THE UNIVERSITY OF HULL

Effect of Resection of Localised Pancreatic Cancer on Tissue-Factor Promoted Pathways of Thrombosis, Cell Invasion and Angiogenesis.

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By

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ABSTRACT

Pancreatic (PC) is the eleventh most common malignancy in the UK but it has the poorest prognosis of all human adenocarcinoma. The autopsy, epidemiological and clinical studies have consistently identified PC as one of the most highly angiogenic and invasive malignancies, with the greatest prevalence and incidence of thrombo-embolism (TE). The incidence of TE in PC has been reported as high as 57%. Tissue factor (TF) bearing microparticles (MP) have recently been shown to promote thrombosis. The biological link between cancer haemostasis, cell invasion and angiogenesis remains unclear. These three indices may be driven by PC cells directly, be a reflection of the individual tumour stromal microenvironment and/or a result of the inflammatory response of the host. The hypothesis of the thesis is that factors directly attributed to the cancer promote the observed pathophysiology and that the removal of the tumour should result in reversal of these abnormalities.

Flow cytometry was used for the evaluation of MP in plasma and quantification of surface-expressed TF, VEGFR-1 and-2 and EGFR. Cellular TF activity and pro-coagulant activity (prothrombin time) of PC patients were measured using a coagulometer. Matrigel™ Invasion Chambers and Boyden chambers with collagen IV were used to measure cellular invasion. A two dimensional angiogenesis assay was used to evaluate tubule formation *in vitro* in response to PC sera. Relative levels of protein expression of 55 angiogenic markers in the sera of PC patients were evaluated using a human angiogenesis array kit. Enzyme-linked immunosorbent assay was undertaken on VEGF, TF, TFPI, Leptin and annexin autoantibodies using sera or plasma from PC patients as appropriate. Immunohistochemical analysis of key markers of angiogenesis and thrombosis was also undertaken on resected PC samples.

The *in vitro* optimisation experiments revealed that the cell invasion was significantly correlated with TF antigen expression and activity on PC cell lines (MIA-PaCa-2, AsPC-1 and CFPAC1) and that blocking TF on these cells decreased cell invasion. In the same manner neutralising soluble TF in PC serum samples also significantly decreased cell invasion, as did spiking of the serum with low molecular weight heparin. Analysis of sera

from patients showed that TF bearing MP, pro-coagulant activity, cell invasion and angiogenesis (total length and number of capillaries) of PC cases were significantly higher than the control. Furthermore, the post-operative median number of TF bearing MP, procoagulant activity, cell invasion and angiogenesis (total length and total number of capillaries) were all significantly lower compared with pre-operative samples. Out of 55 angiogenic markers studied in 6 PC patients, pre- and post-operatively there was a significant decrease of angiopoietin-1, angiostatin/plasminogen, PDGF-AA, PDGF-AB/PDGF-BB and VEGF post-operatively. This result was supported by ELISA analysis of 29 samples and 14 controls that also showed significantly higher levels of VEGF in pancreatic cancer sera versus control groups, and that there was a significant decrease observed post-operatively only in the cancer patients. Furthermore both angiogenesis array and ELISA showed increased leptin levels post-operatively.

Immunohistochemical analysis of the pancreatic tissue sections revealed that TF was expressed on 62 % of PC samples. There was significant correlation between TF expression on the tissue and procoagulant activity. Also, there was a significant correlation between tissue-expressed TF and *in vitro* angiogenesis, i.e. total length and number of capillaries. Furthermore, there was a significant correlation between TF expression on tissue with intratumoural microvascular density (MVD) and tumour-expressed with VEGFR 2. As expected, high levels of MVD correlated with high levels of tissue-expressed VEGF. Finally serum from patients who showed a high level of tissue-expressed VEGF also induced the greatest level of *in vitro* angiogenesis, i.e. number of capillaries.

In summary, it was shown that TF expression on cell lines was significantly correlated with TF activity and cell invasion, and that TF expression in plasma and on tissue from PC patients was significantly correlated with procoagulant activity, cell invasion and angiogenesis. PC tissue-expressed VEGF was significantly associated with the angiogenic activity of PC sera and tissue MVD. Thus, the pathophysiology represented by a high procoagulant state, elevated cell invasion and angiogenic properties seen in PC patient sera appears to be driven by the malignant cells, as removal of the tumour causes a return towards the normal state.

DEDICATED TO

MY MOTHER

MY WIFE, MY

CHILDREN

AND TO THE

MEMORY OF

MY FATHER.

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ABBREVIATIONS

µ	Micro
^{o}C	Degree Celsius
%	Per cent
AA	Amino Acids
ABC	Avidin-Biotin Complex
ADAMTS-1	A Disintegrin and Metalloproteinase with Thrombospondin Motif
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
APC	Advanced Pancreatic Cancer
AsPC1	Ascitic Pancreatic Cell 1
ATCC	American Type Cell Collection
BSA	Bovine Serum Albumin
CAM	Cell Adhesion Molecule
CD	Cluster of Differentiation
CFPAC-1	Cystic Fibrosis Pancreatic Adenocarcinoma-1 Cell Line
CT	Clotting Time
CTAOR	Cell Titer Aqueous One Reagent
CXCL16	Chemokine -X-Cmotif
DAB	Diamino Benzidine
DMSO	Dimethyl Sulphoxide
DPP-IV	Di-peptidyl peptidase-4
DW	Distilled Water
E-cad	E-cadherin
ECL	Chemiluminescent Agent
EGF (R)	Epidermal Growth Factor (Receptor)
EG-VEGF	Endocrine gland-derived VEGF
EHS	Engelbreth-holm-swarm

ELISA	Enzyme-Linked Immunosorbent Assay
EMP	Endothelial Microparticles
EMT	Endothelial Mesenchymal Transition
Erk-1/2	Extracellular Signal-Regulated Kinases-1/2
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocaynate
5FU	5-Fluorouracil
GFL	Glial Family Ligands
GFR	Growth Factor Receptor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
H	Hour
HB-EGF	Heparin-Binding Epidermal Growth Factor
HDMEC	Human Dermal Microvascular Endothelial Cells
HGF	Hepatocyte Growth Factor
H&E	Haematoxylin and Eosin
HIER	Heat-induced Epitope Retrieval
HPF	High Power Field
HUVEC	Human Umbilical Vascular Endothelial Cells
H_2O_2	Hydrogen Peroxide
IGFBP	Insulin-Like Growth Factor Binding Protein
IL-1B, 8	Interleukin 1B, 8
IQR	Interquartile Range
JAKS	Janus Kinases
KDa	Kilo Dalton
KSR1	Kinase Suppressor of Ras1
LAP	Latency-Associated Peptide
LMWH	Low Molecular Weight Heparin
mAb	Monoclonal Antibody
MAPK	Mitogen-activated Protein Kinase

MCP1	Monocyte Chemotactic Protein-1
MMP	Matrix Metalloproteinases
mM	Millimolar
MoMP	Monocytes bearing MPs
MP	Microparticles
MVD	Microvascular Density
MW	Molecular Weight
NaCl	Sodium Chloride
NF-κB	Nuclear Factor kappa B
nM	Nanomolar
NO	Nitric Oxide
NRG1-B1	Neuregulin-1-B1
PAR	Protease Activated Receptor
PBS	Phosphate Buffered Saline
PC	Pancreatic Cancer
PD-ECGF	Platelet-derived Endothelial Growth Factor
PDGF	Platelet Derived Growth Factors
PE	Phycoerythrin
PF4	Platelet Factor 4
PIER	Protease-induced Epitope Retrieval
PI3K	Phosphatidylinositol 3 Kinase
PS	phosphatidyl serine
PL	Phospholipid
PMP	Platelet Microparticles
PoAB	Polyclonal Antibody
PPP	Platelet-poor Plasma
PRP	Platelet-rich Plasma
PSGL-1	P-selectin Ligand-1
PT	Prothrombin Time

PTX3	Pentraxin 3
SAPK	Stress Activated Protein Kinase
SFM	Serum-free Medium
Cryo-SFM	Serum Free Freezing Medium
TBS	Tris buffered Saline
TE	Thromboembolism
Trypsin/EDTA	Trypsin/Ethylene Diamine Tetra Acetic Acid
TF	Tissue Factor
TFG-β	Transforming Growth Factor Beta
TF+MP	TF Bearing MP
TFPI	Tissue Factor Pathway Inhibitor
TNM	Tumour/ Node/ Metastasis
TIMP 1	Tissue Inhibitor of Metalloproteinase 1
TMB	Tetramethyl Benzidine
TNF-α	Tumour Necrosis Factor Alpha
TNS	Trypsin Neutralising Solution
Trypsin/EDTA	Trypsin/Ethylene Diamine Tetra Acetic acid-Trypsin
TSP-1	Thrombospondin-1
VEGF (R)	Vascular Endothelial Growth Factor (Receptor)
v/v	Volume/Volume
WAD	Weight Adjusted Dalteparin
w/v	Weight/ Volume

CHAPTER ONE: INTRODUCTION

1.1 Anatomy, Histology and Physiology of the Pancreas

The pancreas is a relatively large gland about six inches in length, which lies in the superior part of the abdomen, just behind the stomach (Fig. 1.1A). It consists of three parts: head, body and tail. The hormones and digestive juices, which are secreted by the pancreas, flow into the duodenum via the pancreatic duct. The lobular units of acini of the exocrine part of the pancreas drain into ducts of increasingly bigger size, and finally collect into the main pancreatic duct (Duct of Wirsung), which combines with the common bile duct and ends in the papilla of Vater. Some people have an accessory pancreatic duct which ends in minor duodenal papillae (Fig. 1.1B). Numerous anastomotic communications join the Wirsung and Santorini ducts (Delhaye and Cremer, 1992).

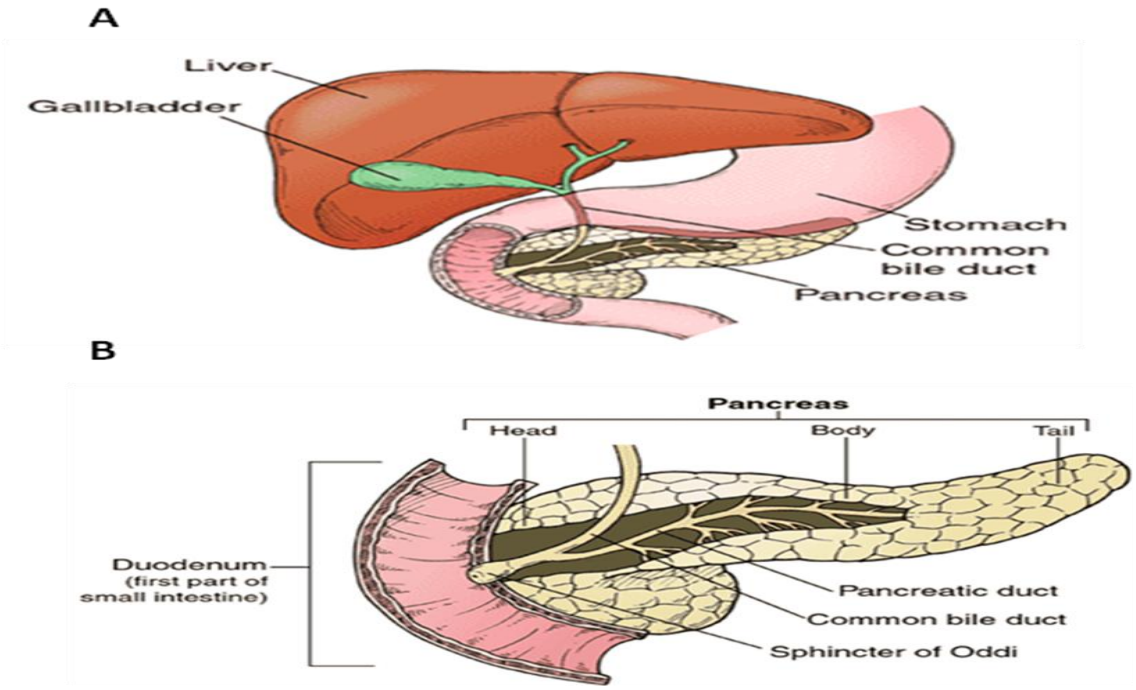

Figure 1.1 Anatomy of the pancreas (Web reference 1).

Histologically, the pancreas consists of two main components: the endocrine and exocrine system (Fig. 1.2). The endocrine portion is known as the Islets of Langerhans and although this structure receives 10-15 per cent (%) of the pancreatic blood flow, it comprises only 1-2% of the total pancreatic mass (Hoppener *et al.* 2008).

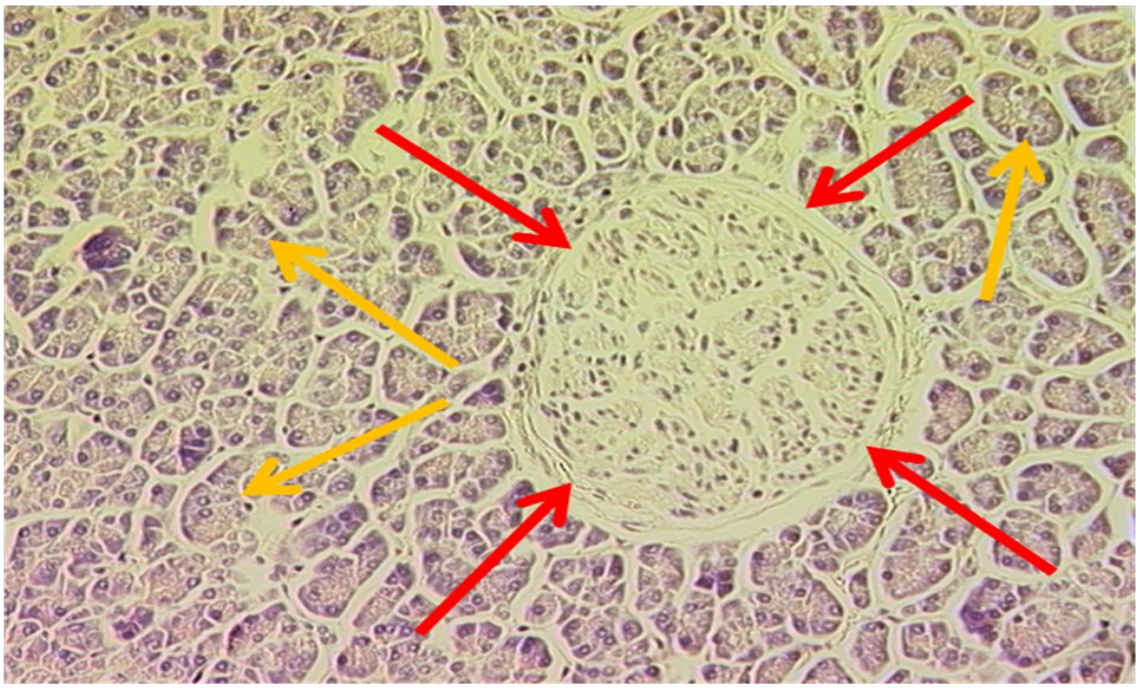

Figure 1.2: Histology of the pancreas. The endocrine part is indicated by red arrows, the exocrine part by orange arrows (x 100).

There are four major types of cells in the Islets of Langerhans, each of which produce different endocrine products (Hoppener *et al.* 2008):

1. **Alpha cells** produce glucagon which converts stored hepatic glycogen into glucose that is then released into the blood stream. The cells are situated in the periphery of the Islet cells and form 15-20 %. The secretory granules are characterised by an eccentric electron-dense core (Brelje *et al.*, 1989).

2. **Beta cells** produce insulin, which causes the body's cells to take up glucose from the blood, storing it as glycogen in the liver and muscle and stopping the use of fat as an energy

source. These cells are situated mainly in the centre of the Islet and form 65-80% of the cell mass. The secretory granules typically have a crystalline appearance.

3. **Delta cells** produce somatostatin which regulates the endocrine system and affects neurotransmission and cell proliferation via interaction with G-protein-coupled somatostatin receptors and inhibition of the release of numerous secondary hormones. These cells are spread through the Islets and form 3-10 % of the total Islet cell number.

4. **Pancreatic Polypeptide-Secreting cells** produce pancreatic polypeptide, a hormone that regulates appetite and food intake. Pancreatic polypeptide-secreting cells are located at the periphery of the Islet cells and form 3-5 % of Islets cells (Hoppener *et al.,* 2008).

The main endocrine functions of the pancreas are mediated by insulin and glucagon which regulate blood glucose. In addition to the hormonal function, the pancreas also secretes digestive enzymes such as proteases, amylase and lipase from the exocrine part and therefore plays a major role in the digestion of food (Mitropol'Skii, 1966). Islet cell tumours are called pancreatic neuroendocrine tumours. These tumours are divided into functional (which secrete hormones) and non functional. The most common functional pancreatic neuroendocrine tumour is insulinoma which secretes an excess of insulin causing hypoglycaemia (Kimura *et al.* 2011).

1.2 Prognosis and Mortality of Pancreatic Cancer

Pancreatic cancer (PC) is the eleventh most common malignancy in the United Kingdom. The male to female ratio is 1.6:1 and 63% of patients are over 70 years (Gold *et al.,* 1985), while Jemal *et al.* (2010) reported the male to female ratio is 0.98:1. Worldwide, more than 270,000 people were diagnosed with PC in 2008 and 266,000 people died from this malignancy in the same year. In comparison, 7690 and 7680 PC cases were diagnosed in 2007 and 2008 respectively in the USA (Jemal *et al.*, 2010).

PC has the poorest prognosis of all adenocarcinoma in the human body (Jemal *et al.*, 2003). Although most PC patients die within one year of diagnosis (Jemal *et al.*, 2010, Hillen, 2000, Olsen *et al.*, 1989), recently it has been shown that the five year survival rates differ from one country to another: in England in the period of 2001-2006 the rate was 3%, compared with 6%

in the USA for a similar time period (1999-2005; Jemal *et al.*, 2010). Successful surgical removal of PC tumours improves five year survival rates, which can reach up to 40% when there is no lymph node. However the five year survival rate drops to 20% when there is lymph node metastasis (Yao and Qian, 2010, Lieberman *et al.*, 1995). Unfortunately, surgical removal of the PC can only be performed in 20% of PC cases because of the presence of distant metastasis (Hackert *et al.*, 2009). Furthermore, approximately 30% of PC patients who submitted to the surgical removal of the tumour died in the first year following the operation and had the same prognosis as those who were treated by anti-tumour therapy alone (Kennedy and Yeo, 2007, Burris, 2005).

1.3 Risk Factors of Pancreatic Cancer

It has been reported that intraductal papillary mucinous neoplasm (IPMN) is a precancerous cystic type tumour that grows within the pancreatic ducts (intraductal). This type of the tumour associated with mucinous secretion (Verbeke 2010). Furthermore, there is a strong relationship between PC and tobacco usage, whether by cigarette/cigar smoking or chewing tobacco (Ahlgren, 1996). Carcinogenic materials, in particular nitrosamines, are found in cigarette smoke (Potter, 2002). PC is also increased among people who regularly consume foods containing high levels of sugar, fat, large quantities of red meats and low levels of fresh fruit, vegetables and vitamin C (Olsen *et al.*, 1989).

Chronic pancreatitis increases the risk of PC, but chronic pancreatitis that is due to alcohol consumption does not increase the risk as much as other types of chronic pancreatitis like idiopathic pancreatitis and pancreatitis that has been caused by taking steroid and anti-inflammatory drugs (Kalapothaki *et al.*, 1993). Moreover, genetic factors may also play a role in PC, because some PC occurs more frequently in certain families (Hruban *et al.*, 1998). Although the risk of PC increases among diabetic patients, diabetes mellitus in PC might be the result of the cancer rather than the cause (Gullo *et al.*, 1994).

1.4 Histopathology of Pancreatic Cancer

Exocrine tumours are the commonest type of PC and adenocarcinoma represent 90% of exocrine PC (Morohoshi *et al.*, 1983). PC can be classified into three main types (Tanase *et al.*, 2006, Yamasaki *et al.*, 2002, Terada *et al.*, 1998):

1. Low papillary: This type is characterised by low papillary projections and the lack of a fibro-muscular core. It forms about 39% of all PC.

2. Tubular: This includes tubular and solid types. It forms 56% of all PC.

3. Mixed: This consists of malignancies containing both low papillary and tubular types and composes a maximum of 5%.

There are three grades of pancreatic carcinoma: well, moderately and poorly differentiated (Morohoshi *et al.*, 1983). The number of intraductal carcinoma foci is correlated with tumour grading (Yamasaki *et al.*, 2002).

Staging of PC depends on Tumour/Node/Metastasis (TNM) classification (Rindi *et al.*, 2006).

1.4.1 TNM Classification

T- Tumour:

T0: Tumour in situ / dysplasia (0.5 cm).
T1:Tumour invades lamina properia or subserosa and the size is less than 1 cm.
T2:Tumour more than 1 cm in diameter and invades muscularis properia or subserosa.
T3:Tumour penetrates serosa.
T4:Tumour invades the adjacent structure.

N-Regional lymph node:

N0: No involvement of regional lymph nodes.
N1: Involvement of regional lymph nodes.

M-distant metastasis:

M0: No distant metastasis
M1: There is distant metastasis.

1.4.2 Staging:

Stage I = T1 N0 M0

Stage IIa=T2 N0 M0

Stage IIb =T3 N0 M0

Stage IIIa=T4 N0 M0

Stage IIIb = any T N1 M0

Stage IV = any T any N M1

1.5 Symptoms and Signs of Pancreatic Cancer

The early symptoms of PC are usually non-specific and are commonly ignored by the patient and doctor. Such symptoms include epigastric discomfort, vomiting, diarrhoea, general malaise and flatulence. The late signs and symptoms include painless jaundice, abdominal pain and weight loss (DiMagno, 1999). The presence of jaundice depends on the location of the tumour, as jaundice in patients with body and tail carcinoma tends to be later than the jaundice caused by the cancer of the head of the pancreas; the latter is usually associated with inoperability because of liver metastasis (Takhar *et al*, 2004).

1.6 Treatment of Pancreatic Cancer

Pancreaticoduodenectomy (Fig. 1.3), with or without preservation of the pylorus, is the procedure of choice for tumours in the head of the pancreas. In contrast the most common operation for malignant tumours of the tail or body of the pancreas is a distal pancreatectomy with splenectomy (Fernandezdelcastillo *et al.*, 1995).

In the Whipple's operation, described by Allen Whipple in 1935, the head of the pancreas, a portion of the bile duct, the gallbladder, the duodenum and occasionally a portion of the stomach are all removed (Whipple *et al.*, 1935). After removal of these structures the remaining pancreas, bile duct and the intestine are sutured into the intestine to direct the gastrointestinal secretions back into the gut (Rivera *et al.*, 1996, Fernandezdelcastillo *et al.*, 1995). Biliary and/or gastric bypass is recommended in patients with obstructive jaundice.

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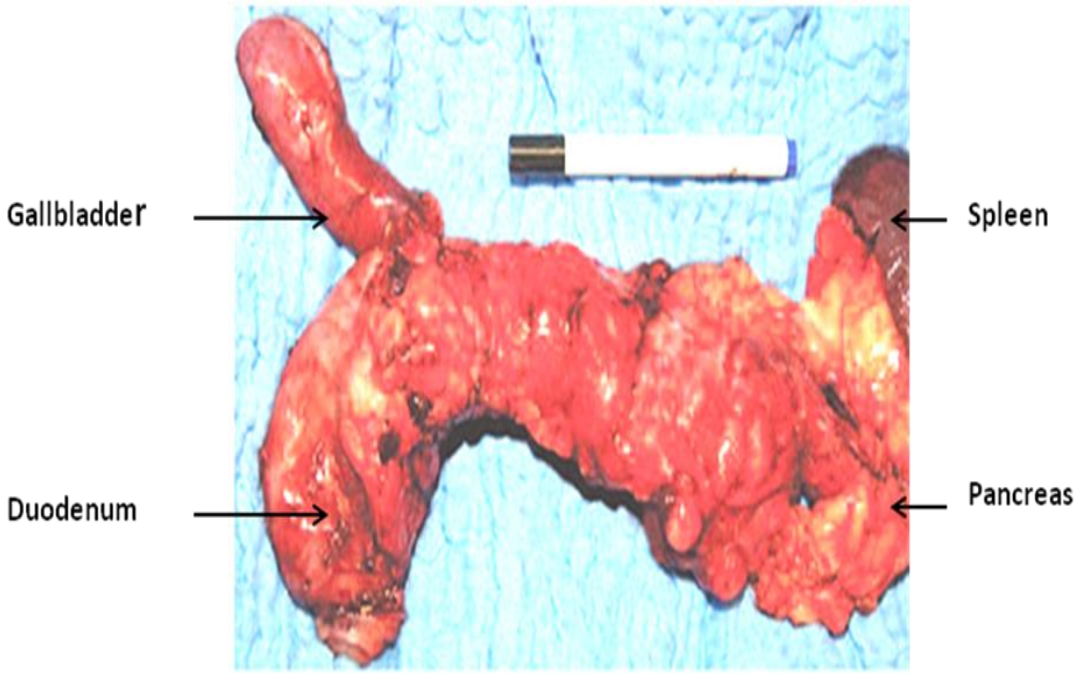

Figure 1.3: Picture showing total pancreatectomy with total duodenectomy, splenectomy and cholecystectomy (Hackert *et al.*, 2009).

Chemotherapy is routinely given after a surgical operation (Lillemoe, 1995) and it is recommended as a palliative treatment with unresectable tumours. In the absence of an available clinical trial, gemcitabine is the evolving standard treatment (Nakchbandi *et al.*, 2008, Fernandezdelcastillo *et al.*, 1995, Lillemoe, 1995) and is used widely in the treatment of PC. Gemcitabine has replaced 5-Fluorouracil (5FU) as the preferred drug due to its efficacy and lower side effects. Although the median survival improvement in favour of gemcitabine compared with 5FU is slight (5.7 *vs.* 4.4 months), the one-year survival rate is more encouraging (18% *vs.* 2%), and most importantly the toxicity is relatively mild and clinical response has been found to be better (24% *vs.* 5%, respectively) (Burris *et al.*, 1997). The potential benefit of combining gemcitabine with other drugs such as low molecular weight heparin (LMWH) and/or anti-angiogenic drugs shows a slight improvement in the survival rates. This will be described later (section 1.17).

1.7 Tissue Factor

Tissue factor (TF), designated cluster of differentiation (CD) -142; thromboplastin or factor III is a 47 kilo Dalton (KDa) glycoprotein receptor (Spicer *et al.*, 1987). In addition to its principal role in the initiation of the extrinsic pathway of coagulation (Nemerson, 1988, Paborsky *et al.*, 1989,

Gouaulthelimann and Josso, 1979, Fig.1.4), TF also has a major role in angiogenesis, tumour growth (Khorana *et al.* 2007, Rak *et al.*, 2006, Kim *et al.*, 2004, Nemerson, 1988) and embryogenesis (Carmeliet *et al.*, 1996). It has been shown that TF plays an essential role during embryo formation as a deficiency of TF in transgenic mice causing death by day 10.5 due to impaired vascular function and abnormal development of the yolk sac (Carmeliet *et al.*, 1996).

TF is expressed in a number of vital organs including the brain (meninges and cerebral cortex), lung (bronchial mucosa, alveolar epithelial cells, aleveolar macrophages and alveolar septa), heart (myocardium), kidney (glomeruli), placenta and blood vessels (advential cells surrounding the blood vessel walls (Drake *et al.*, 1989). TF expression on these organs has a prophylactic effect to inhibit and/or stop bleeding which has dangerous sequellae for these organs. Furthermore it has been reported by Drake *et al.* (1989) that TF is also expressed in a number of non vital organs such as skin (epidermis), gut (mucosa), spleen (trabeculae and capsule) and the peripheral nerve (Schwan cells). Moreover, TF is also expressed normally in host cells such as endothelial cells, monocytes, macrophages and fibroblasts but only in response to inflammatory stimuli or remodelling signals in malignant cases (Ruf *et al.*, 2000). The plasma of healthy individuals contains relatively minor amounts of biologically active TF (Wada *et al.*, 1995), but this increases in various conditions including cancer (Rak *et al.*, 2006). PC shows high plasma TF concentration which is positively correlated with the incidence of thromboembolism (TE) (Khorana *et al.*, 2008). See section 9.6 for details.

TF consists of 295 amino acids (AA): a 32 AA leader sequence (264-295; Paborsky *et al.*, 1989) which is cleaved to produce the protein, 263 AA which is formed of three domains (extra-cellular, transmembrane and cytoplasmic) that have distinct roles (Nemerson, 1988). The extra-cellular N-terminal domain (AA 1-219) comprises two fibronectin type III domains, which interact with FVIIa to initiate the coagulation cascade and bind factor VIIa and factor Xa (Coughlin, 2000, Martin *et al.*, 1995). The transmembrane domain contains 23 AA (220-242), and the short C-terminal cytoplasmic domain (21 AA 243-263) contains three serine residues (Nemerson, 1988, Neuenschwander and Morrissey, 1992, Fig. 1.5).

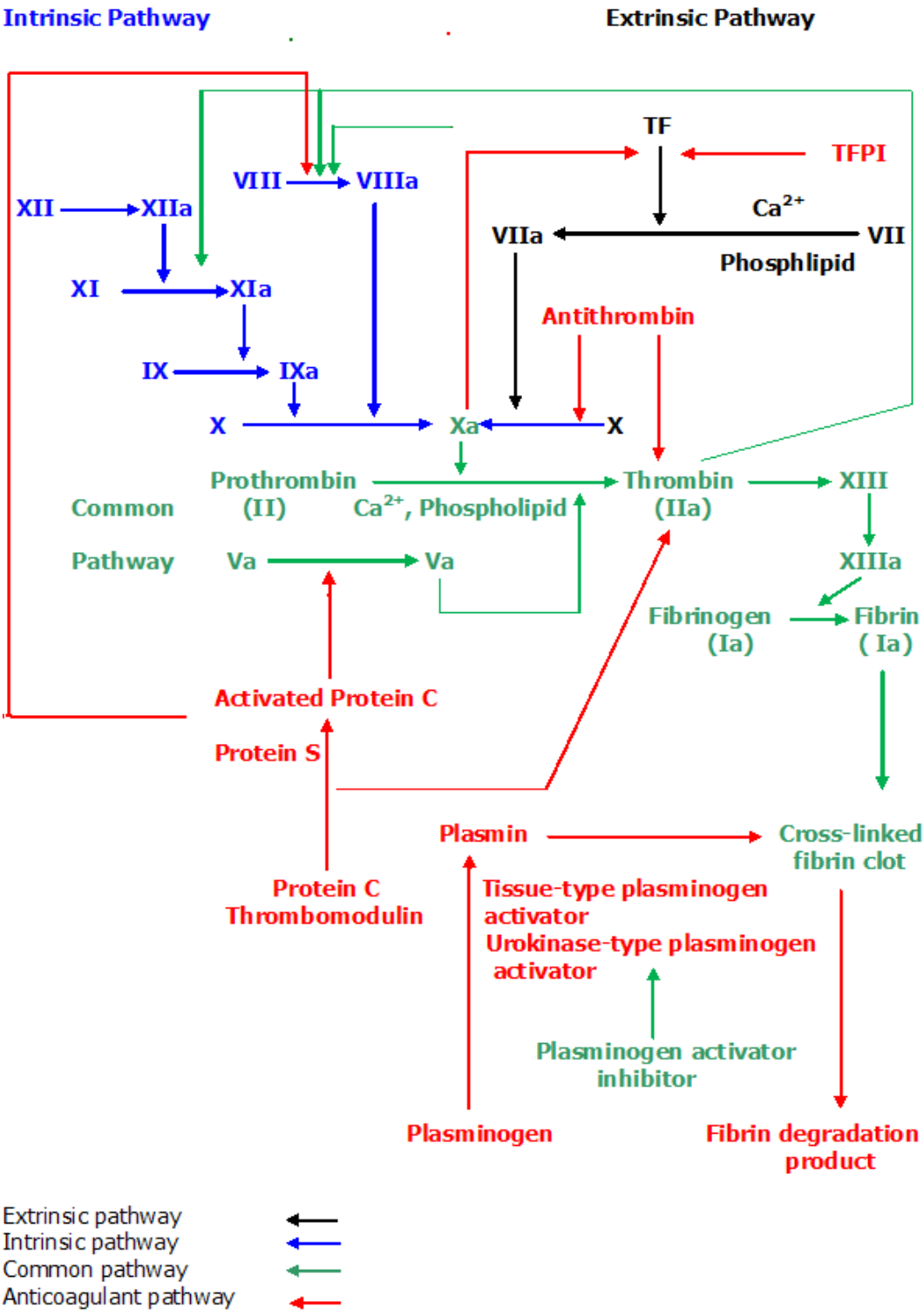

Figure 1.4: Coagulation cascade. Both extrinsic and intrinsic pathways lead to the activation of factor X which combines with activated factor V in the presence of calcium and phospholipid (PL) to produce thrombin. Anticoagulant pathway (fibrinolytic pathway) will be activated after vessel integrity has been restored.

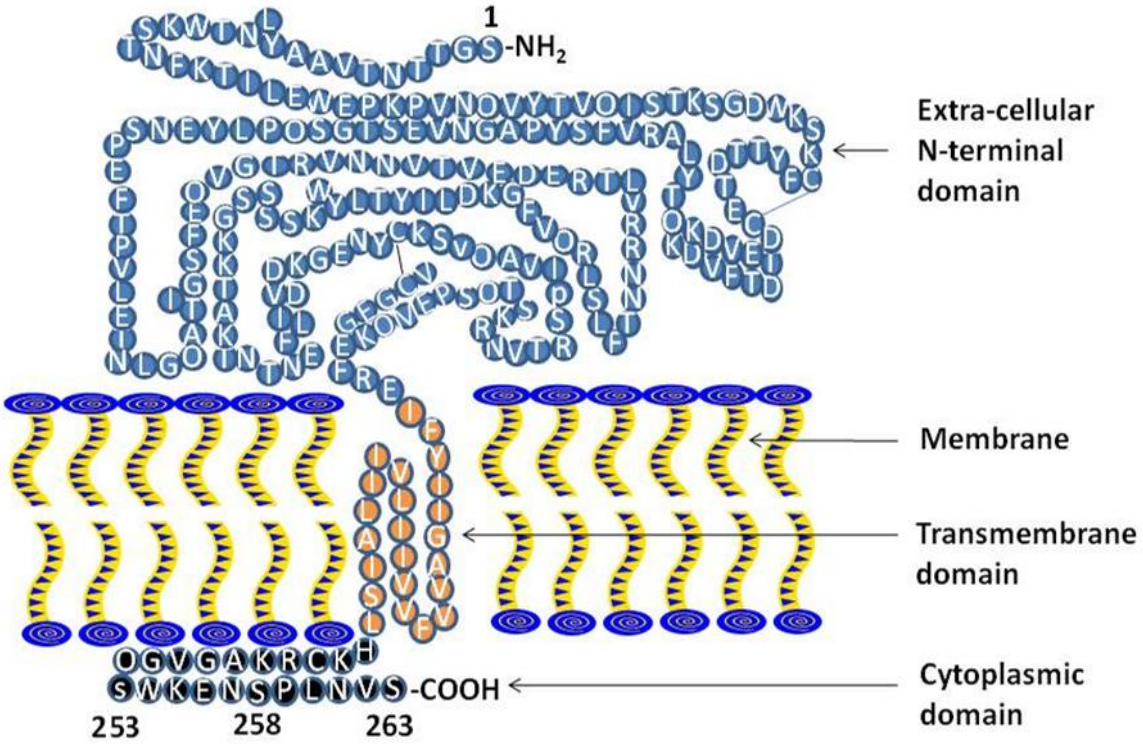

Figure 1.5: Schematic representation of tissue factor adapted from (Nemerson, 1988). The three intracellular serine residues are highlighted.

TF shows similarity to a class II cytokine receptor (Bazan, 1990). However, TF does not exhibit all the classical features of a cytokine receptor; firstly its C-terminal intra cellular domain is short and lacks the membrane proximal motif for binding of the non-receptor Janus kinases (JAKs) (Kotenko and Pestka, 2000, Ruf, 1999). Under the effect of phorbol ester, the cytoplasmic domain can become phosphorylated (Zioncheck *et al.*, 1992). The synthetic cytoplasmic domain has been phosphorylated at multiple sites when incubated with cell lysate (Mody and Carson, 1997). Furthermore, it has been shown that serine 253 (Dorfleutner and Ruf, 2003) and serine 258 may be phosphorylated via a mechanism involving protein kinase (Dorfleutner and Ruf, 2003, Mody and Carson, 1997; Zioncheck *et al.*, 1992). These phosphorylations are necessary for cancer metastasis (Bromberg *et al.* 1999) due to involvement of the cytoplasmic domain of TF in TF-VIIa signalling which increases the chemotaxis ability of malignant cells (Bromberg *et al.*, 1999).

The tendency for aberrant expression of TF on the tissue alters the behaviour of cells through FVIIa/TF signalling (Peppelenbosch and Versteeg, 2001). Furthermore, it has been noted that high TF expression is associated with more growth of primary PC (Kakkar *et al.*, 1999) and sequentially more FVIIa/TF signalling (Peppelenbosch and Versteeg, 2001). It has been postulated that there is an aberrant expression of TF on tumour cells, particularly those of epithelial origin (Callander *et al.*, 1992), one of which is PC. In the latter study, Callander *et al.*, (1992) studied 61 epithelial tumours, 3 of which were pancreatic adenocarcinoma, 19 non epithelial malignant cases and 6 benign cases. The authors postulated that there is an aberrant TF expression on tumour cells, particularly those of epithelial origin and they noted that 41 (68%) cases out of 60 epithelial malignant cases, 3 (16%) cases out of 19 non-epithelial malignant cases and 1 of 6 benign tumours expressed TF. The three PC cases showed the highest level of TF staining. Callander and his colleagues used rabbit polyclonal anti human antibody but most of the specimens were frozen tissues; some samples were taken on the day of surgery and others from autopsy. All the samples were embedded in optimum cutting temperature compound and stored in liquid nitrogen and stored at -70^{o}C in air-tight bags which has less effect on masking of the antigen. Furthermore it has been noted that the increase of lymphatic and vascular cell invasion of the pancreatic cancer cells is correlated with the histological grade (Nitori *et al.*, 2005). In the latter study paraffin embedded tissues were used.

Moreover, it has been noted that positive expression of TF has been found in glioma (Guan *et al.*, 2002) as well. In this study 34 glioma cases were involved, of which 10 were grade IV, 12 were grade III, 7 grade II and 5 were grade I. The authors evaluated expression of TF antigen and von Willebrand factor using immunohistochemistry. They found that 90 % of grade IV cases strongly expressed TF, 58 % of grade III cases strongly expressed TF, 43% of grade II cases strongly expressed TF and 20% of grade I cases strongly expressed TF. Positive TF expression has been reported in breast cancer as well. Contrino *et al.* (1996) noted that TF was expressed in the endothelial and tumour cells of breast cancer while it was not in 10 cases of breast fibrocystic disease. Nakasaki *et al.* (2002) reported that TF expression was correlated to the Ducke's stage and the clinical staging in colorectal cancer. The authors noted that TF expression was positive in 57% of all cases.

It has been reported that normaly some of circulating TF is potentially active and the TF that come from leukocytes and monocytes are completly encrypted (Giesen, *et al.* 1999). More growth of primary PC and an increase of procoagulant activity of the tumour cells correlates with the histological grade (Kakkar *et al.*, 1999). The details are discussed in section 8.6. Another source of TF in the blood is circulating microparticles (MP, Yu & Rak, 2004) and this will be discussed in section 1.8. Under the effect of cytokines and inflammatory molecules such as bacterial lipopolysacharide, endothelial cells (Parry and Mackman, 1995) and monocytes (Mackman, 1996) express TF.

1.8 Microparticles

1.8.1 Definition of Microparticles

MPs are membrane vesicles of approximately 0.1-1 μm in diameter shed from the plasma membrane of healthy, apoptotic and stimulated cells (Freyssinet, 2003, VanWijk *et al.*, 2003). MPs were discovered by Wolf in 1967 but he regarded them as "platelet dust" (Wolf, 1967).

1.8.2 Origin and Structure of MP

MP originate from different types of cells including monocytes (Satta *et al.*, 1994), platelets (Pasquet *et al.*, 1996), granulocytes (Berckmans *et al.*, 2002), endothelial cells (Combes *et al.*,

1999), smooth muscle cells (Leroyer *et al.*, 2007) and tumour cells (Yu and Rak, 2004). The latter are considered as a major source of TF-bearing MP (Yu & Rak, 2004). It appears that MPs differ in their size, phospholipid (PL) and protein content, depending on their origin (Morel *et al.*, 2004). The MP is composed of a cytoplasmic component and membrane elements such as PL and cell surface receptors. Following stimulation, the plasma membrane is rearranged resulting in an outer leaflet rich in phosphatidylserine, essential for TF activity (Fig. 1.6). This is followed by release of MP into the circulation. Furthermore, p-selectin dependent interaction among platelets, macrophages and granulocytes determines expression of TF activity (Scholz *et al.*, 2002). Moreover, MP express antigenic markers distinctive of the parent cell (Abid Hussein *et al.*, 2003).

