.67-123.33
	Mem	40.00	17.50-71.67
	Nuc	-	-
P.STAT3 Ser727	Cyto	23.33	6.67-54.58
	Mem	-	-
	Nuc	43.33	23.75-80.00
P.STAT3 Tyr705	Cyto	13.33	3.75-26.67
	Mem	-	-
	Nuc	66.67	26.67-126.67

3.4.5 Protein Expression and Survival

To assess whether expression levels of proteins in the JAK-STAT pathway were associated with poorer survival in pancreatic cancer, Kaplan-Meier graphs were plotted for patients expressing low levels (below cut off)and high levels (above cut off) of staining in both the PC and NPPC groups and were compared using the log rank test. Table 3.17 shows survival in patients with PC for both low and high levels of staining for each of the tested antibodies in each of the cellular compartments. Table 3.18 shows the corresponding values for patients with NPPC tumours.

At first glance there appear to be statistically significant results in both the PC and NPPC patients (cytoplasmic STAT3 and nuclear Phospho.STAT3 Tyr 705 respectively). However when multiple testing is taken into account using Bonferroni correction, there are no statistically significant results in either the ductal or non-ductal groups at the 5% level[271].

Table 3.17: Difference in survival between low and high protein expression in the PC group.

Protein		Median Survival (days)		P-value
		Low expression	High expression	
IL-6R	Cyto	345	370	0.6988
	Mem	430	279	0.0549
	Nuc	430	304	0.3769
JAK 1	Cyto	405	339	0.6106
	Mem	405	345	0.4487
	Nuc	-	-	-
P.JAK 1	Cyto	407	339	0.2498
	Mem	374	345	0.3382
	Nuc	407	267	0.0853
STAT 3	Cyto	345	801	0.0286
	Mem	370	512	0.5725
	Nuc	-	-	-
P.STAT3 Ser727	Cyto	411	304	0.847
	Mem	-	-	-
	Nuc	430	304	0.1894
P.STAT3 Tyr705	Cyto	389	345	0.5874
	Mem	-	-	-
	Nuc	407	271	0.2428

Table 3.18: Difference in survival between low and high protein expression in the NPPC group.

Protein		Median Survival (days)		P-value
		Low expression	High expression	
IL-6R	Cyto	572	618	0.2931
	Mem	582	1432	0.2924
	Nuc	582	848	0.3263
JAK-1	Cyto	572	811	0.2669
	Mem	572	1423	0.0623
	Nuc	-	-	-
P.JAK-1	Cyto	618	811	0.5617
	Mem	618	1055	0.9913
	Nuc	618	582	0.8709
STAT 3	Cyto	477	1423	0.0528
	Mem	572	1055	0.1982
	Nuc	-	-	-
P.STAT3 Ser727	Cyto	582	618	0.9536
	Mem	-	-	-
	Nuc	632	618	0.2880
P.STAT3 Tyr705	Cyto	572	655	0.1878
	Mem	-	-	-
	Nuc	529	848	0.0446

3.4.6 Further Statistical Analysis

The analysis above was carried out by the author (SD) turning what is continuous data (a histoscore of between 0 and 300) into binary data by creating cut-off points to differentiate between high and low levels of scoring. Although this is an accepted method of statistical analysis the author was keen to explore ways in which the data could be analysed in a continuous way. The raw scoring data from each of the JAK STAT proteins for the 90 PC patients was sent to JP a statistician at the Beatson Oncology unit in Glasgow who prepared the following analysis.

The fractional polynomial approach was used in the analysis to establish the best shape of relationship between the protein measurements and survival[272]. Fractional polynomials explore a wide range of plausible polynomial relationships between the predictor and outcome in a statistically controlled manner which avoids the problems of exploring arbitrarily chosen cut-off levels. Because of the large number of variables assessed, the false discovery rate has been used to assess the final level of statistical significance[273].

When the data is analysed in this fashion on first inspection there appears to be a number of significant results (Table 3.19). However when taking into account the number of variables assessed, none are significant.

Table 3.19: Results of fractional polynomial modelling with Cox regression.

Protein		Hazard Ratio(*) (HR)	95% Confidence Interval for HR	P-value(**)
IL-6R	Cyto	6.3	1.22-32.60	0.037
	Mem	1.014	0.999-1.029	0.085
	Nuc	1.017	1.002-1.033	0.037
JAK-1	Cyto	1.004	0.997-1.011	0.245
	Mem	1.004	0.996-1.012	0.321
	Nuc	1.001	0.989-1.014	0.827
P.JAK-1	Cyto	1.004	0.997-1.011	0.246
	Mem	1.002	0.996-1.008	0.477
	Nuc	1.003	0.998-1.009	0.217
STAT 3	Cyto	0.995	0.990-1.000	0.035
	Mem	1.000	0.994-1.007	0.930
	Nuc	-	-	-
P.STAT3 Ser727	Cyto	1.001	0.993-1.009	0.839
	Mem	-	-	-
	Nuc	1.003	0.996-1.011	0.361
P.STAT3 Tyr705	Cyto	0.997	0.983-1.011	0.676
	Mem	0.995	0.952-1.039	0.807
	Nuc	1.002	0.998-1.006	0.352

*- Relative change in hazard for unit change in the best fitting polynomial.

**- Adjusting for multiple comparisons none using the false discovery rate of these are statistically significant at 5%.

3.5 The MAPK Pathway

3.5.1 Antibody Selection

For a review of the MAPK pathway see section 1.4, a short summary follows. Inactive Ras protein is anchored to the cellular membrane hence its ability to take part in cellular signalling. Ras is activated by multiple extra-cellular growth factors including IL-6. Ras activation occurs via interaction with the membrane bound gp130 receptor subunit with phosphorylation, causing Ras to change from its inactive GDP-bound state to its active GTP-bound state[274]. Active Ras then binds to Raf-1 at the cell membrane and undergoes a complex series of events leading to Raf-1 activation. The first stage of activation involves de-phosphorylation of an inhibitory site (serine259), followed by phosphorylation at serine338 which is crucial for Raf-1 activation[247].

Activated Raf-1 then interacts with MEK, a protein serine/threonine kinase in the cytoplasm that has three isoforms (MEK 1a/MEK 1b/MEK 2)[248]. Activated (phosphorylated) Raf-1 goes on to phosphorylate MEK 1/2 at 2 serine residues. Activated MEK 1/2 then goes on to interact with MAPK (sometimes known as ERK). There are three members of this group of protein kinases, but are generally considered together as they are functionally similar. MAPK activation requires phosphorylation at threonine and tyrosine residues and this is accomplished by interaction with activated MEK, again in the cytoplasm[248]. Phosphorylation of MAPK is the main requirement for its translocation to the nucleus where it regulates genes controlling growth, proliferation, differentiation and apoptosis[249].

The most suitable antibodies to test the hypothesis were chosen (KRas, Raf-1, Phospho.Raf-1 Ser259, Phospho.Raf-1 Ser338, MAPK, Phospho.MAPK). These antibodies were also chosen as they had been used successfully for immunohistochemical analysis of the MAPK pathway in breast and prostate tissue within the same laboratory and were all available (Table 3.20). In addition the specificity of all of these antibodies was confirmed within the same laboratory by Western Blotting (see section 2.3.2).

Table 3.20: Antibodies Used in Analysis of MAPK Pathway

Antibody	Manufacturing Company
K-Ras	Sigma
Raf-1	Santa Cruz
Phospho.Raf-1 Ser259	Cell Signalling
Phospho.Raf-1 Ser338	Upstate
MAPK	Cell Signalling
Phospho.MAPK	Cell Signalling

3.5.2 Optimisation of Antibodies

As stated in section 3.5.1, all six of the antibodies had been previously used for immunohistochemistry on breast and prostate tissue within The University Department of Surgery Laboratory, Glasgow Royal Infirmary. The IHC protocols used for breast tissue were taken as the starting point for IHC protocols by the author of this thesis (SD) and used on the pancreatic TMA's with permission of author LM.

Using a practice TMA described earlier in this thesis, key variables, primary antibody dilution, blocking agents and epitope retrieval method were altered until optimal staining was achieved. Once optimal staining was achieved on the practice TMA, the protocol was repeated on the actual TMA's. With every IHC run, a practice TMA slide was included that underwent the same protocol but without incubation with the primary antibody as a negative control. Positive control cores were already incorporated onto the TMA's as described earlier. The quality of the staining for each antibody was assessed by the author (SD) and confirmed by the senior scientist within the laboratory (JE). Representative immunohistochemical staining patterns for each of the antibodies is shown in figure 3.15.

3.5.2.1 K-Ras

This K-Ras monoclonal mouse antibody (Sigma) had been previously used successfully on breast tissue and this protocol was followed exactly using the practice pancreatic TMA. This protocol used EDTA buffer and 1:10 dilution and gave excellent results and was therefore repeated exactly on the full TMA's with no staining seen on the negative control slide. Staining was seen in all three cellular compartments.

3.5.2.2 Raf-1

The Raf-1 antibody used was a mouse monoclonal antibody that had been used successfully in the same laboratory on breast and prostate tissue. The author (SD) used the breast protocol as a starting point with the practice TMA. This protocol involved citrate buffer and 1:40 antibody dilution. The staining was extremely strong and a second identical run took place but with 1:60 dilution of the primary antibody. Again the staining was strong and a further run with 1:80 dilution took place using the practice TMA. This gave excellent staining and was repeated using the full pancreatic TMA's. Results were good with staining seen in the cytoplasm and at the membrane and there was no staining seen on the negative control slide. The datasheet for the antibody stated that cytoplasmic and membrane staining was expected.

3.5.2.3 Phospho.Raf-1 Ser259

A protocol previously used on breast tissue with citrate buffer and a 1:25 dilution of the monoclonal rabbit Phospho.Raf-1 Ser259 antibody. Although specific staining was seen it was extremely weak and a second run with 1:10 dilution was performed. Excellent staining was achieved and the protocol repeated on the full TMA's. Staining was excellent and seen in the cytoplasm only with no staining on the negative control.

3.5.2.4 Phospho.Raf-1 Ser338

A previously used breast protocol was again used as the starting point for staining using Phospho.Raf-1 Ser338 (Upstate), a monoclonal mouse antibody. EDTA buffer along with antibody dilution of 1:250 was used on the practice TMA with excellent results. This protocol was repeated on the full TMA's but one of the TMA's (TMA 2) showed no staining at all. This was thought to be a technical error and was repeated, however on this run the negative control slide showed evidence of staining. Again this was thought to be due to a technical error and a repeat run took place without any changes to the protocol. Satisfactory staining was seen in all three cellular compartments and crucially not on the negative control slide.

3.5.2.5 MAPK

The previously used breast protocol employing citrate buffer and a 1:200 dilution of the monoclonal rabbit MAPK antibody was used on the practice TMA. Very weak staining was seen and therefore the protocol was repeated with a dilution of

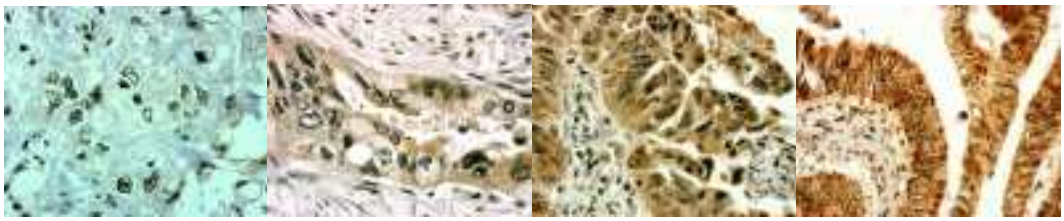
1:100. The staining was still very pale and was considered very difficult to score so a repeat run with 1:50 dilution was performed. On analysis of the 1:50 run staining was still pale so the author (SD) consulted the senior scientist in the laboratory (JE) and a change of buffer was used. When EDTA buffer was used with a 1:50 antibody dilution good staining was seen and a repeat run on the full TMA's took place. Good staining was seen in all three cellular compartments, however the datasheet showed expected staining in only the cytoplasm and the nucleus. No staining was seen on the negative control slide.

3.5.2.6 Phospho.MAPK

As a starting point a protocol used on breast tissue was again used. Citrate buffer along with an antibody dilution of 1:200 gave very pale staining and the protocol was repeated with dilution of 1:100. The stronger antibody gave improved results but staining was still pale and the dilution was changed to 1:50 for a third run. This third run gave stronger staining but it was patchy and after discussion with JE a change of buffer was used. When EDTA buffer was used with a 1:50 antibody dilution good, specific staining was seen and the full TMA's underwent the same protocol. Although the datasheet showed expected staining in the cytoplasm and the nucleus, staining was seen in all three cellular compartments but not on the negative control slide.

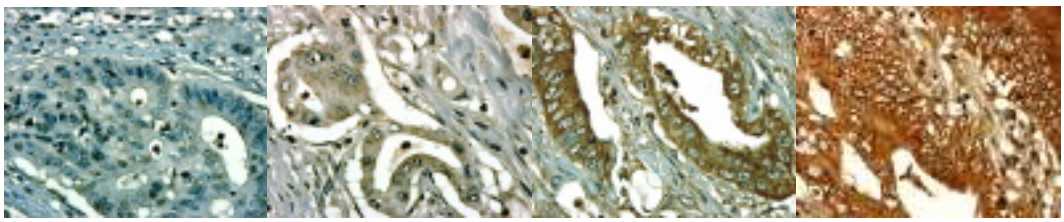
Figure 3.15: Representative Immunohistochemical Staining of MAPK Proteins (0=no staining, 1=pale staining, 2=moderate staining, 3=strong staining)

K-Ras Staining



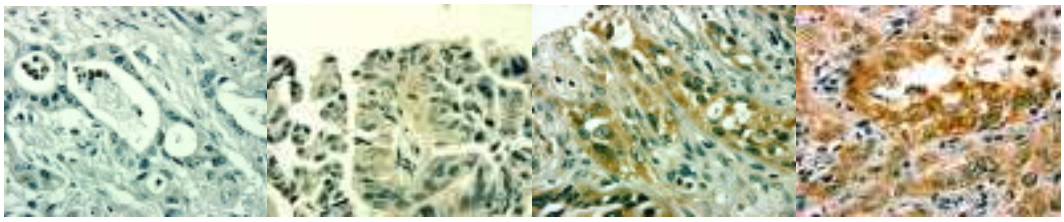
0 1 2 3

RAF-1 Staining



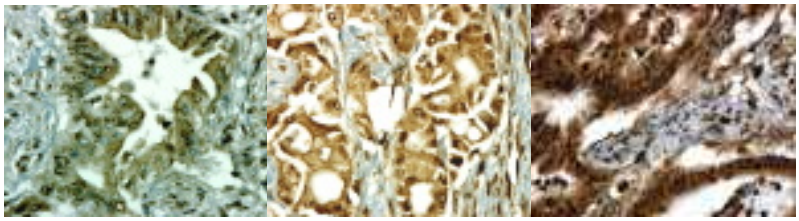
0 1 2 3

Phospho.RAF-1 Ser259 Staining



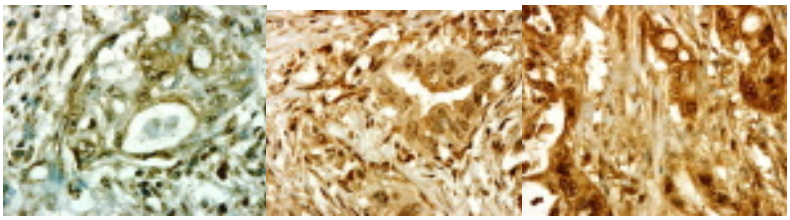
0 1 2 3

Phospho.RAF-1 Ser338 Staining



1 2 3

MAPK Staining



1 2 3

Phospho.MAPK Staining

0 1 2 3

3.5.3 Variation in Scoring

The weighted histoscore (discussed in section 2.5) was used for all of the immunohistochemical staining. As discussed earlier, all scoring was performed by the author of this thesis (SD). The senior scientist within The University Department of Surgery, Glasgow Royal Infirmary (JE) independently double-scored 10% of the cores. The variation in observer scoring was calculated by the interclass correlation coefficient (ICCC). An ICCC of 0.5-0.6 is considered acceptable, 0.6-0.7 good and >0.7 is considered excellent with an ICCC of 1 indicating identical scores. Table 3.21 below shows the individual ICCC scores for each of the antibodies.

Table 3.21: ICCC scores for MAPK IHC scoring.

Antibody	**ICCC score**
K-Ras	0.82
RAF-1	0.637
P.RAF-1 Ser259	0.904
P.RAF-1 Ser338	0.809
MAPK	0.532
P.MAPK	0.872

3.5.4 Protein Expression

For the six MAPK proteins included in this study staining was seen in all three compartments in four antibodies (K-Ras, Phospho.Raf-1 Ser338, MAPK and Phospho.MAPK). Raf-1 showed staining in the cytoplasm and membrane only and Phospho.Raf-1 Ser259 showed staining only in the cytoplasm. The distribution of scoring for each of the antibodies in each of the cellular compartments was then examined. The distribution of scoring was demonstrated by plotting frequency histograms (SPSS 15.0, Chicago). These histograms are shown in full in Appendix 2.

When these histograms are analysed the majority of these graphs demonstrate skewing to the right. Before further statistical analysis took place the continuous data needed to be dichotomised, splitting the data in to high and low staining before survival analysis could take place. Following statistical advice the upper quartile was chosen as the cut-off between high and low scoring. Table 3.23 shows the median and inter-quartile ranges of each antibody in each cellular compartment and hence the cut-off values used for subsequent survival analysis.

Table 3.22: Median histoscores and interquartile range

Protein		Median Histoscore	Interquartile range (Lower Quartile to Upper Quartile)
K-Ras	Cyto	63.33	30.00-96.667
	Mem	20.00	6.667-50.00
	Nuc	33.33	6.667-76.667
Raf-1	Cyto	100.00	56.667-140.00
	Mem	30.00	10.00-60.00
	Nuc	-	-
P.Raf-1 Ser259	Cyto	30.00	10.00-56.667
	Mem	-	-
	Nuc	-	-
P.Raf-1 Ser338	Cyto	70.00	43.33-100.00
	Mem	30.00	15.00-63.33
	Nuc	135.00	86.667-175.00
MAPK	Cyto	50.00	20.00-85.833
	Mem	40.00	20.00-60.00
	Nuc	70.00	40.00-109.167
P.MAPK	Cyto	13.33	3.75-30.00
	Mem	3.33	0-16.25
	Nuc	95.833	50.833-139.167

3.5.5 Protein Expression and Survival

To assess whether expression levels of proteins in the MAPK pathway were associated with poorer survival in pancreatic cancer, Kaplan-Meier graphs were plotted for patients expressing low levels (below cut off)and high levels (above cut off) of staining in both the PC and NPPC groups and were compared using the log rank test. Table 3.23 shows survival in patients with PC for both low and high levels of staining for each of the tested antibodies in each of the cellular compartments. Table 3.24 shows the corresponding values for patients with NPPC.

At first glance there appear to be two statistically significant results in the patients with ductal tumours. Differences in cytoplasmic MAPK and cytoplasmic phosphorylated MAPK staining appear to have a significant effect on survival ($p=0.0229$ and $p= 0.0342$ respectively). However when multiple testing is taken into account using Bonferroni correction, there are no statistically significant results in the PC group at the 5% level[271]. In those patients with NPPC there were no significant effects on survival.

Table 3.23: Difference in survival between low and high protein expression in the PC group.

Protein		Survival (days)		P-value
		Low expression	High expression	
K-Ras	Cyto	405	150	0.1340
	Mem	330	411	0.8597
	Nuc	339	389	0.5168
Raf-1	Cyto	389	370	0.9712
	Mem	407	374	0.1065
	Nuc	-	-	-
Raf-1 Ser259	Cyto	345	430	0.3245
	Mem	-	-	-
	Nuc	-	-	-
Raf-1 Ser338	Cyto	407	339	0.9915
	Mem	407	345	0.5337
	Nuc	444	279	0.2194
MAPK	Cyto	339	635	0.0229
	Mem	405	312	0.4514
	Nuc	407	253	0.0777
P.MAPK	Cyto	370	389	0.0342
	Mem	370	389	0.4046
	Nuc	405	304	0.6661

Table 3.24: Difference in survival between low and high protein expression in the NPPC group.

Protein		Survival (days)		P-value
		Low expression	High expression	
K-Ras	Cyto	572	622	0.4099
	Mem	618	811	0.5926
	Nuc	618	632	0.9215
Raf-1	Cyto	618	582	0.6924
	Mem	618	398	0.6478
	Nuc	-	-	-
Raf-1 Ser259	Cyto	582	618	0.5205
	Mem	-	-	-
	Nuc	-	-	-
Raf-1 Ser338	Cyto	572	811	0.3583
	Mem	618	535	0.2800
	Nuc	618	582	0.6425
MAPK	Cyto	582	1659	0.3169
	Mem	572	1423	0.4355
	Nuc	582	826	0.3885
P.MAPK	Cyto	826	529	0.1891
	Mem	582	655	0.6657
	Nuc	655	494	0.7049

4.0 DISCUSSION

HYPOTHESIS

The presence of a systemic inflammatory response is associated with a particularly poor prognosis in pancreatic cancer. Pro-inflammatory cytokines, in particular IL-6, are known to induce CRP production and also may influence tumour growth and differentiation through well-described cellular pathways. Upregulation of IL-6 dependent pathways in tumour cells may explain the poor prognosis associated with a systemic inflammatory response.

At the start of this thesis a number of aims were stated to allow testing of the above hypothesis. These aims included:

1/. Establish pancreatic cancer patient database with basic demographics, pathological and survival data.
2/. Confirm the connection between a raised CRP and survival in pancreatic cancer in a larger cohort and establish if there is a relationship between CRP and non-pancreatic peri-ampullary cancers.
3/. Simultaneously create a prospective database and establish the connection between CRP and survival in a prospective cohort.
4/. Create tissue micro-arrays using tissue from the pancreatic cancer database to enable the efficient examination of relevant antibodies using standard immunohistochemical techniques.
5/. Using immunohistochemistry, stain for antibodies from the JAK-STAT and MAPK pathways and establish whether poor survival in these patients with pancreatic cancer is associated with upregulation of these pathways.

4.1 CRP: Results Summary

This thesis has confirmed that pre-operative CRP is a significant prognostic indicator in resectable pancreatic cancer in the largest series to date. Multivariate analysis has revealed that a raised CRP is the only prognostic factor that retains statistical significance, making it more important in outcome in this cohort of patients than the traditional, pathological factors associated with poor survival discussed in section 1.1.7.2. This is highly significant and raises the issue of whether patients with a raised pre-operative inflammatory response who are otherwise found to be operable should be offered curative resections. More information is needed regarding the pathophysiology behind this response and the effects of pharmacologically reducing it however, it is conceivable that CRP may be included in staging of these patients in the future.

This thesis also shows in a prospective group that high levels of plasma IL-6 are associated with poorer post-operative survival and show a statistically significant correlation with plasma CRP levels. This adds to the body of evidence describing an IL-6 driven inflammatory response.

4.2 JAK-STAT Pathway: Results Summary

When the results were analysed by creating cut-off points (Kaplan Meier) and analysing the data in a continuous fashion (fractional polynomial approach), there appears to be no relationship between JAK STAT staining and survival taking into account the multiple tests performed. The IHC in this thesis demonstrates staining in the JAK STAT pathway but crucially this staining does not appear to significantly influence survival in this patient group and therefore the author cannot reject the null hypothesis.

A number of studies looked at STAT3 and its role in pancreatic cancer. Huang et al looked at the effects of blocking JAK proteins and was able to show a resulting decrease in phosphorylation of STAT3 in addition to a reduction in tumour invasion and metastases[275]. Wei et al showed constitutive STAT3 activation and resulting increased growth and metastatic potential[238]. Both of these studies however were in-vitro studies on pancreatic cancer cell lines and the value of their results is therefore limited. Greten et al were able to demonstrate that activation of STAT3 appeared to inhibit apoptosis, but this was a study on mice and there was no mention of a resulting effect on survival[239]. There are no studies in the published literature that can provide any evidence for the JAK STAT pathway influencing outcome in pancreatic cancer. The vast majority of studies that suggest that the JAK STAT pathway may have a role to play in pancreatic cancer are experimental, often in-vitro or are they are performed on animals. No real conclusions can be drawn about the role that this pathway plays in human pancreatic cancer from these studies and the results of this thesis demonstrate that although there is activity in human pancreatic cancer cells, it is not responsible for poor outcome.

4.3 MAPK Pathway: Results Summary

As with the JAK STAT pathway, there are no significant differences in survival between low and high levels of staining in any of the MAPK antibodies stained in this thesis. Similar to the JAK STAT pathway, there are a multitude of studies that show that the MAPK pathway may have a role to play in pancreatic cancer but these results do not appear to be bourne out by results of clinical trials on humans. As described in section 1.4.2, at least 25 potential therapies aimed at various proteins in the MAPK pathway have been trialed and none have yet to show any significant difference in outcome, supporting the results described here[252].

4.4 Justification of Methods

4.4.1 Tissue Microarrays

The completion of the human genome sequence has provided a colossal amount of information about human gene structure. To go along with this explosion in genetic information cDNA microarrays have been developed, allowing simultaneous expression analysis of thousands of genes in one experiment[276]. Performing traditional methods of molecular pathology in large scale analyses are in comparison incredibly slow and labour intensive meaning that valuable human tissue resources are rapidly exhausted. A method of analyses that preserved human tissue and allowed large cohorts of patient specimens to be analysed quickly was required.

In 1998 Kononen and colleagues developed the tissue microarray (TMA) for the high throughput molecular profiling of tumour samples[277]. This technique is now standard and described in detail in section 2.2.

4.4.2 Advantages of TMA's.

Other than the obvious major advantage of human tissue preservation, there are a number of other advantages associated with the use of TMA's. As all tissue samples on the TMA are treated at the same time they are all exposed to identical conditions during immunohistochemical analysis enabling standardisation of testing. With conventional techniques sections are exposed to a multitude of slide to slide variations including reagent concentrations, incubation times, temperatures and wash conditions as well as antigen retrieval. Using TMA's means every tissue sample is treated in an identical manner. With the use of TMA's only a small amount of reagent is required to analyze an entire cohort of samples.

There were initial concerns about the preservation of antigenicity in samples preserved over long periods of time in paraffin. A number of studies have looked at this potential problem. Camp et al showed that there was no significant decrease in antibody staining in blocks from pathology archives up to 68 years old[278]. Another study by Rhodes again showed no deterioration in antigenicity of samples dating back to the 1930's[279].

4.4.3 Limitations of TMA's

The major potential limitation of TMA's is the lack of tissue volume used for analyses. Sceptics claim that the tiny amount of tissue used is too small to be representative of an entire tumour, particularly those tumours that exhibit heterogeneity. It is not known how much tissue however is required to be truly representative of a tumour. This problem is normally addressed by taking multiple cores from each tumour block when making up the TMA. There has to be a trade off however between taking increasing numbers of cores to be representative of the whole tumour and enjoying the advantages of TMA technology. So how many cores are needed to be representative of a tumour?

There have been a number of studies validating the use of TMA's and suggesting the optimal number of cores. Hoos et al showed that three cores (of 0.6mm diameter) showed concordance with standard techniques in more than 90% of cases when they examined breast tumours staining with a number of antibodies[280]. Camp et al took 38 cases of breast carcinoma and compared the staining of three common antigens on 2-10 microarray discs and the whole tissue section from which they were derived[278]. With one microarray disc only there was over 90% accuracy for all three antigens. When three discs were analyzed, accuracy increased to over 97% for all three antigens when compared to the full tissue section. These studies suggest that although the tissue used in microarrays is small, it is enough to be representative of the tumour when compared to standard tissue sections.

Other potential drawbacks of using TMA technology are core loss or damage and a lack of identifiable tumour in the core. This can occur during sectioning if a section passes through a gap in a core (gaps can occur if the original paraffin block is thin meaning multiple thin cores are placed on top of each other to fill the space in the recipient block) or due to sub-optimal sectioning technique. The impact of these problems can be overcome by including multiple tissue cores and careful technique.

4.4.4 TMA's and Pancreatic Tumours

Pancreatic and biliary tumours are commonly heterogeneous and often associated with a significant surrounding desmoplastic stromal reaction. It is now widely accepted that pancreatic cancers originate from the ductal cells of the pancreas and not the more abundant acinar tissue.

Swierczynski et al looked at novel tumour markers in pancreatic and biliary tumours[281]. Prior to the main analysis they used 8 markers shown to be over-expressed in whole tissue sections of pancreatic adenocarcinoma and compared them to their own TMA's. The labelling of these markers on the TMA's was concordant with the whole tissue sections but used far fewer slides and reagents. Maitra et al looked at pancreatic intraepithelial neoplastic (PanIN) lesions using TMA's whilst analysing the multistep progression model for pancreatic cancer[39]. They found that when compared with previous data published using routine histology sections, their results on the PanIN specimens using TMA's were comparable. These studies suggest that analyzing pancreatic tissue using TMA's gives comparable results to standard histology sections with all of the advantages already mentioned above.

4.4.5 TMA Quality in this Thesis

There is adequate evidence to justify the use of TMA's in immunohistochemical analysis. However, successful use of TMA's does depend on their construction and more specifically the quality of that construction. There is an accepted loss of cores during the manufacturing of TMA's that occurs usually during sectioning but can less commonly occur as a consequence of double or triple stacking (described earlier). This loss has been described by a number of authors looking at the validity of TMA use in immunohistochemistry and cancer research[282,283]. Both studies describe a loss of 5-10% of cores during sectioning when the cores are 0.6mm in diameter (as they are in this thesis). During scoring of the TMA sections in this thesis a record was kept of the number of missing or damaged cores that could not be scored. Out of a total of 8880 cores 182 (2%) were missing or damaged, comparing favourably with accepted losses in the literature.

Not mentioned in the papers described above however were the cores that were included on the TMA's that did not contain the required tissue. If the initial cores are not taken from the desired tissue then despite a high quality of construction, the subsequent analysis will be affected as inappropriate tissue cores will have to be excluded.

During scoring of the TMA's in this study in addition to missing cores, a record of the number of cores that did not contain the correct tissue type and therefore were not included in analysis was kept. As discussed above, the number of missing cores in the TMA's used was well within acceptable limits but no where in the literature is the mention of the expected loss of cores due to incorrect tissue. In this thesis a total of 2324 cores out of a total of 8880 were excluded from analysis

due to incorrect tissue type. In the case of tumour cores the reason for exclusion was the lack of tumour within the cored specimen (usually stroma, pancreatitis or normal pancreatic tissue). In the cores of normal ductal tissue there was a wide range of other tissues seen leading to exclusion (acinar tissue, pancreatitis, pancreatic tumour, pancreatic islet tissue, stroma).

When the excluded cores are broken down it is clear that the majority occur in the normal ductal cores (750 cores of tumour excluded compared with 1574 of normal ductal cores). It would appear logical in this study that the majority of incorrect cores would occur in the normal tissue for a number of reasons. All the patients included on the TMA's in this thesis had resections for cancer and therefore the amount of genuinely normal pancreatic tissue was minimal. In addition, the amount of normal ductal tissue within the small areas of normal pancreas was extremely small making coring of normal pancreatic ducts very difficult. The technique of TMA manufacture requires the use of H&E slides as a guide to enable coring of a specific area. It must be remembered that having identified a small area on the H&E slide with the aid of a microscope, to accurately core the exact area on the original tissue block is difficult, hence the large number of normal ductal cores that actually contained pancreatic acinar tissue in this study. The same problem is applicable to the tumour cores but as all the resections were for cancer there were larger areas of tumour which were more easily identified and therefore with the aid of H&E slides, cores are less likely to contain inappropriate tissue. As normal pancreatic tissue was not included in the analysis in this thesis then the loss of cores in this group is irrelevant.