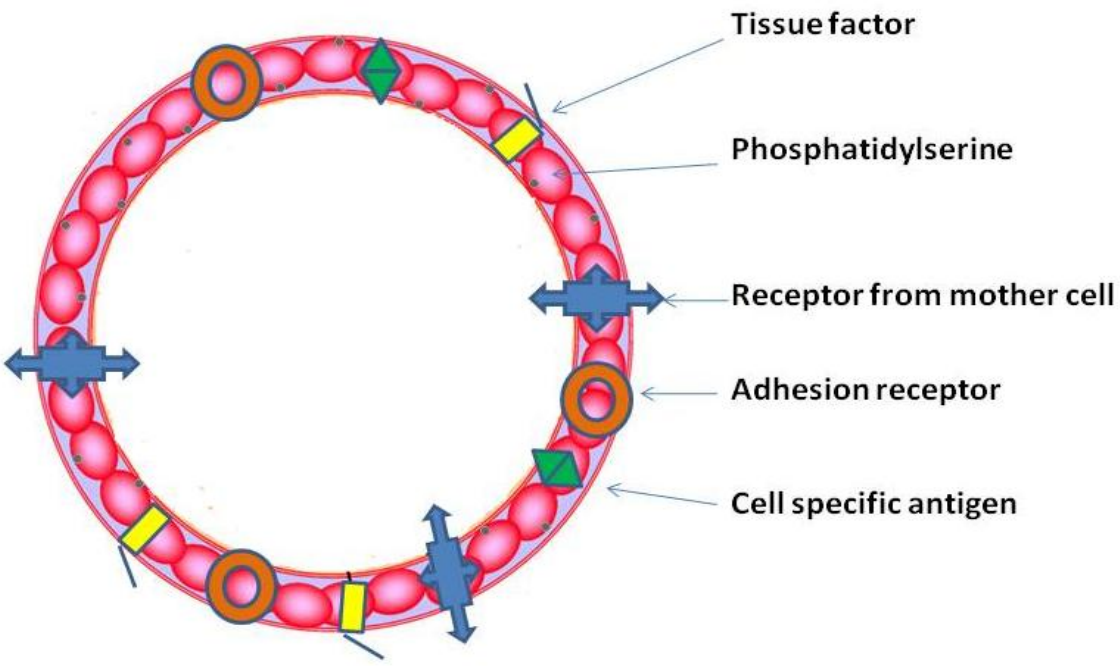

Figure 1.6: Schematic Presentation of Microparticle.

MP might express P-selectin glycoprotein ligand-1 on their surfaces (Lopez *et al.*, 2005, Vandendries *et al.*, 2004). Endothelial MP (EMP) are also known to contain matrix metalloproteinases (MMP) in addition to procoagulants that are involved in angiogenesis (Taraboletti *et al.*, 2002). MMP play an important role in the cell invasion processes. MMP

degrades the basement membrane allowing the invasion of the interstitial stroma by migrating endothelial cells (Taraboletti *et al.*, 2002).

Furthermore, CD-62P (P-selectin), CD-42a (GPIX), CD-42b (GPIb), CD-41 (GPIIb/IIIa, αIIbβ3) and CD-61 (GPIIIa) are expressed on platelet derived MP (Baj-Krzyworzeka *et al.*, 2002); CD-4, CD-8 and CD-3 are expressed on MP derived from leukocytes (Martin *et al.*, 2004), CD-14 (Endotoxin receptor) is expressed on monocytes derived MP (MoMP) and CD-62P, CD-31 (PECAM-1), CD-51 (Vitronectin receptor, αvβ3); CD-54 (ICAM-1); CD-62E (E-selection), CD-105 (Endoglin); CD-144 (VE-Cadherin); CD-146 (MeICAM) and CD-106 (VCAM-1) are expressed on endothelial cell derived MP (Nomura *et al.*, 2008). These characteristics allow individual sub-populations of MP to be enumerated by flow cytometry.

In vitro, MP induction has been induced by chemical stimuli, such as incubation with tumour necrosis factor alpha (TNF-α), Interleukin 1 (IL-1) and bacterial polysaccharides and by external causes such as hypoxia, oxidative injury and chemotherapy due to the damage to endothelial cells (Lechner *et al.*, 2007).

1.8.3 Pro-coagulant Activity of MP

The pro-coagulant activity in cancer patients depends mainly on numbers of circulating TF and PL bearing MP (Debaugnies *et al.*, 2010, Zwicker *et al.*, 2009, Del Conde *et al.*, 2007, Tilley *et al.*, 2008) as both these factors (TF and PL) are important to convert FVII to FVIIa which is essential for extrinsic pathway of coagulation. Furthermore, there is accumulating evidence of a correlation between TF activity expressed on MP and thromboembolism (TE) in cancers (Tilley *et al.*, 2008). It has been reported by some investigators that TF bearing MP (TF+MP) involved in coagulation result in an increase of blood clot size rather than initiating coagulation (Giesen *et al.*, 1999), because the TF within the MP is thought to be either at too low a level to trigger clot formation itself or encrypted; other investigators, however, suggest that TF+MP initiate coagulation and have an effect on vascular function (VanWijk *et al.*, 2003). The TF bearing MP might have both, initiating and increasing the clot if there is a high level of TF bearing MP.

1.8.4 Clinical Experience of MP Number and Activity in Pancreatic Cancer

Madden *et al.*, (2008) suggested the existence of a circadian variation of TF-bearing MP in normal individuals and postulated a role in haemostasis. Furthermore, MPs have been detected in the blood of healthy individuals but their level is increased in malignant cases where the risk of thrombosis is increased (Biro *et al.*, 2003).

The suggestion that the tumour may be the main source of TF+MP comes from the demonstration that TF+MP reduce substantially after successful surgery to remove tumours (Zwicker *et al.*, 2009) and the fact the correlation between the level of TF+MP activity and incidence of thrombosis in cancer has been demonstrated (Khorana *et al.*, 2007). It has also been demonstrated that tumour growth, metastasis, and angiogenesis can be stimulated by TF+MP (Debaugnies *et al.*, Yu and Rak, 2004). Furthermore, increased levels of MP can be found in patients with a number of cancer types but the highest levels of TF activity are those in PC patients (Tesselaar *et al.*, 2009, Tilley *et al.*, 2008). Tesselaar *et al.*, (2007) reported that 34% of PC patients and 29% of advanced breast cancer cases involved in this study had TF activity above the upper limit of the normal range. Furthermore, it has been noted that vascular functions in particular processes involved in cardiovascular diseases can be affected by TF expression; this is mainly due to apoptotic, survival, remodelling, pro-coagulant and pro-inflammatory effects of microparticles (Wada *et al.*, 1995). In conclusion, a review of the literature on MP supports the idea that there is an increase of MP number and activity in some types of cancer including PC.

1.9 Angiogenesis

Angiogenesis is defined as new blood vessel formation from the pre-existing vascular network, consisting of three stages: migration, proliferation and differentiation. It can be physiological as in wound healing, tissue remodelling, regeneration and the menstrual cycle or patho-physiological as in cancer, rheumatoid arthritis and atherosclerosis. Tumours can only grow up to 1-2 mm^3 independent on angiogenesis. For growth bigger than this size, angiogenesis is necessary (Folkman, 1995a).

Recently, Lomberk (2010) suggested that PC is one of the more angiogenesis-driven tumours, as he showed that rapid tumour growth with a poor prognosis was positively correlated with increased angiogenesis. There are two principal mechanisms for promotion of angiogenesis; clotting dependent and clotting independent (Rickles *et al.*, 2003, Shoji *et al.*, 1997).

1.9.1 Clotting Dependent Mechanism of Angiogenesis

The extrinsic pathway of the coagulation cascade and clotting dependent mechanism of angiogenesis is initiated by activation of TF receptors via ligand binding; TF binds with FVIIa to form the TF/ FVIIa complex (Coughlin, 2000, Martin *et al.*, 1995). This complex triggers the coagulation cascade (Fig. 1.4) which involves activation of FX to FXa in the presence of Ca^{2+} and PL, followed by the conversion of prothrombin to thrombin (Gilbert and Arena, 1995) which is crucial in clot mediating formation due to fibrin formation and platelet activation (Falanga and Rickles, 1999) (Fig. 1.4). TF-FVIIa has also been reported to induce activation of all three mitogen-activated protein kinase (MAPK) pathways, p44/42 (Poulsen *et al.*, 1998), p38 and c-Jun N-terminal kinases/stress activated protein kinases (JNK/SAPK) (Camerer *et al.*, 2000) in different cell types. TF-FVIIa also promotes the activation of the src family of proteins (Versteeg *et al.*, 2008). The src family proteins are known to increase phosphatidylinositol 3 kinase (PI3K) activities, which in turn activates protein kinase B. Activated platelets can then promote angiogenesis by releasing a number of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), beta fibroblast growth factor (β-FGF) and platelet derived growth factor (PDGF) (Battinelli *et al.,* 2011, Fig. 1.7).

1.9.2 Clotting Independent Mechanism of Angiogenesis

In addition to its role in the clotting dependent mechanism, thrombin also has a role in the clotting independent mechanism of angiogenesis through proteolytic cleavage of protease-activated receptors (PAR). There are four PAR of which PAR1, PAR3 and PAR4 are cleaved by thrombin while the proteases trypsin, tryptase, TF-FVIIa and FXa can activate PAR2 (1998, Ishihara *et al.*, 1997, Nystedt *et al.*, 1995). There are several different pathways for clotting independent mechanisms of angiogenesis (Fig. 1.7), including a direct effect of TF-FVIIa that is dependent on phosphorylation of the cytoplasmic domain of TF, as mediated by PAR2. TF cytoplasmic domain may contribute to cell signalling by regulating TF-FVIIa signalling.

In fact, the activation of PAR2 and PAR1 by TF-FVIIa-FXa induces the phosphorylation of the cytoplasmic tail (Ahamed and Ruf, 2004). In support of the co-operative involvement of the cytoplasmic domain with PAR signalling, a study demonstrated that PAR2 dependent angiogenesis is tightly controlled by the TF cytoplasmic domain (Belting *et al.*, 2004). Other pathways are mediated by FVIIa and FXa activation of PAR (Camerer *et al.*, 2000). For example the TF/FVIIa complex reacts with FXa to form TF/FVIIa/FXa, and this complex triggers protease-activated G protein-coupled receptors, through PAR1 and PAR2 (Camerer *et al.*, 2000). Furthermore, thrombin increases cell invasion by its direct effect on the integrins or indirectly through increase of proangiogenic markers through clotting dependent and independent mechanisms. All of these mechanisms could contribute to the control of angiogenesis.

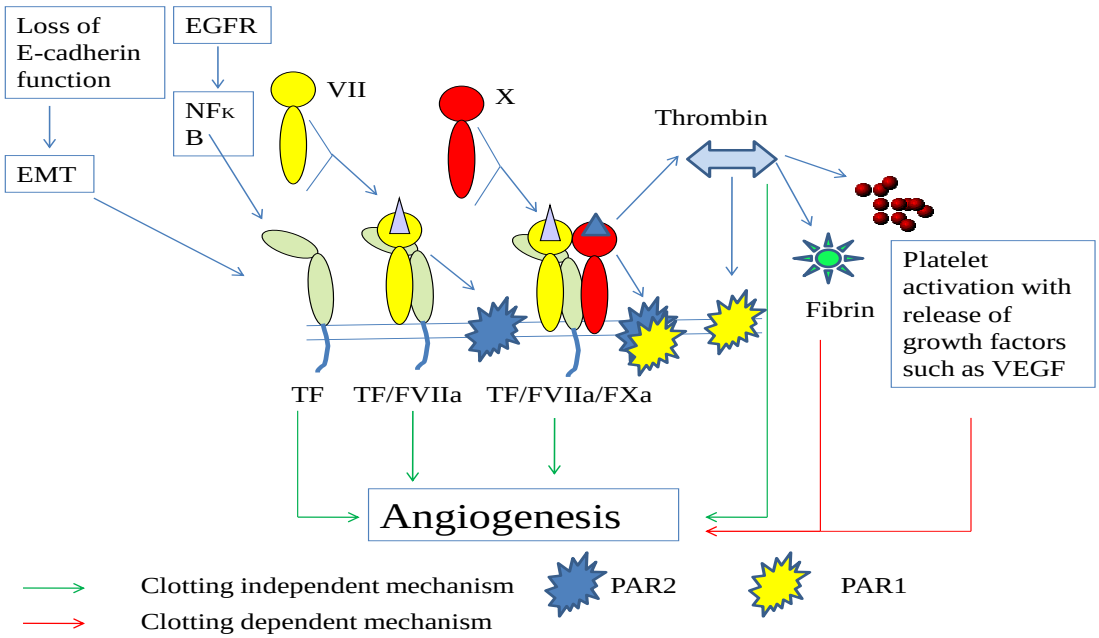

Figure 1.7: Representation of clotting dependant and clotting independent mechanism of angiogenesis. EMT: Endothelial Mesenchymal Transition.

1.9.3 Capillary Formation and Angiogenesis

To study angiogenesis *in vitro*, different types of endothelial cells as well as cells that have endothelial characteristics and behaviours have been utilised. One of the characteristic features of these endothelial cells is a cobblestone appearance in culture medium supported by growth factors, but on removal of these growth factors or when the endothelial cells are seeded on or

embedded in the collagen and fibrin, they form tubes which are similar in nature to capillaries (Smith and Staton 2006).

In vitro, the number, length and diameter of these capillaries can be measured, which therefore provides a quantitative method to evaluate the angiogenic activity in PC sera. This method is employed in the thesis as described in section 2.2.7.2. Although three dimensional (length, width and depth) capillary formation is more closely similar to *in vivo* angiogenesis than two dimensional (length and width) capillary formation and their analysis takes a longer time compared with two dimensional (length and width) tubule formation (1-3 days *vs.* more than 7 days; 1 day *vs.* 5-15 days, respectively). Moreover, tubule analysis of three dimensional methods is more complicated as the gel should be fixed with 10% formaldehyde, dehydrated with graduated alcohol and imbedded in paraffin block. The block should be then sectioned horizontally and vertically in 3-5 micro metre (µm) thickness using microtome and stained with anti CD 31 and analysed using a digital analysis software (Smith and Staton 2006).

1.10 Relationship between Cancer and Thrombosis

In 1865 Trousseau described thrombosis as a major complication of cancer (Trousseau, 1865), the risk of thrombosis being increased 2-7 fold in patients with cancer compared with a non-cancer population (Stein *et al.*, 2006, Blom *et al.*, 2005, Heit *et al.*, 2002). Furthermore, Prandoni *et al.* (2002) noted that the rate of recurrence of TE is higher in cancer patients than in non-cancer patients (1.9% *vs.* 5.4%). Aberrant coagulation is commonly detected in many patients with malignancy, especially in advanced or disseminated stages (Kirwan *et al.*, 2003). The higher the prevalence of abnormalities in coagulation, the greater the likelihood a recognisable haemostasis-related clinical syndrome will occur. The most common coagulation abnormalities observed in solid malignancies are TE and disseminated intravascular coagulation (Kakkar *et al.*, 2003). A seminal post-mortem study reported in 1938 showed that TE is a major complication of PC with the highest incidence being found in pancreatic tail tumours (Sproul, 1938). Many recent studies have verified that PC is one of the malignancies (Khorana and Fine, 2004, Ogren *et al.*, 2006a, Shah and Saif, 2010) with the highest prevalence of TE (up to 57% of PC cases, Khorana and Fine, 2004) and that a patient's risk of developing thrombosis is further increased with chemotherapy treatment (Khorana *et al.*, 2008, Ogren *et al.* 2006b, Moore *et al.*, 2005, Heit *et al.*, 2000b, Wall *et al.*, 1989).

The presence of TE in PC patients is correlated with a shorter survival period (Mandala *et al.*, 2007) and the associated early mortality may be directly related to the incidence of TE (Sgouros and Maraveyas, 2008). There are three, non-mutually exclusive, explanations for the relationship between thrombosis in PC and reduced survival. The first is that the thrombosis itself is a potentially lethal event that causes mortality (Maraveyas and Johnson, 2009) and the use of many of the conventional therapies, including chemotherapy (Heit *et al.*, 2000a, Wall *et al.*, 1989), anti-angiogenic drugs (Nalluri *et al.*, 2008, Heit *et al.*, 2000a) and erythropoietin (Bennett *et al.*, 2008) directly increases this risk. Secondly malignant cells exhibiting enhanced ability to shed MP and promote thrombosis are inherently or phenotipically more aggressive (Khorana *et al.*, 2007) and finally the targeting of an aberrant coagulation exacerbates the malignant phenotype by sustaining a continuous loop of factors that promote trafficking and anchoring, invasion, tumour growth and metastasis, or a combination of one or more processes.

1.11 Regulation of Tissue Factor

There are several mechanisms that control TF activity; however the TF pathway inhibitor (TFPI) (Fig. 1.4), a serine protease inhibitor (Broze, 1995), acts as the principal endogenous regulator of TF (Sandset, 1996). TFPI is produced mainly by the microvascular endothelium and up to 85% of it is bound to these cells (Bajaj *et al.*, 1990). Ten percent is associated with lipoprotein in plasma and the remainder is stored within platelets. Small amounts of TFPI are released from platelets (Novotny *et al.* 1991). It has been reported that the median concentration and range of TFPI in 35 healthy people was [90 (75-120) ng/ml] while the median concentration of TFPI and range in 22 patients with solid malignancy was [128 (47-286) ng/ml]. However, the median TFPI concentration and the range of 52 patients with haematological malignancies was [68 (23-133), Iversen *et al.*, 1998].

TFPI is composed of three Kunitz type protease inhibitor domains and a C-terminal polybasic motif. The first domain reacts with and inhibits FVII in the TF-FVIIa complex; the second domain reacts with and inhibits FXa in the TF-FVII-FXa complex (Sevinsky *et al.*, 1996, Broze, 1995, Girard *et al.*, 1989) and the third domain has an unknown function at this time. However Sameera *et al.* 2011 (Conf reference 1) suggested the Kunitz 3 has a role in inhibition of FXa. Therefore, TFPI reacts with TF and inhibits its pro-coagulant activity (Bach and Moldow, 1997). In addition to the anti-coagulation effect, recombinant TFPI (rTFPI) has been shown to decrease

cell proliferation (Hamuro *et al.*, 1998) and to induce apoptosis of human umbilical endothelial cells (HDMEC) due to its inhibitory effect on TF (Hamuro *et al.*, 1998). Furthermore, Ahamed *et al.* (2004) noted that the TFPI could prevent the PAR1 signalling pathway in Chinese hamster ovary cells. However the concentration of TFPI used to prevent the PAR1 signalling is 30-fold higher than the concentration of TFPI for anticoagulation. In phase III clinical studies, TFPI modified the coagulation system but it had disappointing results in improving the survival rate in patients with sepsis (Polderman and Girbes, 2004, Abraham *et al.*, 2003). There was no clear explanation for these results.

It is reported that TF expression can also be regulated signalling via the epidermal growth factor receptor (EGFR1) through nuclear factor kappa B (NF-κB), loss of E-cad and subsequent EMT (Fig. 1.7, Milsom *et al.*, 2008). It has been noted recently that LMWH may impact on TF level through NF-κB by EGFR1. Furthermore It has been noted that the LMWH effect on NF-κB may be mediated via effects on growth factor signalling (Ettelaie *et al.*, 2011b). The signals through these pathways enhance activation of TF. Another mechanism that controls TF is FXa, which has a negative feedback effect on the regulation of TF (Ettelaie *et al.*, 2007, Fig. 1.4). Furthermore, there are some biological changes which are responsible for the up-regulation of growth factors in general, including TF. These include phosphatidylinositol kinase (PI3K) which controls cellular activities and properties including proliferation, survival, motility and morphology , p38 and Extracellular Signal-Regulated Kinases-1/2 (Erk-1/2) Mitogen-Activated Protein Kinase (MAPK) which have a critical role in chemo-attraction and angiogenesis (Schett *et al.*, 2008). In tumour cells, the activity of PI3K is decreased; in contrast p38 and Erk-1/2MAPK are increased (Blum *et al.*, 2001). Consequently, apoptosis will decrease and proliferation and angiogenesis will increase. Thrombin can activate PAR signalling directly and some transcription factors such as specific protein-1 , activator protein-1 and NF-kB bind to corresponding sites in TF after cell activation (Martin *et al.*, 1995). As a conclusion, there are several signalling pathways that could control TF.

1.12 Vascular Endothelial Growth Factor

VEGF is a 40 kDa glycoprotein critically important in controlling angiogenesis. The VEGF family includes VEGF A, B, C, D, E, G and the placental growth factor (Ogawa *et al.*, 1998). VEGF A and B are two of the most angiogenic proteins, whereas VEGF C and D have been shown to act principally as lymphangiogenic factors. Placental growth factors control angiogenesis in ischemic heart and limb (Autiero *et al.*, 2003, Nagy *et al.*, 2002). VEGF ligands bind to two main groups of receptors, tyrosine and non tyrosine kinase receptors. There are three types of tyrosine kinase receptors: VEGFR 1, also called Flt-1 (fms-like tyrosine kinase-1), VEGFR 2 also called KDR (Kinase insert domain-containing receptor) and VEGFR-3, also called Flt-4 (fms-like tyrosine kinase-4). Angiogenesis is mainly controlled by signalling through VEGFR 2 (Autiero *et al.*, 2003) and its ligands (Milkiewicz *et al.*, 2004) which depends on many factors such as hypoxia, hypoglycaemia stress and the presence of transcription factors such as signal transducer and transduction of transcription-3 (Stat-3).

VEGF A, B and placental growth factor bind to VEGFR 1 receptor. VEGF A and VEGF E bind to VEGFR 2. VEGF C and D bind to VEGFR-3 (Olsson *et al.*, 2006). VEGF A has an important role on vascular-angiogenesis, while VEGF C has an important role on lymph-angiogenesis (Eriksson and Alitalo, 1999). VEGF A and VEGF C are commonly expressed by malignant pancreatic cells.

There is a direct link between VEGF and TF expression. High expression of TF is associated with raised expression of VEGF (Abe *et al.*, 1999, Nakasaki *et al.*, 2002). In an immunohistochemistry study Nakasaki *et al.* (2002) noted a significant correlation between expression of TF with VEGF and microvascular density. The data is described in relation to this thesis work in section 9.4. It has been noted that VEGF upregulation can be controlled by the cytoplasmic domain of TF in a mechanism that is independent of FVIIa (Abe *et al.*, 1999). Abe *et al.* (1999) reveal that tansfection of HT144 a (low TF and VEGF producing melanoma cell line) with a TF cDNA containing the full-length sequence produce increased levels of both TF and VEGF on the HT144 while transfection with a cytoplasmic deletion TF cDNA produced an increase of TF with little or no increase in VEGF.

In summary, VEGF is an important signalling protein involved in angiogenesis. As its name implies, VEGF activity is restricted mainly to cells of the vascular endothelium, although it does have effects on a limited number of other cell types (e.g. stimulation monocyte/macrophage migration) and it seems to be a direct line between VEGF and TF expression.

1.13 Angiogenic Markers Commonly Studied with Relevant Pancreatic Cancer

The angiogenic markers are divided into two groups; proangiogenic and antiangiogenic markers. It has been postulated that angiogenic markers play a major role in angiogenesis, as the up-regulation of proangiogenic markers enhance angiogenesis and is associated with poor prognosis. It has been hypothesised that a tumour is the main source of the serum angiogenic markers. Furthermore, evaluation of these markers in the serum is a rapid, relatively non-invasive and convenient method in comparison to surgical resection and diagnostic pathological techniques, such as taking a fine needle aspirate (Poon *et al.*, 2001). Increased expression of several angiogenic markers such as VEGF (Chang *et al.*, 2008, Pistol-Tanase *et al.*, 2008), EGFR (Yamanaka *et al.*, 1993) and platelet-derived endothelial cell growth factor (Ikeda *et al.*, 1999) has been noted in PC tissue and or in the serum of PC patients. Furthermore, clinical and laboratory studies suggest that high expression of pro-angiogenic markers is associated with an increase of angiogenesis and poor prognosis (Lomberk, 2010, Fujioka *et al.*, 2001). It has been reported that pro-angiogenic factors enhance angiogenesis due mainly to decrease of apoptosis, increase of survival and cell proliferation (Herbst, 2004, Neutzner *et al.*, 2007). Although the mechanism by which pro-angiogenic factors control survival and apoptosis is not clear, it is thought that the binding of these factors results in PI3K/Akt, Ras/MAPK up-regulation (Suhara *et al.*, 2001), and p38 MAPK-dependent apoptosis pathways down-regulation (Nicholson and Anderson, 2002, Gratton *et al.*, 2001,Yu and Sato, 1999).

Furthermore, VEGF can regulate αvβ3 integrin which enhances cell migration and survival when it is associated with VEGF receptor-2 (VEGFR 2) and defective tyrosine phosphorylation resulting in impaired adhesion, spreading, and migration (Mahabeleshwar *et al.*, 2006, Hood *et al.*, 2003). Other contributing mechanisms could include the Ras/MAPK, phosphatidylinositol 3 kinase (PI3K) /Akt, Janus kinase (JAK)/Stat and phospholipase C /protein kinase C pathways which are the main signalling pathways activated by EGFR 1, resulting in the activation of genes that cause

over-expression of angiogenic factors, increased cell proliferation, migration, adhesion, differentiation and apoptosis (Yu *et al.*, 2005).

The families of molecules studied to-date are briefly reviewed with investigations of particular relevance to PC being highlighted (Tables 1.1 and 1.2).

Table 1.1: Pro-angiogenic factors.

Proangiogenic Factor	**Main Functions**
Amphiregulin	In normal tissue, amphiregulin is expressed in the nuclei, while in normal PC is expressed in the cytoplasmic part of the cells (Yotsumoto *et al.*, 2008, Ebert *et al.*, 1994,).
Angiogenin	Over expression of angiogenin is related to aggressive PC, bad prognosis (Shimoyama *et al.*, 1996) and increased angiogenesis by inducing nitric oxide (NO) through PI-3 and Akt Kinase (Trouillon *et al.*, 2010). In a series of 100 cases of lung cancer, high nuclear expression of angiogenin was found in 67% of cases (Yuan *et al.*, 2009). Furthermore, it has been postulated that angiogenin increases in the advanced stage of breast cancer (Duranyildiz *et al.*, 2009) and melanoma (Vihinen *et al.*, 2007) and high expression is linked to poor prognosis.
Ang-1	Angiopoetin-1 plays an important role in endothelial cell survival and inhibits apoptosis (Papapetropoulos *et al.*, 2000).
Artemin	Artemin belongs to Glial cell line-derived neurotrophic factor (GDNF). It promotes cell survival and differentiation and down regulates apoptosis (Dong *et al.*, 1996).
TF	Also called coagulation factor III or CD 142, induces angiogenesis through clotting dependent and clotting independent mechanisms (Rickles *et al.*, 2003, Shoji *et al.*, 1997).
CXCL 16	Chemokine (C-X-Cmotif) ligand 16. It induces a strong chemotactic response and it guides migration of activated T-cells into inflamed tissue (Abel *et al.*, 2004).
DPP-IV	Di-peptidyl peptidase-4 or CD-26. It is an intrinsic membrane glycoprotein and a serine exopeptidase that cleaves X-proline di-pepides from the N-terminus of polypeptides (Yazbeck *et al.*, 2009).
EGF	Epidermal growth factor regulates cell proliferation, differentiation and

	growth by binding to its receptor (EGFR) (Camaj *et al.*, 2009).
EG-VEGF	Endocrine gland–derived VEGF, it is restricted to the steroidogenic glands, ovary, testis, adrenal and placenta (Hoffmann *et al.*, 2009).
Endoglin-1	Also called CD-105. It is involved in cytoskeletal organisation, affecting cell morphology and migration (Duff *et al.*, 2003, Cheifetz *et al.*, 1992). In a series of 30 cases, endoglin was detected in all cases and it was expressed mainly in small blood and lymphatic vessels (Yotsumoto *et al.*, 2008)
FGF	Fibroblast growth factors-acidic, basic, 4-7 play a role in angiogenesis; wound healing, cell proliferation (Gospodarowicz *et al.*, 1989) and differentiation of cells and tissues. Wang *et al.* (2009b) suggested that bFGF is a negative regulator of angiogenin and the expression of angiogenin decreases when bFGF is over-expressed.
GFL	Glial family ligands play a role in cell survival, cell growth, cell differentiation and cell migration (Lieu *et al.*, 2011).
GM-CSF	Granulocyte-macrophage-colony—stimulating factor is a cytokine that has a major role in the production of white blood cells, particularly macrophages, granulocytes and megakaryocytes (Uchida *et al.*, 2007).
HB-EGF	Heparin-binding epidermal growth factors act on EGF receptor in smooth muscle cells and fibroblast cells but not endothelial cells. It has a major role in cell tumourgenesis and survival (Takemura *et al.*, 1999) and wound healing (Homma *et al.*, 1995).
HGF	Hepatocyte growth factor stimulates mitogenesis, cell motility, matrix invasion and tissue regeneration (Otte *et al.*, 2000).
IGFBP	Insulin-like growth factor binding proteins -1, -2, -3, are made up of six distinct subgroups of protein, IGFBP-1 to IGFBP-6. IGFBPs play a chief role in the regulation of the autocrine/paracrine and endocrine functions of IGF. Furthermore, IGFBPs play a role as a growth factor independent of IGF (Conover *et al.*, 1993, Conover, 1992).
IL-1B	Interleukin-1B has role in white blood cell aggregation, cell proliferation, differentiation and apoptosis (Liu *et al.*, 2009).
IL-8	Interleukin-8 a chemokine (IL-8) that acts as a chemo-attractant and potent pro-angiogenic marker (Waugh and Wilson, 2008).
Lep	Leptin regulates energy intake and expenditure (Brennan and Mantzoros, 2006).
MCP1	Monocyte chemotactic protein-1 acts as a white blood cell chemo-attractant agent, and inhibits stem cell proliferation (Cook, 1996).

MMP	Matrix metalloproteinases 8, 9 have a vital role in metastasis due to the role they play in breaking down the cellular matrix (Page-McCaw *et al.*, 2007).
NRG-B1	Neuregulin-1-B1 EGF has an important role in increasing the level of tyrosine phospherlisation of focal adhesion kinase (Ritch *et al.*, 2003).
PD-ECGF	Platelet-derived endothelial cell growth factor plays a role in angiogenesis (Fujimoto *et al.*, 1999). In immunohistochemistry study, Ikeda *et al.* (1999) noted that an immunohistochemistry that 75% were positive to platelet derived endothelial growth factor (PD-ECGF) and 25%.
PDGF	Platelet derived growth factor plays an essential role in capillary formation and in vascular ageing process of the tumour and also has role in cell growth, division and angiogenesis. PDGF is composed of two chains, so has 3 forms (AA), (BB), or (AB) (Turner *et al.*, 2009, Fruttiger *et al.*, 1996).
PIGF	Phosphatidylinositol-glycan has a major role in protein synthesis and enhancement of angiogenesis (Fox *et al.*, 1987).
Prolactin	Stimulating proliferation of oligodendrocyte precursor cells (Web reference 2, 2011).
VEGF	Acts as a general activator to the endothelial cells (Ferrara *et al.*, 2003).

Table 1.2: Anti-angiogenic factors.* means factors have been reported to have pro- and anti-angiogenic factors

Anti-angiogenic Factors	**Main Functions**
Activin A *	Decrease of pancreatic cell (Zhang *et al.*, 2004) and endothelial cell survival (Chen *et al.*, 2002)). Also shown to regulate cell differentiation cell proliferation, and apoptosis (Galloway *et al.*, 2000, Dong *et al.*, 1996).
ADAMTS-1	A Disintegrin and Metalloproteinase with ThromboSpondin motif-1 has an inhibitory effect on VEGF (Turner *et al.*, 2009, Vazquez *et al.*, 1999). Higher expression has been noted in lymph nodes and peritoneal secondary of pancreatic primary origin (Masui *et al.*, 2001).
Ang-1	Angiopoietin-1 (Ang-1) plays an important role in endothelial survival and inhibits apoptosis (Papapetropoulos *et al.*, 2000). However, other reports have shown that it prevents hepatic growth, vascular permeability and angiogenesis (Stoeltzing *et al.*, 2003).
Ang-2	Angiopoietin -2 is a natural antagonist of Ang-1 binding to the same receptor but not causing a signal (Davis *et al.*, 1998, Maisonpierre *et al.*, 1997).
Angiostatin	Angiostatin is a protein that acts as an inhibitor of angiogenesis (Kirsch *et al.*, 1998, Oreilly *et al.*, 1994).
Endostatin/ CollagenXVIII	Has an anti-angiogenic activity and tumour growth inhibitory effect (Oreilly *et al.*, 1997).
LAP (TGF-B1)*	Latency Associated Peptide (Transforming growth factor beta) acts as a pro-angiogenic factor in low doses because it up-regulates angiogenic factors and proteinases, while in high doses it acts as an angiogenic inhibitory factor because it prevents endothelial cell growth, stimulates differentiation of smooth muscle cells and enhances formation of the basement membrane (Pardali and ten Dijke, 2009, Carmeliet, 2003).
Endothelin	Acts as a vasoconstrictor and inhibitor of angiogenesis. It suppresses growth and metastasis of cancer including PC (Weydert *et al.*, 2009).
PTX3	Pentraxin 3 (PTX 3) impedes endothelial cell proliferation and vascularisation that depends on bFGF because it has a high binding affinity for bFGF receptors (Davis *et al.*, 1998, Maisonpierre *et al.*, 1997).
PF4	Platelet factor 4 is released from alpha granules of platelets during platelet aggregation and promotes clotting. PGF 4 has an inhibitory effect on endothelial cell proliferation and angiogenesis (Gupta *et al.*, 1995, Kolber *et*

	al., 1995, Luster *et al.*, 1995)).
Serpin B5, E1 and F1	Serpins are a group of proteins that inhibit chymotrypsin-like serine proteases. They have an anticoagulant, anti-tumour and anti-angiogenic activity (Web reference 3, 2011).
TIMP-1, -4	Tissue inhibitors of metalloproteinase 1-4 are glycoproteins which act as natural inhibitors of the MMP (Hornebeck 2003 and Green 1996). TIMP4 regulates platelet aggregation and recruitment and may play a role in hormonal regulation and endometrial tissue remodelling (Greene *et al.*, 1996).
TSP-1	Thrombospondin-1 is a natural inhibitor of neovascularisation, tumourigenesis, and angiogenesis (Rastinejad *et al.*, 1989).
TSP-2	Thrombospondin-2 is a matrix glycoprotein and has a potent function against tumour growth and angiogenesis (Strieter *et al.*, 2006).
Vasohibin	Acts as an anti-angiogenic marker (Kaya *et al.*, 2004).

1.14 Factors Influence Thrombosis in Pancreatic Cancer

There are two groups of factors that affect the thrombosis; extrinsic and intrinsic.

1.14.1 Extrinsic Factors

Increased TE in PC is associated with the appearance of distant metastases (Blom *et al.*, 2006). Superimposed risk factors such as acute medical conditions (e.g., concurrent infection, heart failure and chronic obstructive pulmonary disorders (Offord *et al.*, 2004) and surgery can further exacerbate this risk of TE (Bergqvist *et al.*, 2002). Moreover, the systemic treatments used in PC patients may also have a significant prothrombotic effect; chemotherapy for example increases the incidence of TE in PC up to 4.8 fold (Moore *et al.*, 2005). There are distinct mechanisms which may be responsible for increase TE occurrence in cancer treated with chemotherapy: platelet activation (Togna *et al.*, 2000), decrease of the natural inhibitor factors (such as antithrombin III, protein S and protein C; (Feffer *et al.*, 1989), increase of cell adhesion molecules (Sgouros and Maraveyas, 2008, Mandala *et al.*, 2007) and damage of the blood vessel wall/endothelium (Mandala *et al.*, 2006). Any number of these factors may be acting in combination of two or more. More recently it has been postulated that chemotherapy increases apoptosis in PC (Yao and Qian, 2010). The latter process would lead to apoptotic cells and cellular fragments being increased within the circulation, both having a generally pro-coagulant surface due to TF and PL/Phosphatidylserine (PS) exposure.

1.14.2 Intrinsic Factors

PC cells secrete mucin which then enters the circulation. Although most of the mucin that enters the blood circulation is cleared by the liver, some of it remains in the circulation and can react with P-selectin on platelets, L-selectin on leukocytes and P- and E-selectin on the vascular endothelium leading to formation of platelet-rich thrombi (Wahrenbrock *et al.*, 2003). Therefore, pancreatic tumour cells could directly activate the host cells (endothelial cells, platelets, leukocytes) by mucin secretion.

Furthermore, in a 23796 autopsies from general population studied between 1970 and 2006 (Ogren et al. 2006a) reported that the incidence of portal thromboemolism was in 254 (1%), of which 44% were associated with malignancy, 28% with liver cirrhosis and 10 % with abdominal infection and inflammatory disease. In another study using the same data Ogren *et al.* (2006b) reported that association between cancer and thrombosis depends on the histological type as the incidence of thrombosis was more with adenocarcinoma and metastatic cancer.

1.15 Epidermal Growth Factor Receptor in Haemostasis and Angiogenesis

It has been noted that over-expression of EGFR 1 was seen in 43% of human PC cases (Friess *et al.*, 1999) and its expression appears to be correlated with poor prognosis, increased tumour aggressiveness and enhanced angiogenesis (Fujioka *et al.*, 2001, Friess *et al.*, 1999). EGFR 1 expression on PC differs according to the part of the pancreas that is cancerous. For example, cancer of the papilla of Vater did not show an over-expression of EGFR 1 compared with a normal control, while cancer of other parts of the pancreas showed over-expression of EGFR 1, reaching up to 60% (Friess *et al.*, 1999). A range of expression for EGFR2 has also been reported in PC (Koka *et al.*, 2002, Dergham *et al.*, 1997); however, no association with prognosis was observed.

Milson *et al.* (2008) postulated that TF expression could be regulated by EGFR, controlled at least in part via NF-κB signalling. Kinase suppressor of Ras1 (KSR1) is involved in the control of both pro-coagulant and aggressive phenotypes of cancer cells by the up-regulation of TF and downstream of Erb (EGFR) oncogenes (Yu *et al.*, 2010). Therefore the use of KSR1 targeting agents is being explored as a therapeutic strategy (Mostefai *et al.*, 2008a, Zhang *et al.*, 2008,

Xing *et al.*, 2003). Furthermore it has recently been reported that LMWH downregulation of the transcriptional activity of NFκB and interfere with the function of growth factor and sequentially down-regulates TF expression (Ettelaie *et al.*, 2011b). Based on these studies, EGFR-driven up-regulation of TF (and therefore targeting of EGFR receptor directly or indirectly through KSR1) is a potential target for the treatment of PC.

1.16 The Clinical Experience of Targeting Growth Factor Receptors in Pancreatic Cancer

From a clinical perspective two recently completed trials of antiangiogenic agents (bevacizumab or Avastin®) and axitinib (S1005 and AG-013736), when used as a first line treatment for PC combined with standard therapy yielded disappointing results. Firstly, gemcitabine in combination with axitnib resulted in a median overall survival of 7.4 months, compared to 8.2 months for gemcitabine alone, in 630 patients (Kindler *et al.*, 2009). Secondly, gemcitabine in combination with bevacizumab also failed to confer any significant increase in overall survival (5.8 months) when compared to gemcitabine alone (5.9 months) in a study of 535 patients (Kindler *et al.*, 2009).

Furthermore, in a large (607 patients) randomised phase III clinical trial, where a triple combination of bevacizumab, Tarceva® (erlotinib) and gemcitabine was compared to gemcitabine, Tarceva® and placebo, no statistical difference in the median overall survival rate was found (7.1 *vs.* 6.0 months respectively; p = 0.209) (Van Cutsem *et al.*, 2009b). A concern remains that these agents also drive thrombosis and whatever potential benefit from anti-cancer treatments may exist may be lost through excess thrombotic events (Maraveyas and Johnson, 2009).