On further analysis of the excluded cores there is a clear difference in numbers between the JAK-STAT and MAPK pathways, with a lot more cores excluded from analysis in the latter. There are a number of reasons why this may be the case. Firstly, as the MAPK scoring took place after the JAK-STAT, the author who performed the scoring may have gained in experience and therefore was able to exclude a number of cores that previously would have been scored. Secondly, immunohistochemical staining of antibodies from the MAPK pathway took place after staining of JAK-STAT antibodies, using TMA sections from deeper into the TMA. The H&E slides used as guidance on coring represent the surface of the original tissue block. The further into the original tissue block a core goes, the less representative the H&E slide becomes and hence as deeper sections of the TMA are cut the less likely the deeper sections are going to represent the tissue on the superficial sections.

These are all established problems with the use of TMA's as described earlier. The use of multiple cores (3 tumour and 2 normal for each patient in this study) not only allows for representation of heterogeneous tumours but also for the loss of cores. This means that despite significant losses of cores virtually every patient will have appropriate tissue scored for analysis.

One criticism that could be levelled at this thesis is that the creation of the TMA's was done entirely by the author. Before the selection and marking of slides took place, the author underwent an intensive period of histology training under the guidance of a consultant pathologist (KO). After the marking of slides took place, a selection was reviewed by the same consultant pathologist who agreed with the

author who went on to manufacture the TMA's. Therefore the TMA's created for this thesis were done under the continual guidance of a consultant pathologist.

4.4.6 Immunohistochemistry

Antibody specificity was confirmed in 11 of the 12 antibodies used, by scientific staff within the department using Western Blotting, described in section 2.3.2.2. The Phospho.JAK 1 antibody had not been used for IHC before and despite multiple attempts the author was unable to demonstrate specificity but analysed and included the results for completeness.

The IHC protocols used were initially based on protocols used successfully in prostate and breast tissue. The author (SD) used these protocols as a starting point before optimising them on a practice pancreatic TMA over a period of months under the guidance of a scientist within the department (JE) with years of experience of IHC. Once satisfactory staining had been achieved using the practice pancreatic TMA, staining of the original TMA's took place.

4.4.7 Scoring

All patients in the cohort included in this thesis had tissue samples prepared in the same department following the same protocols. The use of tissue micro-arrays (TMA) means the area to be assessed is identical for all patients. Each patient has three 0.6mm diameter cores of tumour and two 0.6mm cores of normal pancreatic tissue included on the TMA's, meaning all areas of the five cores could be scored for each patient. The heterogeneous nature of pancreatic cancer was taken into account when selecting the areas to be cored from the original paraffin blocks. For each patient areas of different differentiation were marked meaning the three cores taken were a representation of the whole tumour.

The weighted histoscore is an immunohistochemical scoring system that enables qualitative as well as quantitative data to be collected and has been used extensively, mainly in the scoring of breast tissue, but also in prostate and colonic tissues[284-290].

The author (SD) underwent a period of training in scoring tissue and then scored a sample TMA slide. A scientist in the department with years of experience using the weighted histoscore (JE), double-scored the same TMA slide and an ICCC of >0.7 (rated as excellent) confirmed consistency of the authors scoring was achieved. Only after this training period did the author go on to score the actual TMA's. All 12 of the antibodies were double scored by the same scientist (JE) and scoring consistency was again confirmed using the ICCC. All 12 antibodies had an ICCC of >0.5 or higher indicating a minimum of satisfactory scoring consistency.

4.4.8 Statistics

All statistics were calculated using the SPSS statistical software package as described in section 2.6. Statistical analysis was performed under the guidance of (WA) a statistician in The University of Glasgow Department of Surgery. All survival analysis was performed using Kaplan Meier curves and the log-rank test. Multivariate analysis was performed using Cox's regression analysis on variables identified as significant on univariate analysis.

Following scoring of antibody staining, frequency graphs were plotted (appendices 1 and 2) demonstrating the distribution of scoring for each antibody. These graphs demonstrate skewing to the right. To dichotomise data and allow Kaplan Meier survival analysis, cut-off points between what is considered low and high levels need to be chosen. If it is required to test whether two groups (low and high scoring groups) differ in some other variable y (survival in this case) then for a given sample size, the greatest power to detect differences between 2 groups is usually achieved if they are of equal size. That is why the median is so often used as a cut-off. However, if the distribution is asymmetrical (skewed) then a cut-off that separates the long tail of the distribution from the remainder clustered about the modal value may be best. Hence, in a distribution skewed to the right (the majority of antibodies in the JAK STAT and MAPK pathways in this thesis), an upper quartile is a more appropriate cut-off than the median.

A second statistical analysis was performed on the data from JAK-STAT staining by (JP), a statistician at the Beatson Oncology Unit, Glasgow to see if analysing the data in a continuous fashion gave different results. The fractional polynomial approach was used in the analysis to establish the best shape of relationship

between the protein measurements and survival. This statistical technique allows data to be analysed continuously without the need for creating arbitrary cut off points. The results of this second analysis confirm the survival analysis done using Kaplan Meier curves and the log-rank test.

4.4.9 Justification of Methods: Conclusions

The author believes that the scientific methods and techniques used in this thesis are justifiable and scientifically sound. The use of TMA's is now commonplace and backed by a significant body of evidence. The creation of the TMA's, done entirely by the author was supervised throughout by appropriately qualified people with experience in TMA manufacturing. The quality of the TMA's matches that described within the literature and the immunohistochemical techniques employed by the author were standard and supervised by a scientist with multiple publications using similar techniques. Use of the weighted histoscore has been published widely in multiple tumours and scoring consistency (as measured by the ICCC) was in the main graded as excellent, never falling below satisfactory following double scoring. The statistics used in this thesis have been employed in numerous publications and were supervised by statisticians with extensive experience in medical statistics. The author maintains that the techniques used in this thesis and therefore the results are reproducible and confirm that the initial hypothesis stated in section 1.5.1 has been disproved.

4.5 Recently Published Data

During the writing of this thesis there have been a number of relevant publications which are summarised below.

Raised CRP levels have been associated with poor prognosis in metastatic prostate cancer[291]. Raised pre-operative CRP predicts poor outcome in operable gastric and oesophageal (squamous cell carcinoma and adenocarcinoma) cancers[292,293]. Hashimoto et al showed that pre-operative serum CRP is an independent and significant predictor of poor outcome and early recurrence in patients with hepato-cellular carcinoma[294].

The importance of CRP in cancer and more specifically its ability to predict outcome in most of the common solid tumours has led to the development of a prognostic scoring system involving CRP. The Glasgow Prognostic Score (GPS) is based on the inflammatory response, with serum CRP (>10mg/l signifying a raised level) and albumin (<35g/l signifying hypoalbuminaemia) being measured. The presence of both a raised CRP and hypoalbuminaemia gives a GPS of 2, the presence of either, a score of 1 and neither a score of 0. This scoring system has been used in advanced colo-rectal cancer[295], inoperable gastro-oesophageal cancer[296], inoperable non-small cell lung carcinoma[297], metastatic renal cancer[298] and inoperable pancreatic cancer[299]. In all cases the GPS was shown to be a significant and independent predictor of survival. This scoring system has yet to be applied to operable cancers but based on these studies it may only be a matter of time before CRP becomes part of pre-operative staging in cancer.

In terms of pancreatic cancer and CRP very little new data has appeared in the literature since Jamieson et al in 2005 showed that pre-operative CRP was a significant predictor of outcome in operable ductal adenocarcinoma of the pancreas[14].

The evidence surrounding an inflammatory response in cancer and more specifically a pre-operative inflammatory response in pancreatic cancer has been discussed in detail in the introduction and support the authors findings in this thesis. The role of pre-operative CRP in cancer and more specifically pancreatic cancer now appears to be beyond doubt.

Also discussed in the introduction is the role of IL-6 as the major inducer of plasma CRP levels[183-186]. CRP is synthesised predominantly in hepatocytes under the influence of cytokines, with Interleukin-6 (IL-6) the major inducer of hepatocyte CRP production[184]. Other sites of CRP production include smooth muscle cells, macrophages, kidney tubular cells, neurons, and lymphocytes, however, these sites contribute only a small proportion of the CRP value and plasma CRP measurements reflect hepatocyte production. There have been a number of key papers involving IL-6 and cancer published during this thesis and they will be discussed in detail later in this discussion.

4.6 What are the Factors Behind Inflammation and Poor Prognosis in Pancreatic Cancer?

The role of the inflammatory response in cancer and specifically pancreatic cancer is now well established and the results of this thesis support this. The results reported here do not show any evidence for involvement of two major signalling pathways, both shown to be involved in inflammation and carcinogenesis in numerous other publications. So what are the explanations for this poor prognosis?

There appear to be a number of possibilities that may explain the role of inflammation and poor prognosis and fit with the results reported here. One is that other signalling pathways, maybe multiple signalling pathways or signalling proteins are responsible for this poor prognosis. The other possibility is that the inflammatory response rather than just being the result of another process is itself responsible for poor outcome. Whether it is the tumour or the reaction of the host to the tumour that induces this response also needs to be addressed. These possibilities will be explored in the next section.

4.6.1 The SOCS Gene

When STAT3 is activated in response to cytokine stimulus, this activation is rapid and transient. Even if the cytokine stimulus persists, STAT3 activation only lasts a few hours[231]. This suggests a feedback mechanism that in normal physiological conditions leads to the deactivation of STAT3 even under persistent cytokine stimulation. One such mechanism that terminates STAT3 activity is by the activation of a group of proteins known as the suppressors of cytokine signalling, or SOCS family.

SOCS proteins, of which there are currently eight identified, were first discovered in 1997 when they were identified as JAK regulatory proteins[300]. Of the 8 SOCS proteins, SOCS-1 appears to be involved with the inhibition of IL-6 signalling. These proteins bind to JAK's, halting their activation and the subsequent activation of STAT3, essentially switching off cytokine signalling via the JAK STAT pathway. A number of cytokines, including IL-6 are responsible for the activation of SOCS proteins and there is some evidence that SOCS genes are actually among the target genes of STAT proteins creating a negative feedback loop[231,301]. Following the identification of the JAK STAT pathway as having a role in carcinogenesis and the progression of tumours, attention more recently has turned to the SOCS family of proteins in their role as negative regulators of JAK STAT signalling. As constitutive activation of STAT proteins, in particular STAT3 has been reported in a number of cancers, could it be loss of this negative feedback that is responsible, meaning a potential role in cancer for SOCS proteins?

Yoshikawa et al published their work on SOCS-1 in hepatocellular carcinoma (HCC) in 2001[301]. They were able to identify aberrant methylation of the SOCS-1 gene in HCC cell lines and subsequent transcription silencing. Following the restoration of SOCS-1 there was suppression of growth seen in the cell lines. They identified that this growth suppression was caused by apoptosis and this apoptosis was recreated by using the JAK inhibitor AG490. The authors also demonstrated that in 26 HCC tissue samples removed at surgery, this same methylation of the SOCS-1 gene was seen in 65%. In non-tumour samples from the same patients, no methylation was seen. The authors conclude that constitutive activation of the JAK STAT pathway as a result of methylation of the SOCS-1 gene and the subsequent lack of growth suppression is a key step in the development of HCC.

Multiple Myeloma (MM) is a malignancy where cytokines and particularly IL-6 are known to have a crucial role in pathogenesis. Following the study of SOCS proteins in HCC, Galm et al looked at SOCS-1 methylation in MM cell lines and primary MM samples[302]. Aberrant methylation was identified in both IL-6 dependent MM cell lines but not in acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), and lymphoma cell lines, nor in normal peripheral blood mono-nuclear cells. This methylation was seen to coincide with SOCS-1 gene transcription silencing. Reversal of this methylation led to up-regulation of SOCS-1 transcription. The authors then went on to demonstrate similar methylation of the SOCS-1 gene in 63% of MM patient samples. Comparative assays on samples of various lymphomas identified SOCS-1 methylation in only 3% of patients and no methylation in normal peripheral blood leucocytes or bone marrow. In their conclusion the authors state that methylation of the SOCS-1 gene in MM may contribute to cell survival by removing the negative feedback from

the JAK STAT pathway and increasing cellular sensitivity to cytokines and particularly IL-6.

As inflammation has been shown to influence survival in pancreatic cancer in this thesis in addition to other publications, may the SOCS protein have a role to play in the poor prognosis of patients with an inflammatory response? Komazaki et al examined a number of pancreatic tumours, looking for methylation of the SOCS-1 gene[303]. They demonstrated methylation of the SOCS-1 gene in 50% of the cancers included in the study and resulting suppression of SOCS-1 mRNA.

Fukushima et al also looked at the SOCS-1 gene in pancreatic cancer and went on to examine if there was any resulting affect on the JAK STAT pathway[304]. Similar to the previous studies outlined above the incidence of SOCS-1 gene methylation was initially established in both cell lines and tissue specimens. Aberrant methylation was seen in 31% of pancreatic cancer cell lines, which was associated with loss of gene transcription in 83% of cases. When 60 pancreatic cancer specimens were similarly examined, methylation was seen in 13 (22%) and in 6% of the intra-ductal papillary mucinous neoplasms (IPMN). However methylation was not seen in normal ductal epithelium, in pancreatic intra-epithelial neoplasia (PanIN) or in IPMN's without invasive cancer. This study then went onto show that methylation of SOCS-1 gene in pancreatic cancer cell lines was associated with an IL-6 dependent, statistically significant increase in cellular growth.

Although these studies are relatively few in number, some authors are actually describing the SOCS-1 gene as a possible tumour suppressor gene, as methylation is a common mechanism by which other established tumour suppression genes are

affected in cancer. Does this evidence for the methylation of SOCS genes fit with the results from this thesis? Could it be that a loss of negative regulation rather than increased activation leads to increased signalling via the JAK STAT pathway? This is certainly one possibility that would fit with the results reported in section 3. Those patients with a raised pre-operative CRP and therefore raised IL-6 may have increased transcription of STAT3 target genes via relatively weak signalling due to the removal of the negative feedback of SOCS proteins and may explain why there was no obvious evidence of upregulation of the JAK STAT pathway in this patient cohort. One other possibility is that inflammation and IL-6 negatively effects survival via JAK STAT independent mechanisms and this will be explored later in this discussion.

4.6.2 NF-κB and Cancer

NF-κB is an important protein in both the inflammatory response and tumourogenesis. It is a ubiquitously expressed transcription factor and regulates the expression of various inflammatory, apoptotic and oncogenic genes[159]. The active form of NF-κB is a heterodimer with two sub-units p65 (also called RelA) and p50 which are both members of the Rel family of transcription factors. Activation of NF-κB occurs by a number of stimuli including IL-1, protein kinase C, reactive oxygen species, viruses, TNF and radiation[160,161]. Once activated at sites of inflammation NF-κB causes increased transcription of 27 different genes encoding for cytokines, chemokines and receptors crucial for neutrophil adhesion and migration including IL-2, IL-2R, IL-6, IL-8, IL-12 and TNF[162]. These cytokines can in turn activate the NF-κB pathway establishing a positive autoregulatory loop that sustains and amplifies the inflammatory response.

Cancer is a hyper proliferative disorder resulting from the inability of cells to undergo normal programmed cell death or, apoptosis. One of the most potent well studied inducers of apoptosis in mammalian cells is tumour necrosis factor (TNF)[305]. Many members of the TNF superfamily induce apoptosis, but in addition, various chemotherapeutic agents and ionising radiation work by inducing apoptosis. Most agents that induce apoptosis are also known to activate NF-κB. A number of publications have shown that activation of NF-κB induces resistance to apoptosis induced by TNF, chemotherapeutic agents and radiotherapy[305-309]. NF-κB induces inhibition of apoptosis by upregulating anti-apoptotic genes including Bcl-2, cIAP-1, cIAP-2, TRAF-1, TRAF-2 amongst others[305]. The ability of NF-κB to inhibit apoptosis has been demonstrated in many different cell types including myeloid cells, hepatocytes, melanoma cells,

prostate cancer cells, head and neck squamous cell carcinoma, and pancreatic cancer cells[163,310-314].

In the treatment of cancer, chemotherapeutic agents and radiotherapy are employed to induce apoptosis in abnormal cancerous cells, but paradoxically they both induce activation of NF-κB which acts as an inhibitor of apoptosis. This process is thought to be one of the ways that tumours become resistant to chemo and radiotherapy.

Kim et al examined the effect of inhibiting NF-κB in human prostate cells, demonstrating increased sensitivity to radiation when it was blocked[309]. The authors also were able to show that this increased sensitivity was in cells that had mutated K-Ras, a relationship explored further below.

In terms of chemoresistance, Wang et al were able to demonstrate increased chemosensitivity in chemoresistant tumours by inhibiting NF-κB[315]. They demonstrated tumour regression in cells with NF-κB inhibition and this was due to increased TNF mediated apoptosis.

Pancreatic cancer is resistant to almost all cytotoxic drugs but Gemcitabine appears to have activity in some patients without having a significant effect on their survival. Arlt et al looked at the effects of Gemcitabine on a number of pancreatic cancer cell lines, some with and some without high basal NF-κB activity[316]. They were able to demonstrate that treatment with Gemcitabine led to a dose dependent increase in NF-κB and that treatment led to a significant induction of apoptosis in those cell lines without high basal NF-κB, but not in

those with high basal activity. They also showed that inhibition of NF-κB strongly diminished the resistance of those cell lines to Gemcitabine treatment. They conclude that constitutive activation of NF-κB in pancreatic cancer cell lines leads to resistance against Gemcitabine by the inhibition of apoptosis and that modulation of this activity leads to chemo-sensitivity. This then begs the question is there any evidence for upregulation of NF-κB in pancreatic cancer cells?

Wang et al were able to demonstrate constitutive activation of NF-κB in 67% of human pancreatic cancer tissue and in 81% of pancreatic cancer cell lines (there was no activity in normal pancreatic cells or non-cancerous pancreatic cell lines)[317]. Interestingly, constitutive activation of NF-κB was found in 65% of pancreatic cancer tissue that had a K-Ras mutation and in 89% of pancreatic cancer cell lines that contained the K-Ras mutation. This study along with the study described above by Kim et al suggests a possible link between mutated K-Ras (present in 90% of pancreatic cancers) and NF-κB activation. Kim et al also published another study looking specifically at the role mutated K-Ras (constitutively active) had in the activation of NF-κB in prostate tissue[318]. They were able to show that mutated K-Ras lead to the activation of NF-κB and inhibition of K-Ras lead to a corresponding reduction in phosphorylation of NF-κB.

To summarise, NF-κB has been shown to be constitutively active in pancreatic cancer cells and in turn this leads to increased transcription in multiple genes involved in inflammation including IL-6. These inflammatory growth factors in turn lead to further activation of NF-κB causing a pro-inflammatory autoregulatory loop. Activated NF-κB is also known to lead to increased

transcription in a number of genes involved in the inhibition of apoptosis which in addition to the pro-inflammatory conditions creates an environment ideal for tumour growth. NF-κB, as discussed above appears to aid tumour resistance to chemo and radiotherapy by this inhibition of apoptosis. With K-Ras being mutated (constitutively active) in around 90% of pancreatic cancers and known to activate NF-κB, could this pathway be responsible for the poor prognosis associated with an inflammatory response in patients with pancreatic cancer?

K-Ras, as discussed earlier is known to activate the MAPK pathway. In this thesis it has been demonstrated that there is no relationship between poor survival and upregulation of the MAPK pathway and therefore if the above theory is true and the results in this thesis are to be believed then K-Ras must activate NF-κB via an MAPK-independent manner. A number of publications show that K-Ras can activate a number of downstream effector pathways, but of interest is its activation of the PI3K/Akt pathway.

Chen et al published their findings after looking at the effects of lidamycin (an antibiotic) on apoptosis in human pancreatic cancer cell lines[319]. They describe mutant K-Ras leading to activation of the Akt pathway which in turn leads to activation of NF-κB. They were able to show that lidamycin treatment led to a dose dependent inhibition of Phospho.Akt and NF-κB along with down-regulation of K-Ras mRNA and protein expression in both of the two examined pancreatic cancer cell lines. Following this a remarkable improvement in chemo-sensitivity to mitomycin, adriamycin, taxol and gemcitabine was seen in both cell lines. In those cells treated with lidamycin there was also evidence of growth inhibition, apoptosis induction and cell cycle arrest. This study shows that NF-κB is not only

a potential therapeutic target in pancreatic cancer but its activation appears to be as a result of mutated K-Ras acting via the Akt pathway. K-Ras activating the Akt pathway is an established process that has been demonstrated in a number of other studies[320,321].

The above process would lead to a raised CRP and would support the findings in this thesis, explaining why activation of the JAK STAT and MAPK pathways was not seen. Clearly more research is needed, but this hypothesis needs to be explored.

4.6.3 The Systemic Inflammatory Response and Cancer

When a literature review of the systemic inflammatory response syndrome (SIRS) or acute phase response (APR) and cancer is carried out, there is a huge amount of research detailing the relationship between inflammation and weight loss. Cancer-related weight loss is more commonly referred to as cancer-related cachexia. In patients with pancreatic cancer the development of cachexia is a major therapeutic challenge and along with gastric tumours, pancreatic cancer has the highest rate of cachexia of all cancers at well over fifty percent[322].

Cancer-related cachexia is a syndrome and is difficult to define being more complex than just simple weight loss. It is characterised by a combination of some, if not all of the following signs and symptoms: anorexia; early satiety; weight loss; weakness; anaemia; oedema. Fearon et al looked at 170 patients with advanced pancreatic cancer who were losing weight to try and establish if weight loss alone or a three factor cachexia profile (weight loss, decreased intake, inflammatory status) best predicted functional and prognostic outcome.[323]. Patients were included if they had lost >5% of their pre-illness weight in the preceding six months and excluded if they had undergone surgery, stenting, radiotherapy or chemotherapy in the last four weeks. Through statistical analysis the authors were able to demonstrate that in that particular patient cohort, weight loss alone did not separate patients with poor function and prognosis sufficiently. However if the patients had all three of the cachexia factors present then there was a significant negative impact on survival and function emphasising that the negative impact of cancer-related cachexia may be due to more than just weight loss. This paper also highlights the involvement of inflammation and cancer-related cachexia.

A much referenced paper by Dewys et al published in 1980 looked specifically at the prognostic role of weight loss in 3047 patients awaiting chemotherapy for cancer[324]. They described the rates of weight loss from as low as 31% in non-Hodgkins lymphoma up to 87% in gastric cancer patients. This study demonstrates that patients with weight loss had a shorter median survival, poorer chemotherapy response rates and poorer overall performance status. This study did not analyse inflammation but clearly demonstrates in a large group of patients that weight loss alone can impact on survival.

O'Gorman et al examined the relationship between weight loss, appetite, performance status and the inflammatory response in 50 patients with advanced GI malignancies who were losing weight[325]. Patients were observed for a six week period and then grouped into those who had lost weight (>3%), had stable weight (<3% change) and those who had gained weight (>3%). At baseline those patients who subsequently lost weight had higher CRP and lower albumin levels ($P<0.05$). During follow up the weight loss group had increased CRP levels, reduction in skin-fold thickness and reduction in performance status ($P<0.05$). Conversely, performance status improved in the group that gained weight. This study suggests that the presence of an inflammatory response causes increased weight loss and is associated with deterioration in performance status in patients with GI malignancy. This paper also outlines a relationship between weight loss, inflammation and albumin levels.

The role that serum albumin plays in patients with cancer and the relationship it has with an inflammatory response has been explored by a number of authors. In a review article on serum albumin, Margarson stated that hypoalbuminaemia is

almost inevitable in disease states and is associated with poorer prognosis [326]. They also stated that the systemic inflammatory response was in part responsible for the low serum albumin. The cause of hypoalbuminaemia associated with cancer was investigated by Fearon et al comparing six patients with pancreatic cancer and six healthy controls[327]. They were able to demonstrate that cancer patients had a higher serum CRP and a lower serum albumin ($P<0.01$ for both), but showed that the rate of albumin synthesis was similar between both groups. A review article by the same authors looking at pancreatic cancer, the inflammatory response and cancer cachexia, discusses the possibility that the APR leads to a reprioritisation of body protein metabolism which may be significant in the loss of lean tissue in these patients[328].

McMillan et al examined the relationship between albumin, weight loss, body cell mass (as measured by total body potassium) and the presence of an inflammatory response in 40 male patients with either advanced GI or pulmonary malignancy[329]. They demonstrated that lower albumin levels significantly correlated with weight loss ($P<0.01$), with total body potassium ($P<0.001$) and inversely with CRP levels ($P<0.001$). They concluded that the inflammatory response is not only associated with lower serum albumin levels but also with the loss of body cell mass. They hypothesise that the inflammatory response creates an increased demand for specific amino acids and promotes the degradation of body proteins including albumin. Due to the relatively small pool of albumin when compared to body cell mass, hypoalbuminaemia is noted before weight loss which is likely due to loss of skeletal muscle mass. The inverse relationship between an APR (as measured by CRP) and albumin levels has been used to create a prognostic scoring system in patients with inoperable pancreatic cancer, discussed earlier in section 4.5.1[299].

If the inflammatory response is responsible for hypoalbuminaemia, loss of body mass and possibly cancer-related cachexia, can attempting to abolish this response potentially reverse weight loss and improve outcome? A number of studies have attempted to manipulate the inflammatory response and assess whether weight loss or deteriorating function can be reversed.

Lundholm et al retrospectively analysed 702 patients with cancer who were losing weight and had undergone no treatment[330]. They compared these patients with 132 undernourished non-cancer patients and were able to demonstrate that at baseline the cancer patients had a significantly increased resting energy expenditure (REE) ($P<0.001$). This difference became significantly reduced by the administration of indomethacin (anti-inflammatory drug) ($P<0.003$). They also showed that total body fat was more preserved in those treated with indomethacin ($P<0.005$) although lean body mass was not affected. These changes were observed in addition to a significant reduction in the inflammatory response as measured by CRP in those patients treated with indomethacin ($P<0.0004$). Systemic inflammation and resting energy metabolism predicted weight loss in cancer patients ($P<0.0001$).

Another study by Wigmore et al examined the effect of ibuprofen (anti-inflammatory drug) on REE in patients with irresectable pancreatic cancer who were losing weight[331]. Ten patients with pancreatic cancer were given 1200mg of ibuprofen daily and their REE and CRP compared with six similar patients who were given a placebo over a 7 day period. Prior to treatment the pancreatic cancer patients had a significantly higher REE when compared with 17 healthy controls

(P<0.02). Following treatment the mean REE of the ibuprofen group fell significantly compared with pre-treatment values (P<0.02), but no difference was seen in the placebo group. Serum CRP also fell significantly in the ibuprofen group (P<0.05).

These two studies by Lundholm and Wigmore demonstrate that in addition to causing hypoalbuminaemia and altering protein metabolism, an inflammatory response also leads to an increase in resting energy expenditure and that manipulating this response with simple anti-inflammatory medication can reduce a catabolic process which will undoubtedly contribute to weight loss and the cancer-cachexia syndrome.

Both of the aforementioned studies show that anti-inflammatory drugs can reduce the abnormally high REE associated with cancer patients by decreasing the inflammatory response, but they don't reveal if there is a resultant increase in weight or improved outcome for these patients. McMillan et al performed a prospective randomised trial examining the effects of giving patients with GI cancer and weight loss a combination of megestrol acetate (a progesterone derivative and known appetite enhancer) and ibuprofen or placebo[332]. The authors assessed CRP, appetite, performance status, quality of life, weight and albumin at baseline, 6 weeks and 12 weeks comparing both the ibuprofen and placebo groups (38 and 35 patients respectively). Analysis of the results revealed no significant difference between the two groups at baseline. After 6 weeks there was a weight gain seen in the ibuprofen group and a weight loss in the placebo group (P<0.01). At twelve weeks this difference was highly significant (P<0.001) with a median weight gain of 2.3kg in the ibuprofen group and median weight loss of 2.8kg in

the placebo group. There was also a significant improvement in the quality of life score in the ibuprofen group ($P<0.05$). This study shows that reducing an inflammatory response using anti-inflammatory drugs can actually reverse the weight loss suffered by patients with advanced cancer and improve their quality of life.

These studies demonstrate the link between an inflammatory response and mechanisms which are likely to contribute to cancer-related cachexia including increased resting energy expenditure, altered protein metabolism and hypoalbuminaemia. A number of questions are still unanswered. Why do some patients with cancer get this inflammatory response which is responsible for the development of a cachexia syndrome? Is it the tumour itself or the reaction of the host to the tumour that leads to cancer cachexia? Do the results reported in this thesis fit with the theory that an inflammatory response causes cachexia in pancreatic cancer patients leading to a poorer prognosis?

Falconer et al looked at the APR, REE and cytokines in cachectic patients with pancreatic cancer[333]. They looked at 21 patients with pancreatic cancer and compared them with 16 age-related healthy controls assessing REE, body composition, CRP, IL-6 and TNF. In addition they also designed an in vitro experiment on peripheral blood mononuclear cells (PBMC's) examining their production of cytokines. Patients with pancreatic cancer had a significantly elevated REE compared to healthy controls ($P<0.003$) and that within the cancer group those with an inflammatory response ($CRP>10mg/l$) represented a subset of cancer patients who were significantly hypermetabolic ($P<0.04$). Serum levels of IL-6 were detected but there wasn't a significant difference between cancer

patients with and those without an inflammatory response. TNF was not detected in the serum of any of the cancer patients. However, spontaneous production of TNF and IL-6 by PBMC was significantly greater in cancer patients with an APR than in those without ($P<0.003$). The authors conclude that the presence of an inflammatory response separates a subgroup of cancer patients who are markedly hypermetabolic. They also suggest that as serum IL-6 is not significantly different in the inflammatory subset but PBMC production of IL-6 is significantly raised, that local rather than systemic cytokine production may be important in regulating the APR.

The findings from Falconers paper appear to be supported by O'Riordain et al who also looked at PBMC, APR and IL-6 in weight-losing cancer patients[184]. They were able to demonstrate that PBMC from cancer patients induced a significantly higher CRP than healthy controls ($P<0.005$). CRP production in vitro correlated with IL-6 production from PBMC in patients with pancreatic cancer ($P<0.0001$). When IL-6 neutralising antibodies were applied the CRP production was reduced by 84% ($P<0.001$). There was a statistically significant negative correlation between PBMC-induced CRP production and survival ($P<0.01$). This paper suggests that PBMC production of IL-6 leads to the hepatic acute phase response seen in cancer patients and that this APR is significantly associated with poor survival.

A study looking specifically at patients with operable pancreatic cancer and the development of cachexia was published in 2005[322]. The aims were: to establish which factors were associated with the development of cachexia; to analyse whether the tumour or the reaction of the host to the tumour is the source of or the

reason for induction of cachexia; to establish whether the tumour or the host is the production site of the cachexia-mediating factors. This study is in multiple parts and each will be discussed in turn.

In order to establish which factors were crucial to the development of cachexia, tissue from 8 patients with pancreatic cancer (4 with and 4 without cachexia [defined as >10% weight loss in preceding 6 months]), from 8 patients with alcohol induced chronic pancreatitis (CP) and 8 normal pancreatic specimens were obtained. DNA microanalysis was then carried out on all specimens and after analysing over 5600 human genes four showed significant differences in expression between the above three groups and between those patients with and those without cachexia. One of these four was the IL-6 gene.

The authors then further analysed these four significant genes by quantitative reverse transcription-PCR analysis. RT-PCR was done in tumour samples of non-cachectic (14) and cachectic (19) patients in addition to patients with CP (34) and normal controls (21). This analysis in a larger sample size revealed that only IL-6 mRNA expression was significantly increased in tumour samples of cachectic patients compared with non-cachectic cancer patients, CP patients and healthy controls ($P<0.01$).

Having established IL-6 as the key factor in those patients with cachexia, immunohistochemistry (IHC) was performed using paraffin embedded tissue from cancer patients without (9) and those with cachexia (11). Non-cancerous tissue from the surrounding area showed no IL-6 immunoreactivity, but in contrast it was strongly present in the cytoplasm of pancreatic cancer cells. When

comparison was made between those patients with and those without cachexia, much stronger IL-6 staining was seen in the cachectic patient samples. Next serum taken from 27 pancreatic cancer patients with and 14 without cachexia underwent analysis for IL-6 levels. A significant difference was seen between the groups, with levels being higher in cachectic patients ($P<0.04$).