However, a large randomised clinical trial of Tarceva® and gemcitabine *vs.* gemcitabine alone produced one of the few positive results in APC (Moore *et al.*, 2005). In this series of 569 cases, the median overall survival was significantly prolonged with Tarceva® (100 mg/ daily) and gemcitabine compared with gemcitabine only (6.24 *vs.* 5.91 months, p = 0.038); a marginal clinical improvement that was seen only in the stage IV patients. There was no significant difference in the objective response rate and there were many grade I and II side effects in the group treated with Tarceva® plus gemcitabine, such as rash, diarrhoea, stomatitis and infection

(Moore *et al.*, 2005). However, one recently concluded trial of anti-EGFR agent [cetuximab (Erbitux), (IgG1 monoclonal antibody (mAb)], in a larger randomised clinical trial of cetuximab and gemcitabine *vs.* gemcitabine alone was negative. In this randomised controlled trial of 745 patients with APC, gemcitabine and cetuximab did not demonstrate a survival prolongation compared to gemcitabine alone (6.3 months for gemcitabine plus cetuximab v 5.9 months for gemcitabine alone; p = 0.23, Philip *et al.*, 2010). To date therefore, there is little positive support for the targeting of growth factor receptors in PC.

1.17 Clinical Evidence of Benefit from Direct Interference with the Coagulation Apparatus

Natural heparin (unfractionated heparin) is a highly-sulfated glycosaminoglycan that has varying molecular weights (3-40 kDa) (Weitz1997). Low molecular weight heparin (LMWH) is defined as heparin salts having an average Molecular Weight of less than 8 kDa and a mean MW of about 5 kDa. Clinically, LMWH has been used for treatment and prevention of thrombotic disorders (Linhardt and Gunay, 1999) with a lower risk of bleeding tendency, thrombocytopenia and osteoporosis than natural heparin (Linhardt and Gunay, 1999, Weitz, 1997). One of the most widely used forms of LMWH is dalteparin (Fragmin), which is a nitrous acid depolymerised type of heparin with mean MW of 6 kDa (Fig. 1.8, Weitz, 1997).

Heparin can potentially act as an anti-cancer agent via a variety of mechanisms. It inhibits several coagulation factors such as thrombin, FXa, FIXa, FXIa, FXIIa (Palumbo *et al.*, 2000) and inhibits cell proliferation, particularly of those cells that over express PAR1 (Balzarotti *et al.*, 2006). Heparin also interacts with VEGF-165 and VEGF-189 expressed on malignant cells (Yu *et al.*, 2002) and could inhibit angiogenesis via blocking of P- and L-selectin (Borsig, 2010). It has been recently shown that LMWH can decrease the angiogenic and chemo-attractant activity of PC patients' sera (Maraveyas *et al.*, 2010a). A recently completed randomised phase IIb study in APC of gemcitabine *vs.* weight-adjusted dalteparin (WAD), a type of LMWH, demonstrated a significant decrease in the overall incidence of venous TE from 31% to 12%, with a reduction of recorded lethal TE and sudden death from 9% to 0%. These differences were significant but the trial was too small to demonstrate an overall survival advantage (Maraveyas *et al.*, 2010b) .

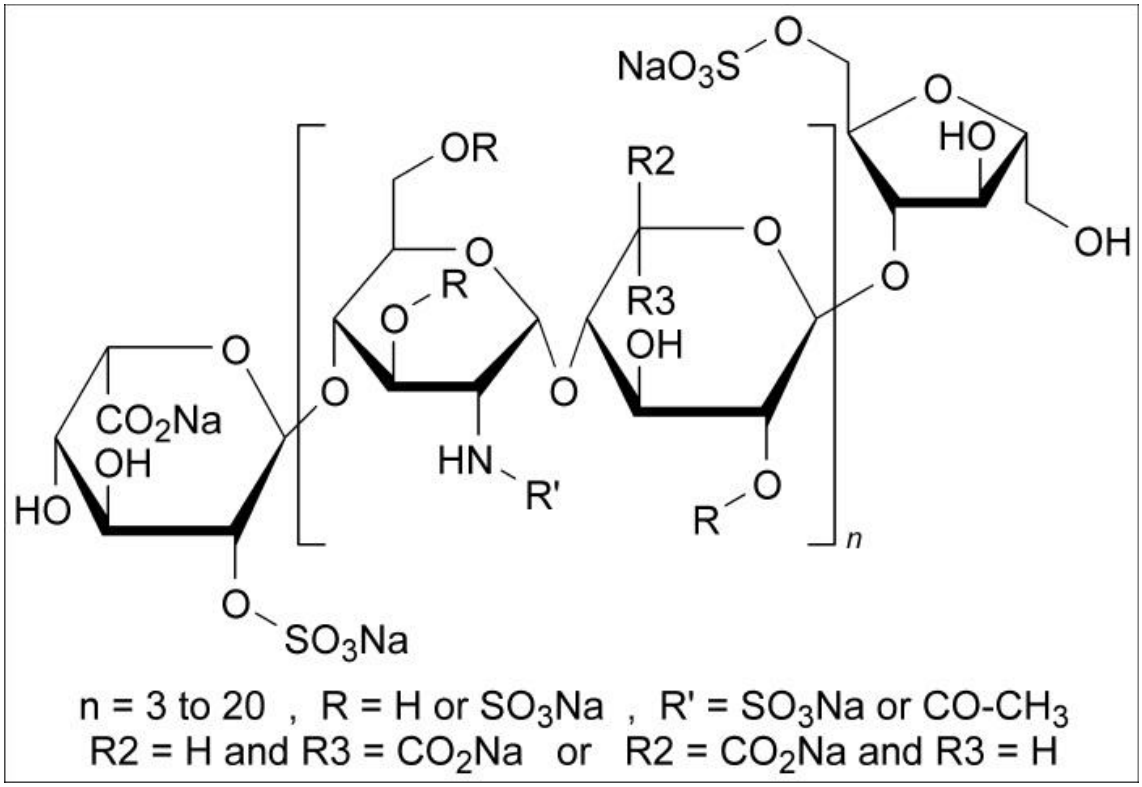

Figure 1.8: Schematic representation of dalteparin structure adapted from European Pharmacpoeia.

In a second larger randomised trial, Riess *et al.* (2010) studied 312 APC patients receiving chemotherapy randomised into two groups, those with enoxaparin (Clexane which is another type of LMWH have MW of 4.5 KD and the ratio of anti-Xa/anti-IIa activity is 3.9 while the MW of dalteparian is 6 KD and the anti-Xa/anti-IIa activity is 2.5) those without. Similar to Maraveyas *et al.* (2010a), a significant decrease of clinical TE was found, 15% *vs.* 5%, respectively in the group treated with LMWH. However, once again no overall survival benefit was documented (Riess *et al.*, 2010). Most recently, Epstein *et al.* (2010) studied 6870 PC cases of which 19% of patients suffered TE. Ninety five percent of PC patients that developed TE were treated with chemotherapy and LMWH and the survival time of PC patients who developed thrombosis at the time of the diagnosis was 6.2 months, while those with secondary thrombosis after PC diagnosis was 13.7 months (Epstein *et al.* (2010).

1.18 Pancreatic Cell Invasion:

Although there have been advances in surgical techniques (Hines and Reber, 2009) and chemotherapy (Hines and Reber, 2009, Willett and Czito, 2009), still PC has very poor prognosis, due mainly to cell invasion. Tumour cell metastasis depends on stages that precede the actual invasion, which are self-sufficiency in growth signals, insensitivity to growth-inhibitory (ant-igrowth) signals, evasion of programmed cell death (apoptosis), and limitless replicative potential sustained angiogenesis.

The first step of invasion is loss of cell to cell adhesion (Kominsky *et al.*, 2003), often followed by impairment of intercellular connection (Czyz, 2008). The most common changes in cell-environment interaction in malignancy are loss of E-cadherin (E-cad) function (Kominsky *et al.*, 2003, Christofori and Semb, 1999) and E-cad expression changes to other integrins such as α3β1 and αVβ3 that bind the degraded stromal materials (Varner and Cheresh, 1996). Furthermore, neural cell adhesion molecule (CAM, CD-56) decreases either in its level, as in PC and colorectal cancer (Fogar *et al.*, 1997) or in activity, as in Wilms' tumour and neuroblastoma (Johnson, 1991). The aggressiveness of the cells depends on the presence of abnormal mitotic activity in the nucleus of invaded cells. Integrin β1 regulates several aspects of mitosis in centrosome function cells and cytokinesis (Revert Carlos *et al.* 2006) and the disruption of this integrin inhibits pancreatic cell invasion (Lee *et al.* 2011). On genetic bases, erythropoietin-producing human hepatocellular carcinoma (Eph receptors) with their ephrin ligands (Eph family receptor interacting proteins) form an important cell-cell communication system capable of bi-directional signalling (Heroult et.al. 2006, Kullander and Klein, 2002). It has been reported that Eph genes play an important role in control of growth factors and their receptors which are involved in the regulation of cell proliferation. They also play a key role in oncogenesis (Hirai *et al.*1987). There are two subclasses of Eph family receptor and ligands; the ephrin A ligands bind to EphA receptors, while ephrin B ligands bind to EphB receptors (Heroult et.al, 2006). Although the bulk of research has studied the EpH & Ephrin role in development, new evidence suggests strong involvement in tumourigenesis, including cell invasion and angiogenesis (Wimmer-Kleikamp and Lackmann, 2005; Dodolet and Pasquale, 2000). Through the use of multiple cell lines, including PC cells, it was demonstrated that the C-terminus of ephrin B1 was responsible for conferring the increased invasive properties through activation of matrix metalloproteinase-8 secretion (Tanaka et. al., 2007b).

It has been postulated that there are several oncogen mutations and their signalling pathways play an essential role in PC, of which K-RAS, which is a member of the RAS family of GTP-binding proteins, mediates several cellular activities including cell survival, proliferation and differentiation (Malumbers and Barbacid 2003).

The next step of cell invasion is degradation and destruction of the extra-cellular matrix (Ellenrieder *et al.*, 1999, Mignatti and Rifkin, 1993 Clark, 1979). ECM proteinases, such as tryptase or thrombin, act on PAR. This interaction releases a blocking peptide, allowing the internal ligand to bind and activate a G protein–coupled signalling cascade. When the GPI-anchored uPA-R binds uPA, it interacts with integrins and activates signalling pathways mediated by growth factor receptor-bound protein 2 (Grb2). The transmembrane A disintegrin and metalloproteinases (ADAM) proteinases target substrate such as receptors in the plasma membrane. Proteolysis of ECM proteins alters integrin-mediated anchorage, focal adhesions, cytoskeletal architecture, and signalling molecules. Binding of cleaved ECM fragments by integrins also activates different pathways than binding of intact molecules. Proteolysis can activate growth factors, such as TGFb, by liberating them from binding proteins in ECM. The growth factors then can bind to and activate their plasma membrane receptors, and initiate the downstream signalling cascades (Werb 1997, Fig. 1.9).

It has been noted that the thrombophilic state of PC patients may be an accelerant of this process. One of the most important steps is up regulation of proteases, which have an important role in membrane degradation (Ellenrieder *et al.*, 2000, Koshiba *et al.*, 1998, Werb, 1997, Sporn, 1996). TF-FVIIa-PAR2 signalling regulates αvβ3 integrin which enhances cell migration and survival when it is associated with VEGFR 2. Furthermore the chemo attractive activity of pro-angiogenic factors plays an important role in enhancement of cell invasion (Mahabeleshwar *et al.*, 2006, Hood *et al.*, 200). Furthermore, protease inhibitor is down regulated, protease is unregulated and the inactive form of protease converted to the active form of the enzyme. Protease binds its protease receptor on the surface of the membrane or interacts with integrins (Werb, 1997, Sporn, 1996). High levels of active MMP-2 have been associated with metastasis and the aggressiveness of PC (Ellenrieder *et al.*, 2000, Koshiba *et al.*, 1998).

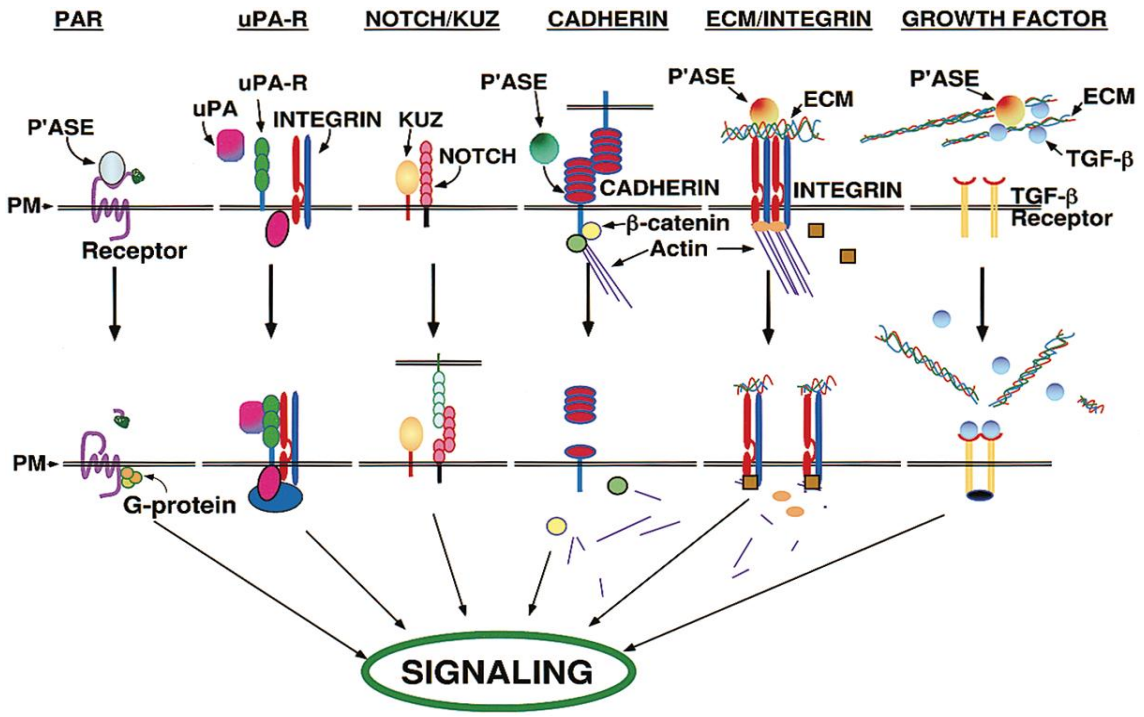

Figure 1.9 Signalling pathways involving in cell invasion (adapted from Werb 1997)

Thrombin stimulates adhesion of PC cells to endothelial cells and extracellular matrix (Rudroff *et al.*, 1998) and also stimulates gelatinase matrix metalloproteinase-2 (MMP-2), which is a collagen type IV degrading enzyme (Page-McCaw *et al.*, 2007), therefore enhancing invasion of the basement membrane. In addition, thrombin mobilises adhesion molecules like CD-40 ligand, P-selectin and αIIbβ3 integrin to the cell surface of endothelial cells. Furthermore, it has been reported that substitution of the three serines of TF the cytoplasmic domain and substitution of key residues of the extracellular domain decrease cell invasion (Bromberg *et al.*, 1999).

TF-FVIIa-PAR2 signalling that is produced by the clotting-dependent mechanisms induces the production of pro-angiogenic factors including VEGF (Albrektsen *et al.*, 2007). VEGF is produced by clotting-dependent mechanisms and by clotting- independent mechanisms through TF-FVIIa-PAR2 signalling regulates $\alpha_{v}B_{3}$ integrin which enhances cell migration and survival when it is associated with VEGFR 2 (Hood *et al.*, 2003, Mahabeleshwar *et al.*, 2006). Furthermore, thrombin mobilises adhesion of molecules like CD-40 ligand, P-selectin and αIIbβ3 integrin to the cell surface of endothelial cells. Moreover, endothelial cells (Haralabopoulos *et al.*, 1997) platelets, tumour cells and the extra-cellular matrix are adhered to each other by thrombin (Maragoudakis *et al.*, 2001).

Fibrin clots coat TF expressing tumour cells. This coat could allow the tumour cells to escape surveillance (Palumbo *et al.*, 2000) and would also aid the adhesion of metastatic cells to the endothelium (Biggerstaff *et al.*, 1999), leading to colonisation of a new site in the body. Metastasis will start when malignant cells adapt to the microenvironment away from the primary tumour cells (Gupta and Massague, 2006). Furthermore, vascular permeability is increased in cancer to cover the nutritional demands (Utoguchi *et al.*, 1996) facilitating blood and lymphatic vessel invasion (Ellenrieder *et al.*, 1999, Fig. 1.10).

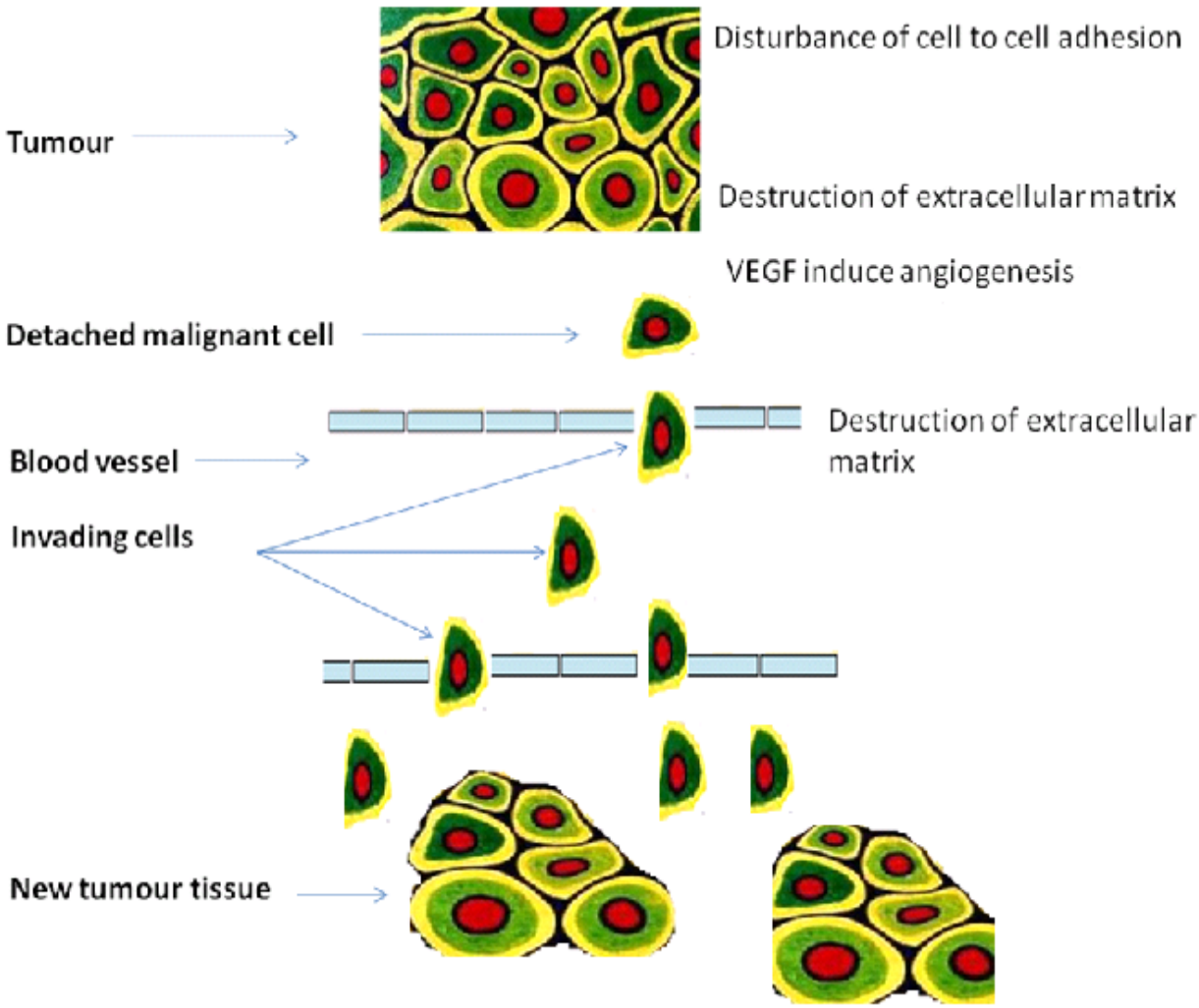

Figure 1.10: Schematic representation of the process of cell invasion (adapted from Martin and Jiang 2009).

In summary, the autopsy, epidemiological and clinical studies identified pancreatic cancer as one of the most highly invasive and angiogenic cancers. There is a significant correlation between pancreatic cancer and TE. Furthermore, the incidence of thrombosis significantly correlated with TF bearing MP. However, the link (s) between cell invasion, angiogenesis and haemostasis are still unclear. The hypothesis of the thesis is that factors directly attributed to the cancer promote the observed pathophysiology and that the removal of the tumour should result in reversal of these abnormalities.

1.19 The Aims of the Study

1- To optimise and standardise the methodology for the clinical part of the study.

2- To study the expression levels of angiogenic soluble markers pre-and post-operatively in patients with operable PC.

3- To study whether increased levels of these soluble angiogenic markers are associated with *in vitro* motility and chemotaxis of cancer cells (cell invasion assays) or increased haptotaxis of endothelial cells (tubule angiogenesis assays). Also to correlate the cell invasion with angiogenic results to see how these change.

4- To study the levels of pre-operative MP in plasma by flow cytometry and investigate changes to these (quantitative) before and after surgery and correlate the results with cell invasion and angiogenesis.

5- To evaluate the procoagulant activity of PC patients pre- and post-operatively and correlate the results with TF-bearing MP.

6- To correlate serological findings with immunohistochemical parameters such as VEGF, VEGFR1, VEGFR2, TF, EGF and EGFR.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials

All the reagents and chemical used in this thesis were of cell culture grade as appropriate.

2.1.1 Reagents

Reagents were obtained from the companies described in Table 2.1

Table 2.1: Materials used in the study.

Companies	Reagent	Catalogue Number
abcam plc, Cambridge, UK	Goat F (ab)2 Poyclonal (Po) Antibody (Ab) anti Rabbit IgG (Biotin) Rabbit PoAb to human CD-34 Rabbit PoAb to human EGF Rabbit PoAb to human EGFR Rabbit PoAb to human TF Rabbit PoAb to human VEGF Rabbit serum (sterile)	ab6111 ab64480 ab9695 ab2430 ab62251 ab46154 ab7487
AbD Serotec Ltd., Oxford, UK	Mouse anti-human CD-31: FITC Mouse anti-human CD-42b: RPE Mouse anti-human CD-14 : RPE Mouse anti-human tissue factor: FITC Mouse IgG1 Negative control: FITC Mouse IgG1 Negative control : RPE Rabbit anti mouse HRP Rabbit anti rat FITC	MCA1738F MCA740PE MCA2185PE MCA2548F MCA928F MCA928PE STAR13B STAR17B
American diagnostica Inc, Stamford, USA	FITC mAb mouse anti-human tissue factor Rabbit PoAb anti-human tissue factor IgG (1 mg)	4507CJ 4502
Aniara, Mason, Germany	Zymuphen MP-Activity	A521096
ATCC, Teddington, UK	AsPC1 CFPAC1 MIA-PaCa-2 Iscove's Modified Dulbecco's Medium	CRL-1682™ CRL-1918™ CRL-1420™ 30-2005
AXIS-SHIELD diagnostics Ltd, Dundee, UK	Gluteraldehyde (70%) Tween®20	G7776 P1379
BD Bioscience, Oxford, UK	BD Matrigel™ invasion chamber 24- well plate 8 Microns. Collagen type I	354480 354236
Biocytex, Marseille – France	Megamix beads	7801

BD Pharmingen ^{TM}, Oxford, UK	PE Annexin V apoptosis detection kit I	559763
Biosera East, Sussex, UK	Foetal Bovine serum	S1870
Caltag Laboratories, Buckingham UK	Caltag Counting Beads	PCB-100
Dade Behring, Milton Keynes, UK	Dade ^{R} Innovin	B421-50
eBioscience, Hatfield, UK	Human anti-annexin V platinum ELISA	BMS247 TEN
GE Healthcare, Bucks, UK	Chemiuminescent (ECL western blotting detection reagent and analysis)	RPN2106
GIBCO Invitrogen Corporation, Paisley UK	MEM 10x with + Earle's with -L-Glutamine and $NaHCO_3$	21430
Helena Bioscience Europe, Tyne and Wear , UK	Normal-Trol 1 Coagulation Control	5186
Hyphen BioMed, Versailles, Germany	Zymuphen MP-Activity	521096
Invitrogen Ltd, Paisley, UK	AccuCheck counting beads for count & pipetting accuracy	PCB 100
National diagnostics, Georgia, USA	Histoclear II	HS-200
PAA the cell culture company, Yeovil, Somerset, UK	Bovine serum albumin DMEM High Glucose Without L-Glutamine HEPES Buffer Solution (1M) L-glutamine Medium 199 With Earle's Salts and L-Glutamine Penicillin/Streptomycin 100X RPMI 1640	K45-001 E15-009 S11-001 M11-004 E15-834 P11-100 E15-840
Pharmacia Ltd., kent, UK	Fragmin	P10229
Promega Corporation, UK	CeII Titer 96 Aqueous One Solution Cell Proliferation Assay	G3581
Promo Cell, Heidelberg, Germany	Endothelial Cell Growth Medium(MV) Free Freezing Medium (Cryo-SFM) HDMEC Hepes Buffer Saline HUVEC Trypsin/EDTA Trypsin Neutralising SolutionTNS	C-22020 C-29910 C-12210 C-40010 C-12200 C-41010 C-41110
R&D system Europe, Ltd. Abingdon UK	Human angiogenesis array (proteome profiler^{TM} antibody arrays) Human leptin immunoassay Human VEGF immunoassay Human VEGF R1/Flt-1	ARY007 DLP00 DVE00 AF321

	Human VEGF R2/KDR/Flk-1 antibody Monoclonal anti h-VEGFR-RPE Mycoplasma detection kit TF ELISA TFPI ELISA	AF357 FABSP3P CUL001B DCF300 DTFP10
Scientific laboratory supplies LTD	BD cell falcon™ culture insert	353097
Sigma-Aldrich Company, Poole, UK	Accustatin® Harris (Haematoxylin solution) Bovine serum albumin Collagen, type IV; from human placenta, Acid Diamino benzidin (DAB) Dimethyl sulphoxide (DMSO) Ethanol Magnesium sulphate Methanol Sodium Chloride (NaCl) Potassium bicarbonate Sodium pyruvate Soluble Phosphate Buffered Saline Trypan blue solution (0.4%) Trypsin	HHS A2153-50G S3014 D4293-50SET D2650 32221 M7506-500G 24229 C5533-5MG P9144-500G S8636 P4417-100TAB T8154 T7168-50TAB
Trizma Sigma, Poole, UK	Tris	T 1503
Vector laboratories, Inc., Burlingame U.S.A	Antigen unmasking solution Avidin/Biotin Blocking kit Biotinylated anti-goat IgG (H+L) TMB Substrate kit for peroxidise Vectostatain ABC kit	H-3300 SP-2001 BA-9500 SK-4400 PK-6200
VWR International, Leicestershire, UK	Calcium chloride 2-hydrate Methylene blue	100704Y 3019270

2.1.2 Equipment

Equipment and Materials used are described in Table 2.2.

Table 2.2: Equipment used in the study.

Companies	**Equipment and Materials**
Axis-Shield Diagnostics Ltd,	Cuvettes 500 pieces Gluteraldehyde (70%) Steel balls
BD Bioscience	25 cm^2 flasks with red cup 75 cm^2 flasks with red cup 150 cm^2 flasks with red cup Flow cytometry (FACSCaliur™), CellQuest software version 3.3 24-well plates 8 µm pores Boyden chambers Plasma tubes Serum tubes
BD Vacutainer, Franklin Lakes, UK	Serum tubes Sodium citrate tube
Biotek Instrument, Winooski, USA	ELISA plate reader
Epi Chemi II,Cambridge, UK	Transmission mode scanner / UVP gel doc system / Darkroom Analysed on Labimage Aquisition and software/ version 4.6 / Cambridge, UK)
ELWD 0.3 T1-SNCP- Nicon JP	Imaging RTTIGA 2000R, Camira attached to Leitz Labrluxs
Heraeus centrifuge, Buckinghamshire, UK	Centrifuge
Fishe Thermo Scientific,Roskilde, DK	Freezing container (Mr Frosty)
Progen, Mexborough, UK	Gen Fuge Ultracentrifuge
Sarstedt, Leicester, UK	25 cm^2 flasks with yellow cup 75 cm^2 flasks with yellow cup Tissue Culture Plate 24-Well Flat Bottom with Lid
Sartorrius stedim, biotech, Aubagne, FR	0.10 µm filter
Scientific laboratory supplies Ltd, East, Yorkshire, UK	BD falconTM cell culture insert pH meter
Thermo Shandon Ltd, Cheshire, UK	Sequenza rank
Weber Scientific International Ltd., Teddington, UK	Haemocytometer

2.2 Methods

2.2.1 Culture of the Cell Lines

All experiments and procedures were carried out in a sterile environment in a class II biological safety cabinet. Ethanol [70% volume/ volume (v/v)] was used for cleaning all surfaces before commencing work. Only sterile plastics were used and all media were pre-warmed by placing in a 37 degree Celsius (^{o}C) water bath for 30 minutes before use.

2.2.2 Endothelial Cell Lines Used in the Study

A panel of cell lines were used in this study.

2.2.2.1 ECV 304

2.2.2.1.1 Maintenance and Adaptation to Serum Free Medium

ECV 304 cells are a human bladder cancer cell line initially used as a model for studying endothelial angiogenesis. ECV 304 is a cell type characterised by a typical cobblestone monolayer growth pattern and a high degree of proliferation without requirement of any specific growth factors (Takahashi *et al.*, 1990). ECV 304 has been used previously to provide insight into the mechanisms governing angiogenesis under both physiological and pathological conditions (Hughes, 1996). ECV 304 was used in this study to evaluate methods of angiogenesis assay as these cells have some similarity to endothelial cells. ECV 304 cells were kindly supplied by Dr C Ettelaie (Department of Biological Science, University of Hull).

ECV 304 cells were cultured in M199 medium supplemented with foetal bovine serum [FBS, 10% volume/volume (v/v), 100 units/ml penicillin 1% weight/ volume (w/v), 100 µg /ml streptomycin (1% w/v) and 2 mM/L-glutamine]. These cells were incubated in a humidified environment at 37 ºC; in the presence of 5% CO_2 (the medium was replaced every 2-3 days). ECV 304 cells were propagated as a monolayer until approximately 95% confluent and then the cells were scraped and sub cultured at a ratio of 1:3. In all experiments, cells were treated with test reagents at 70% confluence. For angiogenesis experiments, the cells need to be adapted to serum free medium by progressive reduction of the serum concentration. The medium was replaced with M199 medium supplemented with FBS (5%; v/v) and antibiotics as listed previously for 48 hours (h) at 37 ^{o}C / 5% CO_2. Finally, cells were cultured in 2% (v/v) FBS plus

antibiotic at 37 °C / 5% CO_2 for 48 h. Then cells were washed three times with phosphate buffer saline (PBS) and transferred to serum-free medium (SFM) and incubated for 24 h, at 37 °C / 5% CO_2 before harvesting.

2.2.2.1.2 Passaging

The medium was removed from the flask and washed with 5 ml of PBS, to remove traces of remaining serum. Trypsin/Ethylene Diamine Tetra Acetic acid (Trypsin/EDTA; 4 ml) was added to the flask which was then incubated for 3 minutes to allow the detachment of cells. Complete medium was then added (4 ml) to neutralise the trypsin. The suspension was centrifuged at 320 *g* for 5 minutes, and the supernatant discarded. The cell pellet was resuspended in an appropriate volume of medium and the cell density determined using a haemocytometer. Cell suspension (10 µl) plus 10 µl of 0.2% (w/v) trypan blue stain were mixed together and 10 µl of mixture were loaded onto the haemocytometer. The concentration of cells per ml was determined using the average total cell count (colourless and blue colour cells) multiplied by 2 x 10^4. The cell viability was determined per ml using the average of colourless cell count multiplied by 2 x 10^4. ECV 304 cells were used at passage 10.

2.2.2.1.3 Storage and Recovery of ECV 304

Cells taken from the flask were centrifuged (320 *g* for 5 minutes), the supernatant was aspirated and the cells re-suspended in complete M199 medium supplemented as described in 2.2.2.1.1 with the addition of 10% (v/v) Dimethyl Sulphoxide (DMSO). One ml of the medium containing 0.5 x 10^6 of cells was kept in 1.5 cryovials labelled with ECV 304 cell type, number and date. The vials were transferred into a freezing container (Mr Frosty) and placed into a -80 °C freezer overnight. It is critical to get a repeatable -1 °C/minute cooling rate, which is required for successful cell cryopreservation and recovery. This was achieved by adding 100% isopropanol to the freezing container. After at least 24 h, vials were transferred to liquid nitrogen and the location was recorded. This methodology has been used by many researchers (Meads and Schroer, 1995, Ihrke *et al.*, 1993, Cassio *et al.*, 1991). When required, cryovials containing the cells were thawed by placing them in a 37 °C incubator. Once defrosted, the cells were transferred immediately to pre-warmed 199 M medium, washed three times to removes traces of DMSO and seeded out into a 25 cm^2 cell culture flask at the required density.

2.2.2.2 Human Dermal Microvascular Endothelial Cells (HDMEC)

2.2.2.2.1 Maintenance and Adaptation to Serum Free Medium

HDMEC are isolated from the dermis of juvenile foreskin and adult skin (Kubota *et al.*, 1988). Since the dermis contains blood and lymphatic capillaries, HDMEC comprise blood and lymphatic micro vascular endothelial cells. HDMEC were purchased from Promo cell. A small sealed flask (25 cm^2) was obtained from the Promo Cell Company. On arrival the flask was incubated for 2 h at 37 ^oC (the lid remained completely closed). The cells were maintained in endothelial cell growth medium (MV type) supplemented with 2% (v/v) FBS, 10 ng /ml EGF provided by the cell provider and antibiotics at the same concentration as described for ECV 304. All HDMEC were used as flasks and became approximately 95 % confluent below passage 6. A similar strategy to that described for ECV 304 was used; the FBS and EGF were depleted to 1% and 5 ng/ml respectively for 48 h and then to 0.5% to 2.5 ng/ml respectively for the next 48 h to adapt them to SFM.

2.2.2.2.2 Passaging

The flask was carefully opened in a class II biological safety cabinet, the medium aspirated and the cells washed with 2 ml of Hepes buffered saline. Trypsin/EDTA solution (2ml) was added for 3-5 minutes, then the trypsin was inactivated by adding 2 ml trypsin neutralising solution. The cells were re-suspended and centrifuged at 320 x*g* for five minutes. The pellet was re-suspended and the cells transferred to a 75 cm^2 yellow cup cell tissue culture flask made from high-quality polystyrene with a positively charged growth surface, created by imitating the amine functional group of proteins. HDMEC were used at passage 5.

2.2.2.2.3 Storage

The old medium was aspirated; the cells were washed with Hepes buffered saline (3 ml), then pre-warmed trypsin/EDTA (3 ml) was added for 3-5 minutes and trypsin neutralising factor (3 ml) was added to neutralise enzyme activity. The cells were pelleted at 320 x*g* for five minutes, and then were resuspended in Cryo-SFM (1 m). Cryovials with HDMEC were labelled as described for ECV 304. Cryo-SFM is a commercial formulation for cryo-preservation of animal and human cells; it contains DMSO, methylcellulose and SFM. Long term freezing and storage

were as described in 2.2.2.1.3. The density and viability were determined as described for ECV 304.

2.2.3 Pancreatic Cell Lines

2.2.3.1 Maintenance and Adaptation of AsPC1 to Serum Free Media

AsciticPC cells-1 (AsPC1) are PC cells derived from asciticfluid (Chen *et al.*, 1982). AsPC1 cells were purchased from American Type Cell Collection (ATCC). AsPC1 cells were maintained in RPMI 1640 medium supplemented with Hepes buffer [final concentration 10 nanomolar (nM)]; sodium pyruvate [final concentration 1millimolar (mM)]; 2 mM/L-glutamine, FBS and an antibiotic with concentration as described for ECV 304 (section 2.2.2.1.1). A similar strategy for adaptation to SFM was followed to that described for ECV 304. For Hepes buffer and sodium pyruvate, the final concentration was depleted to 5 nM and 0.5 mM respectively for 48 h. It was then depleted to 2.5 nM and 0.25 mM respectively in the following 48 h. Other steps were identical to those followed with ECV 304. AsPC1 cells were used at passage 10.

2.2.3.2 Maintenance and Adaptation of CFPAC1 to Serum Free Media

Cystic Fibrosis Pancreatic Adenocarcinoma-1 cell line (CFPAC1) is derived from the liver (McIntosh *et al.*, 1988). The original source of these cells was from a patient with cystic fibrosis and adenocarcinoma of pancreas. CFPAC1 cells were purchased from ATCC. CFPAC1 cells were maintained in Iscove's modified Dulbecco's medium supplemented with FBS and antibiotic as described for ECV 304 (2.2.2.1.1). The growth of CFPAC1 was relatively slow when the cells were cultured in a red cup flask, i.e. the ordinary flask, but when yellow cup tissue culture flasks were used, the growth of CFPAC1 was substantially faster. SFM adaptation of CFPAC1 was similar to that described for ECV 304 (section 2.2.2.1.1). CFPAC1 cells were used at passage 10

2.2.3.3 Maintenance and Adaptation of MIA-PaCa-2 to Serum Free Media

The MIA-PaCa-2 cell line is a cell line from primary pancreatic tumour. MIA-PaCa-2 cells were maintained in Iscove's Modified Dulbecco's Medium supplemented with 2.5% (v/v) horse serum, FBS and antibiotics as described for ECV 304 in section 2.2.2.1.1. The growth of MIA-PaCa-2 was similarly slow when ordinary flasks (with red cup) were used. Therefore, cells were

transferred to yellow cup tissue culture flasks for the same reason as described in 2.2.2.2.2. MIA-PaCa-2 cells were used at passage 10.

Enzymatic detachment of adherent cells, determination of cell density and subculture of CFPAC1 and MIA-PaCa-2 cells were as described for ECV304 (section 2.2.2.1.2). For horse serum, the final concentration was depleted to 1.5% for 48 h. It was then depleted to 0.5% v/v for 48 h. Other steps were similar to those followed with ECV 304 for SFM adaptation.

2.2.4 Mycoplasma Testing

The principle of this technique is detection of hybridisation of 16S ribosomal RNA. The eight most common mycoplasma types are *M.orale, M.pirum, M.hominis, M.salivarium, A.laidlawii, M.hyorhinis, M.arginini, M.fermentans* in the cell culture supernatant with biotin-labelled capture oligonucleotide probe and dioxigenin-labeled detection probes. The solution containing rRNA/probe hybrid was then added to streptavidin-coated microplate and the rRNA/probe hybrid was captured. The plate was washed to remove unbound material. An anti-digoxigenin alkaline phosphatase conjugate, substrate solution and amplifier solution were then added and the colour developed in proportion to the amount of mycoplasma rRNA. Cell culture supernatant was collected from cell lines over a period of five weeks. Samples from the medium of each cell line were collected weekly and kept in a freezer at -80 ^{o}C. The manufacturer's method was carried out as follows: all reagents were brought to the room temperature. Concentrated (10X) Cell lysis diluent was diluted with sample diluents to form 34 ml of Cell Lysis Diluent. Capture probes (1 ml) were mixed with detection probes (1 ml) and sample diluent (4 ml). Wash buffer concentrate (10X) was diluted with distilled water to form 1L of wash buffer. Lyophilized substrate was reconstituted with substrate diluents to form the working substrate solution. Lyophilized amplifier was reconstituted with 6 ml of amplifier diluents to form the working amplifier solution.

The hybridisation plate was washed with wash buffer twice and the excess wash buffer was removed by inverting the plate against clean paper towel. Diluted probes (50 µl) were added to the designated wells. A hundred and fifty µl of positive control (in triplicate), negative control (fresh medium, in triplicate) and samples (duplicate) were added to designated wells. A float collar was applied to the hybridisation plate and the plate was incubated at 65 ^{o}C for 60 minutes in a water bath. The streptavidin plate was washed twice and the excess wash buffer was

removed by inverting the plate against clean paper towel. Then, 150 µl from each well of the hybridisation plate was transferred to the washed streptavidin plate and incubated for 60 minutes at room temperature, on a horizontal orbital shaker set at 500 rounds / minute. Finally the plate was washed four times with wash buffer.

Anti-digoxigenin conjugate (200 µl) was added to each well and the plate was covered with a new sealer; the plate was incubated for 60 minutes on the shaker at room temperature. The plate was washed six times with wash buffer and the excess buffer was removed as described before. Substrate solution (50 µl) was added to each well and the plate was incubated for 60 minutes on the shaker at room temperature. Then, amplifier solution (50 µl) was then added to each well and the plate covered with a new sealer and incubated for 30 minutes on the shaker at room temperature. Stop solution 2 N sulphuric acid (50 µl) was added to halt the reaction and the optical density was measured using an ELISA reader at 490 nm wave length. A correction reading was measured at 650 and subtracted from the 490 reading to correct for optical imperfections in the polystyrene micro-plate.

2.2.5 Flow Cytometry Detection of Growth Factor Receptors

For detection of growth factor receptor, the same principles were used to detect receptors on all cell types. The cells were centrifuged at 320 x*g* for five minutes and washed three times in PBS/bovine serum albumin (BSA)/Azide. At a concentration of 2 x 10^6 /ml, an aliquot (100 µl) of the cell suspension was mixed with test antibody or negative control for 30 minutes. For the experiments with unlabelled test antibodies, samples were washed three times and then secondary fluorochrome conjugated antibody was added, mixed and incubated at dark for 30 minutes. Labelled (fluorescent) test antibodies were added and incubated in the dark for 30 minutes. The cells were then washed three times as previously described to remove excess of unbound antibody. Finally, the cells were re-suspended in 300 µl of PBS/BSA Azide and were analysed by flow cytometry. Antibodies used for detection of TF, VEGFR 1-3, EGFR and their negative controls are summarised in Table 2.3.

Table2.3: Antibodies used for detection of growth factor receptors.

Cell Receptor	Antibody	Negative control
TF	Mouse anti – human TF antibody fluorescein isothiocyanate (FITC) - 1 µg/ml.	Mouse IgG FITC-1 µg/ml-5 µl
VEGFR 1 VEGFR 2 VEGFR 3	Mouse anti-human VEGFR 1, VEGFR and VEGFR 3 respectively Phycoerythrin (PE) labelled- µg/ml-1 µl. Incubation was at 2-8^{o}C	Mouse IgG PE-1 µg/ml-5 µl
EGFR	Rat anti-human EGFR- 5µg/ml-5 µl (unconjugated). Rabbit anti rat (FITC labelled)- 1 µg/ml-5 µl (Secondary conjugated)	Rat monoclonal IgG- 5 µg/ml-5 µl Rabbit anti rat (FITC labelled)- 1 µg/ml-5 µl (Secondary conjugated)

2.2.6 *In Vitro* Model of Angiogenesis

Two-dimensional assay of angiogenesis is a simple and common method that enables *in vitro* study of the formation of capillaries (tubule-like structures). Capillaries develop on an extracellular matrix and are a convenient method for evaluation of the pro-angiogenic activity.

2.2.6.1 Gel Preparation

For reproducibility and optimisation, gel was prepared by two methods.

2.2.6.1. A Method A

All materials were kept on ice. Collagen type I, from rat tails, was mixed with acetic acid (0.02 N) to give 3.41 mg/ml concentration was used as a stock concentration. The gel was diluted (1:8) with cold distilled waster (DW). Further dilutions are not recommended because this decreases the rigidity of the gel. To prepare 1 ml of gel, 10X PBS (100 µl) was added to sterile ice cold NaOH (3 µl) and mixed well using a Whirly Mixer, then 10X PBS/NaOH was added to ice cold DW (771 ml), and the volume was adjusted to 1 ml with diluted collagen type I (1:8, v/v) and mixed thoroughly.

2.2.6.1. B Method B

Collagen type I (0.1% (w/v) in 0.1 M acetic acid), 10 X MEM, neutralised buffer (260 mM Sodium bicarbonate, 1 N sodium hydroxide) in respective proportion 9:1:1. The gel was incubated for 1 h at 37 ^{o}C (Ettelaie *et al.*, 2008, Pradier and Ettelaie, 2008, James *et al.*, 2002, Watanabe *et al.*, 1999).

2.2.6.2 Two-dimensional Assay of Angiogenesis

To set up the assay, the gel (0.5 ml) from either method was added to the wells of 24 well plates and allowed to set at 37 ^{o}C, 5 % CO_2 for 1 h. The cells that were previously adapted in SFM (2 x10^4 cells/100 µl) were seeded sparsely on the top of gel. After 3 h, PC serum (100 µl) and if require 2-10 µl as appropriate, the volume was adjusted to 0.5 ml with sterile PBS. After 3 days incubation capillary formation was observed. The gels were stained after fixation with 3% glutaraldehyde for 15 minutes. This method was used successfully by many previous groups (Ettelaie *et al.*, 2008, Pradier and Ettelaie, 2008, Staton *et al.*, 2004b, Zimrin *et al.*, 1995, Madri and Williams, 1983). Immediate fixation is essential to prevent detachment of capillaries from the matrix due to the release of protease enzymes of the cells.

2.2.6.3 Staining of Capillary Formation

After fixation of the gel, the cells were stained with (0.25 µg/ml) mouse anti human CD-31 antibody for 15 minutes (Pradier and Ettelaie, 2008). The collagen gel was washed twice with PBS 0.05% (v/v) tween 20, then incubated for 1 h at room temperature with rabbit anti-mouse horse radish peroxidase (20 µg/ml). The gel was washed twice with 0.05% (v/v) PBS-tween and incubated with tetramethylbenzidine (TMB) substrate for peroxidase, prepared a maximum of five minutes before use, at room temperature for five minutes. TMB substrate was used to stain membranes allowing visualisation of a blue precipitate. The gel was then observed by microscopy (the number and the length of capillaries were assessed) using an Imaging RETIGA 2000 R camera attached to a LeitZ Laborlux S fluorescence microscope. Images were analysed using the Image – pro plus program (The software is Image pro-plus version 6, Medium Cybernetics). All experiments were carried out in duplicate and 10 fields of high power (x400) view were assessed for each test.

2.2.7 Cell Invasion

2.2.7.1 Types

Two methods were used to assess cell invasion: a BD biocoat™ matrigel™ invasion chamber (Khan *et al.* 2009, Kakkar *et al.*, 1999) and a Boyden chamber coated with collagen IV (Ettelaie *et al.* 2011a, Staton *et al.*, 2004b, Hagan *et al.*, 2003, Miao *et al.*, 2000). The matrigel chamber was covered with a thin layer of basement membrane matrix, mimicking the nature of the *in vivo* basement membrane. The BD matrigel matrix consists of collagen IV mixed with materials extracted from engelbreth-holm-swarm (EHS) mouse sarcoma which contains many pro-angiogenic factors that enhance cell invasion such as heparin sulphate protoglycan, laminin, FGF basic and TGF beta. Therefore, the cell motility does not depend only on the biological factors of malignant pancreatic cells and the chemotaxis effect of angiogenic markers in the sera, but also depends on the effect of those factors in the matrigel matrix.

Boyden chambers coated with collagen type VI were used to evaluate the chemotaxis effect of PC sera without enhancement of the PC cells motility by angiogenic factors. Boyden chambers coated with 50 µl collagen IV [1 mg/ml (w/v)] were placed into a 24-well plate and the excess collagen was then discarded after overnight incubation at 37 ^{o}C in 5% CO_2.

In both methods, pancreatic cell lines (8 x 10^5/ml) adapted previously to grow in SFM as described in sections 2.2.3.1-2.2.2.3, in an aliquot of 250 µl medium were seeded into the upper compartment of each chamber. Medium in the base of the well was supplemented with patients' serum (10% v/v; 250 µl total volume). The plates were incubated at 37^{o}C under 5% CO_2 for 24 h. Following incubation, the medium was removed from the upper compartment of each chamber and the cells on the upper side of the filter chamber were scraped off, using a sterile cotton swap. Non-invasive cells cannot infiltrate the membrane, therefore will remain in the upper part of the membrane while invasive cells can detach from the membrane and pass through the pores (Fig. 2.1). The principles of cell migration have been described by several groups (Ettelaie et al. 2011a, Staton *et al.*, 2004b, Hagan *et al.*, 2003, Miao *et al.*, 2000).

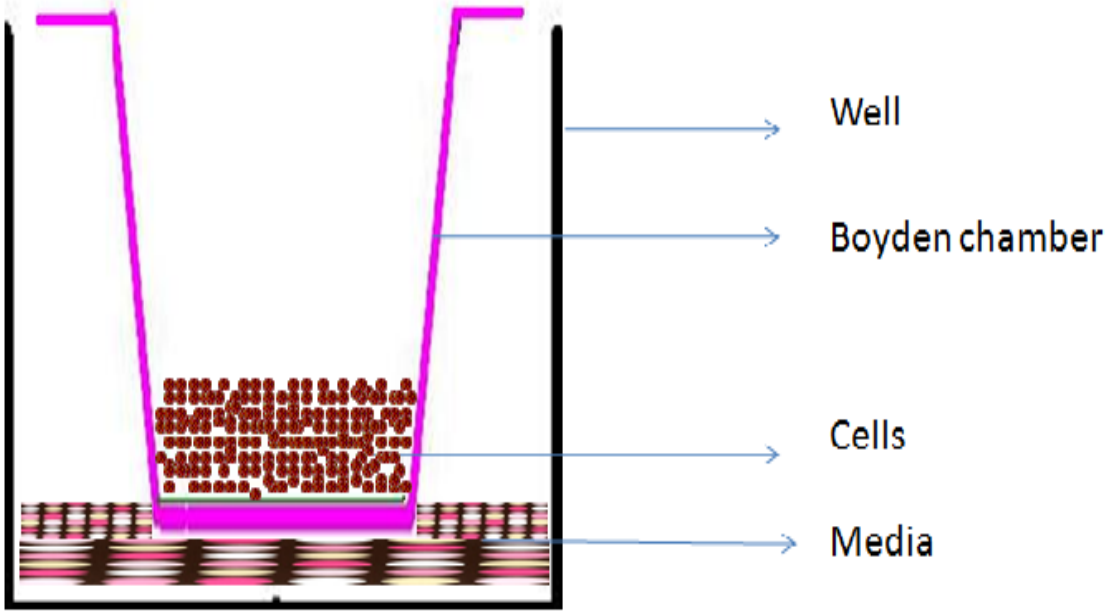

Figure 2.1: Schematic representation of Boyden chamber.

2.2.7.2 Measurement of Cell Invasion

2.2.7.2.1 Cell Titre Aqueous One Reagent (CTAOR) Method

The number of cells that had migrated through the collagen-coated pore was measured by adding CTAOR (40 ml) to the medium in the lower chamber of the plates. A control containing only medium was included. All samples were incubated at 37 °C for 4 h. Finally, 240 µl of each sample was diluted with 360 µl of DW in a plastic cuvette and the absorption values measured against the control samples (known numbers of cells) at 490 nm using an ELISA plate reader. The absorption values were converted to number of cells using a standard curve. CTAOR contains a tetrazolium compound, which is reduced by cells into a coloured formazan product that is soluble in tissue culture medium. The quantity of the formazan product is directly proportional to the number of living cells (Ettelaie, *et al.*, 2011, Maraveyas *et al.*, 2010a).

2.2.7.2.2 Staining Method

Staining was also used to evaluate the cell invasion. The membranes were fixed with 100% ethanol for 2 minutes, stained with methelyne blue for 2 minutes, washed with DW and left to dry. A small drop of immersion oil was placed on the slide, the membrane was put on the slide and then another drop of immersion oil was put on the membrane. The membranes were covered by a cover slip and gentle pressure was applied to expel any air bubbles. Cells were counted in non-overlaping 5 high-power fields of view (x 400) covering the whole membrane. The mean number of these 5 fields was used as a measure of cell invasion. This methodology has been used successfully by many researchers (Tang *et al.*, 2010, Staton *et al.*, 2004a, Koshiba *et al.*, 2000, Kakkar *et al.*, 1999, Miao *et al.*, 2000).

2.2.8 Flow-cytometric Analysis of MP

Blood samples were collected in 5 ml sodium citrate tubes (tubes with a blue top which contain buffer tri-sodium solution and citrate concentration of 0.109 mol/L (3.2%). The details of blood sample collection will be described in section 6.2. Aliquots (50 µl) of platelet poor plasma (PPP) were mixed with target-specific FITC and PE antibodies (1 µg/ml, 5 µl) in polypropylene tubes and incubated at room temperature in the dark for 30 minutes. Filtered PBS (0.1 µm filter) was then added before assay by flow cytometry. An anti-TF conjugated FITC antibody was used to determine the total number of TF bearing MPs; anti-CD-14 PE was used to determine monocyte-derived MP (MoMP), anti-CD-42b PE and anti-CD-31 FITC antibody were added to platelet derived MPs (PMP) and EMP as PMP were positive for both of them and EMP was positive for anti-CD- 31 and negative for anti-CD-42b antibodies. Negative control FITC (mouse IgG) and PE were used to identify the positive expression of the factors mentioned above.

To determine the size of 0.1-1 µm MP, megamix beads (20 µl with 180 µl distilled water; DW) were used. These are a mixture of fluorescent beads with diameter in the range of 0.5 and 0.9 and platelet size that ranged from 0.9-3 µm. This allows the setting of the cytometer to study MPs within a fixed size region and to get reproducible MP counts (Robert *et al.*, 2009, Vince *et al.*, 2009). MP were identified initially according to their scatter properties and further analysed according to provenance into MP by selective antibody labelling measured by flow cytometry. A known volume of counting beads was mixed with the same known volume of PPP. Since the concentration of MP was unknown, the number of MP/µl (the absolute count) was obtained by

relating the number of MP counted to the total number of fluorescent bead events. The MP number was then multiplied by the number of total fluorospheres per unit of volume.

For annexine MP, Two aliquots (25 µl) of PPP were mixed with target-specific PE Annexin V antibody (1 µg/ml, 5 µl) in polypropylene tubes and incubated at room temperature in the dark for 30 minutes, 1X annexin V binding buffer (400 µl) and counting beads (25 µl) were added to one tube (test) and filtered PBS (400 µl) was added to another (control) before assay. Megamix beads were used to identify the site of MP and counting beads were used to count the absolute number of MP as described in section 2.2.8.2.

2.2.9 Measurement of One Stage Prothrombin Time

One stage prothrombin time (PT) time assay can be defined as a measurement of the time in seconds (S) which is required for a plasma sample to clot using a coagulometer. In the current study a modified one-stage PT assay was performed using a Thrombotrank SOLO Coagulometer. A modified one stage PT assay was used in this study as described by other groups (Ettelaie *et al.*, 2008, Ettelaie *et al.*, 1998).

TF concentration used for measurement of samples PT was determined according to the TF concentrations that produce CT of 90 ± 10 when NormTrol control plasma (which has all the clotting factors except TF) was added. For the latter experiment, the coagulometer was switched on 15 minutes before starting the experiment to allow the test chambers to reach 37 ^{o}C. The materials were also all equalibrated at 37 ^{o}C prior to addition of the reagents. A steel ball was put in the central cuvette of the coagulometer. $CaCl_2$ (25 mM; 100 µl) and NormTrol control plasma (100 µl) were incubated together at 37 ^{o}C for two minutes in the central cuvette, then the coagulation was initiated by the addition of TF (100 µl, 10 U/ml). Serial dilutions of the recombinant TF (1000-1 U/ml) were carried out and the PT assay was performed on several dilutions. The experiments were repeated three times for each dilution to generate a standard curve. A CT of 90 S was recorded when 10 U/ml of TF was used and this level of TF was used as standard when PT of test samples was measured.

The samples as described previously were collected in 5 ml sodium citrate tubes (Blue lids). The PT was performed within 4 h of venesection to be sure the integrity of TF-bearing MP. PT assay was performed when blood samples filled the vacuntainer completely as marked on the tube and, which indicates an accurate 1:9 dilution of one part sodium citrate to 9 parts of the blood. One stage PT relies on the activity of the recombinant tissue factor to initiate the coagulation mechanism, whereas the low activity of the 10 U/ml TF allows the values to be altered by the presence of any TF in the added samples. Furthermore, adding of 10 U TF allows the clotting quicker. The preparation of the coagulometer was performed as described in the measurement of CT with NormTrol control plasma. Fresh PPP (100 µl) and recombinant TF (100 µl, 10 U/ml) were added to the central cuvette and incubated at 37 ^{o}C for two minutes, then coagulation was initiated by the addition of 25 mM $CaCl_2$ (100 µl). The experiment was repeated three times for each sample and mean of these readings was recorded.

2.2.10 Human Angiogenesis Array (proteome profiler^{TM} antibody arrays)

2.2.10.1 Principles

The measurement of levels of expression of angiogenic markers is a key step to understanding the role of these markers in angiogenesis. This was done using serum from PC patients using a human angiogenesis array kit. Detection of relative expression of 55 angiogenic markers simultaneously was achieved with this kit. It is a rapid and economical method when compared with other methods such as ELISA, in which the protein expressions are assessed individually; however the latter would give quantitative values. Captured antibodies were spotted in duplicate on nitrocellulose membrane. Diluted serum was mixed with a cocktail of labelled of antibodies specific for angiogenic factor and then incubated with nitrocellulose membrane which contained capture antibodies to bind the antibody/antigen complex. Streptavidin-HRP and chemiluminescent (ECL, which is a western blotting detection reagent and analysis) reagents were added to visualise binding. Bound proteins are detected by the light emitted, which is proportional to the amount of antibody/antigen complex captured from the samples.

The human angiogenesis array kit contains four nitrocellulose membranes; each membrane contains 55 antibodies, six identical positive control antibodies containing protein standards and two negative controls. All antibodies are spotted in duplicate. The membranes were handled with gloved hands and flat-tipped tweezers to avoid contamination. The detection antibody

cocktail was reconstituted with 100 µL of DW. The 25X wash buffer was diluted to 1X wash buffer (40 mL of 25X wash buffer was diluted with 960 ml of distilled water). Array buffers 4, 5 and 7 were ready for use. Streptavidin-HRP was diluted in 1:2000 with buffer 5. The developer and fixative were diluted with distilled water 1:4. All assay reagents were brought to room temperature and the patients' samples were thawed and kept on ice before use.

2.2.10.2 Method

Array buffer 7 (2 ml) was added into each well of the four-well multi-dishes for blocking of non specific protein reaction and incubated for one hour on a rocking platform. At the same time, serum (100 µL) was added to 0.5 mL of array buffer 4 and the volume was adjusted to 1.5 mL with array buffer 5 (i.e. adding 0.9 mL of array buffer 5), then 15 µL of reconstituted labelled antibody cocktail was added to the mixture and incubated for one hour at room temperature. Buffer 7 was aspirated from the wells and the sample/antibody mixture was added to the membrane. The lid was put on the plate, which was then incubated overnight at 2-8 ^{o}C on a rocking platform. The membrane was removed from the well to individual plastic containers and washed three times for ten minutes each. Finally, the membranes were removed and the lower edge was gently blotted on absorbent paper to allow excess buffer to be drained off. The membranes were moved to wash out 4-well multi dish plates and were incubated with 1.5 ml of diluted streptavidin-HRP for 30 minutes. The membranes were moved to individual containers and washed with wash buffer three times for ten minutes for each wash. Again the membranes were carefully removed from the individual containers and blotted on absorbent paper to remove excess buffer.

ECL western blotting detection agents (3 ml) were added to each membrane for three minutes. A transmission mode scanner (UVP gel doc system / Epi Chemi II Darkroom) running on Labimage Acquisition and software/ version 4.6. / Cambridge, UK) was used to detect the light emitted from the spots; the scanner captured four pictures, one picture every five minutes up to 20 minutes. As the time of exposure increases, the density of spots increased (5, 10, 15, 20 minutes). The first picture was chosen for analysis (Fig. 2.2).

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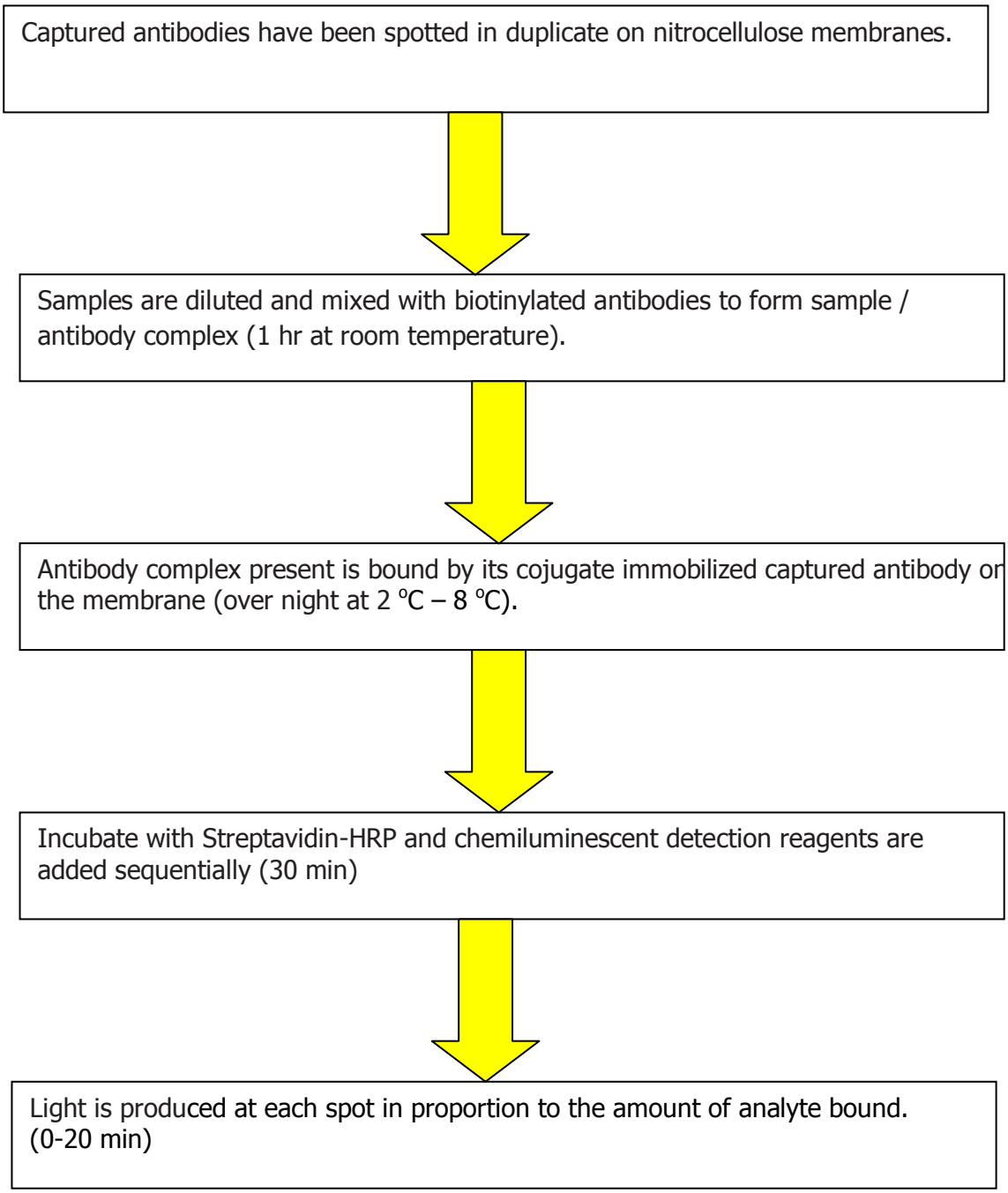


Figure 2.2: Diagrammatic representation of angiogenesis protocol.

2.2.11 MP Activity

To avoid measuring platelet MP PL, frozen PPP samples were used in this experiment. The MP activity kit was removed from 4 ^{o}C storage and kept at room temperature for 30 minutes. Sample diluent (containing FIIa and Xa and Ca^{2++} inhibitor, 380 µl) was added to 20 µl of calibrators, controls and samples. Lyophilised Calibrator was prepared from lysed platelet concentrate and was reconstituted before use in 2 ml of sample diluent. The stock concentration of PS was 2.8 nM. A dilution curve of 2.8, 1.4, 0.7, 0.28, 0.05 and 0 nM was obtained using sample diluent.

Normally prothrombin is converted to thrombin in the presence of calcium, Xa, PL and Va. Bovine Xa, Va, calcium and human prothrombin were added so the reaction depended primarily on the sample PL. The samples (100µl) were then added to the microtitere plate and incubated for 1 h at 37 ^{o}C. The plate was washed five successive times with 50 µl of washing solution. R1 which is bovine FXa-FVa mixture containing calcium (100 µl) and then R2 which contains human prothrombin (50 µl) were added, and then the plate was incubated for 10 minutes at 37 ^{o}C. R3 which is a thrombin specific chromogenic substrate (50 µl) was added, the plate then incubated for three minutes and the reaction was stopped with 3% (v/v) citric acid. The plate was incubated at room temperature for 10 minutes to allow colour stabilisation and then the absorbance was measured at 405 nm using the plate ELISA reader (Fig. 2.1). The samples Xa and IIa were inactivated by the sample diluents. Therefore the limiting factor was PS.

2.2.12 ELISA

2.2.12.1 VEGF ELISA

The plates were coated with monoclonal antibody specific to VEGF. Sera and standards VEGF are then bound with the antibody. Polyclonal antibody and the substrate are then added. The reaction is then stopped and the VEGF concentration which is proportional to the colour intensity was measured.

All reagents were brought to room temperature before use. Wash buffer (20 ml) was diluted with DW to prepare a total volume of 500 ml. VEGF standard was reconstituted with 1 ml of

calibrator diluent to form a stock solution (2000 pg /ml) which was then diluted with calibrator diluent to give a range of standards: 1000; 500; 250; 125; 62.5, 31.2 and 15.6 pg/ml. Assay diluent (100 µl) was added to each well. Standards and samples (100 µl) were added and the plate was incubated for 2 h, followed by three washes with 400 µl wash buffer to remove any unbound VEGF. Then VEGF conjugate (200 µl, a polyclonal antibody against VEGF conjugated to horseradish peroxidase) was added and the mixture incubated for two h. The wash was then repeated 3 times as described before. Substrate solution [colour reagent A (stabilized hydrogen peroxide) and reagent B (stabilized chromogen) were mixed together in equal volumes, 200 µl] was added and incubated for 25 minutes at room temperature in the dark. During this time a blue colour appeared in proportion to the amount of polyclonal antibody. Then stop solution [(2 N sulphuric acid) (50 µl)] was added to each well and the blue colour changed to yellow. The concentration of TFPI was measured using an ELISA plate reader at 450 nm wave length with a 540 nm wavelength correction.

2.2.12.2 TFPI ELISA

The plasma samples were used throughout. Due to a high endogenous TFPI concentration, plasma TFPI required a 100-fold dilution before assay (20 µl of plasma was added to 180 µl of diluents). All reagents were brought to room temperature before use. Wash buffer (20 ml) was diluted with DW to prepare a total volume of 500 ml. TFPI standard was reconstituted with 1 ml of calibrator diluent to form a stock solution (20000 pg /ml) which was then diluted with calibrator diluent to give a range of standards: 2000; 1000; 500; 250; 125; 62.5 and 31.2 pg/ml. Colour reagents A and B were mixed together in equal volumes a few minutes before use.

Assay diluent (100 µl) was added to each well. Standards and samples (50 µl) were added and the plate was incubated for 2 h, followed by four washes with 400 µl wash buffer. Then TFPI conjugate (200 µl, which is polyclonal antibody against TFPI conjugated to horseradish peroxidase) was added and the mixture incubated for one h. The wash was repeated four times as described before. Substrate solution [colour reagent A (stabilized hydrogen peroxide) and reagent B (stabilized chromogen) were mixed together in equal volumes, 200 µl] was added and incubated for 30 minutes at room temperature in the dark. During this time a blue colour appeared. Then stop solution [(2 N sulphuric acid) (50 µl)] was added to each well and the blue

colour changed to yellow. The concentration of TFPI was measured using an ELISA plate reader at 450 nm wave length with a 650 nm wavelength correction.

2.2.12.3 TF ELISA

To evaluate plasma TF, samples were diluted with calibrator diluents (150 µl of plasma + 150 µl calibrator diluent). The TF standard was reconstituted with 1ml of DW to produce a 5000 pg/ml stock solution. The stock solution was diluted with calibrator diluents to form a series of concentrations: 500, 250; 125; 62.5; 31.3; 15.6 and 7.8 pg/ml. The remaining methodology was similar to that with TFPI, except that the standard and sample volumes were 100 µl instead of 50 µl. It has been noted that 1000 U/ml of TF equal 5 ng/ml.

2.2.12.4 Leptin ELISA

The leptin standard was reconstituted with 1 ml DW to produce a stock solution (10000 pg/ml). This was successively diluted to yield concentration of 1000, 500, 125, 31.2 and 15.6 pg/ml. Calibrator diluent served as the zero standard (0 pg/ml). The remaining methodology was similar to VEGF as described in section 2.2.12.1.

2.2.12.5 Human anti-Annexin V platinum ELISA

Annexins are a family of calcium-dependent PL-binding proteins. The *in vivo* function of annexin is still unclear, but it does have a binding site for protein kinase C (Imai *et al.* 1995). Annexin V binds to the PS-exposing apoptotic cells and inhibits the procoagulant and pro-inflammatory activities of these dying cells. The standards reconstituted in 1 ml assay buffer to form 800 ng/ml concentrations, followed by five repeated serial dilutions to form 400, 200, 100, 50, 25, 12.5, 6.3 ng/ml. The remaining methodology was similar to that of TFPI ELISA as described in section 2.2.12.2.

2.2.13 Haematoxylin and Eosin Staining of Paraffin Sections

Paraffin blocks containing pancreatic tumour samples were sectioned (4 µm) in the Department of Pathology in Hull Royal infirmary. The sections were labelled with pencil and placed in a warm histoclear II (60 ^{o}C) pot 1 for 10 minutes for deparaffinization. The sections were then

deparaffinised with the Histoclear II three times using 3 pots of histoclear, for 1 minute each time to ensure that slides were deparaffinised completely. The sections were then rehydrated through graded alcohols 100%, 90%, 70% (v/v) for 2 minutes each, before being transferred into a pot of tap water. The pot was placed under a running tap for 1 minute, without allowing the running water to fall directly onto the sections. The idea behind rehydration was to allow water to enter inside the cells and the latter would react with different types of stain that coloured different cellular architectures (Cook, 2006).

The sections were rinsed under running tap water for 2 minutes. Slides were stained with Harris haematoxylin for 20 S because this formulation contains mercuric oxide, which stains the nucleus a darker blue. The slides were rinsed in running tap water for one minute. Then the sections were differentiated with acid alcohol (10 dips) because acid alcohol removes the excess haematoxylin. The sections were rinsed in tap water and transferred to Scott's tap water substitute for 1 minute to increase the blue intensity. The sections were then counter stained with eosin yellow (0.5% v/v), which stains the cytoplasm a red colour. The sections were rinsed in running tap water for one minute. Then, the sections were dehydrated with 70%, 90% and 100% ethanol for 60 S for each ethanol concentration to get rid of water outside the cells. Then the sections were transferred to three Histoclear II pots (1, 2 and 3) for 60 S before mounting using Histomount (Cook, 2006).

2.2.14 Immunohistochemistry on Paraffin Embedded Sections

Endogenous biotin may be present in these sections. This may cause non-specific background staining, particularly when using a biotin containing detection system. This blocking step is achieved through sequential incubations with avidin and biotin. Normal serum blocking is performed in order to prevent non-specific hydrophobic background staining; an unlabelled protein antibody of the same species as the secondary antibody is used to block the hydrophobic binding sites, which minimises the secondary antibody binding non-specifically.

For protein visualisation, the avidin-biotin complex (ABC) method was used. This method produces a complex with the antibodies which is very stable. Avidin is an egg white glycoprotein which conjugates readily and strongly to biotin which is a vitamin. The primary antibody will be

bound to the target antigen as in the direct technique but then a secondary antibody which has been raised against the primary antibody in a different species of animals will be bound. It is the secondary antibody which has the label attached to it. Advantages of using secondary antibodies include higher sensitivity, because only one primary antibody can be bound to any one antigen but numerous secondary antibodies can be bound to the primary antibody. As the label will be present on the secondary antibody in the indirect method, the intensity of the visualisation will be amplified compared to the direct method. This means that a smaller level of antigen can be detected and this can be achieved with antibody solutions of lower concentration. A further cost saving advantage to the indirect method is that not every antibody needs to be bound in order to visualise the antigens. The avidin-biotin complex can have the label attached to the avidin and the biotin section will bind to the secondary antibody producing an extremely stable complex with high sensitivity (Cook, 2006).

This visualisation method requires a chromogen step. The enzyme which labels the antibodies catalyse a change of the substrate. Diamino Benzidine (DAB) is one of the most common chromogens; it produces a brown end product. The protocol for immunohistochemistry staining was adapted from Cawkwell (1999).

2.2.14.1 De-wax and Rehydrate

The methodology of deparaffinisation and rehydration was similar to that described in section 2.2.13.

2.2.14.2 Endogenous Peroxidase Blocking

The endogenous peroxidase activity was blocked by placing the slide rank into a pot containing 360 ml methanol and 40 ml of hydrogen peroxide (H_2O_2) for 15 minutes. This solution was made fresh each use.

2.2.14.3 Antigenic Site Retrieval

Heat induced-epitope retrieval (HIER) was used for antigen unmasking. During incubation with hydrogen peroxide, the retrieval buffer (1600 ml DW with 15 ml Vector Antigen unmasking solution) was boiled and shaken in a pressure cooker. When the retrieval buffer was boiling vigorously, the slides were added. The lid was fastened quickly and the mixture boiled for three minutes at pressure according to the recommendation of the supplier. The pot was then removed from the heat and run under cold water. The lid was opened when the internal pressure had dropped sufficiently (approximately 2-3 minutes). The sections were rinsed in running tap water as before. Protease induced-epitope retrieval (PIER) was used by incubation of the slides with solution of 1 mg trypsin/ml DW containing 4 mM CaCl2, 200 mM Tris, pH 7.7 at 25°C for 30 minutes. The sections were rinsed under running tap water for 2 minutes. Then slides were transferred to a pot of tris buffered saline (TBS), pH 7.6 for 5 minutes.

2.2.14.4 Normal Serum Blocking

The slides were assembled into the Sequenza ranks by dipping the cover plate in TBS and placing the section faces to cover the plate. The reservoir was filled with fresh TBS and washed for 5 minutes. The sections were incubated for 20 minutes with 3 drops of diluted blocking serum (provided in the Vectorstain). The diluted blocking serum was prepared by adding one drop of stock approximately (50 µl) to 5 ml TBS. The blocking serum was prepared from the species in which secondary antibodies were made.

2.2.14.5 Endogenous Biotin Block

The non-specific binding of the avidin/biotin system reagents was blocked immediately by adding 3 drops of Avidin D solution to the slides and the reagents incubated at room temperature for 15 minutes. The slides were rinsed in TBS for 5 minutes, three drops of biotin solution were added to each section from the same kit, and after this the sections were rinsed in TBS for 5 minutes.

2.2.14.6 Primary Antibody Incubation

The waste was emptied from the bottom of the Sequenza rank. Three drops of diluted primary antibody were added to all except the negative slides. These slides were incubated for one h (or

overnight at 4 ^{o}C if recommended by the supplier) except the negative slides. After the incubation with primary antibody, the slides were rinsed in TBS for 5 minutes. A negative control was included in each batch of slides, in which the primary antibody was omitted and replaced by TBS or 1:5000 dilution of rabbit serum for those antibodies raised in rabbit (VEGF, EGF, EGFR, TF and CD 34) and 1:5000 dilution of goat serum for those antibodies raised in goat (VEGFR 1, VEGFR 2). Primary antibody concentration, diluent, incubation time, and temperature all impact the quality of staining. These variables needed to be optimised for each antibody and sample to achieve specific staining with low background. Often optimisation is approached by maintaining a constant incubation time and temperature, whilst varying the antibody concentration to determine when an optimal signal is achieved with low background noise. For example, if a high affinity antibody was used, then the antibody can be applied at a relatively low concentration for a shorter incubation time. Alternatively, a lower affinity antibody will be applied at higher antibody concentration for a longer incubation time. Longer incubation durations were employed to ensure penetration throughout tissue sections. To minimise non specific staining, longer incubation periods are often conducted at lower temperatures (i.e. 4 ^{o}C versus room temperature). The optimisation of antibodies is summarised in Table 2.4.

2.2.14.7 Antibody Labelling

The slides were incubated with diluted secondary antibody for 30 minutes (provided with the Vectastatin Elite universal kit). For the rabbit anti-human PoAb , two drops (100 µl) of normal blocking serum were added to 5 ml TBS buffer in mixing bottle and then 2 drops (100 µl) of biotinylated stock antibody were added. For the goat anti-human PoAb, biotinylated antibody horse anti-goat was used in 1:150 dilutions.

2.2.14.8 Antibody Visualisation

Further amplification of the signal (Fig. 2.3) was achieved by taking advantage of the strong affinity of avidin and streptavidin to bind biotin. Streptavidin is purified from the bacterium *Streptomyces avidinii*, is not glycosylated, and exhibits lower non-specific binding than avidin. Streptavidin bind four biotins per molecule. Because biotinylated secondary antibody was employed, the signal was significantly amplified by subsequent incubation with an avidin-biotin complex (ABC Method).

During the incubation with antibody labelling, Vectastain Elite ABC reagent (supplied with the kit) was prepared (two drops reagent A to 5 ml buffer in the ABC reagent large mixing bottle). Then two drops of reagent B were added to the same bottle and mixed immediately, and then the mixture was allowed to stand for 30 minutes before use. The sections were rinsed in TBS for 5 minutes. Vectastatin Elite ABC reagent (100 µl) was added to each section and incubated for 30 minutes. The slides were rinsed with TBS for 5 minutes. One "gold" tablet of DAB and one "silver" tablet of urea hydrogen peroxide were added to 5 ml of DW and left to dissolve. Boundaries were drawn around the sections using an isolator pen to keep the reagent covering the section only and prevent spread of the reagent to unwanted areas of the slide. DAB solution was added to the sections (50 µl) for approximately 5 minutes or until the brown colour was sufficiently developed (Fig. 2.3).

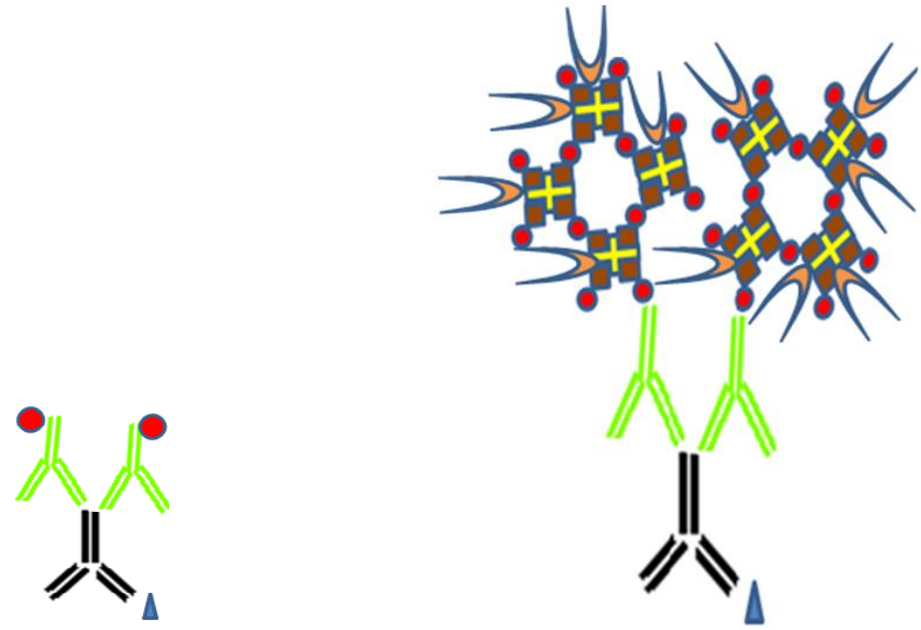
2.2.14.9 Enhance, Counterstain and Differentiate

Slides were stained with Harris haematoxylin for 20 S. The slides were rinsed in running tap water for one minute. The sections were transferred to Scotts tap water substitute for 1 minute to increase the blue intensity.

2.2.14.10 Dehydrate, Clear and Mount

The methodology of dehydration, clearing and mounting was similar to that described in section 2.2.13. Several primary markers were used in this part of this study.

Type, concentration and duration of primary antibody incubation, type and dose of secondary antibodies and the type of antigen retrieval are summarised in Table 2.4.



A B

Figure 2.3: Schematic representation of immunohistochemistry technique, A- Indirect method of imunohistochemistry B- Amplification of the signals using ABC method Antigen , primary antibody , secondary antibody , label , avidin , biotin , diamino benzidine

Table 2.4: Summary of Immunohistochemistry Methodology.