The final part of this study involved the coculture of PBMC from pancreatic cancer patients and healthy controls with pancreatic cancer cell lines. Two cell lines were used, T3M4 (known to produce IL-6) and Panc-1 (known not to produce IL-6). Following coculture IL-6 mRNA was measured. PBMC and Panc-1 cell line coculture resulted in a modest but not significant increase in IL-6 mRNA. PBMC and T3M4 in patients with cachexia led to a 14x upregulation of IL-6 mRNA expression in PBMC's ($P<0.002$). This upregulation was reduced by 60% when IL-6 neutralising antibody was added. In those cancer patients without cachexia and in healthy controls, PBMC and T3M4 coculture did not result in a change of IL-6 mRNA in the respective PBMC's. When the PBMC's from cachectic patients was cultured alone (without tumour cell lines) then IL-6 mRNA was low.

This study confirms that IL-6 is the key cytokine involved in the development of cachexia in patients with pancreatic cancer. It demonstrates that pancreatic cancer cells in those patients with cachexia actually produce IL-6. In addition IL-6 serum levels were significantly higher in the cachectic patients. Using an in vitro model, coculture experiments showed that PBMC of patients with pancreatic cancer with IL-6 positive cells were sensitised to produce IL-6. The PBMC's of those pancreatic cancer patients with cachexia who had IL-6 positive cells were

upregulated 14 times more strongly than non-cachectic patients. The authors hypothesise that cachectic patients PBMC's may be sensitised by the tumour in vivo and trigger further IL-6 overexpression without having any direct contact with the tumour cells. They also observed that IL-6 mRNA is low after 24 hours of culture without tumour cells shows that there appears to be a need for constant tumour-derived stimulus to the PBMC's to maintain IL-6 levels.

Martignoni's paper along with the other studies discussed above demonstrates the crucial role of IL-6 in pancreatic cancer and fits with the results reported in this thesis. A proportion of pancreatic tumours appear to produce high levels of IL-6 and these same tumours sensitise PBMC to produce significantly higher levels of IL-6. With increased levels of serum IL-6 a hepatocyte-driven APR (as measured by CRP) occurs and the development of cachexia with increased REE, hypoalbuminaemia and altered protein metabolism with subsequent poor survival.

Why some tumours produce high levels of IL-6 requires further investigation. Is the poor prognosis associated with an inflammatory response just due to the development of cachexia? This seems unlikely and it would seem probable that cachexia is only part of the story. Some of the possibilities discussed above such as the constitutive activation of NF-κB in pancreatic tumours and the subsequent development of an autoregulatory inflammatory loop leading to high levels of IL-6 fit with the results from this thesis and may explain why some tumours produce high levels of IL-6. Constitutive activation of NF-κB would also lead to the upregulation of genes associated with growth and the inhibition of apoptosis. This would mean the poor prognosis may be multifactorial, a combination of a 'pro-growth' tumour biology and a subsequent APR leading to cachexia, effectively

meaning a ‘double-hit’ for some patients. With around 90% of pancreatic cancers exhibiting mutation of the K-Ras gene and subsequent constitutive activation, further research is required to establish whether this has a role in NF-κB activation as discussed above in section 4.6.1. Further study is also required to establish whether the SOCS-1 gene also has a role to play in the above pathway as removal of negative feedback from the JAK STAT pathway rather than increased signalling may explain why no evidence of upregulation was seen in this thesis. Manipulation of the inflammatory response was examined in a number of studies discussed above. There was evidence for increasing weight, lowering of the REE and some functional status improvement in response to NSAID treatment in cancer patients but none of the studies provided evidence of an improvement in survival. A randomised trial of anti-inflammatory medication against placebo in patients with inoperable pancreatic cancer which is sufficiently powered with a primary endpoint of survival would be easy to set up and due to the dreadful prognosis associated with the disease results would be seen quickly. Even a modest improvement in survival would be important in patients with such a limited life expectancy and virtually no treatment options. A similar study in operable pancreatic cancer would be more difficult as such small numbers of patients have operable disease.

A number of potential questions would need to be considered in a study including patients with operable disease who’s median survival is a little over a year. Would only those patients with a pre-operative inflammatory response benefit from pre-operative anti-inflammatory medication? Would decreasing a pre-operative inflammatory response improve not only long-term survival but also short-term

outcome (operative morbidity and mortality) by effectively helping to optimise a patient nutritionally who may be losing weight?

Other treatment approaches may include manipulation of cellular biology. Could the use of IL-6 neutralising antibody used in vitro by Martignoni et al have any application in vivo? Can further research identify a single common signalling pathway or signalling molecule that triggers the cellular changes leading to increased cellular IL-6 and subsequent inflammatory response and development of cachexia. Is NF-κB this key signalling molecule and can medical manipulation improve the outcome for patients with pancreatic cancer? These are all important questions the answers to which may produce novel treatments for patients with not only pancreatic cancer but all forms of the disease.

APPENDIX 1

The following frequency histograms (figures 1-14) demonstrate the distribution of scoring for each antibody in each of the cellular compartments for the JAK-STAT pathway. As described in section 3.4.4 these graphs were used to select cut-off points prior to survival analysis.

Figure 1: Frequency of histoscores for IL-6R in the cytoplasm.

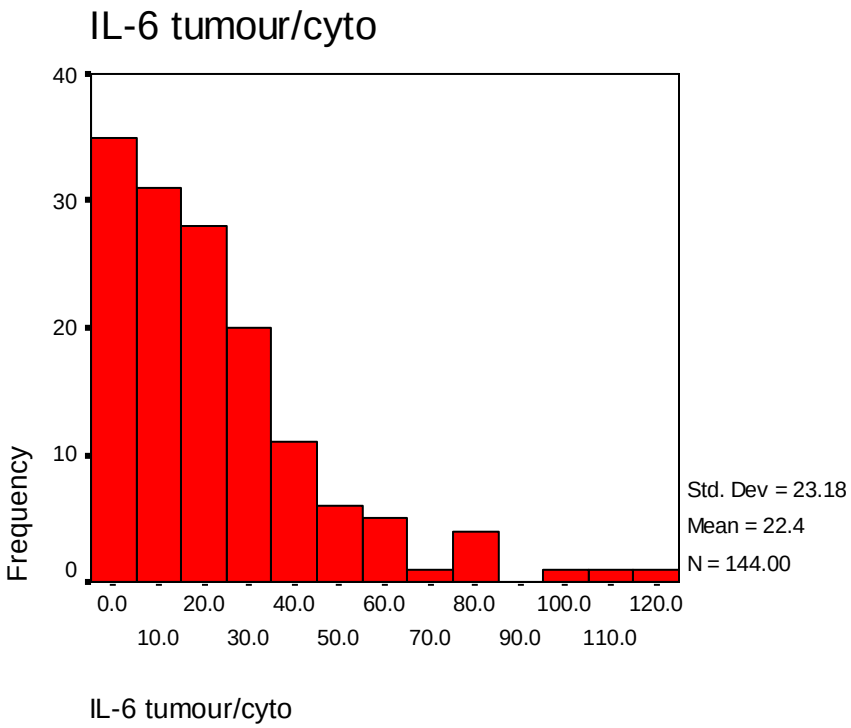


Figure 2: Frequency of histoscores for IL-6R at the membrane.

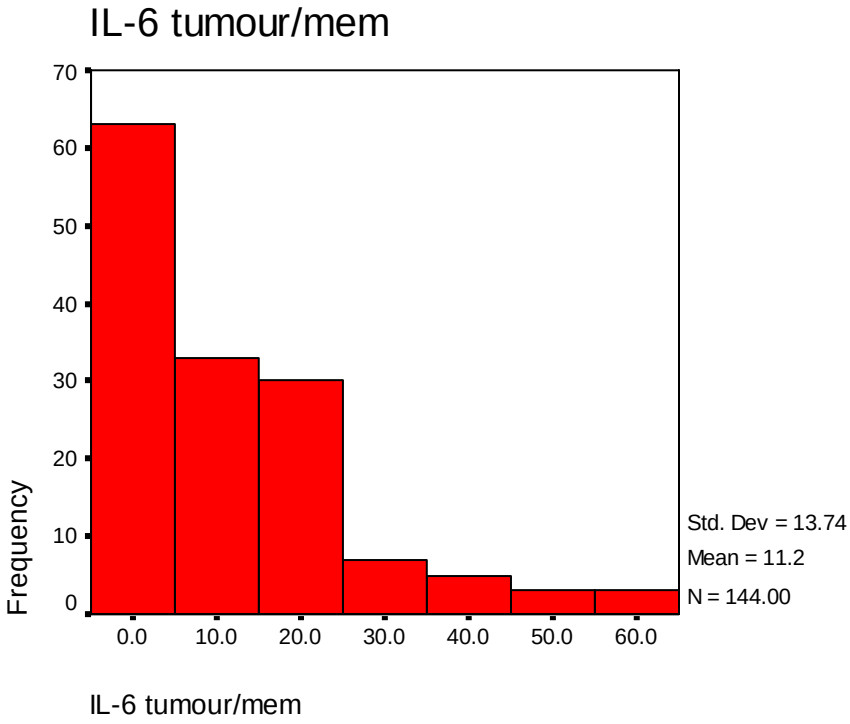


Figure 3: Frequency of histoscores for IL-6R in the nucleus.

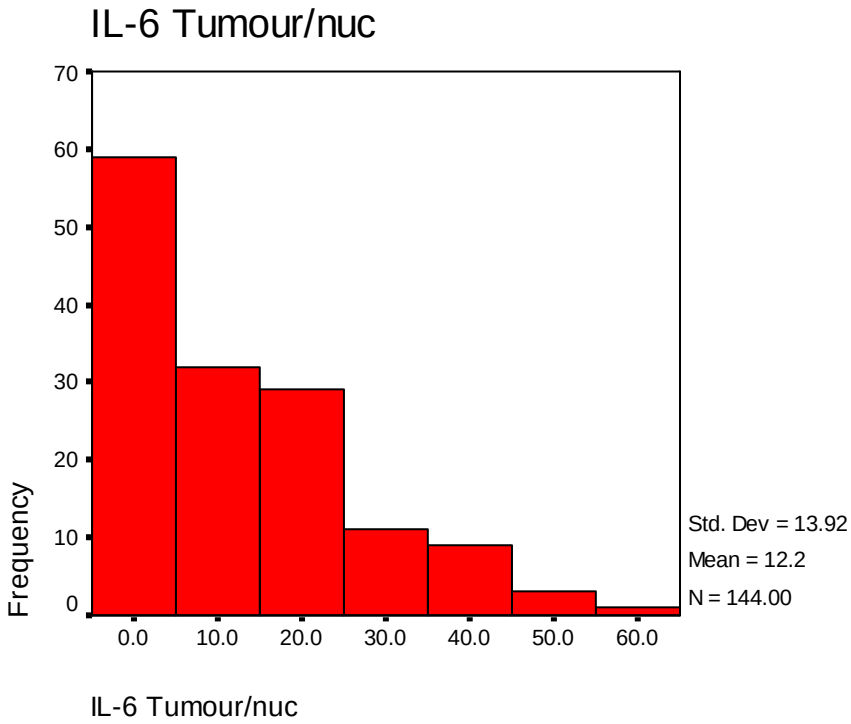


Figure 4: Frequency of histoscores for JAK1 in the cytoplasm.

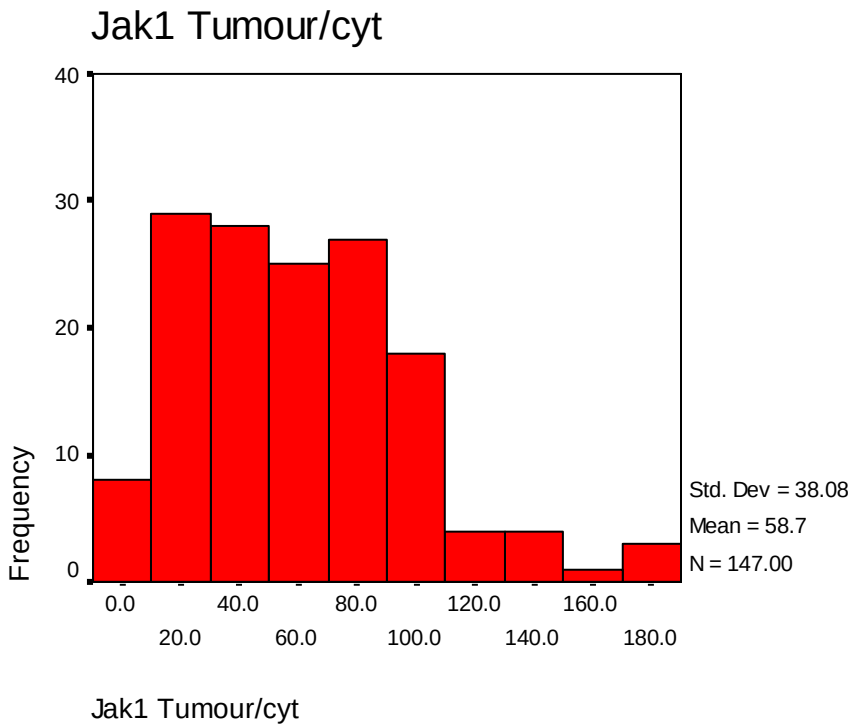


Figure 5: Frequency of histoscores for JAK1 at the membrane.

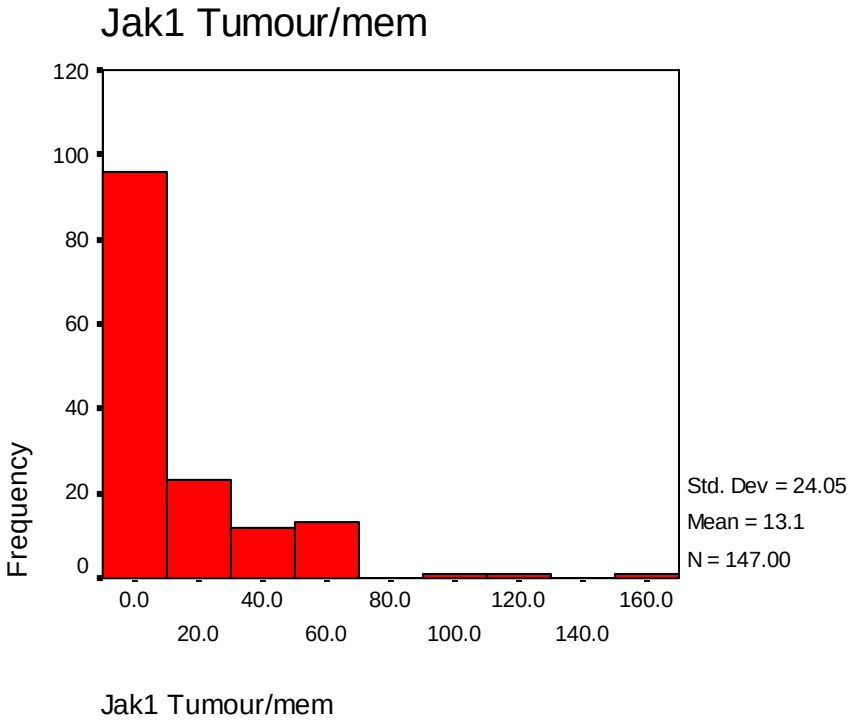


Figure 6: Frequency of histoscores for Phospho.JAK1 in the cytoplasm.