Marker	Primary antibody				Secondary antibody	Antigen retrieval
	Type	Dilution Used	Range	Duration of Incubation	Dilution Used	
VEGF	RPoAB	1:120	1:100-1:200	1 h	1:50	HIER
VEGFR 1	GPoAB	1:20	1:20-1:100	Overnight	1:150	HIER
VEGFR 2	GPoAB	1:20	1:20-1:100	Overnight	1:150	HIER
EGF	RPoAB	1:100	1:500	1 h	1:50	PIER
EGFR	RPoAB	1:30	1:30-1:200	1 h	1:50	HIER
TF	RPoAB	1:50	1:50-1:100	1 h	1:50	HIER
CD34	RPoAB	1:100	1:100-1:500	Overnight	1:50	HIER

Note: [RPoAB: rabbit polyclonal antibody, GPoAB: goat polyclonal antibody, Heat induced-epitope retrieval (HIER), Protease induced-epitope retrieval (PIER)].

2.2.15 Components of Materials used in This Study

1- Acid Alcohol (1% concentration hydrochloric acid in 70% methanol)

Methanol (700 ml)

DW (290 ml)

Concentrated HCL (10 ml)

2- 0.5% (w/v) Eosin Y

Eosin Yellow powder (2.5 g)

DW (500 ml)

3- PBS (isotonic buffer solution, pH 7.3).

1 tablet of PBS

500 ml DW

To form PBS containing 0.14 M NaCl; 0.01 M Phosphate and 0.003 M potassium chloride concentration).

4- PBS/BSA Azide

PBS (1 L)

BSA (2.9 g)

Sodium azide (224 mg)

5- Scotts Tap Water

Potassium bicarbonate (2g)

Magnesium sulphate (20 g)

DW (1 L). (Elangbam *et al.*, 1997)

6- Tris Buffer saline (TBS 20x pH 7.6)

Tris [hydroxymethyl aminomethan] 1 M (121 g)

NaCl 3 M (170 g)

1L DW

pH was adjusted to 7.6 with 80 ml concentrate HCL.

TBS 20x was diluted twenty times to give:

TBS X1 (50 mM Tris, 150 mM)

OPTIMISATION PART

CHAPTER THREE: CELL INVASION

3.1 General Introduction

The following series of experiments was designed to optimise the methodology planned to be used in the analysis of clinical study. Three pancreatic cell invasion experiments were used to evaluate cell invasion and procoagulant activity. A series of stimulation experiments were conducted using extraneous factors to amplify signal. A number of cell invasion experiments were performed to look at the robustness of the assays and the effects of blocking of TF by antibody and LMWH. Different methodologies and different ways of evaluating of cell invasion were investigated.

3.2 Correlation of Tissue Factor Expression and TF Activity with Cell Invasion

3.2.1 Introduction

TF is well known as a principal initiator of the extrinsic pathway of coagulation (Paborsky *et al.*, 1989, Nemerson, 1988, Gouaulthelimann and Josso, 1979) and the relatively high expression of TF on the primary PC is thought to be one of the underlying mechanisms giving rise to the hyper-coagulable state associated with this malignancy (Peppelenbosch and Versteeg, 2001). Therefore, it was postulated that TF expression correlated directly with TE (Khorana *et al.*, 2007) and was associated with an increase of cell invasion and angiogenesis (Nitori *et al.*, 2005, Zhang *et al.*, 1994). The correlation between TF expression and invasion has also been noted with other types of malignancy such as non small cell lung carcinoma (Sawada *et al.*, 1999) and colorectal cancer (Seto *et al.*, 2000). This suggests that the aggressive course, cell invasion and thrombosis may be related to the high expression of TF. In this study three PC cell lines were investigated: MIA-PaCa-2 (low/no TF expression); AsPC1 (low/moderate TF expression) and CFPAC1 (high TF expression).

The one stage PT assay was used to evaluate plasma pro-coagulant activity, dependent on seven coagulating factors [X, VII, V, III (TF), II (prothrombin), I (Fibrinogen), PL, Fig. 3.1]. In the current study, the PT was used to measure the pro-coagulant activity and TF activity of cells.

There are 5 factors [X, VII, V, III, II (prothrombin), I(Fibrinogen)] were present in the fixed concentration within the control plasma but there is no TF. Therefore additional TF present on cells or accelerate clotting. A modified one stage PT for evaluation of procoagulant and TF activity of the cells was used by several researchers (Hobbs *et al.*, 2007, Silberberg *et al.*, 1989).

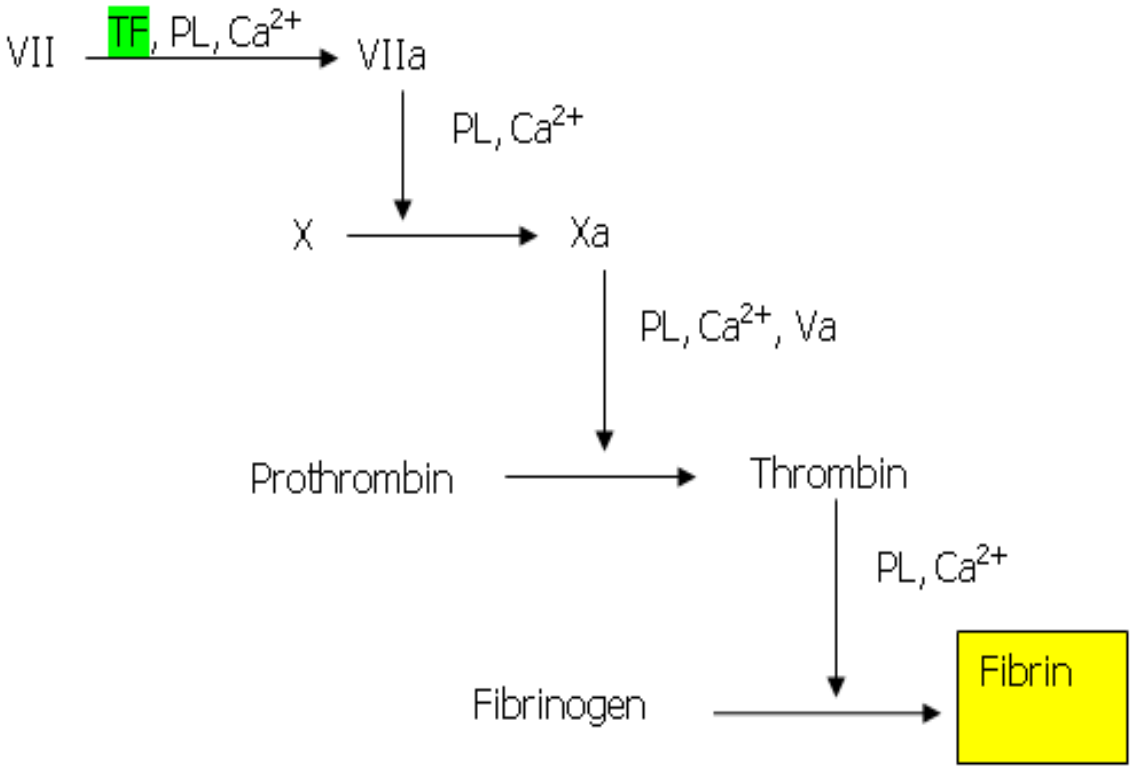


Figure 3.1: Diagrammatic representation of the clotting mechanism . In the assay used in this part of thesis TF is the limiting factor.

The main aim of this study was to evaluate the role of TF expression and activity on PC cell invasion. Before evaluation of the procoagulant activity and cell invasion, all cells were checked for mycoplasma infection and TF receptor expression.

3.2.2 Assay for Mycoplasma Infection

Mycoplasma contamination does not produce visible changes in the medium and resists antibiotics because mycoplasma do not have a cell wall. The incidence of mycoplasma infection in cell culture lines was varied over wide range from 5 up to 94% (McGarrity, 1979). The eight

most common mycoplasma types are *M.orale, M.pirum, M.hominis, M.salivarium, A.laidlawii, M.hyorhinis, M.arginini, M.fermentans*. Mycoplasma and other bacterial infections affect the biological activities and behaviour of the cells. For example mycoplasma infection might change the expression of receptors. Therefore all cell lines were tested for the presence of mycoplasma using a commercial kit which covers the eight most common mycoplasma species listed above.

Cell culture supernatant samples were collected weekly (week 1- week 6) from AsPC1, CFPAC1, MIA-PaCa-2, HDMEC and several other types of cells lines available in the laboratory at the date of experiment and stored at -80 ^{o}C. Samples were analysed as explained in section 2.2.2.7. A representative plate is shown in Fig. 3.2. The mean optical density of six positive controls was 1.7 (yellow box) , the six negative controls (medium only) was 0.6 (black box) and the mean OD of duplicates for week 1-6 culture of AsPC1, CFPAC 1, MIA-PaCa-2 and HDMEC were 0.5 - 0.6 (blue box). The results were considered negative compared to the negative control values (Table 3.1).

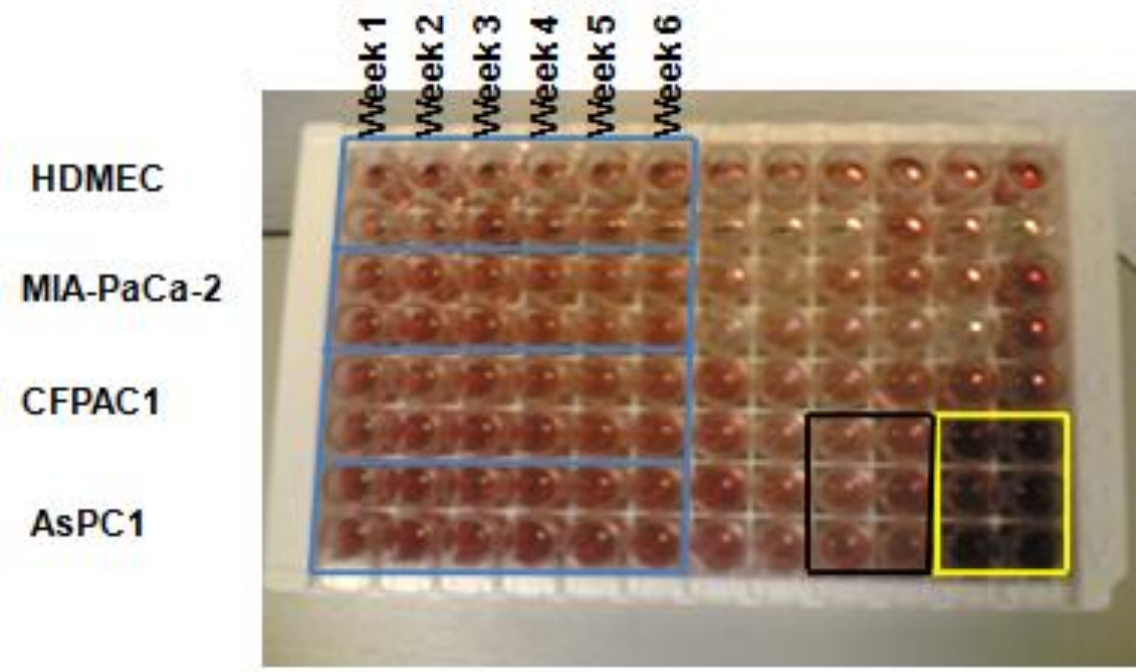


Figure 3.2: Mycoplasma test. Culture medium samples in duplicate from Week1-Week6 (from left - right) were marked with a blue box. Positive controls were repeated six times and marked with a yellow box and negative controls were repeated six times and marked in black. Other cell lines in the laboratory were investigated in other well on the plate.

Table 3.1: Mycoplasma test results.

Sample	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
AsPC1	0.6	0.6	0.5	0.5	0.4	0.4
CFPAC1	0.4	0.5	0.6	0.6	0.6	0.5
MIA-PaCa-2	0.5	0.5	0.5	0.4	0.6	0.6
HDMEC	0.5	0.4	0.6	0.5	0.3	0.6
Positive control	1.7 (mean of 6 positive controls)					
Negative control	0.6 (mean of 6 negative controls)					

Note: The test samples were performed in duplicate, positive and negative controls were performed in triplicate. Average absorbance values are shown.

3.2.3 Growth Factor Receptor Expression of Pancreatic Cell Lines

Cell culture, growth and harvesting of 3 pancreatic cell lines were described in sections 2.2.3.1, 2.2.2.3.2 and 2.2.3.3 respectively. Flow cytometric detection of TF and EGFR was detailed in section 2.2.5. The highest TF expression was noted on the CFPAC1 and no TF expression on MIA-PaCa-2. There was slight TF expression on AsPC1 (4%). Furthermore, the highest EGFR expression was noted on CFPC1, followed by AsPC1 and there was slight expression of TF on MIA-PaCa-2 (Fig.3.3).

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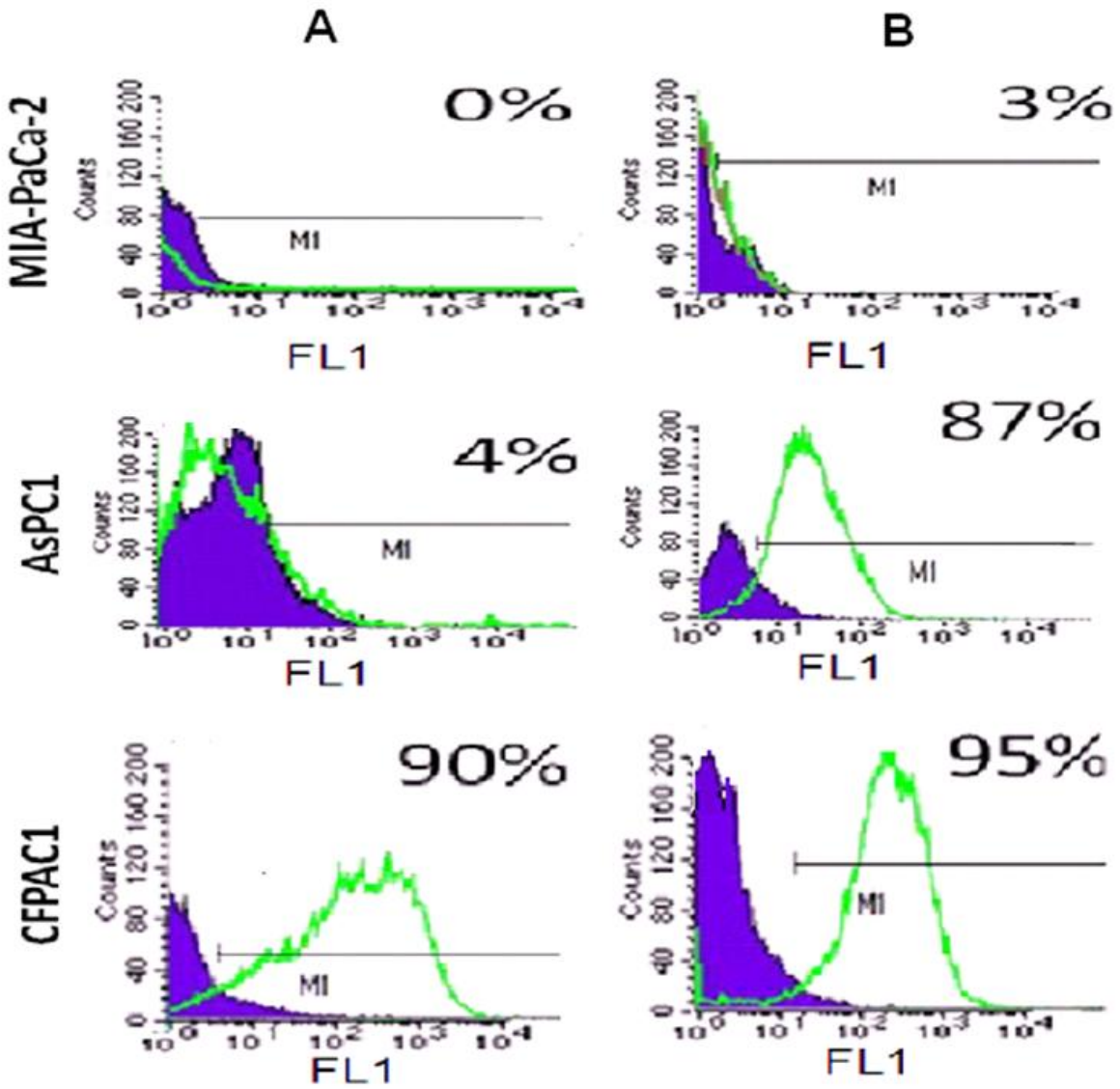

Figure 3.3: Expression of TF (A) EGFR (B) on MIA-PaCa-2, AsPC1 and CFPAC1. Data are representative of three repeats. Blue colour was the negative control. Green colour was the EGFR and TF

3.2.4 Methodology of Procoagulant Activity Cell Invasion

Aliquots of PC cells ($2x10^5$) in 100 µl of PBS were incubated at 37 ^oC with and without 5µl (5 µg/ml) of rabbit PoAb anti human TF for 20 minutes. These aliquots of treated cells were subsequently used in clotting and cell invasion experiments. The one stage PT assay was used to measure the TF activity of PC cell lines using an Axis shield coaguolometer. The limiting factor in the assay was TF on the surface of the cells; more TF expression will lead to shorter CT. The coagulometer and the materials were equilibrated to 37 ^oC before starting the experiment. A steel ball was put in the central cuvette of the coagulometer and mixed with the cell lines and $CaCl_2$ (100 µl, 25 mM). Clotting was induced by the addition of NormTrol control plasma (100 µl), and then CT was recorded.

For invasion experiments, a BD Biocoat^{TM} Matrigel^{TM} Invasion Chamber was used to evaluate the motility of MIA-PaCa-2, AsPC1 and CFPC1 that had previously been adapted to grow in SFM as described in section 2.2.3.1 - 2.2.3.3 in response to sera from patients with advanced PC from a clinical trial investigation (Maraveyas *et al.*, 2010a). In this study, the staining method with methylene blue was used (section 2.2.7.2.2, Staton *et al.*, 2004a, Miao *et al.*, 2000). Pancreatic cell lines were tested both with and without TF blocking. All coagulation and invasion experiments were undertaken in triplicate.

3.2.5 Results

The highest TF expression as assessed by flow cytometry was noted with CFPAC1, and correlated with the shortest clotting time (CT; 28 S), the lowest (undetectable) expression was noted with MIA-PaCa-2 which correlated with the longest CT [120 S]. AsPC1 expression with 4% of TF positive cell showed a CT between these two extremes (74 S). Blocking of TF receptors on these cells showed increased CT for all three cell lines; 95 S for CFPAC1, 119 S for AsPC1 and more than 241 S for MIAPaCa-2 (Table 3.2). The highest cell invasion was achieved with CFPAC1 [240/ high power field (HPF)], followed by AsPC1 (86/HPF) and then MIA-PaCa-2 (34/HPF). Blocking of TF on these cells decreased cell invasion by approximately 50% in both CFPAC1 and AsPC1 but had only a minimal effect on the already low cell invasion potential of MIA-PaCa-2 (Table 3.2, Fig. 3.4, 3.5).

Table 3.2: Expression of tissue factor on pancreatic cell lines: correlation with TF activity and cell invasion.

Pancreatic Cell Line	Mean CT (S)± SD		Mean Cell Invasion/HPF± SD		% of cells expressing TF [MFI]± SD
	Plasma Cells	Plasma, cells with TF blocking	Cells + Serum	With addition of anti TF antibody	
MIA-PaCa-2	120 ± 2	250 ± 1	42±3	34±3	0/[0]
AsPC1	74 ± 2	119 ± 2	86± 2	48 ± 2	4/[8]
CFPAC1	28± 2	95±4	240 ± 8	133 ± 2	90/[135]

Note: The mean fluorescent intensity (MFI) of the negative controls has been subtracted from all values. CT and cell invasion experiments were repeated in triplicate. HPF; high power field. .

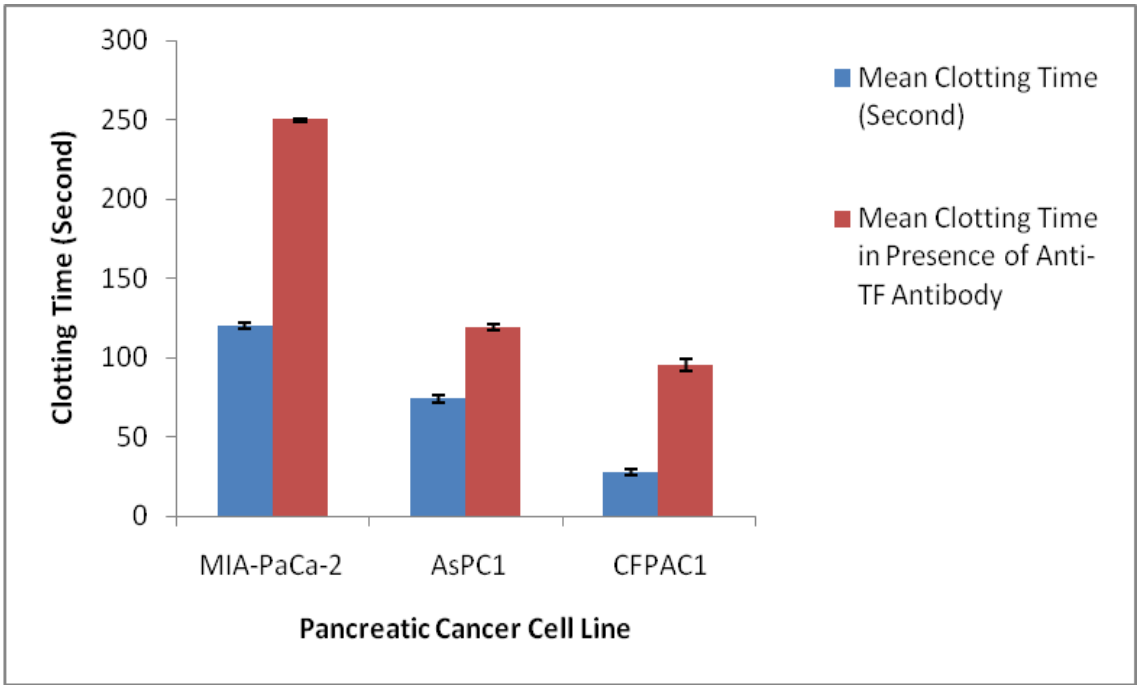

Figure 3. 4: Mean pro-coagulant activity (mean ±SD) of PC cells with and without TF blocking. Data are an average of three independent experiments.

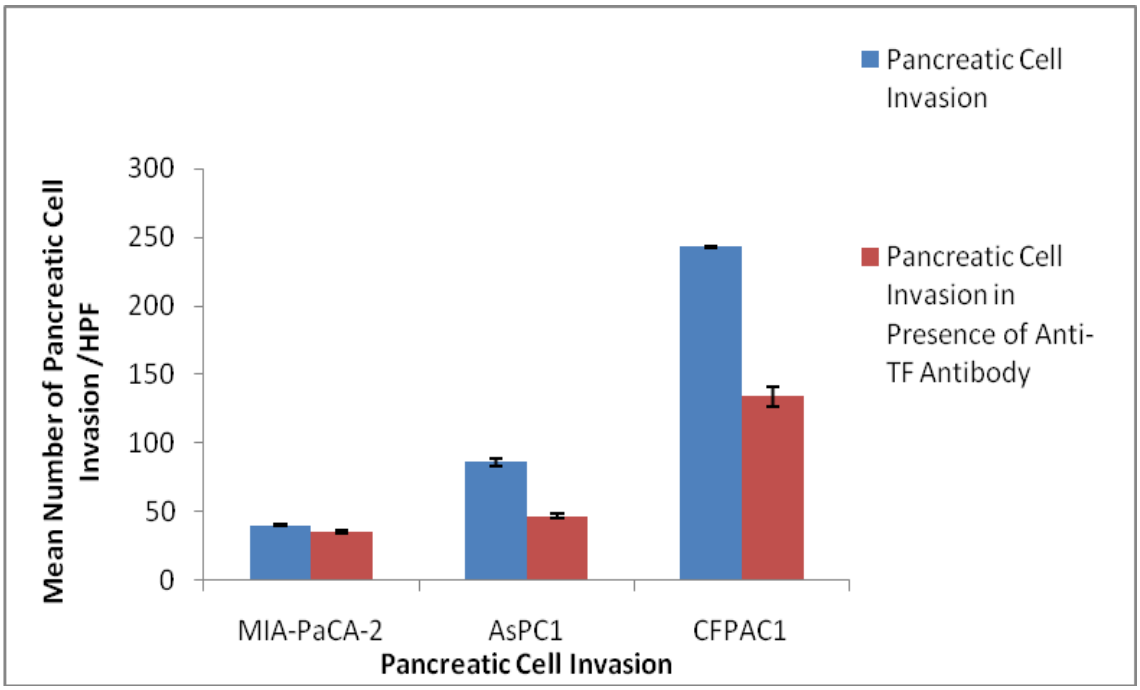

Figure 3.5: Pancreatic cell invasion (mean ±SD) with and without TF blocking in matrigel chamber. Data are an average of three independent experiments.

3.3 Investigation of Induction of Cellular Invasion by Patient Sera

3.3.1 Aim

The principal aim of this investigation was to evaluate the effect of heparin and blocking of serum TF on cell invasion.

3.3.2 Methodology

MIA-PaCa-2 cells were used in this section of the study because they have low endogenous tissue factor (undetectable flow cytometry) and a low inherent invasiveness. Culture, harvesting and adaptation to SFM are described in section 2.2.2.3.

MIA-PaCa-2 cells were used to measure the ability of the patient sera to induce locomotion and cellular invasiveness using Boyden chambers with collagen IV as described in 2.2.8.1. The baseline serum cancer cell invasion induction was assayed from four patients with advanced PC entered into the FRAGEM trial (Maraveyas *et al.*, 2010a). Samples of sera from patients with APC were placed in the bottom chamber (10% v/v) and supplemented with dalteparin (Fragmin) over a range of 0-100 anti-Xa international units/ml (final concentration). Experiments were undertaken in duplicate. To confirm the function of TF in the induction of cell migration, aliquots of patients' sera (n=4) were incubated with a range of concentrations (0-48 μg/ml final concentrations) of a neutralizing PoAb antihuman TF, prior to diluting with base medium. The rate of cell migration was determined following 24 h incubation. CTAOR (section 2.2.7.2.1) was used and the values of absorption were converted to number of cells from a standard curve.

3.3.3 Results

A range of MIA-PaCa-2 (2000- 50000) cells were used to generate a standard curve (Fig. 3.6). The absorbance at 490 nm was recorded using an ELISA plate reader.

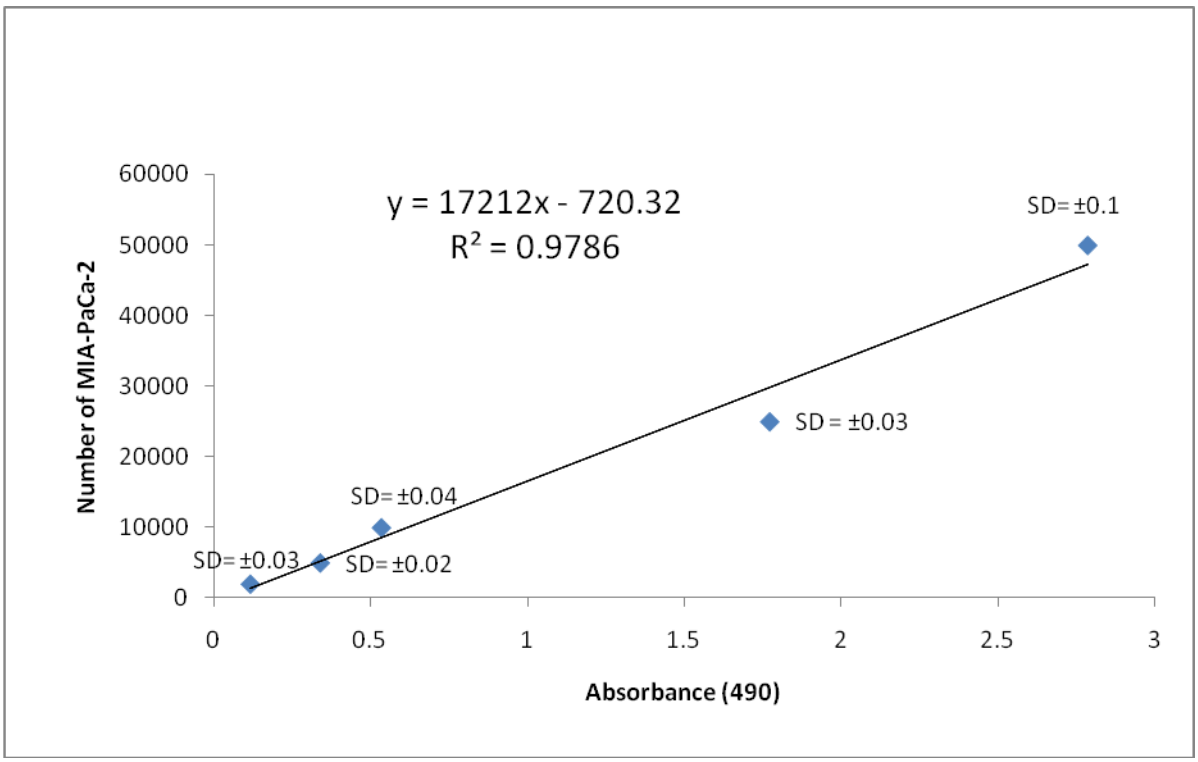

Figure-3.6: Representative standard curve for MIA-PaCa-2 invasion that correlates the invaded cells with the average of absorbance value.

The data presented in Fig. 3.7 suggests that there was no effect of extraneous dalteparin on the cell locomotion invasion of MIA-Paca-2 cells exposed to serum from APC patients (n=4). A test for linear trend in the number of cells migrated with dalteparin level of exposure (U/ml) was made. This was not statistically significant (P= 0.194, Pearson correlation). However, the cell invasion number when 0.01 U/L was significantly lower than control (8023 ± 898 *vs.* 8023 ± 375, p= 0.01, Mann Whitney test).

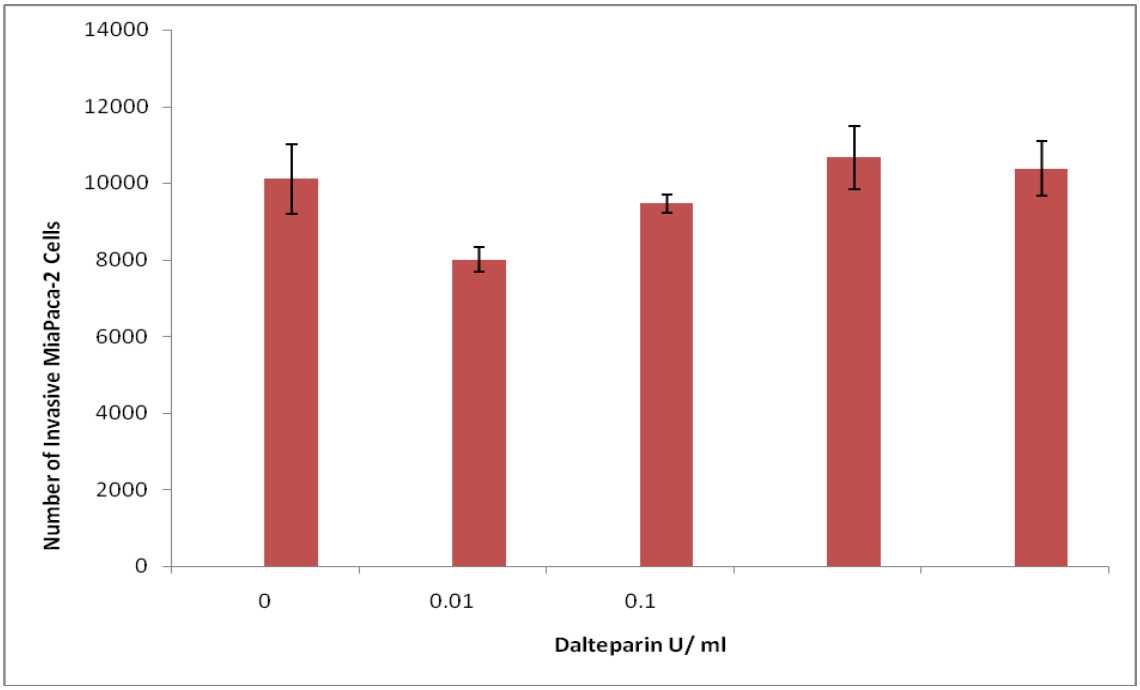

Figure 3.7: Effect of increasing doses of extraneous dalteparin on cellular invasion of in Boyden chamber MIA-Paca-2 cells in response to patients' sera. The results represent the chemotaxis effect of sera from 4 PC sera. The experiment was undertaken in duplicate

Furthermore, the role of TF in inducing cell invasion was demonstrated by pre-incubation of patient's sera with the anti human TF PoAb, which showed a clear reduction in MIA-Paca-2 cellular invasion, paralleling the increase in neutralizing antibody (Fig. 3.8).

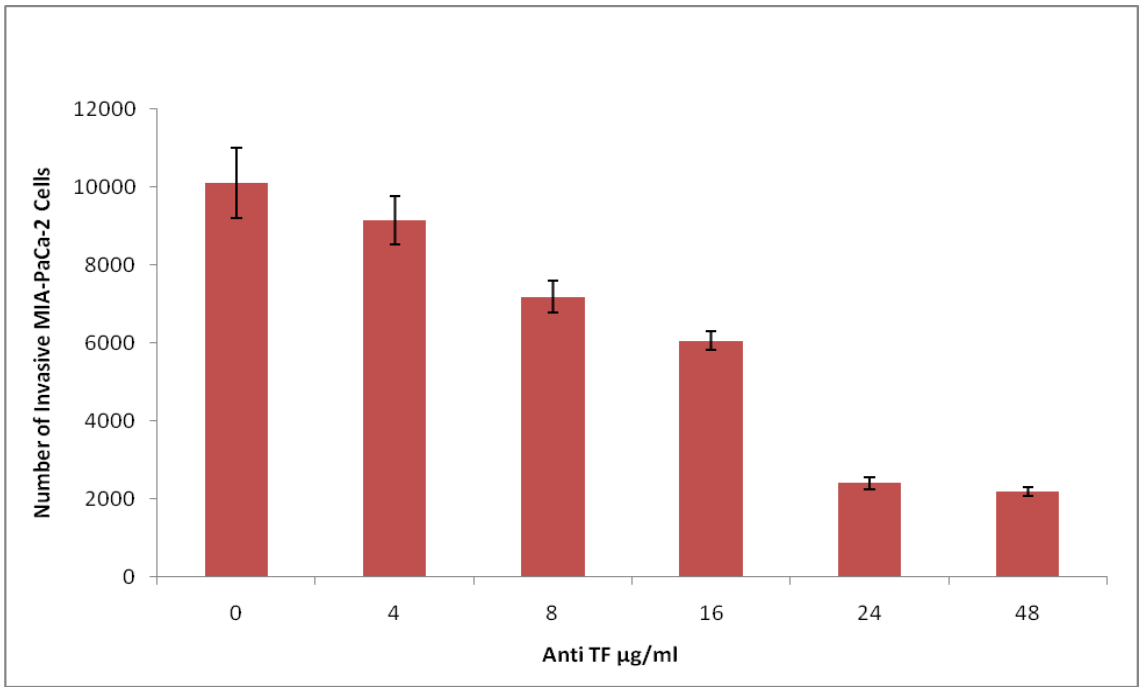

Figure 3.8: Incubation of the patient's serum with anti-TF PoAb demonstrated a dose-dependent inhibitory effect on cell invasiveness of MIA-Paca-2. The results represent the mean ± chemotaxis effect of sera from 4 PC sera. The experiment was undertaken in duplicate

3.4 Effect of Sera from Pancreatic Cancer Patients, TF and VEGF on cell invasion

Poor prognosis of PC cases is mainly due to tumour cell invasion beyond the pancreas (Sporn, 1996). The difficulty of detection and diagnosis of early PC, and the complication of being highly invasive, leave more than 85% of patients inoperable at the time of diagnosis (Muchmore *et al.*, 1996). Despite a general decrease in the rate of mortality and improved survival rate of patients following surgery, the overall 1- year survival rate after diagnosis of PC remains below 20% and the overall 5-year survival rate is around 3% (Web reference 4; 2010).

3.4.1 Aim

The aim of this study was to evaluate the chemotaxis activity of PC sera mediated by TF and VEGF.

3.4.2 Growth Factor Receptors.

Angiogenic receptors are the key targets of angiogenic factors presumed to be present in the serum of PC patients. VEGFR 1, -2 and -3 expression on the pancreatic cell lines is shown in Fig. 3.9.

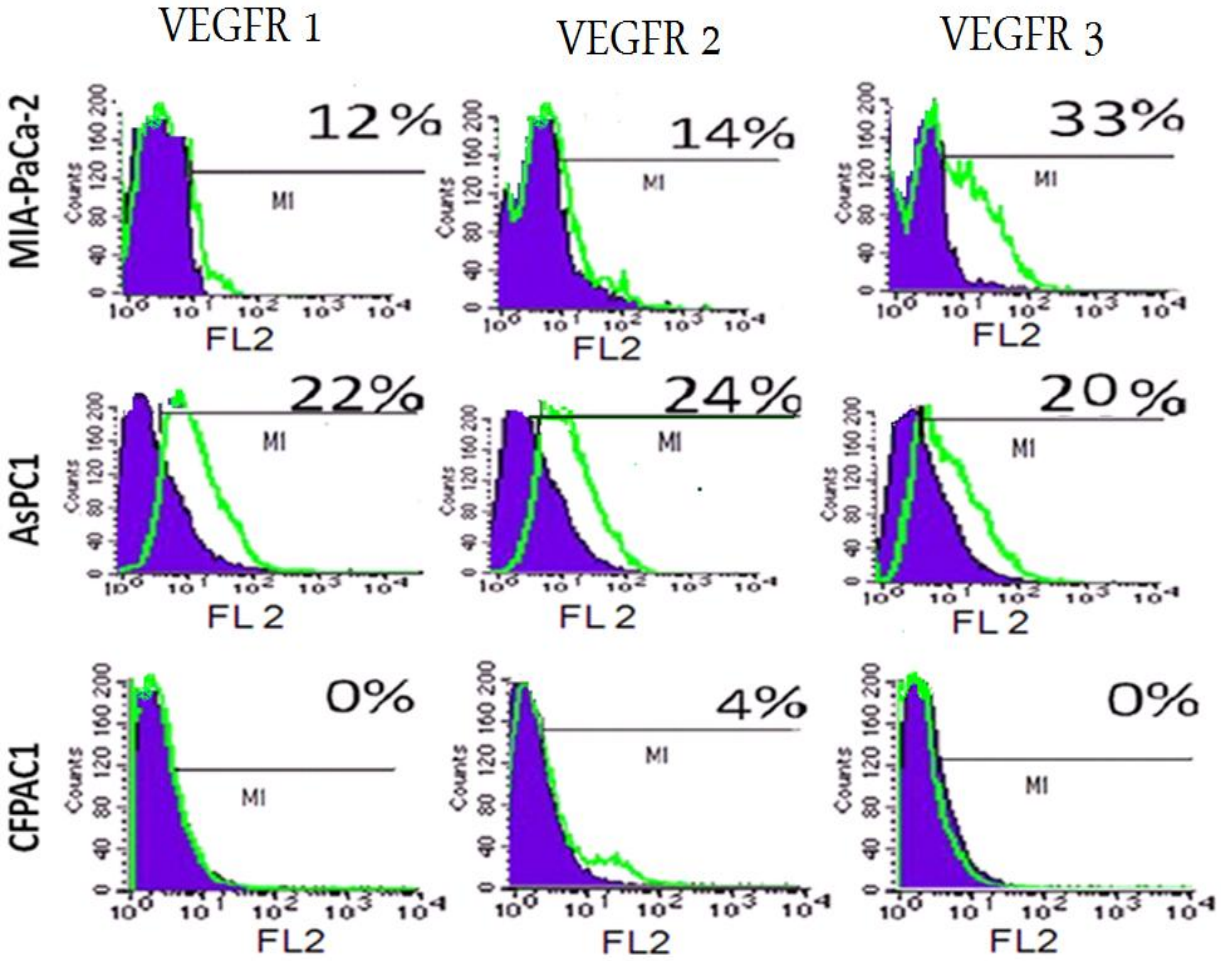

Figure 3.9: Expression of VEGFR 1, -2 and -3 on the surface of MIA-PaCa-2, AsPC1 and CFPAC1 cells. Data are a representative example of three repeats. Solid blue colour is the negative control. Green line was the test antibody.

In conclusion AsPC1 and MIA-PaCa-2 relatively expressed VEGFR 1,-2, -3 at higher levels than CFPAC1.