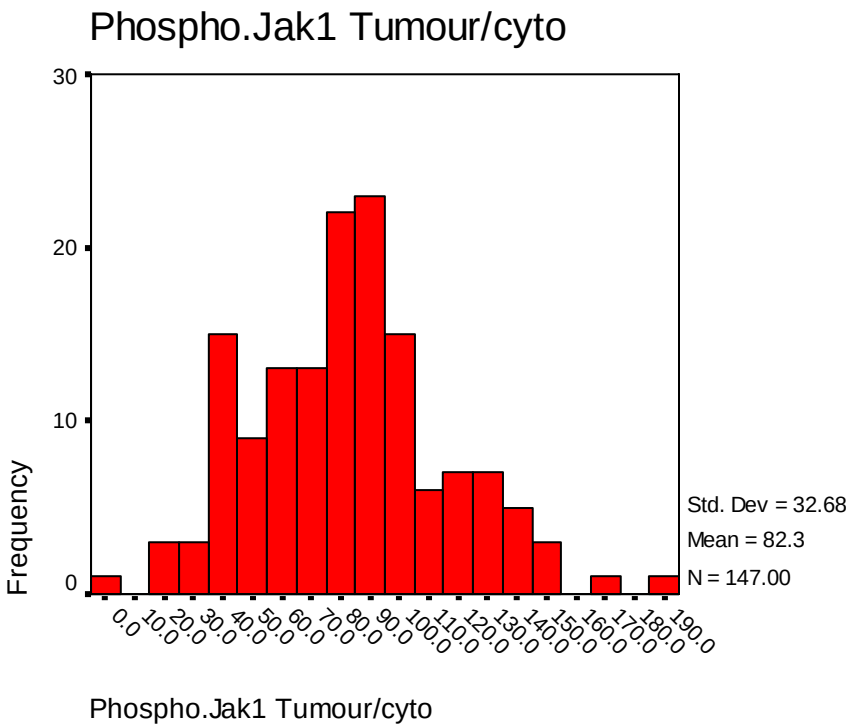


Figure 7: Frequency of histoscores for Phospho.JAK1 at the membrane.

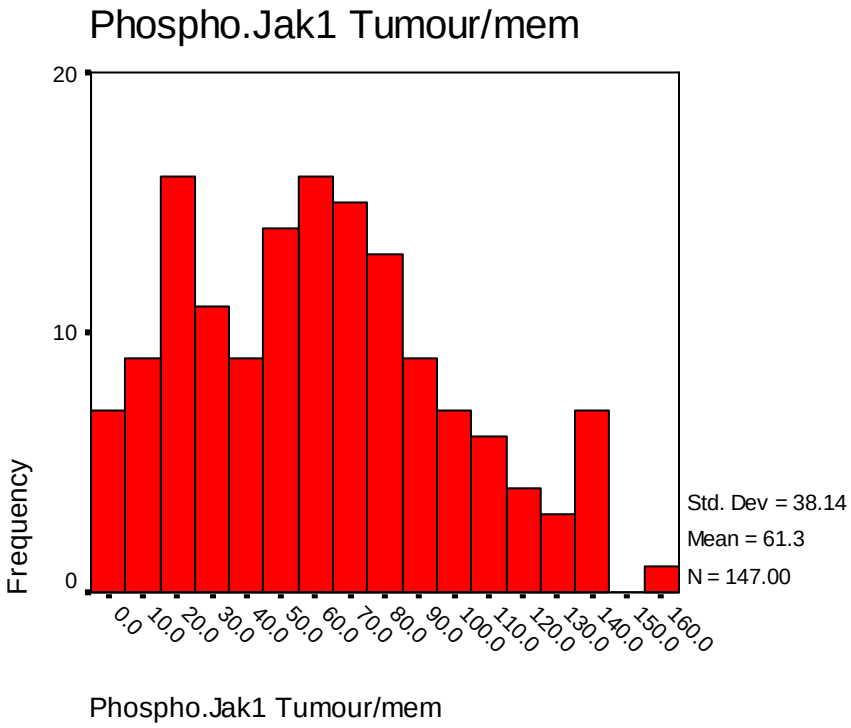


Figure 8: Frequency of histoscores for Phospho.JAK1 in the nucleus.

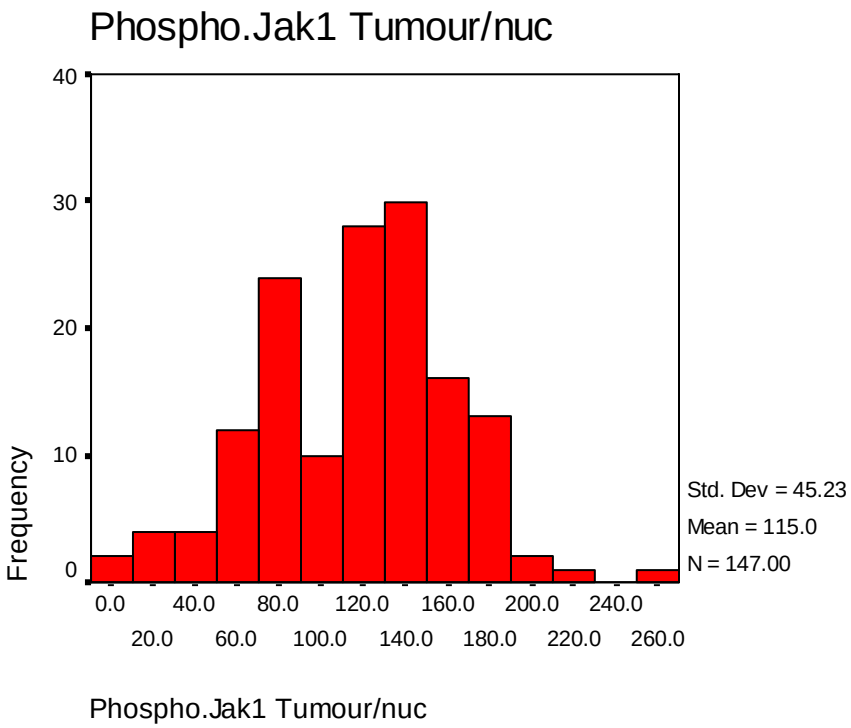


Figure 9: Frequency of histoscores for STAT3 in the cytoplasm.

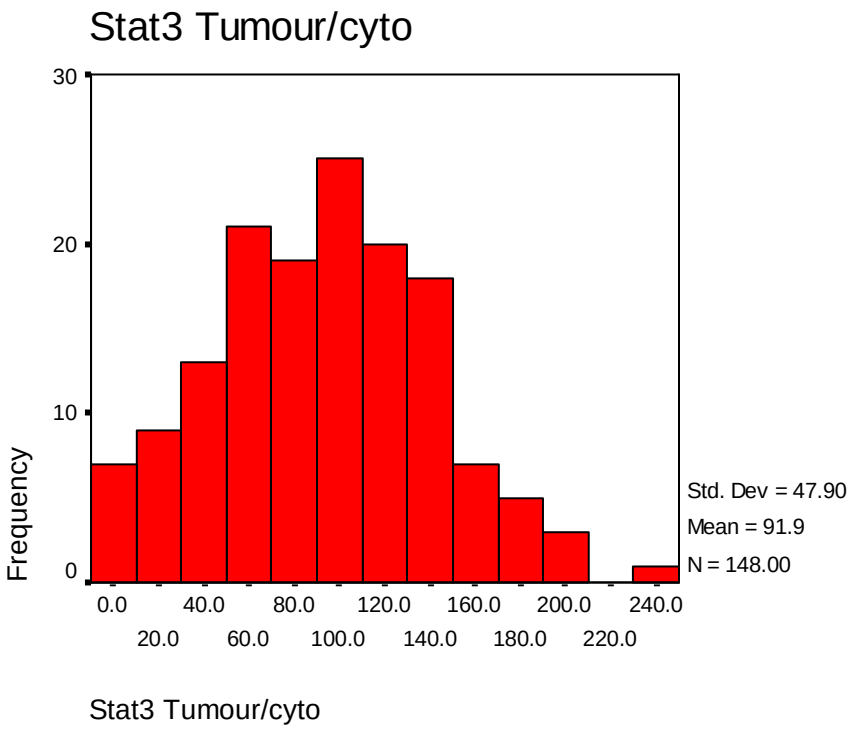


Figure 10: Frequency of histoscores for STAT3 at the membrane.

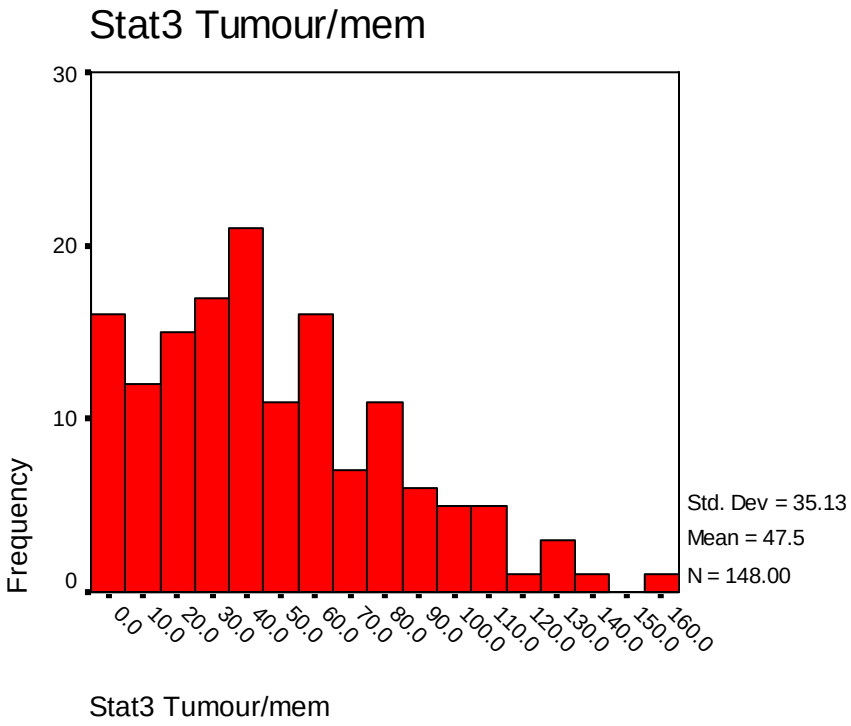


Figure 11: Frequency of histoscores for Phospho.STAT3 Ser727 in the cytoplasm.

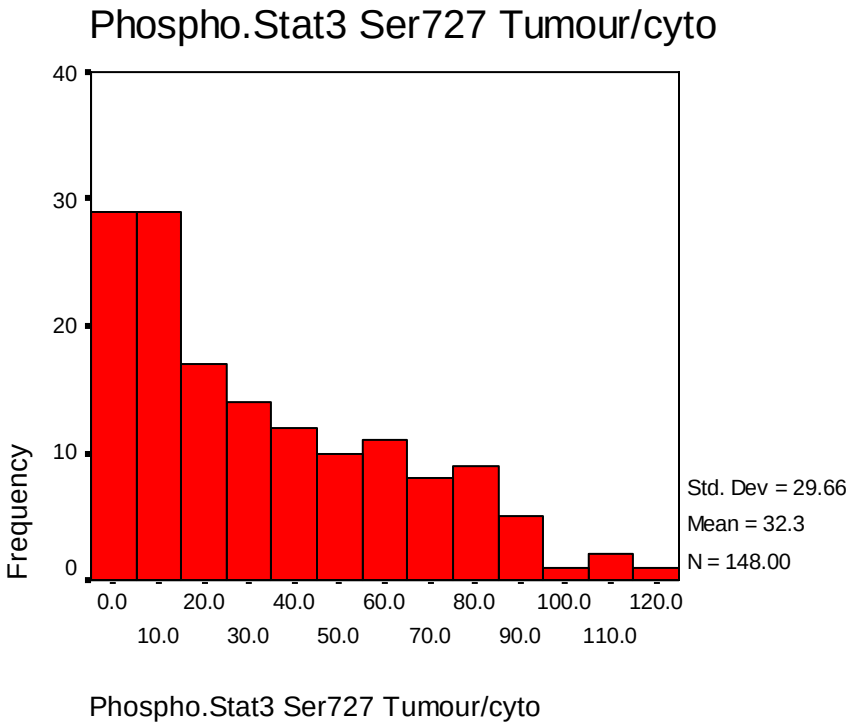


Figure 12: Frequency of histoscores for Phospho.STAT3 Ser727 in the nucleus.

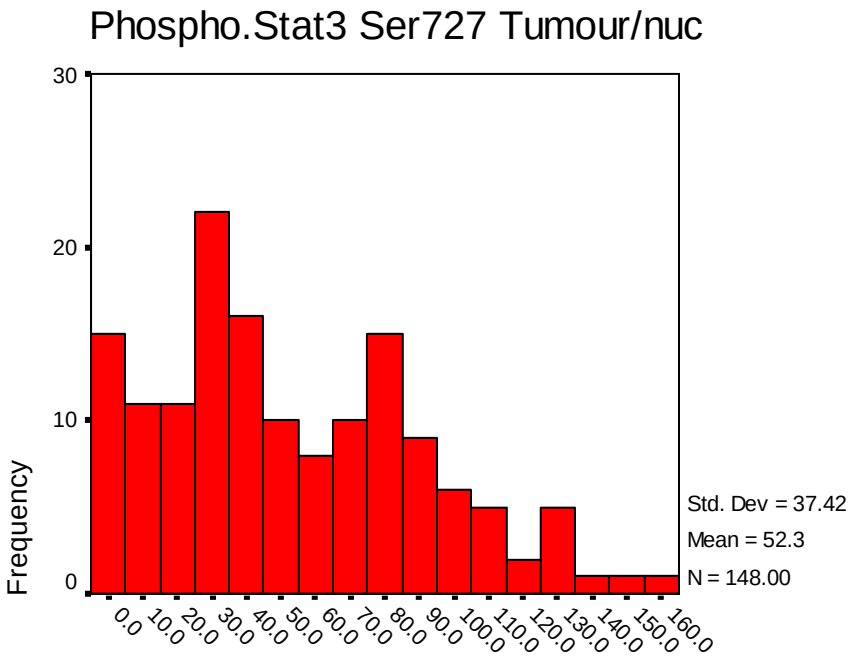


Figure 13: Frequency of histoscores for Phospho.STAT3 Tyr705 in the cytoplasm.

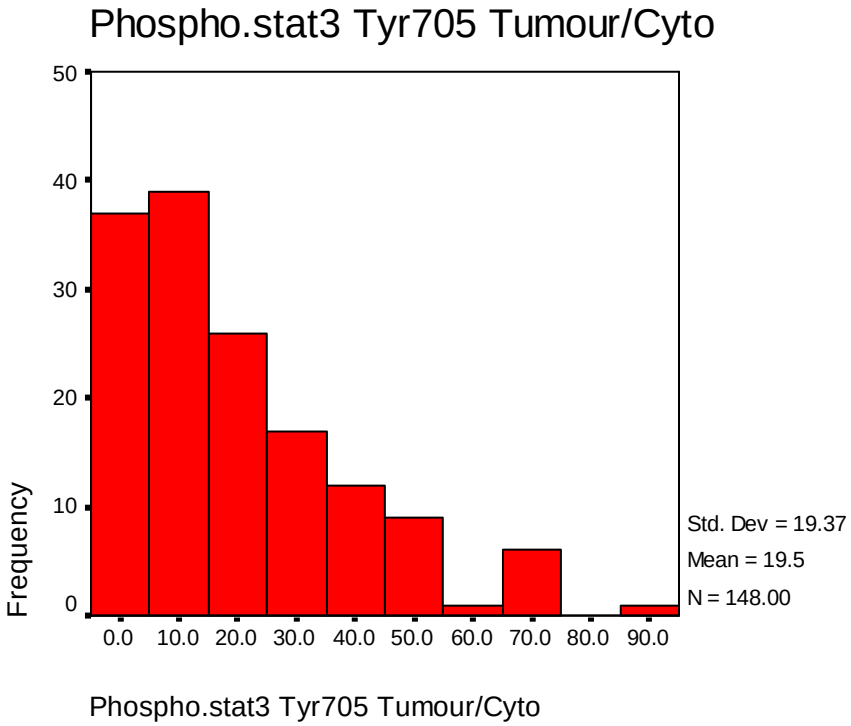
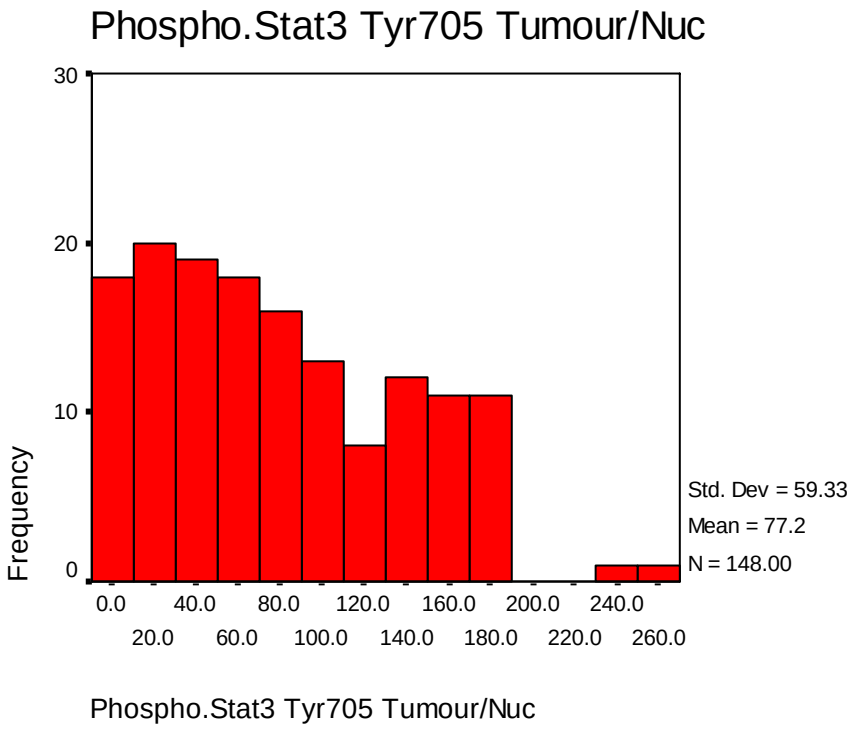


Figure 14: Frequency of histoscores for Phospho.STAT3 Tyr705 in the nucleus.