3.4.3 Materials and Methods

AsPC1 cells were used in this part of study to measure the ability of the TF and VEGF to induce locomotion and cellular invasiveness because it has low TF (4%) as described in section 3.2.3, has invasive properties and all the VEGF receptors are expressed at levels as shown in Fig. 3.9. Matrigel Invasion Chambers were used in this part of the study as described in section 2.2.7.1.

For evaluation of the chemotaxis effect of VEGF and TF, two baseline serums from patients with APC entered into the FRAGEM trial (ISRCTNU76464767, Maraveyas *et al.*, 2010a) were used. Experiments were undertaken in duplicate. AsPC1 cells were adapted to SFM as described in section 2.2.2.3. Cells ($2X10^5$ in 250 µl of SFM) were seeded on the top of the membrane, SFM RPMI 1640 (250 µl) supplemented with 10% v/v serum from APC, TF (a final concentration of 1 unit/ml) and /or VEGF, in a final concentration of 2ng/ml was placed in the bottom. The effect of 10% (v/v) PC serum with or without FBS (10%) was evaluated. The rate of cell migration was determined following 24 h incubation, the CTAOR method was used to evaluate cell invasion assay as described in section 2.2.7.2.1 and the values of absorbance were converted to number of cells from a previously prepared standard curve.

3.4.4 Result

A range of AsPc1 (2000-25000) cells were used to generate a standard curve (Fig 3.10). The absorbance at 490 nm was recorded using an ELISA plate reader.

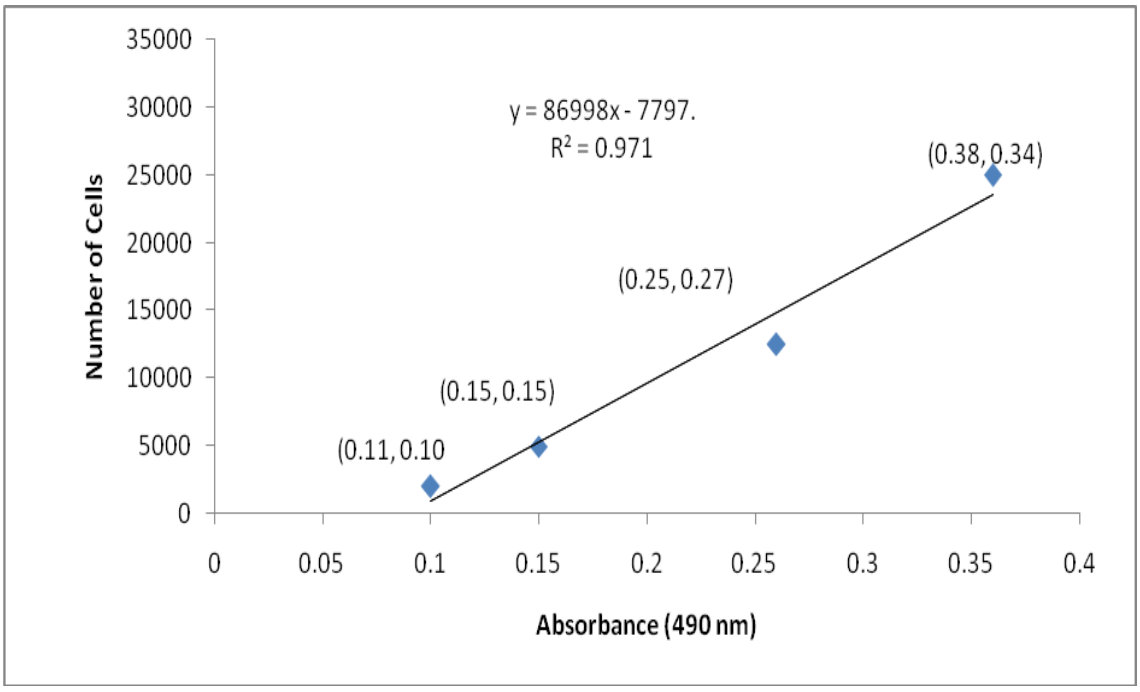

Figure 3.10: A CTAOR standard curve for the number of AsPC1. The experiments were repeated twice and values represent the average.

The highest chemotaxis effect (27002) was achieved when TF (1U/ml), VEGF (2 ng/ml) and PC sera acted synergistically. Ten percent FBS and 10% of PC sera were used as a positive control while PC sera alone showed the lowest chemotaxis effect (903) (Table 3.3).

Table 3.3: The chemotaxis effect of PC sera, TF and VEGF.

10% (v/v) PC sera plus	**Average number of AsPC1 cells**
10 % FBS (v/v)	44402 (41865, 46939)
TF (1 U/ml) and VEGF (2 ng/ml)	27002 (29683, 24321)
VEGF (2 ng/ml)	14822 (12649, 16995)
TF (1 U/ml)	7863 (8345, 7381)
Pancreatic serum (10%) only (v/v)	903 (838, 969)

The values represent mean of chemotaxis effect of two PC sera and each experiment was undertaken in duplicate

3.5 Optimisation of Cell Invasion Method

The aim of this part of the work was optimisation of a methodology to measure cell invasion for analysis of samples from the clinical trial. For evaluation of the chemotaxis effects of serum's proangiogenic factors, matrigel cannot be used because the commercial matrix is supplemented with several known angiogenic factors that will affect cell invasion (according to the manufacturer). Therefore, Boyden chambers coated with highly purified collagen IV were used where the only angiogenic factors are those added to the lower chamber.

AsPC1 was chosen as a model of PC cells for invasion experiment of the clinical part of the study due to its invasive features as it was originally derived from ascetic fluid. Due to these invasive properties, AsPC1 is widely used in cell invasion experiments (Tan and Chu 1985). Furthermore, the expression of TF on these cells was very low TF (4%), with moderate/high expression of several angiogenic receptors (VEGFR 1, 2, 3 and EGFR 1 expression are 22, 24, 20 % and 87%) which are the potential targets of many angiogenic markers in PC sera. AsPC1 adapted to growth in SFM were used to assess (as described in section 2.2.3.1) the chemotaxis activity of 11 PC sera using both CTAOR and slide methods. There was insufficient sample available to repeat analysis in all samples. The materials and methods were as described in sections 2.2.7.2.1 and 2.2.7.2.2 respectively. The values of absorbance with CTAOR were converted to number of cells from a standard curve (Fig. 3.11) over a range of AsPC1 (800-20000). The experiments were undertaken in duplicate. The results are summarised in Table 3.4.

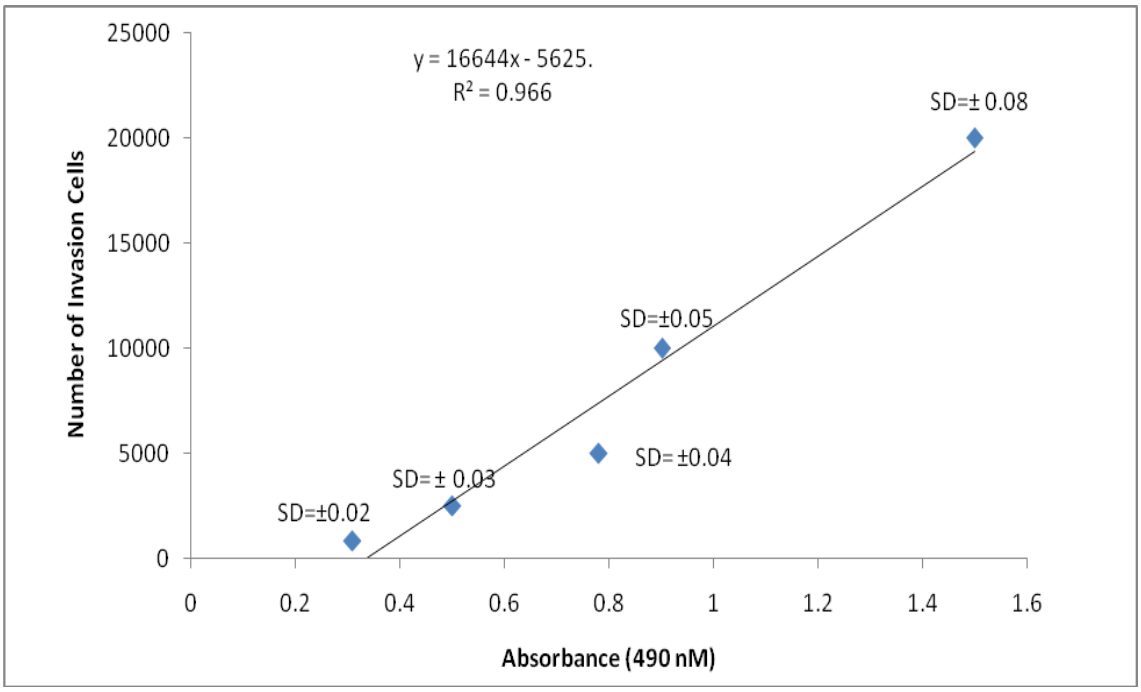

Figure 3.11 A Standard curve of AsPC1 treated with CTAOR. The number represents the mean of three results.

The motility of AsPC1 in response to the serum chemotaxis activity of 11 samples was re-counted using slide methods as described in a methodology described in section 2.2.7.2.2. The experiments were undertaken in duplicate. The results are described in Table 3.4.

Table-3.4: Comparison of CTAOR and slide method to determine invading cells.

Sample	First experiment			Repeat experiment		
	CTAOR	Slide		CTAOR	Slide	
		Mean No/HPF	Total No		Mean No/HPF	Total No
A	27204	121	28435	16594	99	23265
B	11450	107	25145	6727	90	21150
C	4367	92	21620	5483	87	20445
D	2370	93	21855	11311	82	19270
E	756	38	8930	6159	52	12220
F	803	42	9870	5637	43	10105
G	5302	97	22795	-	-	-
H	1722	66	15510	-	-	-
I	76522	124	29140	-	-	-
J	719	105	24674	-	-	-
K	20171	150	35250	-	-	-

The experiment was repeated in duplicate for the cases with sufficient sample available on two separate occasions. The numbers represent the mean of the two results. No: number, HPF: high power field, CTAOR: Cell Titre Aqueous One Reagent.

The results of CTAOR represent total numbers of invading cells while the results of the staining method represent the mean number of invading cells of five microscopical fields of 40 objectives, therefore, to compare these directly, the total number of invading cells on the membrane was calculated thus:

The diameter of the membrane was 7.36 mm. The diameter of the microscopic field of the 40 objective was 480 µm measured using a graticule ruler and stage micrometer. Hence, the radius of the 40 objective field was 240 µm. Because the microscopical field was circular, the area was calculated by $A = \pi r^2$ giving a total area of the field as 180864 μm^2 and the total area of the whole membrane was 42, 523136 μm^2. In summary, total membrane area was 235.1 times the microscopic field area. Therefore the mean number of five HPF was multiplied by 235.1 to quantify the total number of invading cells on the membrane. For standardisation of the methodology, the experiment of AsPC1 motility in response to 6 PC sera (A, B, C, D, E and F) was repeated using slide and CTAOR methods to allow the techniques to be compared for reproducibility. Again the values of absorption with CTAOR were converted to number of cells (Table 3.4) from a freshly standard curve as described previously. Comparing the two methods (CTAOR and slide methods) in the two experiments reveals the difference in the CTAOR method was more than the slide method (Fig.3.12-3.13).

However the statistical comparison of two methods (QTAOR and slide method) reveal no significant difference in both methods as the results of both are within 95% limit of agreement. The statistical comparison of the results of 11 in the first experiment using QTAOR and slide methods revealed that the 95% limit of agreement was from -46511 to 29814. The average and the difference of the results were within this limit except one result of sample I using QTAOR; 76522 (Bland-Altman method comparison, Fig. 3.14). The comparison of the results in repeated experiments in six samples revealed that the 95% limits of agreement average and the difference between the results were between -17882 to -300. Therefore the average and difference of both results were within this limit (Bland-Altman method comparison, Fig.3.15).

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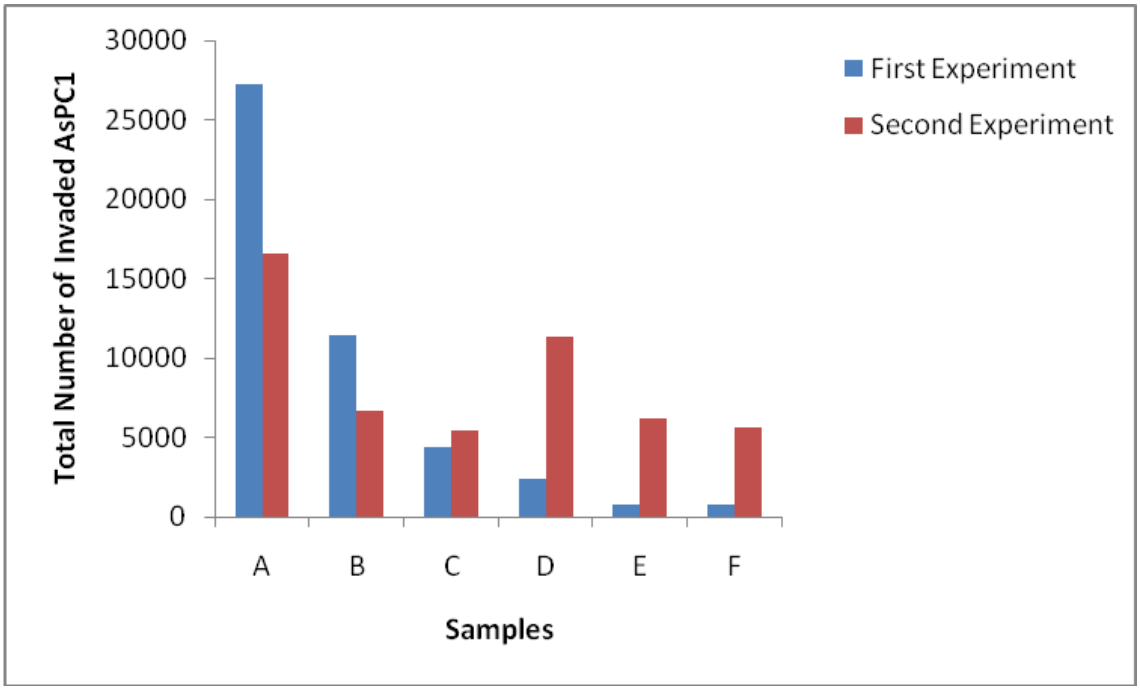


Figure 3.12: Comparison of AsPC1 motility in response to PC sera using the CTAOR method. The experiments were conducted in duplicate.

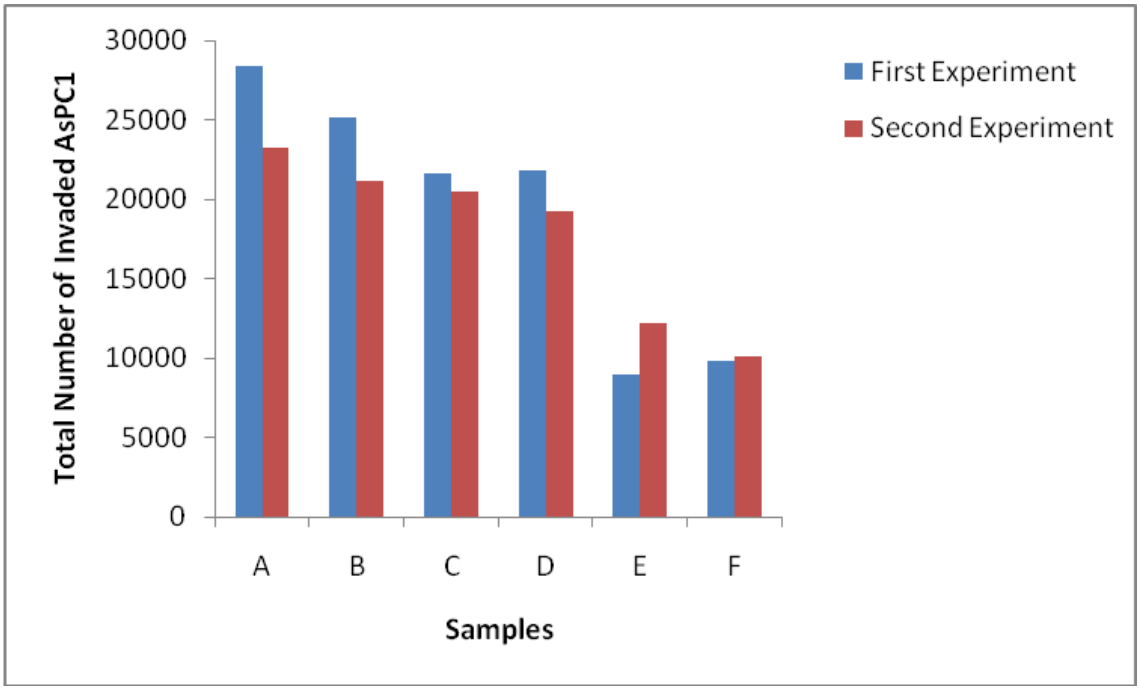


Figure 3.13: Comparison of AsPC1 motility in response to PC sera using the slide method. The experiments were conducted in duplicate.

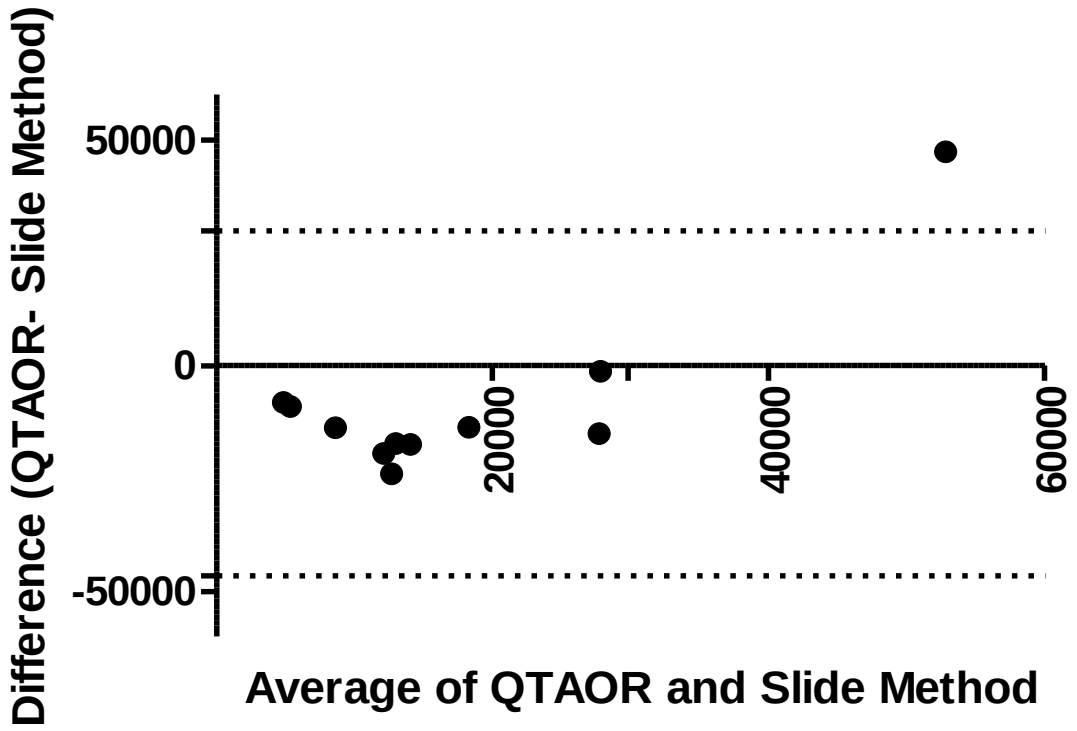


Figure 3.14: Average and the difference of QTAOR and slide method of the first experiment.

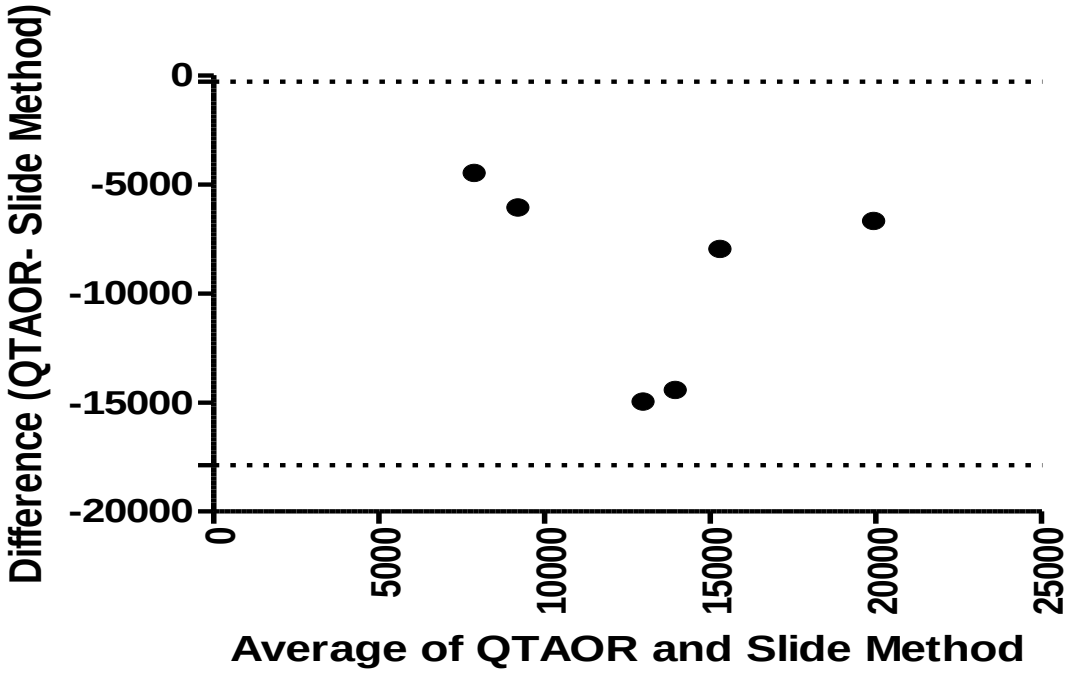


Figure 3.15: Average and the difference of QTAOR and slide method of repeated experiments.

3.6 Discussion

The three PC lines studied varied in their surface expression of TF, as determined by flow cytometry. PC cells grown *in vitro* have a pro-coagulant cell surface that can act as the basis of clot formation. The cells with the highest TF expression (CFPAC1) also showed the fastest CT and highest cell invasion.

The first interesting finding of this study is that TF expression on the surface of PC cell line is associated with cell invasion. This result was in agreement with results of other studies. Nitori *et al.*, (2005) noted that in 113 PC cases, TF expression was associated with PC metastasis. Kakkar *et al.*, (1999) noted that the mean number of MIA-PaCa 2 cell invasion using matrigel was 63 ± 13, the procoagulant activity using a two-stage clotting assay was 7.7 TF Units/ 10^4 cells, total TF content was 377 ± 274 pg/ml (total tissue factor content/mg of protein). The cell invasion, procoagulant activity and TF content of TF transfected MIA-PaCa-2 were significantly higher than those of anti sense TF transfected MIA-PaCa-2 cell (The mean number cell invasion; 210 ± 57 *vs.* 42 ± 6, the mean procoagulant activity was 37.3 *vs.* 6.8 TF Units/ 10^4 cells, the total TF content was 8705 ± 301 pg/ml *vs.* 156 ±50 pg/ml). Sawada *et al.* (1999) noted that there is a correlation between TF expression and invasion in non-small lung cancer NSCLC, Seto *et al.* (2000) postulated that there is a correlation between TF expression and hepatic metastasis of colorectal carcinoma whereas Koomagi and Volm (1998) reported that there is no correlation between TF expression and invasion lung cancer. The latter disappointing results suggest that the relation between TF expression and cell invasion may be cell type specific.

The current study shows a decrease of PC cell invasion by approximately 50% when cellular TF is blocked by anti-TF. This result is again consistent with other studies. Mueller *et al.* (1992), in a mouse model of experimental melanoma metastasis, found that anti-TF antibody decreased pulmonary metastasis. Furthermore, it has been reported that phosphorylation of serines of cytoplasmic domain and the extracellular domain enhances the cell invasion and the substitution of the three serine of cytoplasmic domain decreases the cell invasion (Bromberg *et al.*, 1999). Therefore, phosphorylation has an important role in the involvement of the cytoplasmic domain of TF in TF-VIIa signalling, which increases the chemotaxis ability of malignant cells (Bromberg *et al.*, 1999).

Blocking of CFPAC1 and AsPC1 was shown to result in to prolonged CT and a decrease of the number of invading cells by approximately 50% and this might be anti-TF antibody was not enough to inhibit all the TF activity. Both these cell lines (CFPAC1, AsPC1) were derived from metastatic cancers, whereas MIA-PaCa-2 was derived from primary pancreatic tumour and has very low expression of TF, undetectable by flow cytometry; however blocking of TF was seen to prevent coagulation, suggesting TF is present in relatively low levels on the cell surface. The cellular clotting activity of this study is in agreement with other studies, including Hobbs *et al.*, (2007) who noted that TF transfection into MIA-PaCa-2 cells resulted in a significant increase in procoagulant activity as determined by the one-stage PT method.

The current study supported the hypothesis that the highly aggressive PC cell line derived from the liver metastasis has the highest TF expression, TF activity and the highest cell invasion number. The result of this part of the work is in agreement with the results of other studies such as Silberberg *et al.*, (1989). These authors noted that RWP1 and RWP2 (PC cell lines derived from liver secondary) have the shortest CT (one stage PT was used) compared with NCI-H69 and NCI-HH82 (human small cell lung cancer cells, Silberberg *et al.*, 1989). Furthermore, the result of our study is in accordance with the result of Kakkar *et al.* (1999). These authors showed that over-expression of the TF gene was associated with an increase in TF expression and pro-coagulant activity and an increase in cell invasion. Kakkar and his colleagues used MIA-PaCa-2 pancreatic cell line for this transfection study. Moreover, the result of our study is also in agreement with Moberg *et al.* (2002). The authors noted that blocking of TF with monoclonal anti TF prolonged CT. The pancreatic Islet cells were harvested in dilution medium, mixed with human plasma and the CT was measured using on oscillating rheometer. The CT was 10 minutes without blocking of TF *vs.* 38 minutes with blocking of TF. It can be concluded that the PC cell invasion appears to be correlated with TF expression and activity.

The coagulation abnormalities are driven by the tumour and similarly these trigger the thrombosis events of the underlying malignancy. It has been shown that the *in vitro* incubation of patient's sera with PoAb anti-tissue factor antibody inhibits soluble-TF activity and results in reduction of cellular invasion properties of these sera. This is a reproducible, dose-dependent, *in vitro* effect, which supports the hypothesis that the reduction of circulating-TF antigen in the serum of these patients could be one of the mechanisms that could lead to reduction of cellular invasion. It has been postulated that soluble tissue factor is able to interact with the cell surface (Pradier and Ettelaie, 2008, Ettelaie *et al.*, 2007). This interaction and subsequent activation of

different sets of associated coagulation enzymes results in a varied set of signalling pathways, which result in proliferation, apoptosis (Pradier and Ettelaie, 2008) and cell migration (Satta *et al.*, 1994). Here, we show that TF, as found in patients' sera, has a measurable chemotactic effect on cancer-cell invasiveness that can be modulated by the use of dalteparin. These results are in accordance with Ardissino *et al.* (1997) who noted a significant correlation ($P<0.0001$) in the concentration of TF antigen and TF activity in atherosclerotic plaque. Furthermore, pro-coagulant activity has been linked to the amount of tissue factor antigen (Marmur *et al.*, 1996).

There was no statistical trend effect in the concentration range studied in the dalteparin-spiking experiment. However, statistical power was limited and a decrease was observed at 0.01 U/ml, in comparison to the control. It has been shown in cancer cell lines that there may be a direct effect of dalteparin on TF expression through mechanisms as yet not clear. The possibility can be excluded that the reduction of cellular invasion *in vivo* is the result of the effect of other molecules induced or reduced by the *in vivo* effect of dalteparin such as TFPI, which is known to have direct inhibitory effects on cancer promoting mechanisms (Hembrough *et al.*, 2003). Furthermore, it has been suggested that cellular-invasion assays and soluble-TF antigen may find use as surrogate markers of the anti-malignant effect of dalteparin and other LMWHs and may inform the appropriate dosing of these agents in the use of LMWH for 'oncological' effects. Furthermore, in the current, the maximum AsPc1 cell invasion was achieved when AsPC1 PC sera were treated with TF and VEGF. The dose of 1 U TF/ ml was used in this part of the work because it gives maximum angiogenesis (as will be described in section 3.4.4).

Another interesting result of this study is that considerable chemotactic and angiogenic activities were realised in the serum of patients with PC. This is further evidence that suggests the PC serum may contain considerable amounts of pro-angiogenic factors that could enhance cell invasion. Furthermore, this study shows that TF enhances cell invasion in a mechanism independent of the coagulation cascade and PAR. The current study also shows that VEGF enhances cell invasion in a mechanism in-dependent of the cascade coagulation. The maximum invasion was achieved when both TF and VEGF were added. These results suggest that angiogenic markers including TF and VEGF play an important role in stimulating cell invasion of AsPC1 cells, pointing to the possibility that inhibition of the function of these molecules may be involved in the invasion of PC. In summary, TF that is expressed on the cells and soluble TF in

the serum of PC play a major role in cell invasion. Blocking of cellular and serum TF decreases cell invasion.

For optimisation of the methodology to be used to analyse clinical samples, the slide method was chosen as a method for evaluating the chemotactic activity of PC sera in clinical study because this was easier and did not require formation of a standard curve. Statistically the average and difference of both results (QTAOR and Slide method) reveals that the results of both methods were in 95% agreement except for one result, due to the high number of cell invasion in the QTAOR method in the first experiment. Furthermore, the comparison of the two results (CTAOR and slide method; Table 3.4) reveals unexpected results with CTAOR, such as the result with sample I (76522). CTAOR is time consuming as incubation with CTAOR takes approximately 4 h whilst staining requires 15 minutes. Moreover, when the chemotaxis activity of 6 samples was tested in two experiments on two different days and the results of these two experiments were compared, there was a larger discrepancy in the number of invading cells with the CTAOR method than the slide method.

Therefore it was decided to use AsPC1 pancreatic cell line, Boyden chamber coated with collagen IV and staining of the membranes for evaluation of the chemotaxis activity of the PC sera pre- and post-operatively.

CHAPTER FOUR: ANGIOGENESIS ASSAY

4.1 Introduction

Angiogenesis is a vital step for tumour progression and is correlated with poor prognosis. Therefore, control of angiogenesis represents an alternative approach to the management of PC. Angiogenesis is mediated in part by the secretion of several growth factors, including VEGF. Therefore it may be possible to control angiogenesis by modulating these factors in PC and other solid tumours. In this part of the work TF with serum from patients with APC from the FRAGEM trial (ISRCTNU76464767, Maraveyas *et al.*, 2010a) was used to evaluate angiogenesis. Anti-TF, anti-VEGF and suramin were used to inhibit the angiogenesis. Suramin, a polysulfonated naphthylurea, is well known as treatment in trypanosomiasis and onchocerciasis. It has been reported that suramin has growth factor blocking activity (Middaugh *et al.*, 1992), inhibits cellular proliferation by inhibiting the binding of growth factors to their receptors (Coffey *et al.*, 1987) and inhibits intra-nuclear enzymes and causes partial blocking of EGF signal transduction (Fujiuchi *et al.*, 1997). Therefore, it has been suggested that suramin could have anti cancer and angiogenic inhibition activity.

4.2 Aim

This part of the work was designed to:

1. Optimise a standard methodology for assessing angiogenesis *in vitro* that can subsequently be used to study samples from a clinical trial.
2. Evaluate the effect of pro-angiogenic and anti-angiogenic factors on capillary (tubule) formation.

4.3 Growth Factor Expression

VEGFR are the cellular target of VEGF which are presumed to be available on the serum of PC serum. VEGFR 1, VEGFR 2, VEGFR3 and TF receptors were evaluated on the surface of ECV 304 and HDMEC. The methods of cell culture, growth and harvesting were described in sections 2.2.2.1.1 and 2.2.2.2.1. The method of detection of VEGFR 1-3 and TF was discussed in section

2.2.6. It was noted in the current study that TF was expressed on 2% of ECV 304 and 10% of HDMEC (Fig. 4.1).

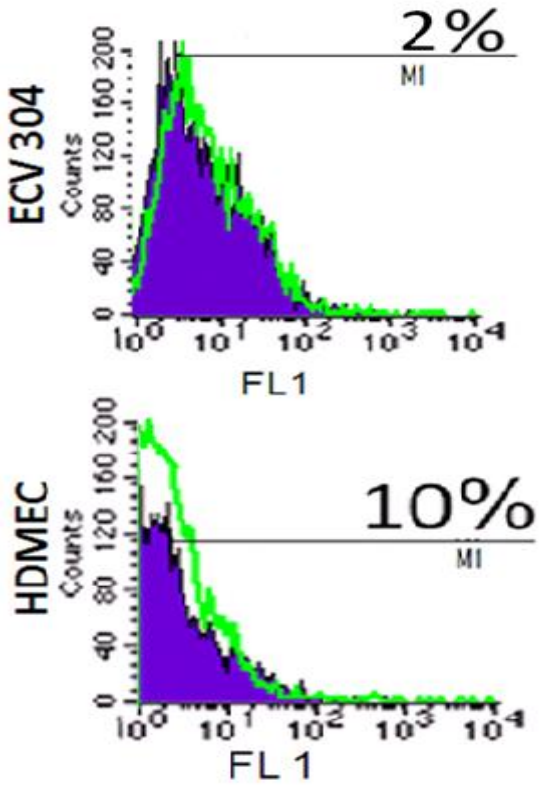


Figure 4.1: TF expression on the surface of ECV 304 and HDMEC. Data is a representative example of three repeats. The blue histogram was the Ig G FITC conjugated control. The green line was cells stained with an anti TF FITC conjugated antibody.

Expression of VEGFR 1, -2 and -3 was in 17, 19 and 22% of ECV 304 respectively and 52, 41 and 65% of HDMEC (Fig. 4.2).

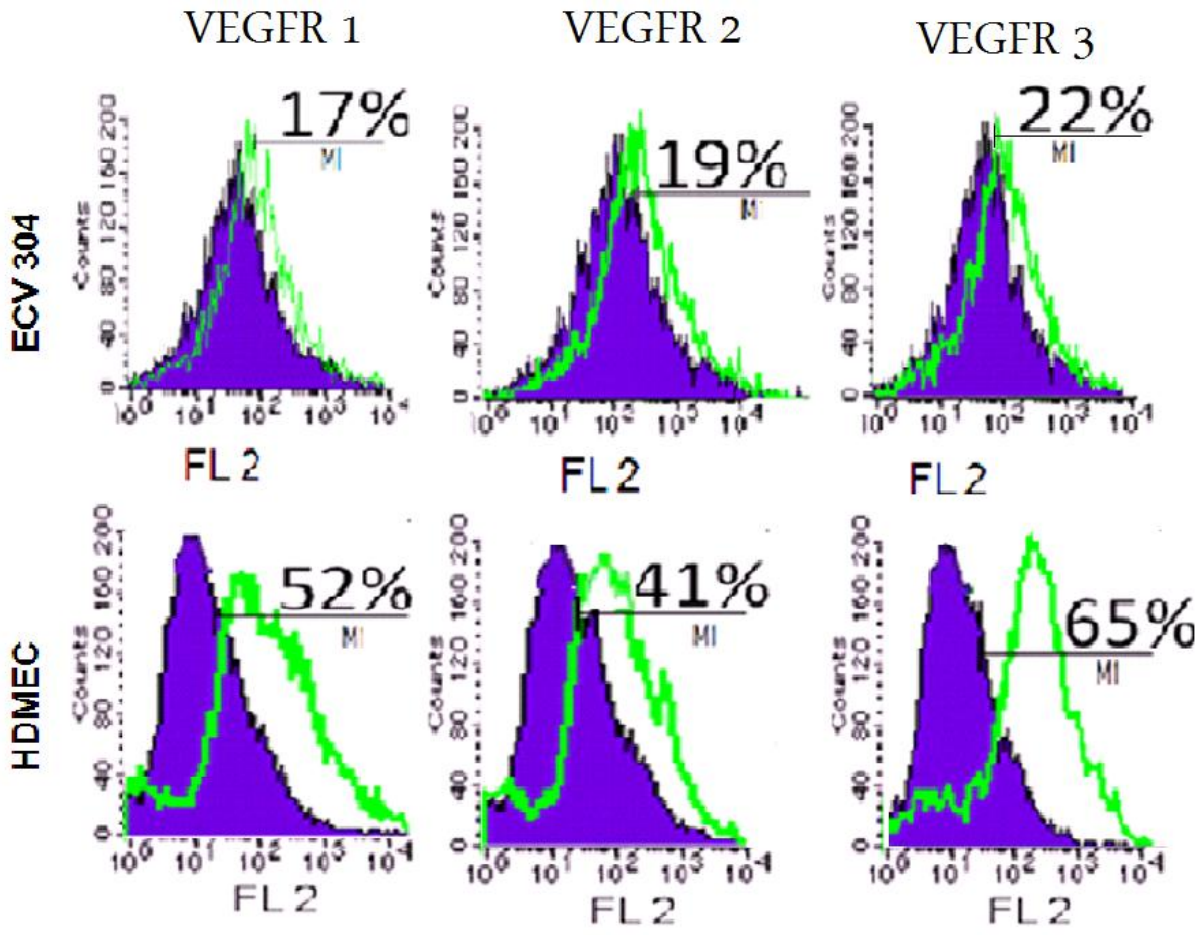


Figure 4.2: Expression of VEGFR 1, 2 and 3 on the surface of ECV 304 and HDMEC. Data are a representative example of three repeats. Blue colour was the negative control. Green colour was the VEGFR expression.

4.4 Materials and Methods

Two types of cells were used; ECV 304 and HDMEC (Fig. 4.3). Gels were formed in two different ways, as described in section 2.2.6.1. The effect of the angiogenic activity of PC serum, pro- and anti-angiogenic factors on angiogenesis was evaluated using collagen type I gel assay. Three baseline sera from patients with APC (from FRAGEM trial) were used in this part of the study. The experiments were undertaken in duplicate. The methodology was described in detail in 2.2.6. ECV 304 cells were treated with TF (1, 4, 10, 20 U/ ml at final concentration), PoAB anti-VEGF (1, 2 and 3.2 ng/ml at final concentration) and suramin (50, 100, 200 and 400 nM as final concentration).

HDMEC were treated with TF (0.2, 0.5, 1, 4, 10, 20 U/ml at final concentration), anti-VEGF (1, 2, 3.2 ng/ml at final concentration), suramin (50, 100, 200 and 400 nM at final concentration) and PoAb rabbit anti-human TF (48 µg/ml at final concentration). Concentrations less than 1 U/ml TF were used to evaluate the effect of low doses of TF on HDMEC angiogenesis. Ten pictures were taken of each well using an Imaging RETIGA 2000 R camera attached to a LeitZ Laborlux S fluorescence microscope. The Image-Pro plus program version 5.1.2 was used to evaluate total length and number of tubule formation, allowing quantitative analysis of angiogenesis.

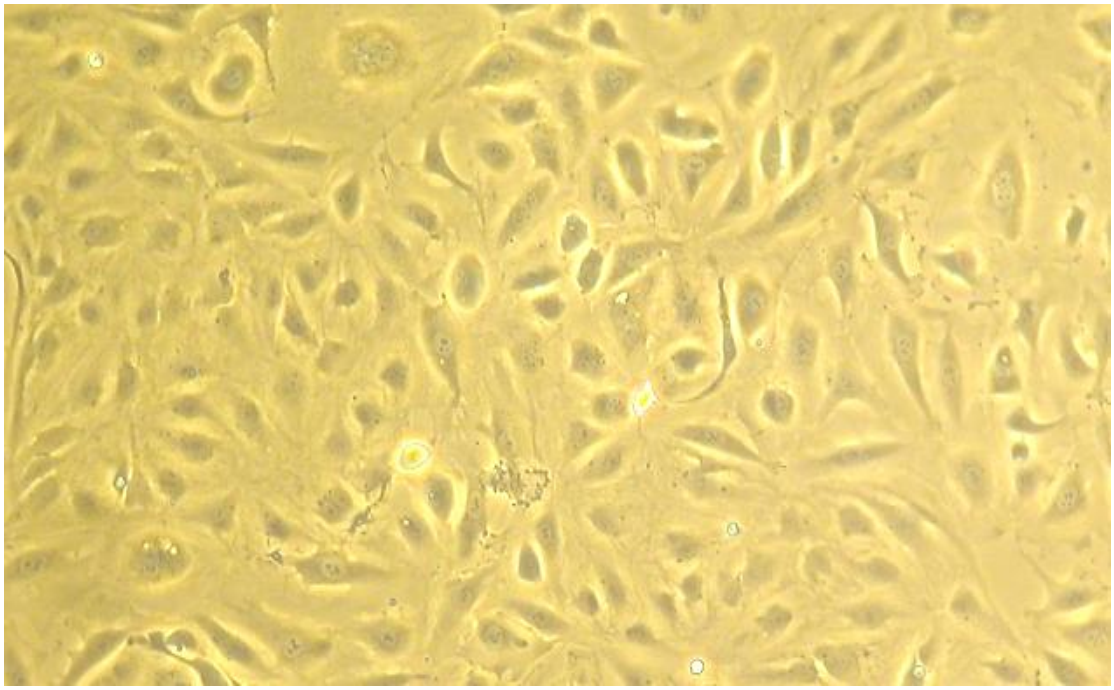


Figure 4.3: HDMEC have a typical cobblestone pattern (microscope magnification x 400).