APPENDIX 2

The following frequency histograms (figures 1-14) demonstrate the distribution of scoring for each antibody in each of the cellular compartments for the MAPK pathway. As described in section 3.4.4 these graphs were used to select cut-off points prior to survival analysis

Figure 1: Frequency of histoscores for K-Ras in the cytoplasm.

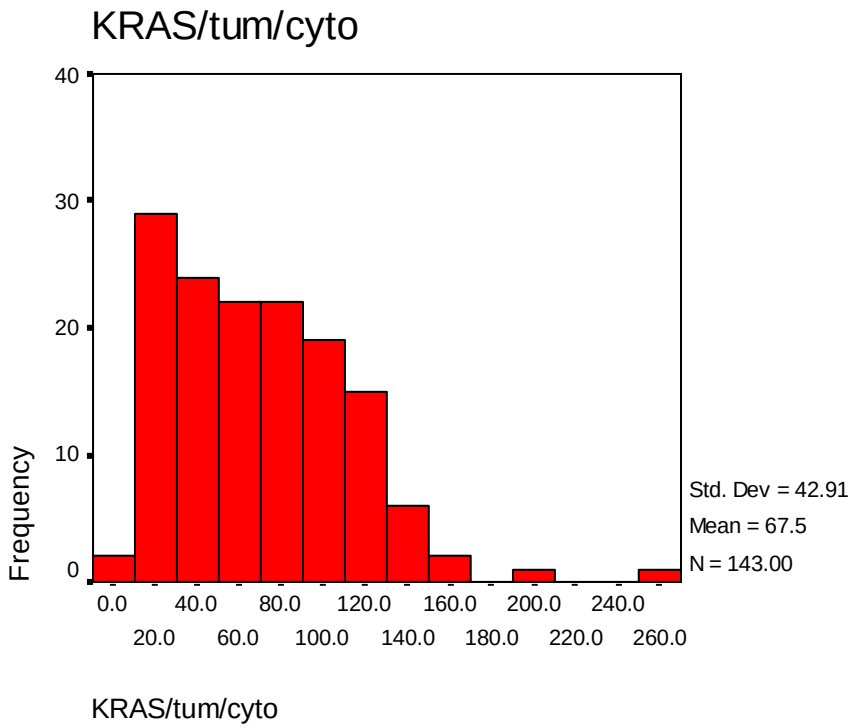


Figure 2: Frequency of histoscores for K-Ras at the membrane.

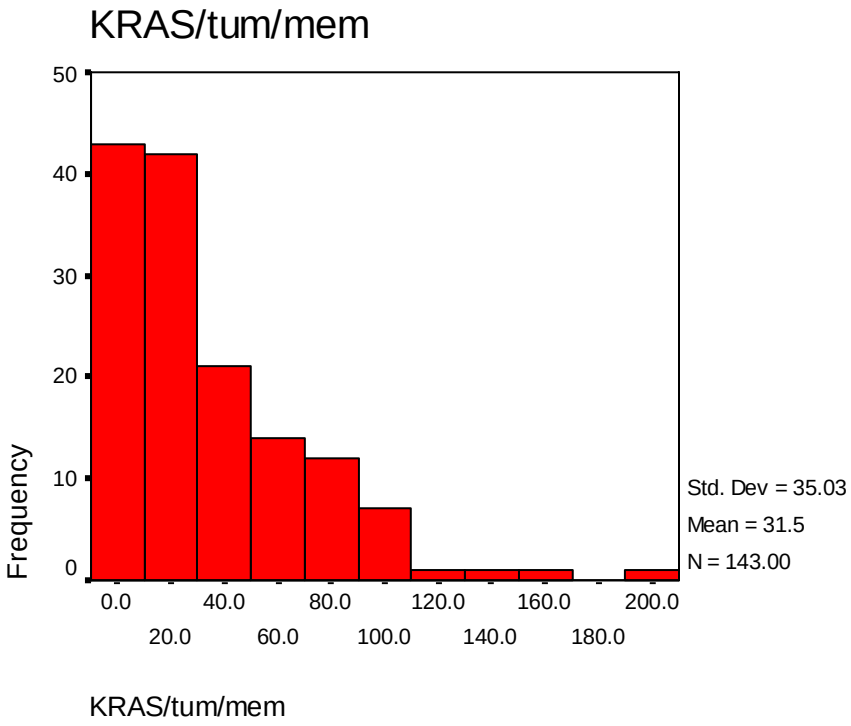


Figure 3: Frequency of histoscores for K-Ras in the nucleus.

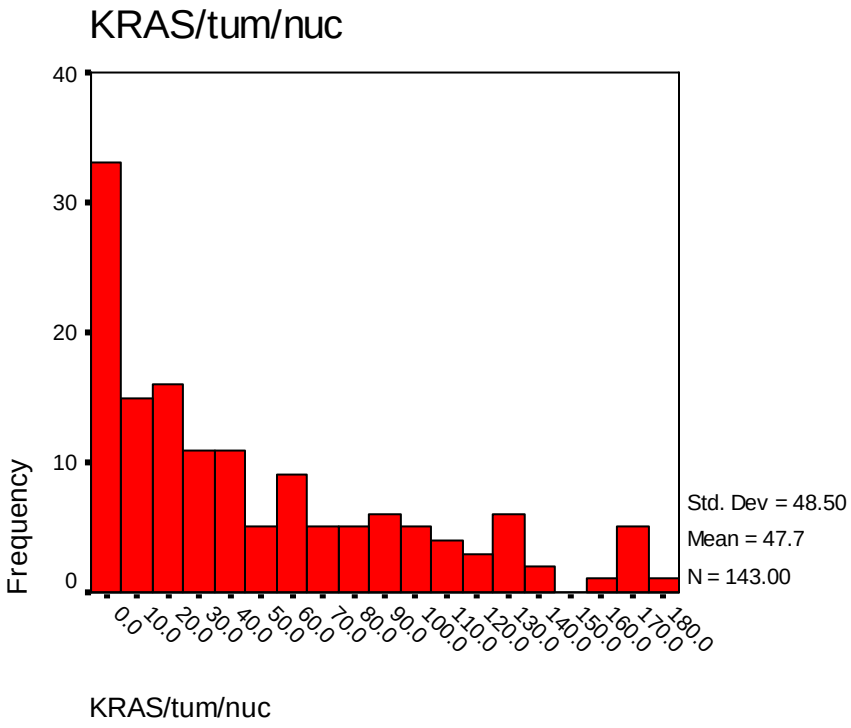


Figure 4: Frequency of histoscores for Raf-1 in the cytoplasm.

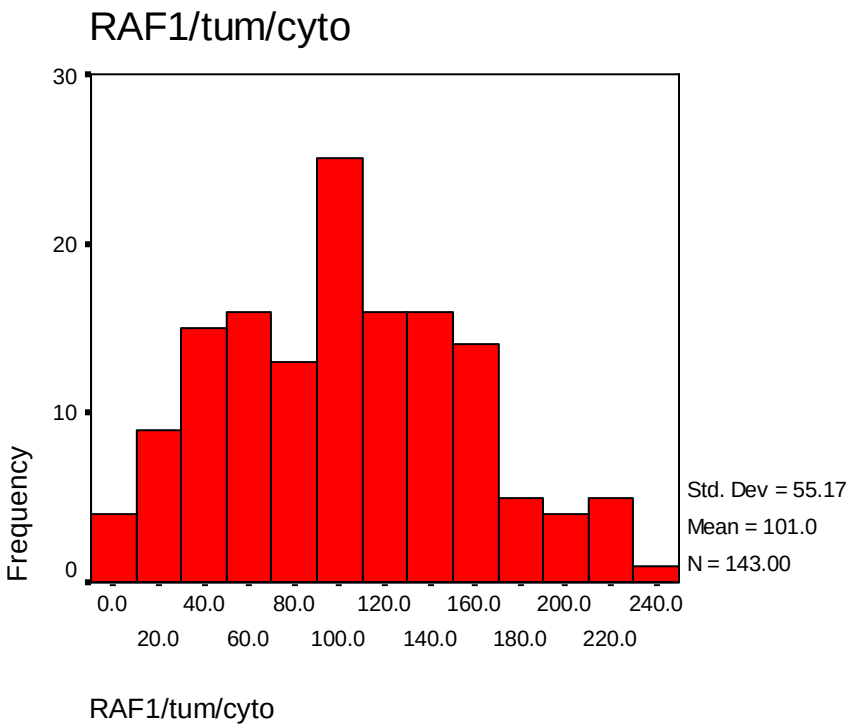


Figure 5: Frequency of histoscores for Raf-1 at the membrane.

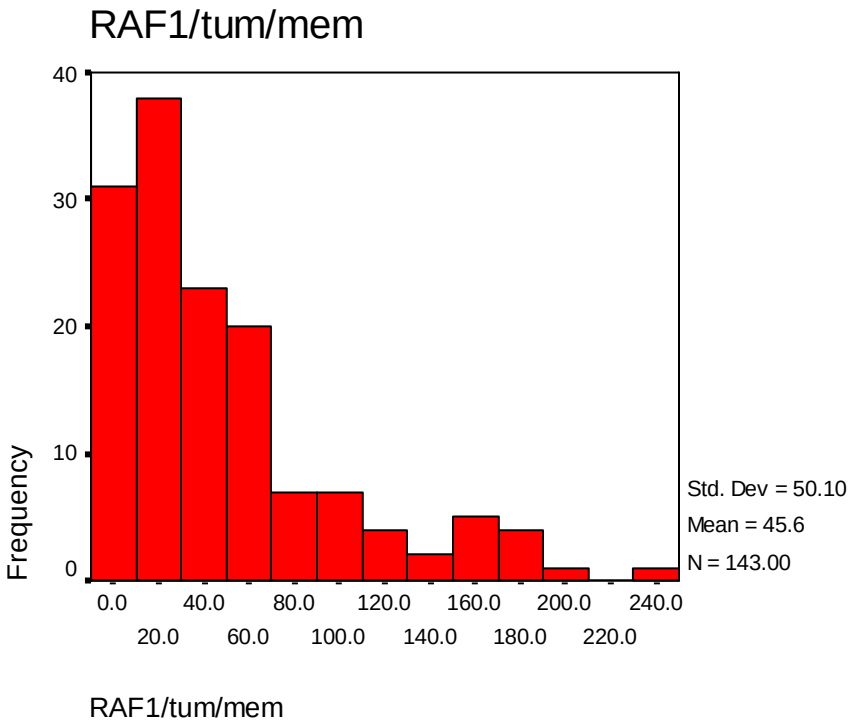


Figure 6: Frequency of histoscores for Phospho.Raf-1 259 in the cytoplasm.

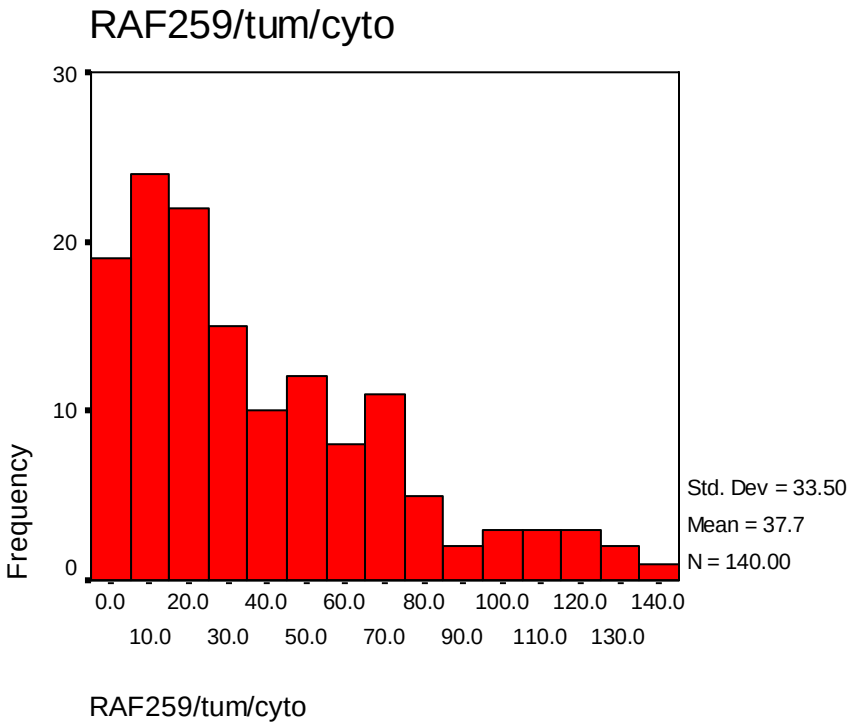


Figure 7: Frequency of histoscores for Phospho.Raf-1 338 in the cytoplasm.

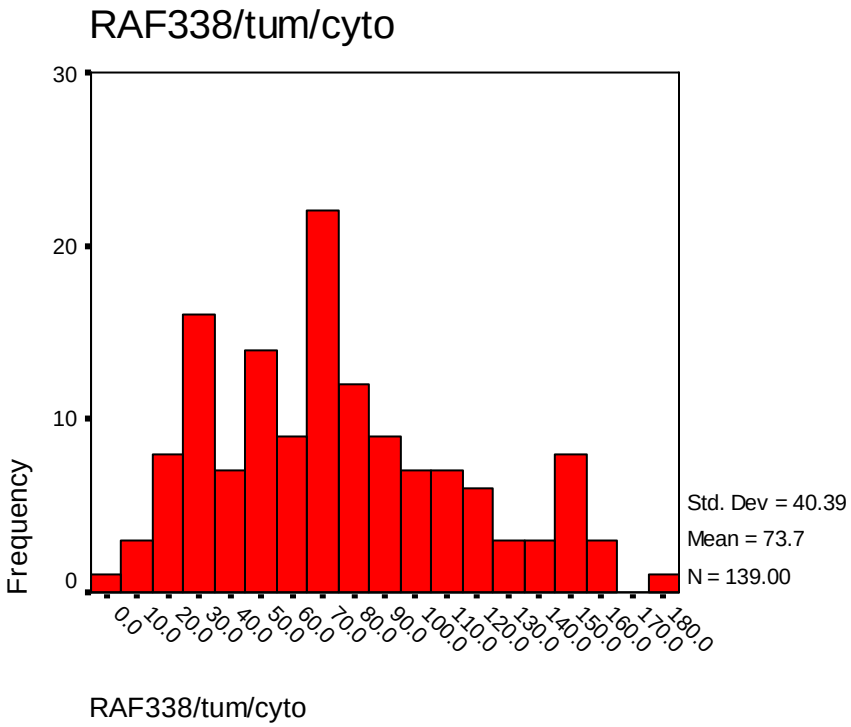


Figure 8: Frequency of histoscores for Phospho.Raf-1 338 at the membrane.