4.5 Results

As described under materials and methods, the angiogenesis assay was performed using gel formed by two different methods. Comparing the two types of gel reveals that the angiogenic tubules were well formed and easier to detect with method B. For this reason and for other technical considerations that will be described in the discussion, method B was chosen as the standard protocol and the results of method A were ignored (Fig. 4.4-4.7).

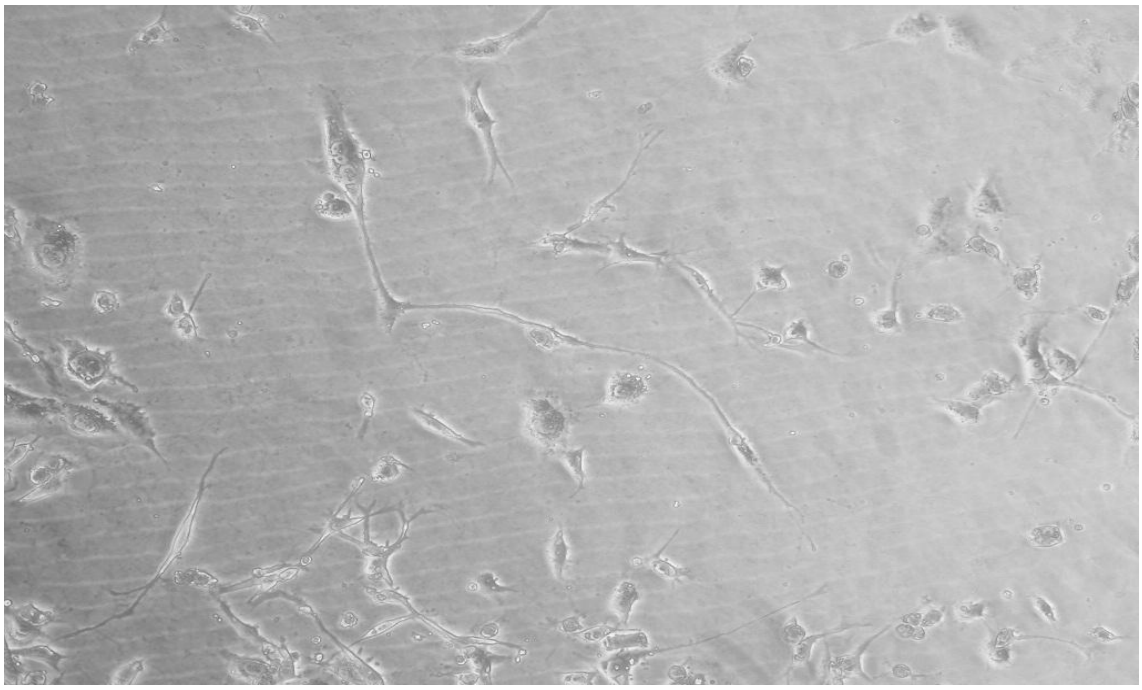


Figure 4.4: Angiogenesis of ECV 304 treated with 1 U TF using method B (microscope magnification x 400).

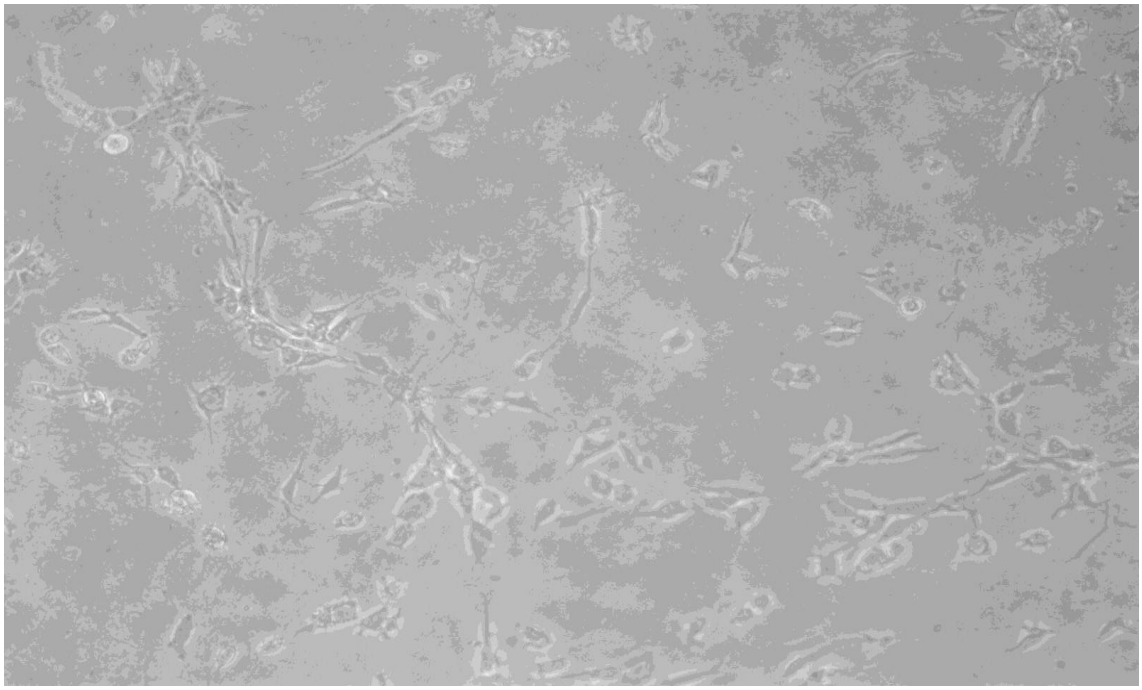


Figure 4.5: Angiogenesis of HDMEC treated with 1 U TF using method B (microscope magnification x 400).

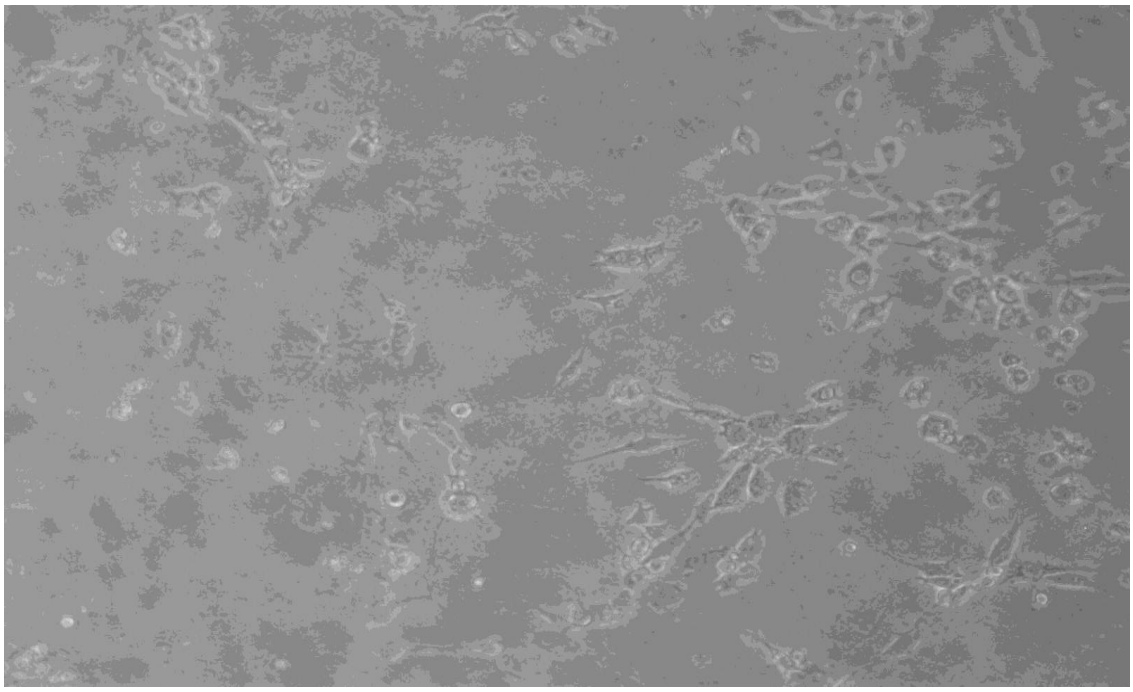


Figure 4.6: Angiogenesis of ECV 304 treated with 1 U TF using method A (microscope magnification x 400).

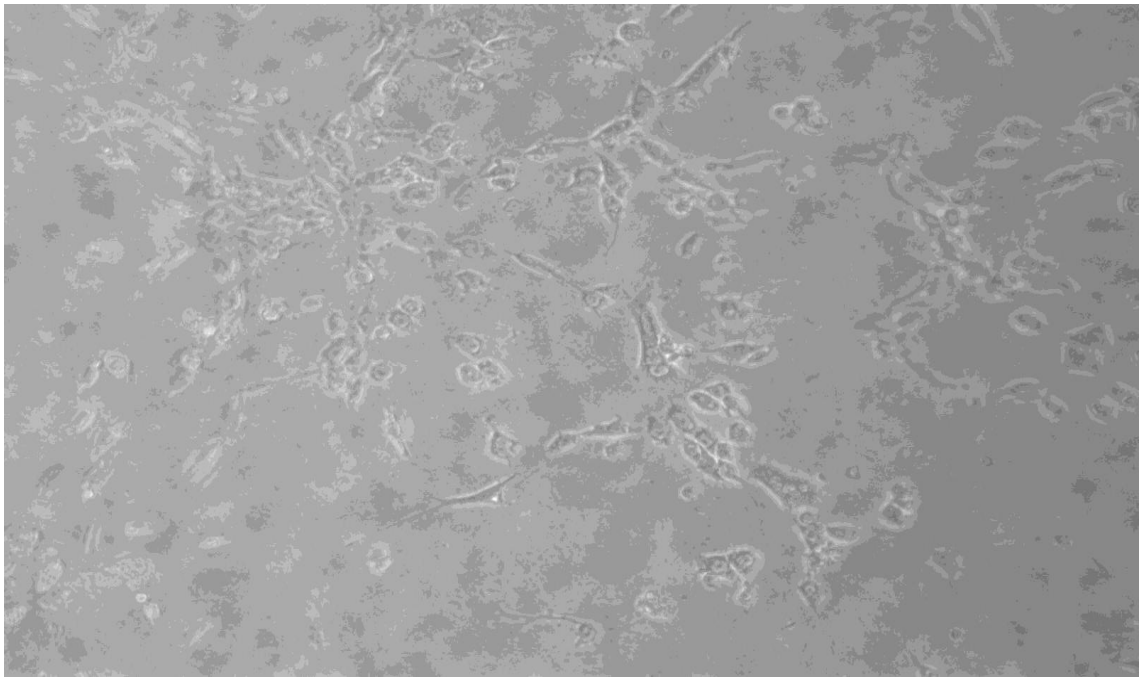


Figure 4.7: Angiogenesis of HDMEC treated with 1 U TF using method A (microscope magnification x 400).

As can be seen in Fig.4.8, maximum total length and number of capillary (tubule) formation were achieved when cells were treated with 1 U/ml final concentration of TF; the mean total capillary length and number of ECV 304 were 1930 ± 270 and 172 ± 16 (Fig. 4.4) respectively while those for HDMEC were 1500 ± 100 and 140 ± 36 respectively (Fig. 4.5). There was a gradual decrease in the mean total length and number of the tubules when TF concentrations higher than 1 U/ml were used.

The mean total lengths of capillaries of ECV 304 were 1700 ± 50, 1300 ± 132, 505 ± 83 when cells were treated with 4, 10 and 20 U/ml TF respectively, while those for HDMEC were 1233 ± 153, 1020 ± 159, 367 ± 29 respectively. The mean total number of capillaries of ECV 304 was 110 ± 26, 75 ± 22 and 30 ± 10 when cells were treated with 4, 10 and 20 U/ml TF respectively while those for HDMEC were 113 ± 15, 70 ± 10 and 27 ± 12 respectively. Furthermore, the mean total length and number of capillaries of HDMEC were 750 ± 50 and 73 ± 13 respectively when cells were treated with 0.2 U/ ml. The mean total capillary length of HDMEC was 1007 ± 178 and the mean total number was 100 ± 10 when cells were treated with 0.5 U/ml. Therefore, when HDMEC were treated with doses less than 1 U/ml (0.2 and 0.5 U/ml), no increase of angiogenic parameters was observed (Fig. 4.8).

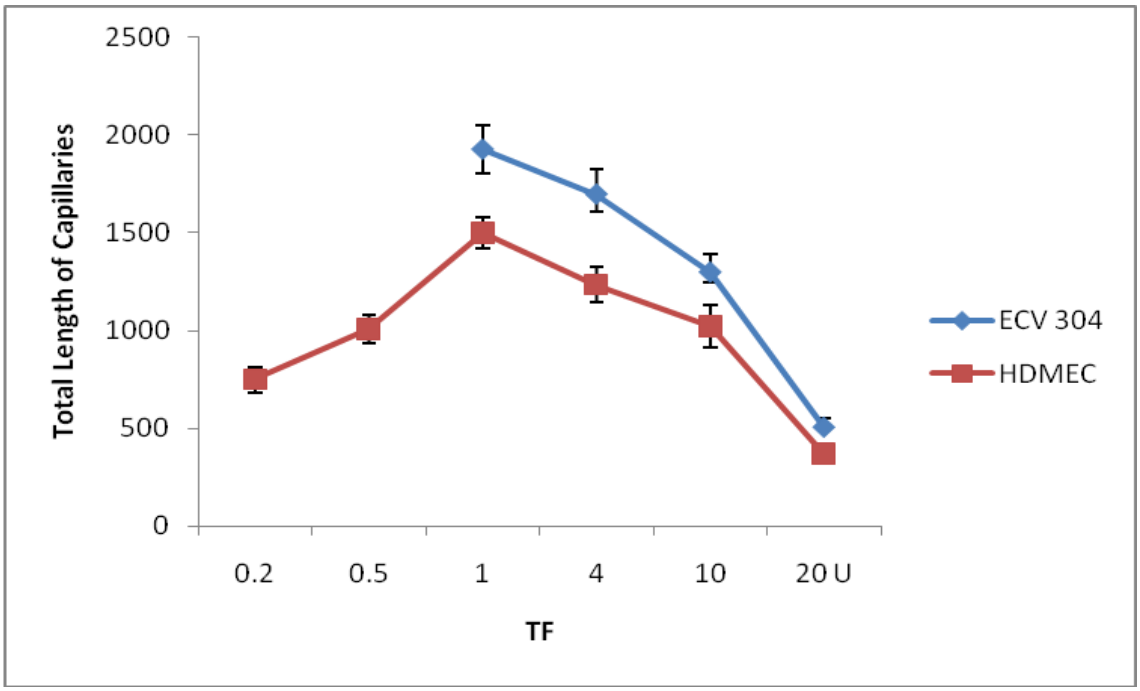


Figure 4.8: Effect of TF on Angiogenesis.

Furthermore, mean total length and number ± SD decreased when either cell type was treated with suramin. The minimum ECV 304 total length and number (80 ± 26, 24 ± 5 respectively) was achieved when the cells were treated with 200 nM while those for HDMEC were 123 ± 31 and 30 ± 9 respectively (Fig. 4.9). Increase of the suramin dose to 400 nM did not appear to decrease the angiogenesis further (Fig. 4.9).

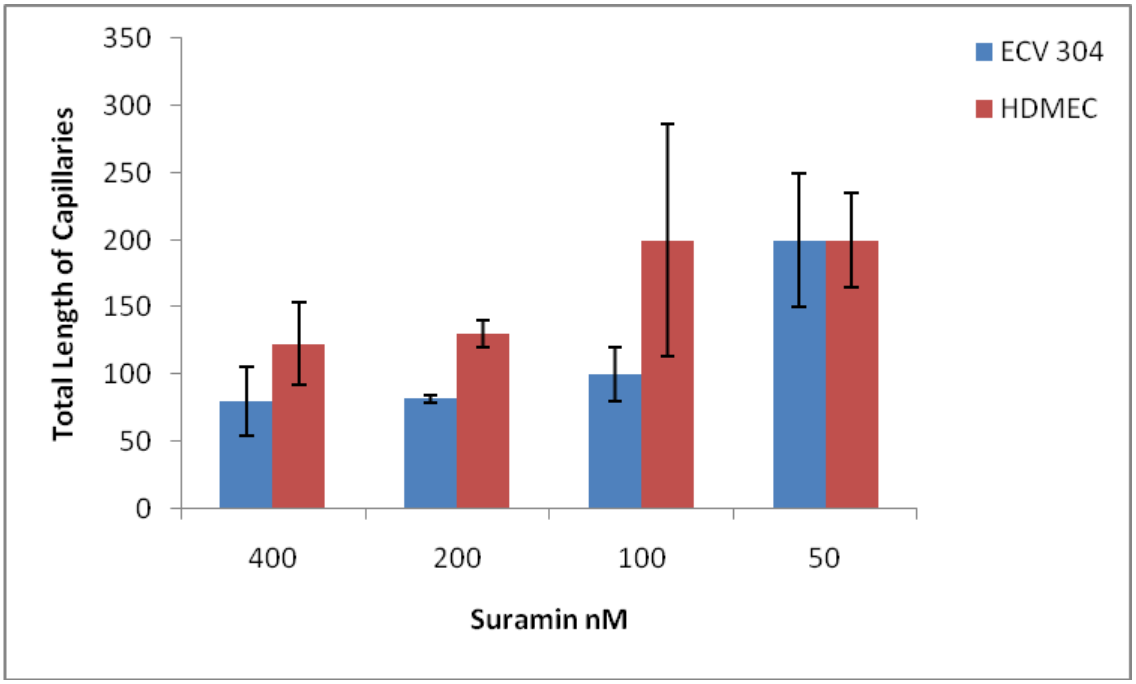


Figure 4.9: Effect of suramin on angiogenesis. The values represent the mean of the total length of three PC sera and each experiment was undertaken in triplicate.

There were similar decreases in the total length and number of capillaries when cells were treated with polyclonal anti-VEGF antibodies. Minimum mean parameters (total length and number) were achieved when cells were treated with 2 ng/ml anti VEGF. The mean ± SD of ECV 304 total length and number were 70 ± 17 and 23 ± 5 when cells were treated with 2 ng/ ml of anti-VEGF while those for HDMEC were 130 ± 10 and 30 ± 5 respectively. Increasing the dose of neutralising anti VEGF up to 3.2 ng/ml did not show further decreases in capillary length and number. Two ng/ml was used in angiogenesis because 2 ng/ml of VEGF increase cell invasion (section 3.4.4)

Inhibition of angiogenesis was achieved when HDMEC were treated with a combination of suramin, anti-VEGF and anti TF in doses of 200 nM, 2 ng/ml, and 48 µg/ml respectively.

4.6 Discussion

As described under materials and methods, the angiogenesis assay was performed using gel formed by two different methods (section 2.2.6.1). In method B, the capillaries were well formed and easier to detect than in method A. Furthermore, method B is widely used and extensively described in the literature as reported in section 2.2.6.1.B, while the first is not. Thirdly it is less complicated than the first method.

There are four main primary variables that are commonly used to determine the extent of capillary formation. They are: total capillary length, total capillary number, average capillary length and average capillary number. Total length and number of capillaries have been used widely to assess tubule formation (Hernandez *et al.*, 2004, Isaji *et al.*, 1997, Wu *et al.*, 1997). Average tubule length is determined by dividing total capillary length by the number of tubules. As it is based on two other variables it cannot strictly be seen as a separate independent variable. Therefore, measurements of total length and number in 10 pictures at 400 X magnification were used as a method for evaluation of the capillary assay and angiogenesis in this study.

The tubules angiogenic assay allows the quantitative detection of endothelial cell capillary formation in response to serum with pro- and antiangiogenic factors. At the beginning of the study, ECV 304 was selected to evaluate the effect of proangiogenic and antiangiogenic factors on angiogenesis. It has been noted that ECV 304 does not express defining endothelial cell markers like Von Willebrand factor (vWF), CD31 and CD34, but it expresses VEGFR 1 and VEGFR 2. Furthermore, treatment of ECV 304 by TNF–α , bacterial lipopolysaccharide (LP) and iterleukin-1B (IL-1B) resulted in expression of ICAM-1 but no E-Selectin and VCAM-1, while HDMEC and other primary endothelial cell lines expressed characteristic key markers such as vWF, CD31, CD34, VEGFR 1, VEGFR 2, ICAM-1, E-Selectin and VCAM-1 (Unger *et al.*, 2002).

In addition, it has been noted in the current study (section 4.3) that the positive expression of TF, VEGFR 1-3 was detected in 2, 17, 19, 22% respectively of ECV 304 while that for HDMEC was 10, 52, 41 and 65% respectively.

Using HDMEC, the maximum total length and number of capillary formations were achieved when cells were treated with low dose (1 U/ml) of TF. Higher concentrations of TF resulted in a decrease of these angiogenic parameters, which may be due to the saturation of endothelial cells and or apoptotic changes with high doses. The current study is in agreement with a study reported by Watanabe *et al.* (1999). These authors noted that capillary formation was achieved when low doses of TF (10-30 µg/ml) were used and higher concentrations did not produce angiogenesis. The maximum increase of capillary formation (250% increase compared to control) was achieved when bovine aorta endothelial cells were treated with 10 µg/ml of TF. This result was in accordance with that of Pradier and Ettelaie (2008) who noted that the maximum capillary formation was achieved when HUVEC were treated with 1 U/ ml of TF due to the activation of caspase-3, expression of p53 and Bax, translocation of p53 into the nucleus and induction of DNA fragmentation. Also, there was no tubule formation when HUVEC were treated with 100 U /ml of TF. Moreover, our results were in accordance with Ettelaie *et al.* (2008) who noted that 13 pg/ml of TF or 2 µM of VEGF resulted in enhancement of HDMEC capillary formation.

It has been reported that TF expression has been significantly correlated with unfavourable prognostic factors such as enhancement of angiogenesis (Lwaleed and Cooper, 2000). In the current study, there were no clotting factors used; therefore enhancement of angiogenesis by exogenous TF is clotting independent. The clotting independent mechanism could be performed through any of four pathways, most of which are initiated by clotting factors. These pathways include TF/FVIIa, TF/FVIIa/FXa, thrombin and directly by TF (as described in detail in section 1.9.2). In the current study, FVIIa/FXa was not used and because the first three pathways depend on TF and other clotting factors. Therefore, the expected mechanism was the angiogenic changes resulted from the effect exogenous TF and soluble angeogenic markers in the serum. Therefore, TF induces angiogenesis in a mechanism independent of coagulation which could be directly through the cytoplasmic domain of TF (Abe *et al.*, 1999, Bromberg *et al.*, 1999,) or through direct interaction with the cell surface protein (Pradier and Ettelaie, 2008). Still there is a possibility that the TF mechanism might be due to the effect of VEGF produced by the effect of TF as aberrant TF expression could enhance VEGF expression (Zhang *et al.*, 2004, Abe *et al.*, 1999).

It has been noted in the current study that anti-VEGF and suramin reduce the angiogenesis. Therefore, blocking of VEGF directly by anti-VEGF or by suramin decreased angiogenesis as expected. Furthermore, a combination of anti VEGF, suramin and anti TF inhibits angiogenesis completely. This result is in accordance with Bhargava *et al.* (2007) who noted that suramin inhibited PC cell proliferation, metastasis and angiogenic activity, represented by a dose dependent decrease of VEGF secretion (10, 100, 200 and 800 µg/ml in a mouse model. Furthermore, Bhargava and colleagues in a later study reported that tumour growth and metastasis of PC were reduced after the mice were treated with suramin when a dose of 60 µg/kg was used. The dose in the current study that decreases angiogenesis is (200 nM/ 20000 cells) which is less than 60 ug/kg. Therefore might low doses of suramin might be enough to decrease the angiogenesis with less side effects.

The results of the current study have suggested some preliminary evidence for the concept of "multitargeted" therapy and the possibility of controlling pancreatic tumour angiogenesis, growth and metastasis through inhibition of the angiogenic factors such as TF and VEGF. While gemcitabine has shown some promising results in PC treatment, chemotherapy in general has been ineffective (Storniolo *et al.*, 1999). Radiation therapy can be used as a treatment in the minority of patients with localised unresectable tumours but it cannot control distant metastases. The pre-clinical evidence of the efficacy of combination of anti-VEGF, anti TF and suramin inhibiting angiogenesis totally need be supported by clinical research and could represent the treatment of choice for this disease in the future. The key mediator of angiogenesis is VEGF and its receptors and the targeting of VEGF may be an optimistic treatment in the future and to overcome resistance multiple combinations are used (Morelli *et al.*, 2009). Ning *et al.* (2010) reported the simultaneous combination of at least two angiogenic inhibitors with chemotherapy to overcome the resistance. Suramin has been used previously in the treatment of prostatic cancer, but it has many serious side effects due to toxicity resulting in nephropathy (Garcia-Schurmann *et al.*, 1999). Therefore, based on the results of the current study, it is suggested that suramin could be used at lower doses and if given in combination with chemotherapy, anti-VEGF and anti-TF might have favourable results in treatment of PC cases. However, anti-TF might has toxic effect.

In the current study, both anti-VEGF and suramin had an anti-angiogenic effect. Maximum anti-angiogenic effects were achieved when the cells were treated with anti-VEGF (2ng/ml) and

suramin (200 mM/ml). Furthermore, increasing the dose of anti-VEGF and suramin had no further effect; this might be due to saturation of the cells.

As a conclusion, TF induced angiogenesis in a mechanism independent of the coagulation cascade. Angiogenesis was almost entirely inhibited when suramin, anti-TF and anti-VEGF were induced together. HDMEC was chosen as a primary endothelial cell model to evaluate the angiogenesis in the clinical part of the study. Method B of gel formation was chosen as the method for gel formation in the angiogenesis experiment of the clinical part of the study. Total length and number of capillaries were chosen as a method for evaluation of the angiogenesis only. The evaluation of clinical samples is important but insurance that the assays and *in vitro* studies are given due credence

CLINICAL PART

CHAPTER FIVE: CLINICOPATHOLOGICAL DETAILS of PATIENTS

The current study is a prospective cohort clinical trial to evaluate the effect of resection of localised PC on tissue-factor promoted pathways of thrombosis, cell invasion and angiogenesis. The trial was approved by the research ethics committee (MREC, number: 08/H1305/59) and by the NHS Trust research and development (R&D) / NHS Trust organisation (number RO721). Forty-one patients underwent attempted pancreatic resection including pancreatic adenocarcinoma (Fig. 5.1), pre-cancerous malignant pancreatic conditions (Fig. 5.2) and cholangiocarcinoma. In addition, 22 cholecystitis patients undergoing laparoscopic cholecystectomy served as controls (Fig. 5.3). A consort diagram of the study is shown in Fig. 5.3. Whipple surgical operation was performed to remove the tumour. For unresectable tumour bypass surgical removal was performed to releave the symptoms (see section 1.6).

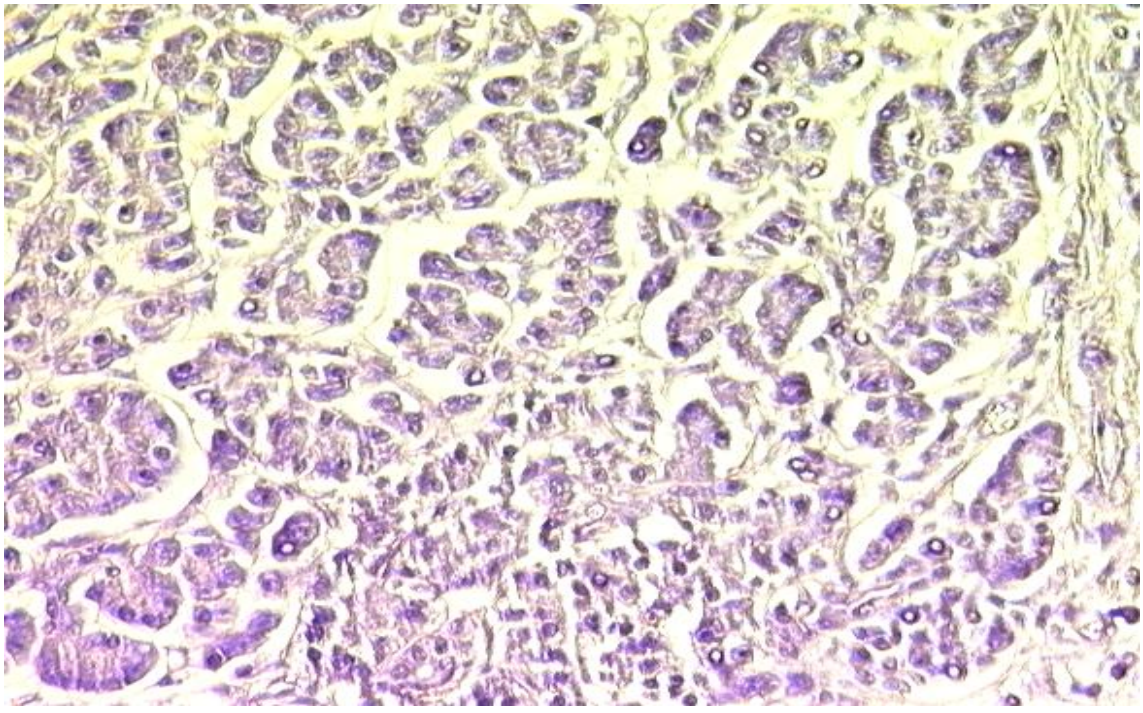


Figure 5.1: Histopathological section of pancreatic adenocarcinoma stained with H&E (x 400).

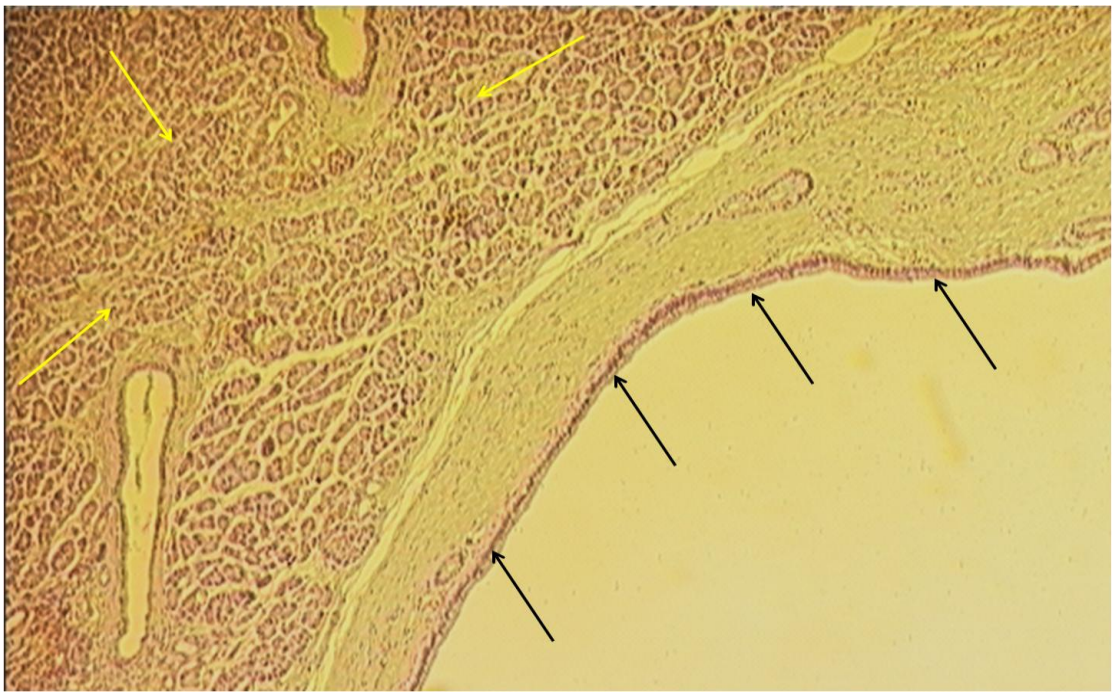


Figure 5.2: Representative example of histopathological section of pre-cancerous mucinous pancreatic cyst stained with H&E (x 100). Black arrows are the columnar epithelium of the cyst. Yellow arrows are the pancreatic tissues.

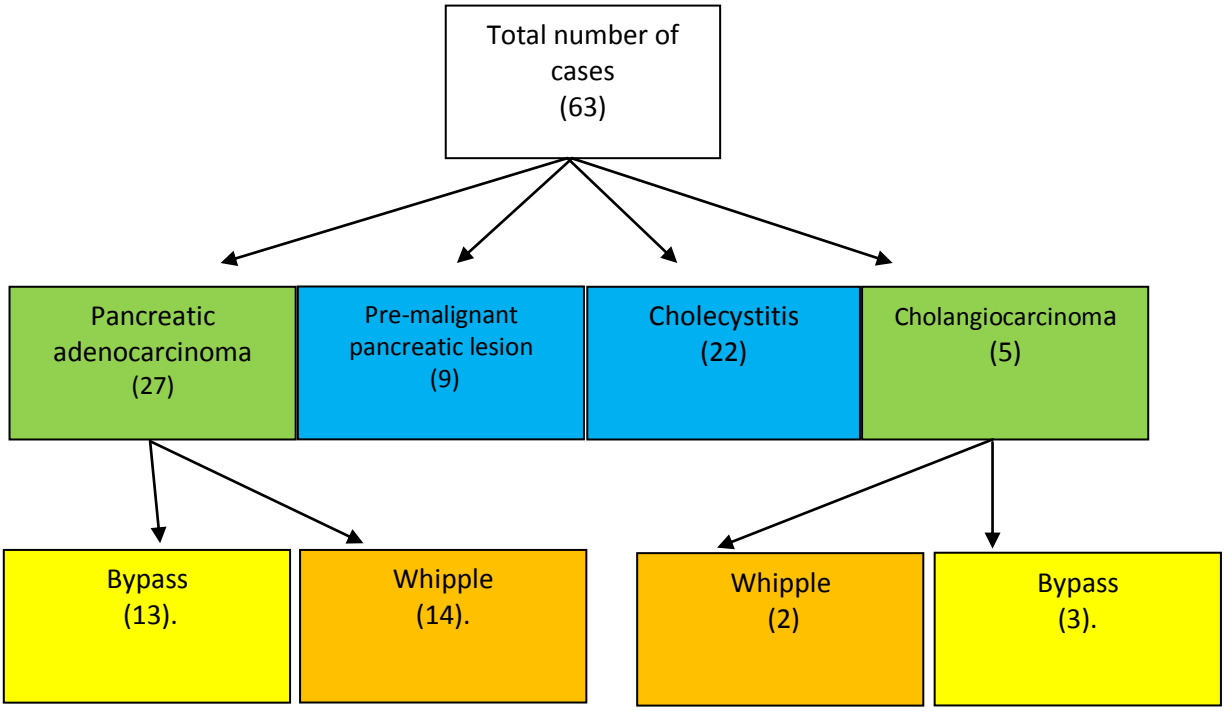


Figure 5.3: Consort diagram of study patients.

Eligible patients for the study were identified, registered with the clinical trial data manager and then approached by the chief researcher or research nurse who explained the purpose of the trial. The relevant patient information sheets (Appendix A) and consent forms (Appendix B) were discussed in detail. Time was given to patients to read them carefully, and to discuss them with friends, relatives and their GP, before written informed consent was obtained. Demographic data were recorded, relating to the conventional pre-operative patient assessment such as haematological tests including haemoglobin, white blood cells, neutrophil and platelet count. Biochemical tests include C-reactive protein; bilirubin and liver enzymes were added to the data collection sheet (Appendix C).

The protocol was to collect blood pre-operatively, post-operatively (6-8 weeks after operation, when the drain tubes were removed, if there was no sign of infection, the patient being in good health or as soon as the patient exhibited of post-operative recovery signes), twice during chemotherapy (week 8 and week 24 during chemotherapy treatment) and then eight weeks after chemotherapy. For the cholecystectomy control group, only pre- and post-operative samples were collected.

All clinical samples were submitted to serological analyses, which included: evaluation of chemotaxis activity; angiogenic activity, TF-bearing MP number; CD14-bearing MP number, PT and VEGF ELISA. A subset cases were submitted to annexin bearing MP, CD 31 bearing MP, CD 42b bearing MP, MP activity (ELISA), auto annexin antibody (ELISA), angiogenesis array using angiogenesis array kit and leptin ELISA. The cholecystectomy group was used as a control for cell invasion and angiogenesis, and where possible the cholecystectomy cases were analysed by ELISA experiments (Fig. 5.4).

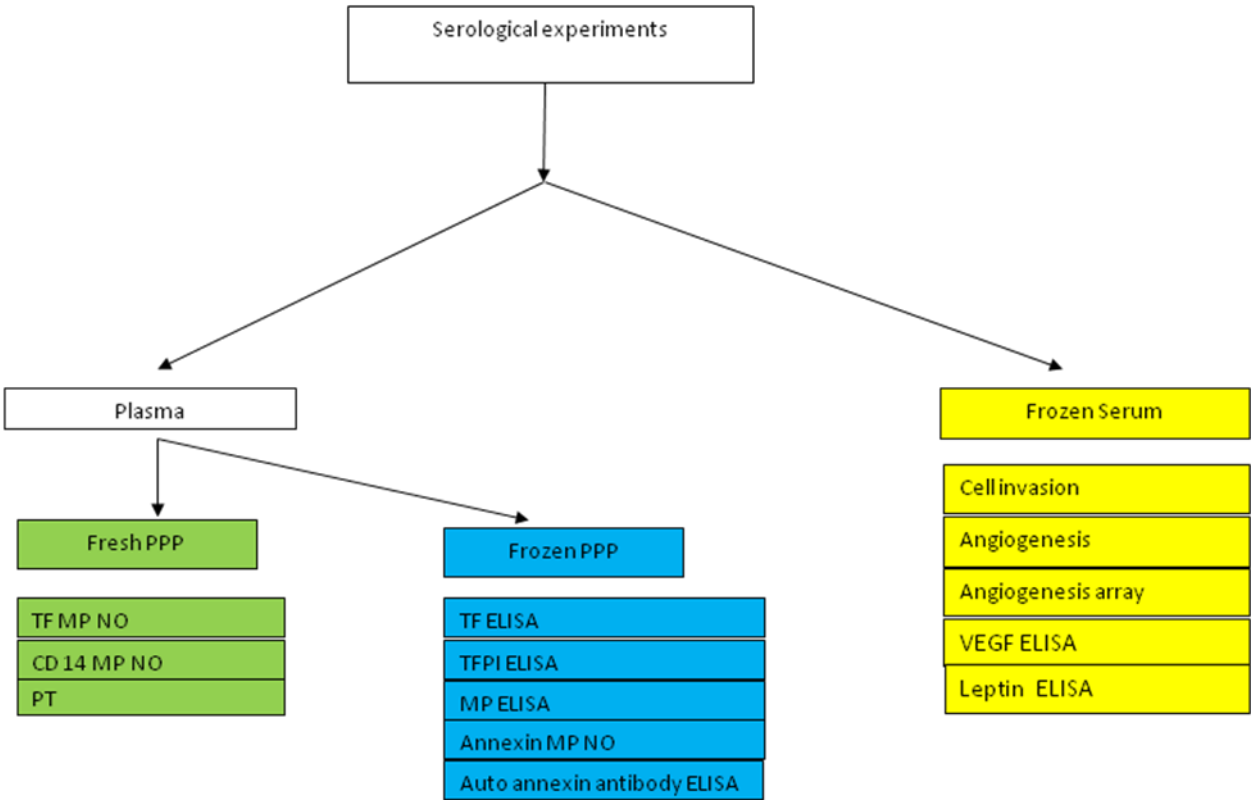


Figure 5.4: Serological experiments. PPP: platelet poor plasma, NO: number, PT: prothrombin time. TF -MP NO, CD 14 MP NO, PT, cell invasion and angiogenesis were performed on all cases. TF, TFPI and VEGF ELISA were performed in all available cases. Angiogenesis array, annexin MP NO and Leptin ELISA were performed in a subset of cases. MP ELISA and auto annexin antibody ELISA were performed in some clinical trial and cholecystectomy cases.