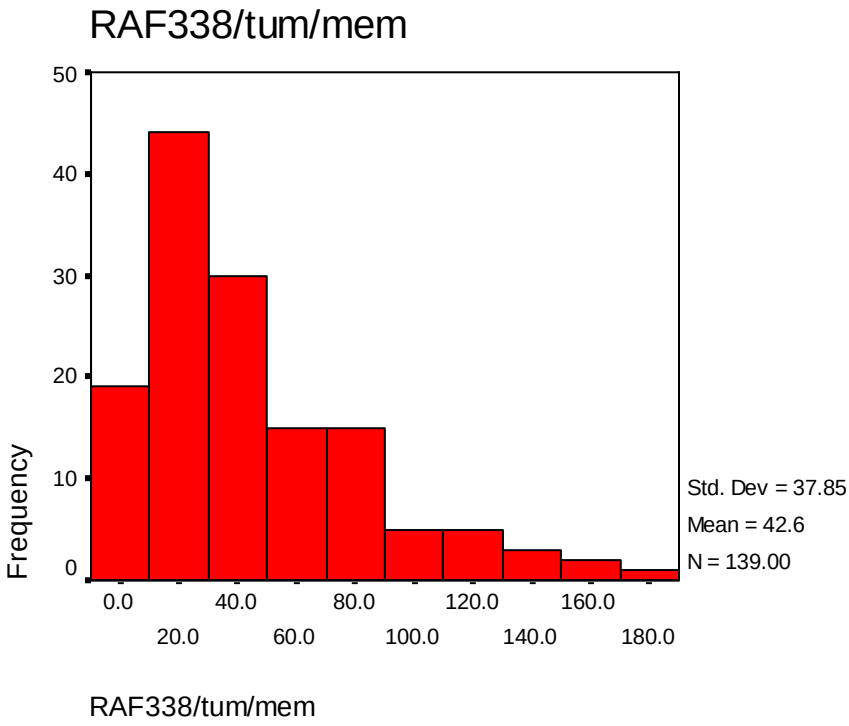


Figure 9: Frequency of histoscores for Phospho.Raf-1 338 in the nucleus.

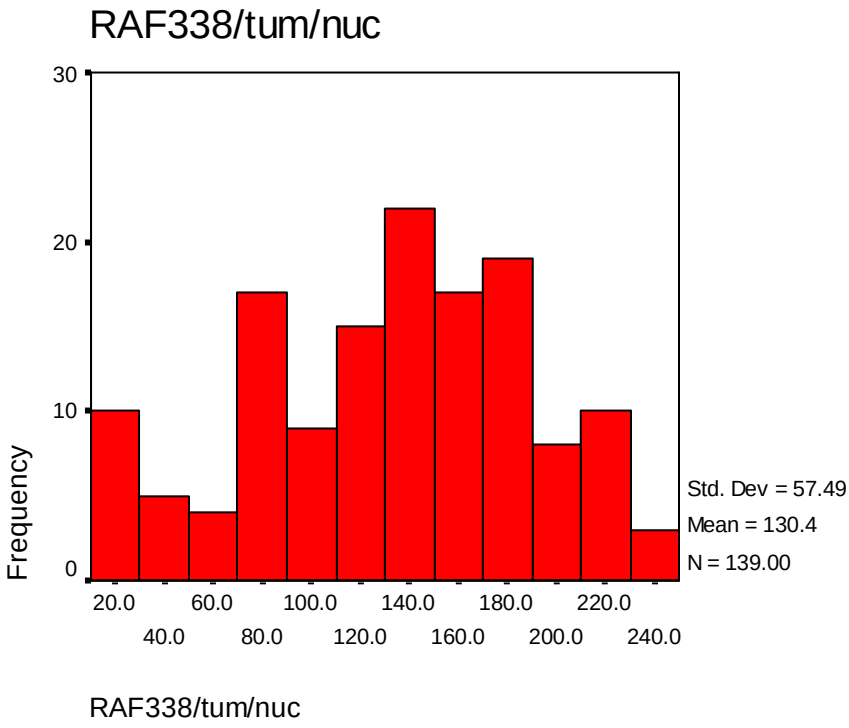


Figure 10: Frequency of histoscores for MAPK in the cytoplasm.

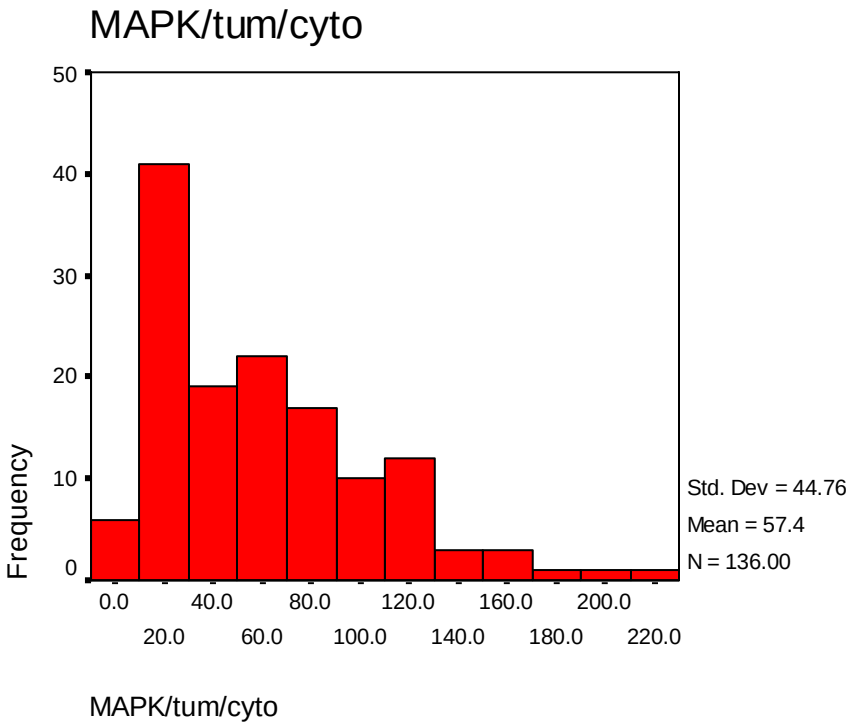


Figure 11: Frequency of histoscores for MAPK at the membrane.

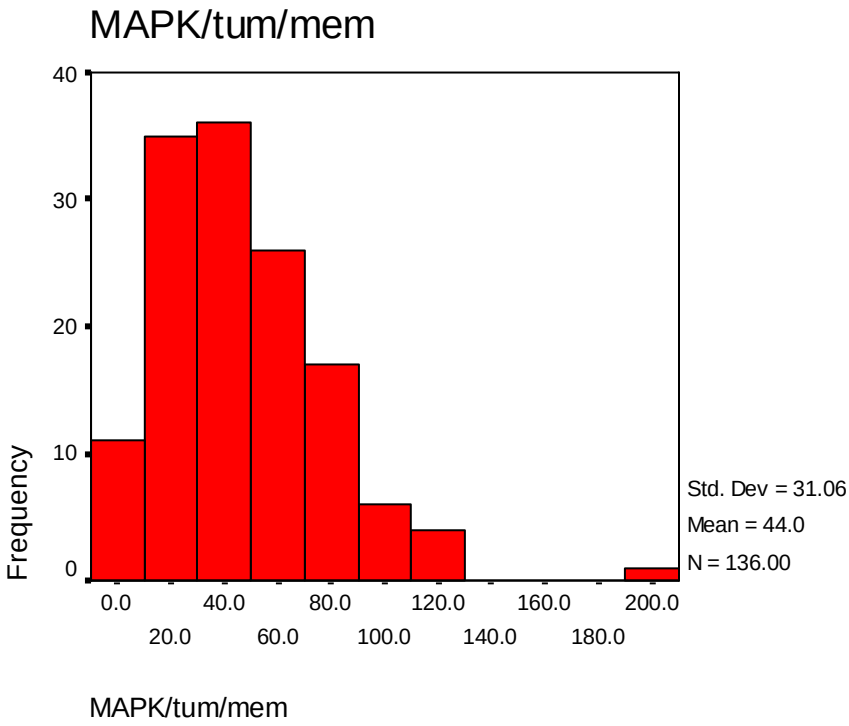


Figure 12: Frequency of histoscores for MAPK in the nucleus.

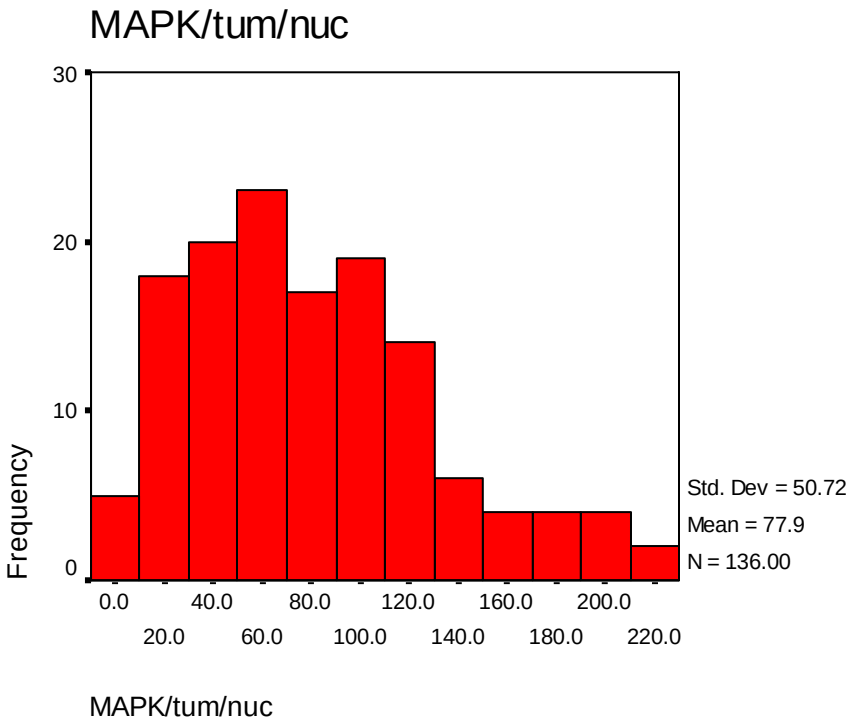


Figure 13: Frequency of histoscores for Phospho.MAPK in the cytoplasm.

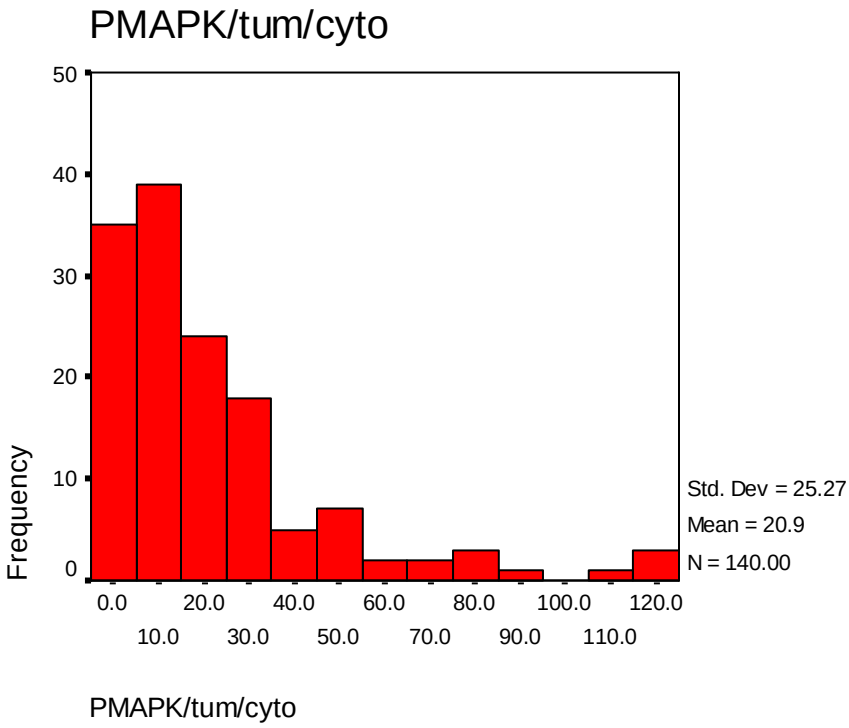


Figure 14: Frequency of histoscores for Phospho.MAPK at the membrane.

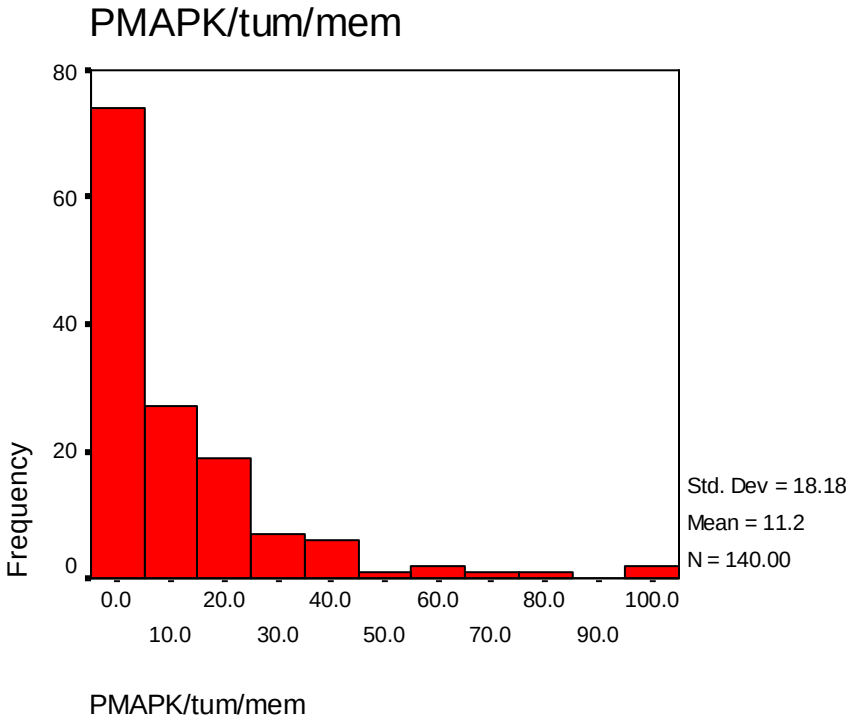
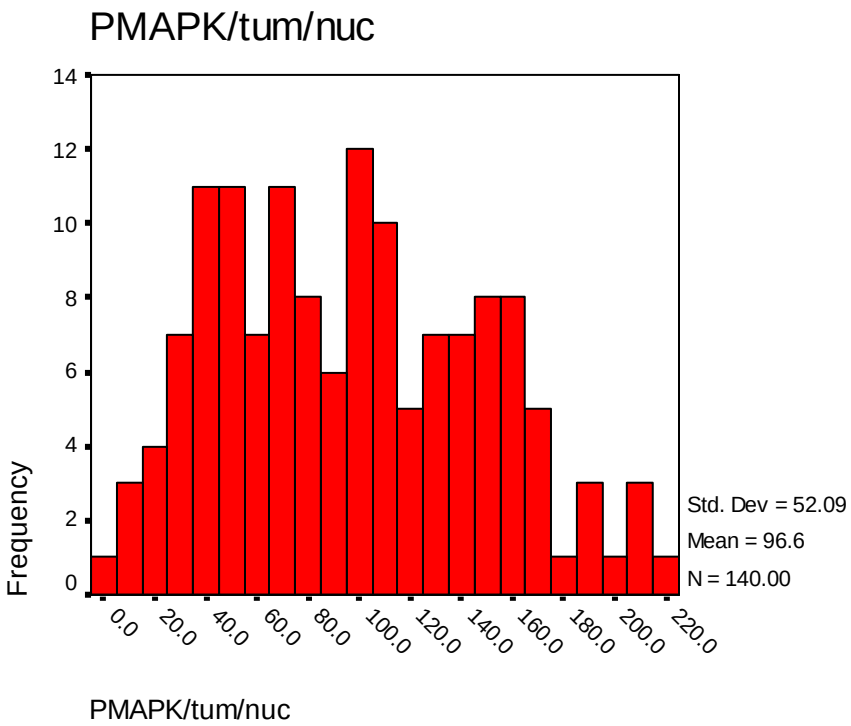


Figure 15: Frequency of histoscores for Phospho.MAPK in the nucleus.



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