Post-operative samples were only collected in approximately one half the cases (Table 5.1). Week 8, week 24 during chemotherapy and week 8 after chemotherapy samples were collected in a few cases (6 cases; week 8 chemotherapy, 5 cases; week 24 chemotherapy and 1 case; week 8 after chemotherapy). The lack of samples was mainly due to high post-operative mortality, withdrawal of the patients and difficult phlebotomy or in some cases results were excluded from the study due to partial or complete haemolysis of blood samples.

Table 5.1: Number of cases with pre-and post-operative blood samples.

Pancreatic Cancers (Group I)	Cholangiocarcinoma (Group V)	Precancerous lesions (Group III)	Cholecystectomy (Group IV)
9	2	5	11

In addition to that, there were 4 cases from group D [all unresected cases of malignant cases (groups II and VI)] with pre-and postoperative blood samples. In this thesis, the data was only related to the pre-operative and post-operative period. The part of the study relating to effects of chemotherapy is on-going. The patient groups and post-operative blood samples collected for this study are summarised in Table 5.1. Laboratory analyses were blinded to clinical provenance of specimens.

- Group I: PC cases successfully resected.
- Group II: PC cases with a bypass
- Group III: Pre-malignant pancreatic cases successfully resected.
- Group IV: Chronic cholecystitis cases treated by laparoscopic cholecystectomy.
- Group V: Cholangiocarcinoma cases successfully resected.
- Group VI: Cholangiocarcinoma cases with bypass.

For the statistical analyses the cancer cases were also summatively grouped as follows:

- Group A: All PC cases (groups I and II).
- Group B: All malignant cases (groups I, II, V and VI).
- Group C: All resected cases of malignant cases (groups I and V).
- Group D: All unresected cases of malignant cases (groups II and VI).
- Group E: All cholangiocarcinoma cases (groups V and VI).

5.2 Blood Samples

The blood was collected in two types of tubes. For plasma, blood was collected in 5 ml sodium citrate tubes [citrate concentration of 0.109 mol/L (3.2%)], in the ratio 1 to 9. These plasma tubes were blue topped vacutainers. The tubes were centrifuged (at 360 x*g* for 10 minutes) to prepare platelet-rich plasma (PRP). The PRP was further centrifuged (15 minutes at 13000 x*g*) to obtain PPP as described previously (Jimenez *et al.*, 2003). TF bearing MP, CD 14 bearing MP (for monocytes) , CD 31 (for endothelial cells), CD 42b (for platelets) count and PT were performed on fresh PPP (within the first four hours of venepuncture). The remaining plasma was kept as 200 µl aliquots in 0.5 ml polypropylene tubes at -80^{o}C. Frozen PPP samples (not thawed previously) were used in ELISA experiments (TF, TFPI, MP activity) and annexin MP count (Fig. 5.4). For the serum, blood was collected in 5 ml serum-separating tubes (also known as yellow topped vacutainers). These tubes contain a gel that separates the clot from serum. The tubes were centrifuged at 360 x*g* for 10 minutes. The serum was aliquot (200µl) in 0.5 ml polypropylene tubes at -80 ^{0}C. Serum samples were used to evaluate the expression of angiogenic markers in the serum of PC, cell invasion and angiogenesis. Frozen samples were used to evaluate angiogenesis array, cell invasion, angiogenesis assay (capillary formation), VEGF and leptin concentration using ELISA (Fig. 5.4). All samples used in the current trial were not thawed previously.

5.3 Age and Sex

The mean age of group B was 61.2 ± 11.1 years. No patient was operated on below the age of 39 years or above 81 years. The Male: Female ratio was 1.2:1. The mean age of group III (premalignant cases; 9 cases) was 58.8 ± 13.1 years. Male to female ratio was 1.2:1. The mean age of group IV (Cholecystectomy group; 22 cases) was 63.7 ± 7.4; male: female ratio was 0.5:1.

5.4 Clinical and Social History

Two cases were hypertensive out of 27 (group B); there was no history of infection or ischemic heart disease. Two cases from group II, one case from group III and 2 cases from group E were on prophylactic dalteparin (5000 U).

Three cases out of 27 of group A were diabetic; one case of group III was diabetic. Two cases of group IV were diabetic.

Four out of 31 of group B were ex-smokers, 5 patients smoked 1-10 cigarettes/day; two patients smoked 11-20 cigarettes/day. One patient out of nine of group III smoked 10-20 cigarette/day. Three patients of group IV were ex-smokers, three patients smoked 1-10 cigarettes/day; two patients smoked 11-20 cigarettes/day.

Four cases in group B drank alcohol 1-7 glasses/week and four cases were drank eight or more glasses/week. Three cases of group III drank 1-7 glasses/week and 3 cases drank 8 glasses or more/week. Three cases out of 22 of group IV drank 1-7 glasses/week and 3 patients drank 8 or more glasses/week.

5.5 Haematological Finding

The mean haemoglobin concentration of group B was 12 ± 2.3 g/L. Mean platelet number of group B was (258.3 ± 66.8) x 10^9/L, WBC count was (6.2 ± 1.4) x10^9/L, neutrophi count was (3.9 ± 1.2) x10^9/L.

5.6 Statistical Analysis

The statistical analysis was performed under supervision of Dr Victoria Alga (Senior Lecturer in Medical Statistic in Hull Yorkshire Medical School). The median was used as the main comparator in this study when the data were asymmetrical (skewed), while the mean was used when the data were normally distributed. GraphPad prism 5 statistic programme was used for statistical analysis.

CHAPTER SIX: ANGIOGENESIS ARRAY

6.1 Introduction

The regulation of tumour angiogenesis in cancers, including PC depends on a delicate balance between pro-angiogenic (angiogenic activators) and anti-angiogenic factors (angiogenic inhibitors) which are secreted by the tumour and surrounding cells (Chang *et al.*, 2008, Hicklin and Ellis, 2005, Poon *et al.*, 2001). The point at which the balance shifts towards pro-angiogenic factors is called the angiogenic switch (Baeriswyl and Christofori, 2009, Abdollahi *et al.*, 2007). The suggestion therefore, is that the on and off of the angiogenic switch plays a crucial role in determining whether a tumour grows or regresses (Chang *et al.*, 2008, Hicklin and Ellis, 2005, Poon *et al.*, 2001). Hence, angiogenesis could be induced by an increase in pro-angiogenic factors, a decrease of anti-angiogenic factors or a combination of both (Sasano and Suzuki, 2005) and in reverse angiogenesis could be inhibited.

In past years many proteins have been categorised as angiogenic activators. Foremost among them are VEGF, PDGF, bFGF, erythropoietin, PDGF, IL-10, TGF-β, CD 34, NO and uPA. Angiogenic markers have been widely used to predict the prognosis, relapse and recurrence of cancers (Achimas-Cadariu *et al.*, 2009, Andersen *et al.*, 2009, Alamdari *et al.*, 2007, Konukoglu *et al.*, 2007). Furthermore, two important anti-angiogenic factors are angiostatin and endostatin; both are produced by proteolytic cleavage of plasminogen or collagen XVIII, respectively (Oreilly *et al.*, 1997, 1994). Moreover, TSP-1, TSP-2 and the chemokines such as CXCL4, CXCL9, CXCL10 and CXCL11 can also inhibit angiogenesis (Strieter *et al.*, 2006). A summary of pro and anti-proangiogenic markers was given in section 1.13.

It has been widely reported that PC is a highly angiogenic tumour (McElroy *et al.*, 2009, Ikeda *et al.*, 1999, Itakura *et al.*, 1997). At the time of diagnosis, most PC patients already have locally advanced or metastatic disease (Yeo *et al.*, 1997). However, the surgical removal of PC followed by chemotherapy is the treatment of choice. Unfortunately this can only be performed in 15-20% of PC cases (Takamori *et al.*, 2006, Zuckerman and Ryan, 2008). Because of this, and because the overall five year survival rate of PC remains disappointing (Sohn *et al.*, 2000), there has been an interest in recent years in understanding the markers that control angiogenesis in

PC and looking to manipulate the balance between pro-and anti-angiogenic markers inducing a shift towards the anti-angiogenic status.

Therefore, manipulation of the angiogenic balance may represent a therapeutic target in the future. Because multiple factors may contribute to angiogenesis in PC, it is critical to determine the serum expression of multiple factors simultaneously. In many studies, the expression levels of multiple angiogenic factors were quantified by ELISA or at the mRNA level by reverse transcription-PCR. The ability of these techniques to measure multiple samples of angiogenic factors simultaneously is limited because they required always large sample volumes, and exhibit low throughput. Therefore, using array technology has several advantages over other techniques, such as detection of expression levels in small serum volume (100 µl); it is cheaper than other techniques and all the markers are tested in a standardised approach.

Angiogenesis arrays (an example of a multiplex assay) have been recently used by several researchers. Byers *et al.* (2010) used this technique in measurement of the effect of hypoxia regulated factors in the treatment of squamous cell carcinoma of the head and neck tumours. Funk *et al.* (2010) used a multiplex assay as well to measure the angiogenic markers in the ocular fluid. Whereas Yurkovetsky *et al.* (2007) used it to evaluate the angiogenic markers in melanoma patients. The authors found that the angiogenic markers were higher in the serum of patients with melanoma *vs.* healthy control. Furthermore, they found that VEGF, EGF and hepatocyte growth factor decreased in melanoma patients after treatment with interferon-A2b. Keyes *et al.* (2003) used multiplex technology to measure angiogenic markers in serum of mice bearing human tumour.

The main aim of this study is to identify angiogenesis-related factors, other than TF, that could potentially influence the result of the angiogenesis. In the current study, array kits from different companies were evaluated and the angiogenesis array of the R&D system company was chosen because the number angiogenic markers (55) are higher than others. Each membrane was coated with duplicate spots of antibodies to activin A; ADAMTS-1; angiopoietin; amphiregulin; artemin; angiogenin; angiostatin; tissue factor; CXCL 16; DPPIV, EGF; EG-VEGF; endoglin-1; endothelin-1; endostatin; FGF; basic FGF, acidic FGF; basic fibroblast growth factor; FGF4; FGF7; the GDNF family of ligands (GFL) consists of four neurotrophic factors:GDNF, NRTN,

ARTN) and PSPN; GM-CSF; HB-EGF); HGF ;IGFBP; IL-1B; IL-8; LAP; leptin; MMP8;MMP9 ;MCP1; NRG1-B1 is active EGF; PTX3; PIGF; PDGF (AA, BB and AB); PF4;Prolactin; Serpin ; TIMP 1) and TIMP4; thrombospondin-1; TFG-β); Vasohibin and VEGF. To the best of the author's knowledge, this study is the first that has used an angiogenesis array in PC to study changes of 55 angiogenicmarkers pre-and post-operatively.

6.2 Materials and Methods

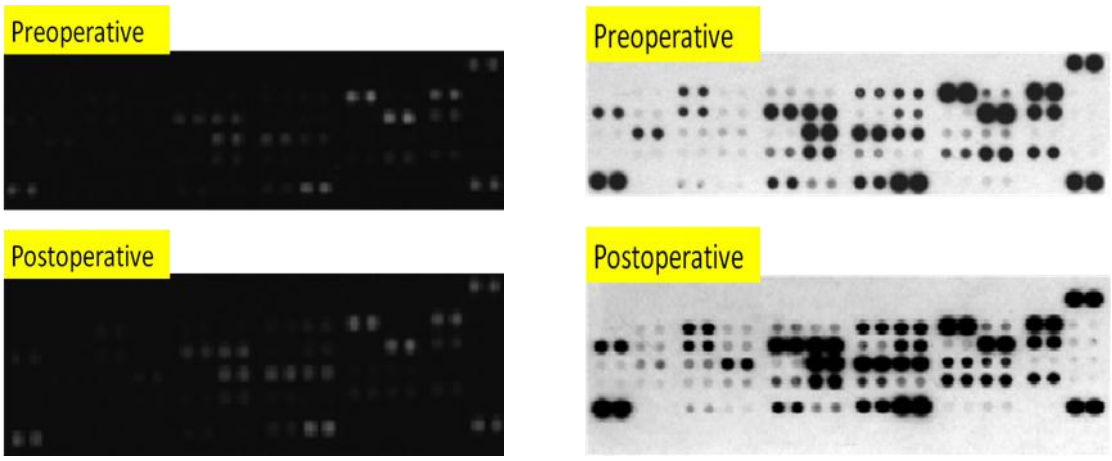
The relative expression of 55 angiogenic markers was evaluated using R&D angiogenic array kit. The angiogenic markers with six positive controls and two negative controls were spotted in duplicate on nitrocellulose membrane. The light emitted after incubation of the membrane with test samples and ECL agents was proportional to the protein expression of the specific markers (see section 2.2.10). The tested pre- and post-operative samples totalled seven; six from group I (1 case; stage I, 1; stage IIIa and 4; stage IIIb) and a single pre-cancerous pancreatic case (group III). The chemotherapy part of the protocol is on-going and not reported here and in all clinical chapters. Two cases of week 8 chemotherapy were studied to evaluate the relationship between angiogenic markers. Methodology was described in detail in chapter 2, section 2.2.10. VEGF ELISA was performed in all available samples and the methodology was described in detail in section 2.2.12.1. Leptin ELISA was performed to validate the angiogenesis results. The methodology of Leptin ELISA was described in detail in section 2.2.12.4.

6.3 Results

6.3.1 Comparison between X-ray and Direct ECL Detection Systems

After incubation of the membranes with sample and ECL reagents, a gel doc system (transmission mode scanner) was used to detect the light emitted from the spots; the scanner captured one picture every five minutes. Pictures were taken at 5, 10, 15 and 20 minutes. As the duration of exposure increased the optical densities of all the dots on the membranes increased for both the test and background. Therefore, the first picture (5 minutes) was chosen for analysis because the dots were clearest against the background (Fig. 6.1A). After the analysis on the gel doc system, the membranes were incubated in fresh ECL reagents. The membranes were again exposed to x-ray films for different durations of 1, 3, 5, 7 and 10

minutes sequentially. Three minutes exposure to x-ray was chosen because the dots were clearest against the background (Fig. 6.1B; 2).



A B

Figure 6.1: Representative example of membrane and X-ray film results of Angiogenesis Array. A- Represents the pre-operative and post-operative protein expression of angiogenic markers measured directly from the membrane after five minutes exposure. B- Represents the pre-operative and post-operative protein expression of angiogenic markers using X-ray film after three minutes.

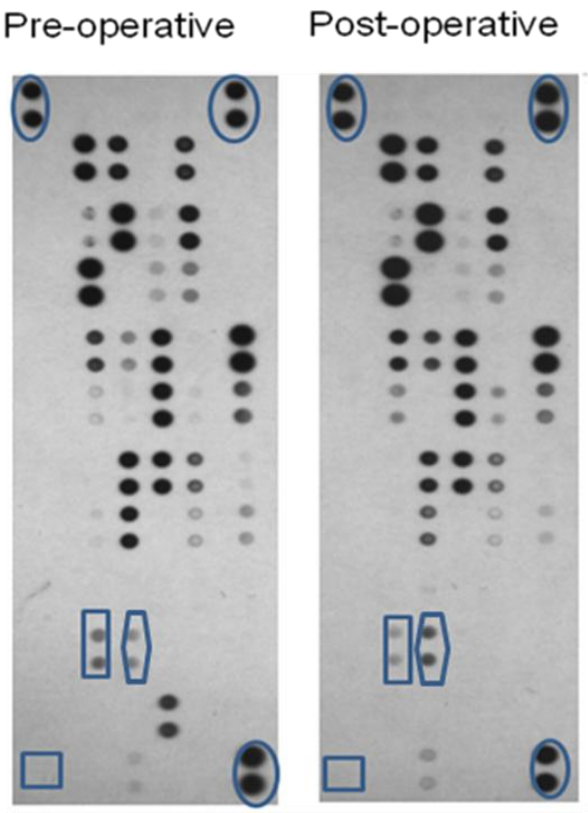


Figure 6.2: Representative example of X-ray film showing pre- and post-operative angiogenic markers on a PC serum. Positive controls are marked by ◯. Negative controls are marked by ▭. VEGF is marked by ▯ PF4 is marked by ⟨⟩.

To assess which method is preferable for analysis, the relative expressions of angiogenic markers were both measured directly from the membrane and from X-ray film. The latter was recommended by the supplier. The analysis was conducted twice on different days using the same samples and different membranes to allow the techniques to be compared for reproducibility. After subtracting the negative control and normalising the results against the mean of 6 positive controls spotted on the membrane, as shown in Fig. 6.3, the difference in the summation of normalised values of optical densities for all 55 values, emitted directly from the membrane, in the first and second experiments was greater than that of the x-ray (Fig. 6.3).

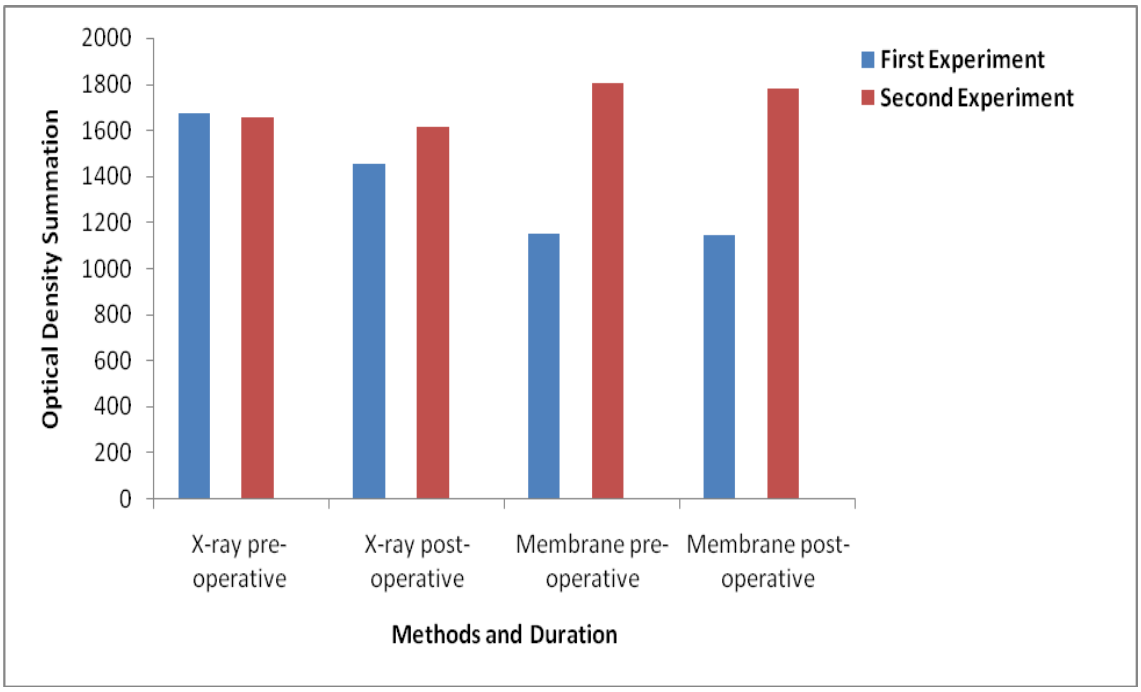


Figure 6.3: Comparison between the reproducibility of X-ray and membrane methods. v The summation of the normalised values of optical densities of all angiogenic markers including the Positive controls have been considered as an indicator. The first experiment is represented in blue colour, the second in red.

Furthermore, the mean normalised values of the duplicate relative expressions of each angiogenic markers were compared individually; the differences between corresponding markers were greater with the membrane method than the X-ray method (Fig. 6.4; 6.5). This difference was seen in both pre- and post-angiogenic factors; however, it must be noted that wide discrepancies were observed in some factors in the x-ray too.

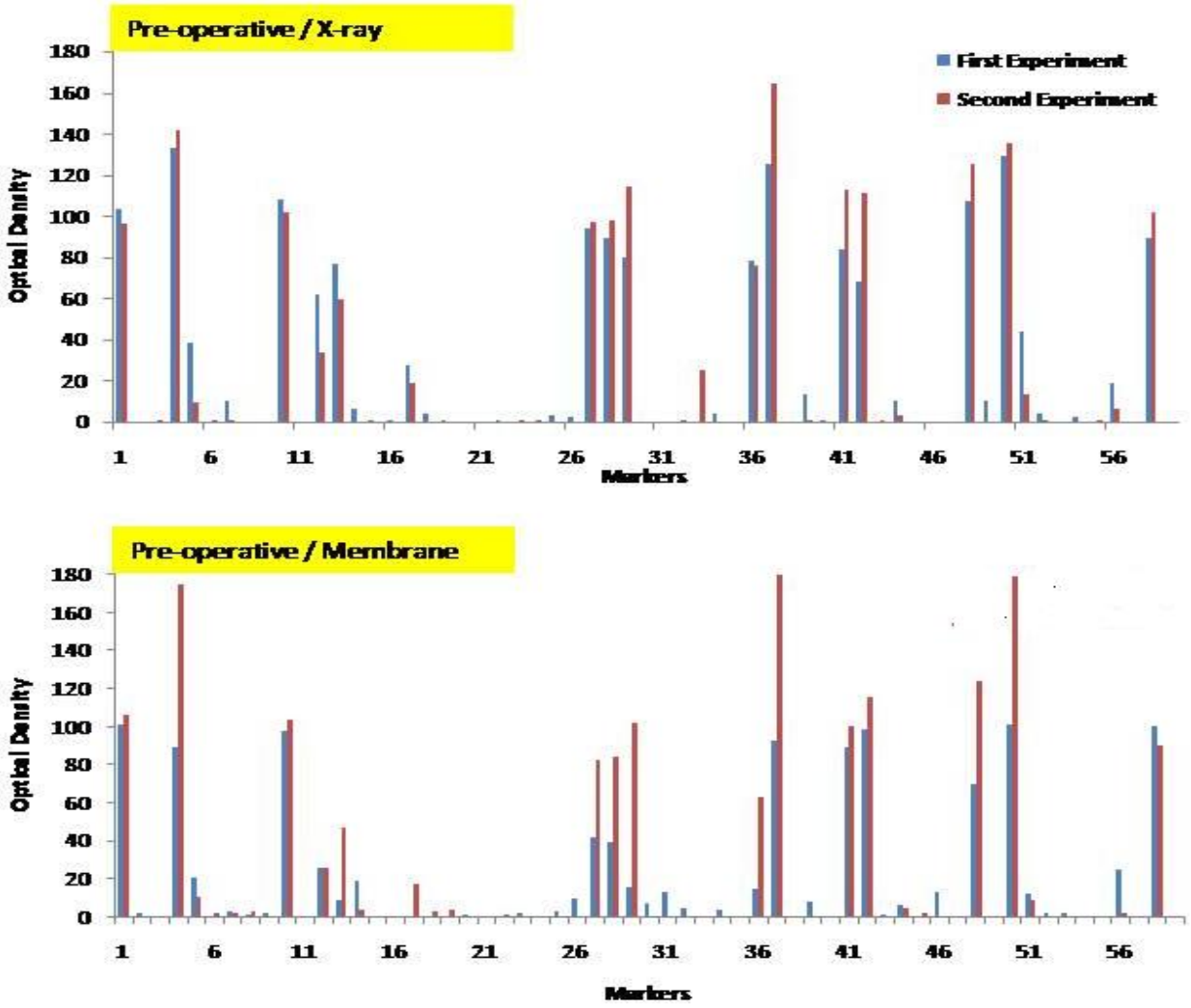


Figure 6.4: Comparison between pre-operative optical densities of the first and second experiment using x-ray and membrane methods. The upper part represents x-ray optical density. The lower part represents membrane optical density. The first experiment is shown in blue, the second experiment in red.

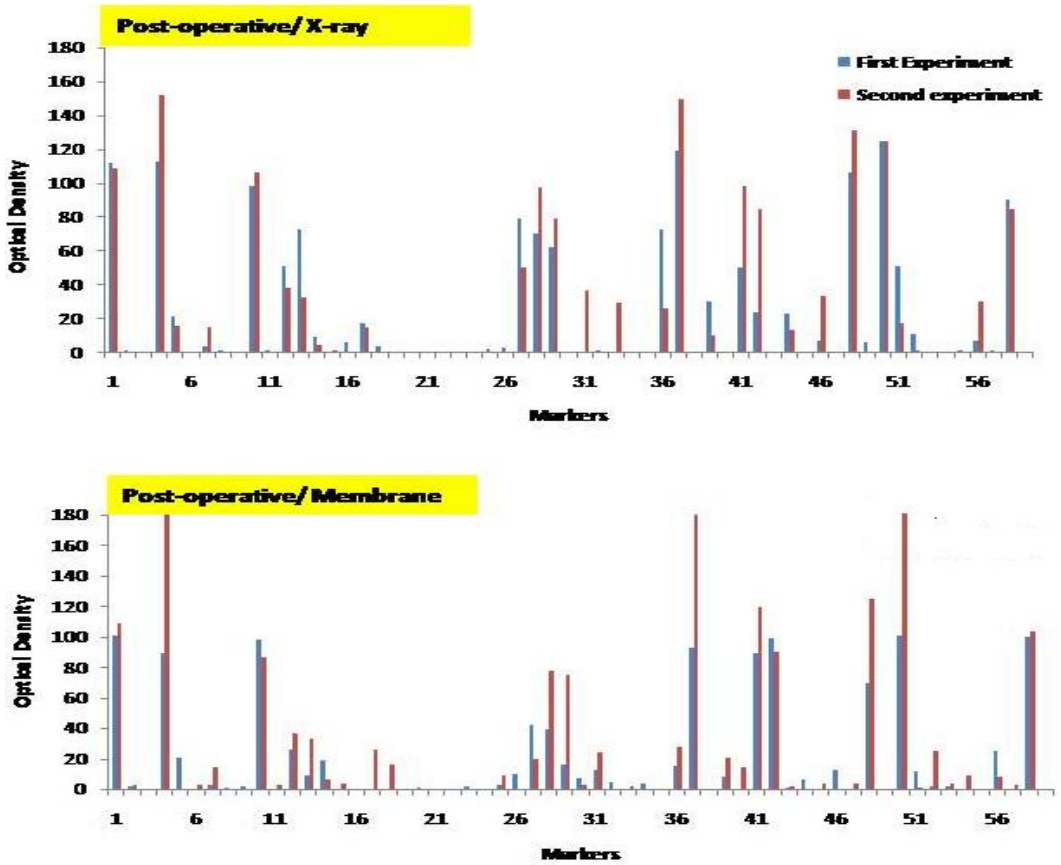


Figure 6.5: Comparison between post-operative optical densities of the first and second experiment using the x-ray and membrane. The upper part represents x-ray optical density. The lower part represents membrane optical density of pre-operative samples. The first experiment is shown in blue, the second experiment in red.

Based on these evaluations, the X-ray method was adopted to assess the angiogenic markers in the study. The difference in the comparability between the two methods may be explained at least in part due to a technical problem with the direct analysis of optical density from the membranes. This is because after making the grid on the membrane, the dots became hazy and unclear. Therefore, it was difficult to recognise and encircle some of the dots in a reproducible manner. This problem was not found when the x-ray film was used and the dots were clear and easy to encircle accurately.

6.3.2 Optimisation and Normalisation of Data

The average optical density of the duplicate negative controls was subtracted from the average optical density of each duplicate positive control on every membrane. The test factor expression value was normalised as a percentage against the average of six positive controls spotted on each membrane. In total, 16 membranes (48 pairs of positive controls) were investigated. As

shown in Fig. 6.6, in 92 % of cases, the optical density of each duplicate positive control was equal to the overall mean of all positive controls ± 10%.

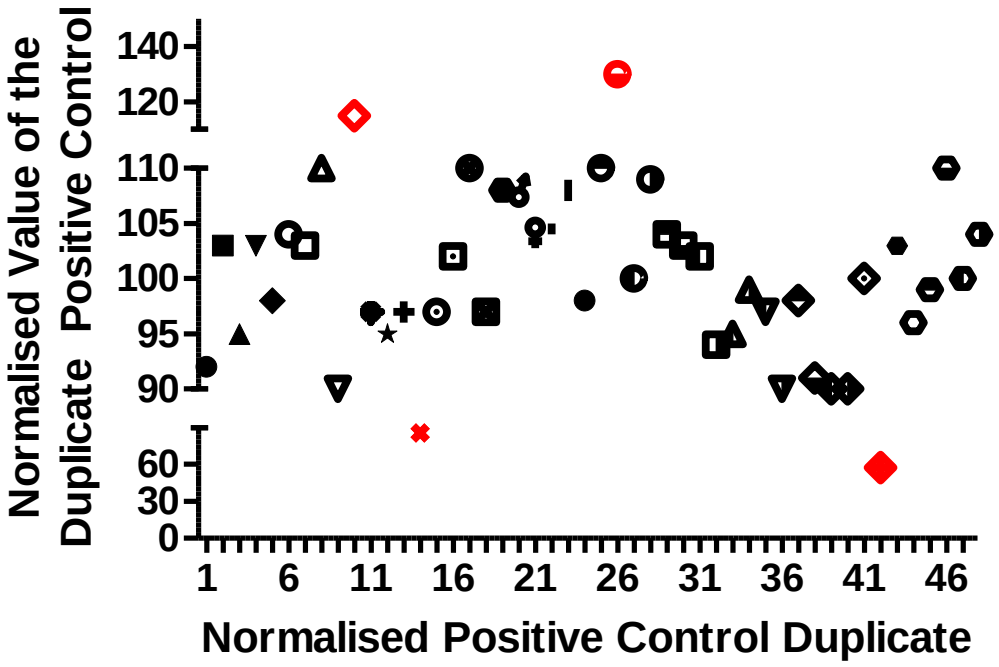


Figure 6.6: The distribution of the average positive control values; estimated from the mean of the six positive controls; were spotted in three pairs on each individual membrane.

Therefore, a normalised value ≤ 10% was considered as not detectable and changes of ≤ 10% between samples were not considered significant due to this level of variation. For example in one membrane the optical densities of the six positive controls were (167, 197), (194, 185), (196, 185). The average of the duplicate negative controls was three. The values after subtraction of the average control were (164, 194), (191, 182), (193, 182): the average of each duplicate was 179, 186.5 and 187.5 respectively. The mean of these values was 184. Thus, each of these normalised values was within the range of positive control ± 10.

In the current study the expressions of 30 angiogenic markers were undetectable in the serums of any of the PC patients because their normalised values of expression were ≤ 10%. For example, the normalised value of artemin on one membrane was 3%, the same value as the average of the six positive controls. Therefore the expression of this marker was considered undetectable. In another example, the normalised value of angiogenin on one membrane was 94% of the average six positive controls; this marker was considered detectable.

The thirty markers that were undetectable in PC sera were: Activin A, ADAMTS-1, Angiopoietin-2; Amphiregulin, Artemin, Coagulation Factor III , EG-VEGF, FGF acidic, FGF basic, FGF 4, 5FGF 7 ,GDNF, GM-CSF, HB-EGF , HGF, ILB , IL8, LAP(TGF-B1), MCP-1, MIP-1a, NRG1-B1,PD-EGF; Preserphin, PIGF , Serpin B5, Serpin F1, TSP-2, uPA, Vasohibin and VEGF C. Twenty five markers were detectable (≥ 11% of positive controls) in the serum of the serum of PC patients. These are listed and grouped into three categories (Table 6.1). TF was not detectable in this experments could be due to several causes such as the samples are frozen sample not fresh plasma, the expression of TF below the threshold of the array kit, the idiotype of the antibody used in microarray could be different from used in other techniques or might be combination of two or more causes. The undetectable expression of ILB and IL6 could be pro-inflamatory process.

Table 6.1: Summary of expression of angiogenic markers in PC sera

Undetectable < 11%	**Low 11-25%**	**Moderate > 25 < 75%**	**High > 75%**
Activin A	Angiostatin/plasminogin	Angiopoietin-1	Angiogenin
ADAMTS-1	Endoglin	CXCL 16	IGFBP-1
Angiopoietin-2	Endothelin	DPPIV	IGFBP-2
Amphiregulin	Platelet factor 4	EGF	IGFBP-3
Artemin	Thrombospondin-1	Endostatin/collagen XVIIII	PDGF-AA
Coagulation Factor III		Leptin	PDGF-AB/PDGF-BI
EG-VEGF		MMP-8	Serpin E1
FGF acidic		MMP-9	TIMP-1
FGF basic		Pentraxin	
FGF 4		Prolactin	
FGF 7		TIMP-4	
GDNF		VEGF	
GM-CSF			
HB-EGF			
HGF			
ILB			
IL8			
LAP(TGF-B1)			
MCP-1			
MIP-1			
NRG1-B1			
PD-EGF			
Preserphin			
PIGF			
Serpin B5			
Serpin F1			
TSP-2			
uPA			
Vasohibin			
VEGF C			

Note: Values expressed as percentages of positive control average.

To semi-quantify the level of expression, the normalised value of the pre-operative expression was considered. If the mean value of % expression of factor of PC cases was below 25%, the expression was considered low. If the mean value was > 25% < 75%, the expression was considered moderate. If the expression was > 75%, it was categorised as high.

For example the pre-operative normalised values of angiogenin in six PC cases were 121%, 120%, 133%, 109%, 90%, 89% giving a mean of 110%. Therefore, the expression was high.

The pre-operative normalised values of TSP-1 in 6 PC cases were 16%, 1%, 4%, 0%, 22%, 37% giving a mean of 13; thus TSP-1 was considered low expression.

Accordingly nine markers showed high expression, eleven factors showed moderate expresion and five markers showed low expression (Table 6.1).

6.3.3 Correlations between Expression of Different Angiogenic Factors

Individual membranes were used to evaluate the angiogenic markers of seven cases (six PC cases pre- and post-operative samples with two week eight, a single non PC case with pre- and post-operative samples). The normalised expression of angiogenic markers was evaluated by investigating correlation in their expression. No correlation was found between any two angiogenic markers in PC except between VEGF and EGF expression when analysed in a pair-wise manner. When all sixteen results were taken into consideration, there was a significant correlation between VEGF and EGF (0.002, corelation test, Fig. 6.7).

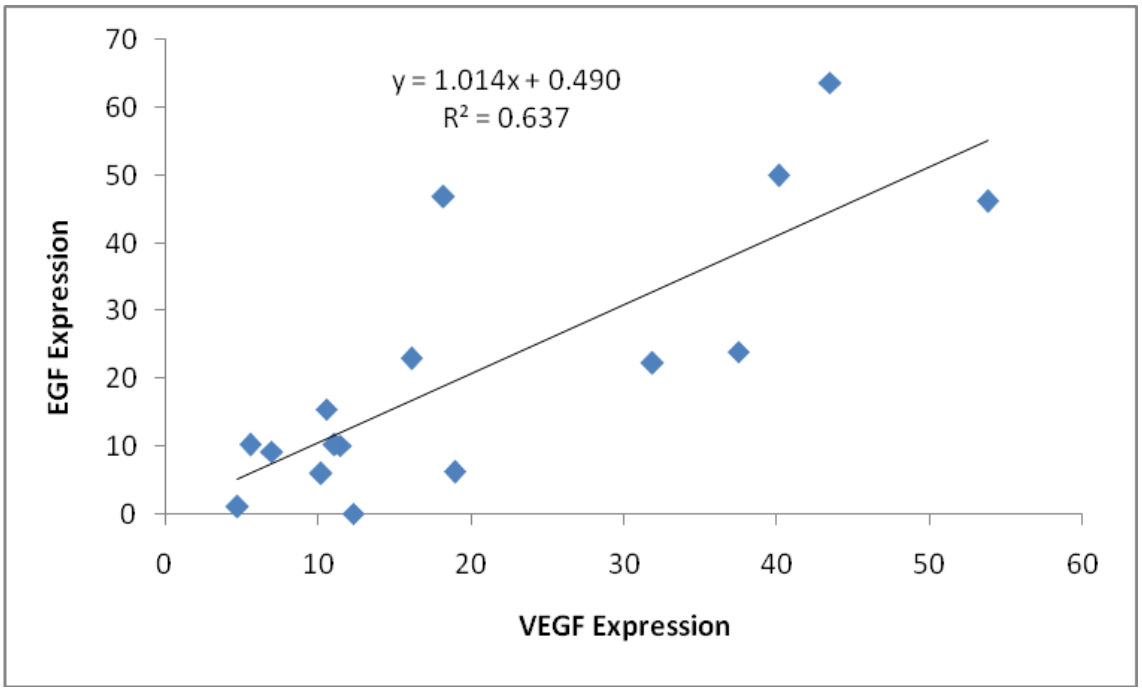


Figure 6.7: The correlation between VEGF and EGF expression in PC cases

6.3.4 Effect of Pancreatectomy on the Expression of Angiogenic Markers.

The mean and median expression of the normalised values for each angiogenic markers pre- and post-operatively are shown in Table 6.2. There was a significant decrease of angiopoietin-1, angiostatin/plasminogen, PDGF-AA, PDGF-AB/PDGF-BB and VEGF (Wilcoxon matched pairs signed rank test). All of these markers are pro-angiogenic except angiostatin/plasminogen, which acts as an angiogenic inhibitor. Furthermore, there was no significant changes in the expression of other angiogenic inhibitor factors such as endostatin, endothelin-1, pentraxin, platelet factor 4, serpin E1, TIMP-1, thrombospondin-1. Moreover, there was no significant increase of any angiogenic marker post-operatively.

Table 6.2: Pre- and post-operative expression of angiogenic markers of PC sera.

Marker	Pre-operative mean		Post-operative mean		P
	Mean ± SD	Median IQR	Mean ± SD	Median IQR	
Angiogenin	115 ± 16	120 (100-127)	101± 34	113 (76-120)	0.56
Angiopoietin-1	43±25	38 (20-68)	28±22	21 (11-50)	0.03
Angiostatin/ Plasminogen	13±11	12 (3-21)	6±6	3.5 (1-12)	0.03
CXCL 16	45 ±25	59 (23-61)	42±19	47 (30-53)	0.4
DPPIV	63±25	74 (37-80)	57±32	67 (31-78)	0.2
EGF	31± 25	34 (5-51)	21 ±17	20 (7-31)	0.43
Endoglin	15±18	6 (2-31)	15±18	7 (1-36)	0.1
Endostatin/collagen XVIII	39±28	43 (8- 65)	31 ±24	28 (11-52)	0.11
Endothelin-1	16 ±21	7 (1-36)	7 (1-28)	126 ± 15	0.5
IGFBP-1	79 ±19	89 (61-93)	64 ±34	80 (37-86)	0.2
IGFBP-2	86 ±7	86 (79-93)	74± 39	81 (53-104)	0.6
IGFB-3	86±13	87 (73-98)	80±21	80 (60-101)	0.2
Leptin	25±31	11 (8-58)	42± 43	42 (0-72)	0.3
MMP-8	62±18	67 (47-77)	57± 26	64 (48-72)	0.4
MMP-9	104±15	105 (93-116)	105±31	116 (93-121)	0.8
Pentraxin	29±25	22 (10- 48)	25±20	23 (8-39)	0.8
PDGF-AA	95±8	94 (88-104)	83±18	86 (73-98)	0.03
PDGF-AB/PDGF-BB	78±9	83 (67-84)	61±20	68 (48-74)	0.03
Platelet factor 4	21±20	16 (2- 41)	25±23	20 (3-50)	0.14
Prolactin	30±27	35 (1-54)	12±10	11 (4- 21)	0.15
Serpin E1	92±13	94 (77-103)	91±27	100 (79-105)	0.5
TIMP-1	111±10	109 (105-116)	102±18	101 (81-116)	0.12
Thrombospondin-1	13±15	10 (1-26)	16±4	12 (3-27)	0.2
VEGF	30± 16	26 (17-46)	19 ±15	14 (6.5-38)	0.03

Note: Wilcoxon signed rank test was used for statistical analysis. IQR is interquartile range. Five highlighted angiogenic markers show significant post-operative changes. The unit of expression is optical density

The results were re-analysed by comparing the mean of six PC cases pre-and post-operatively and using 10 points difference as a cut-off. Therefore, if there was an 11 point change between pre- and post-operative, the expression would be considered as a post-operative increase or decrease accordingly. However, if the difference between the mean pre-operative and post-operative was less than 11%, the results would be considered as no change.

For example: The mean normalised value of PC cases of angiogenin was 115% pre-operatively and 101% post-operatively, so the decrease of post-operative expression was 14 points. Therefore the expression of this marker was considered to have decreased post-operatively.

Conversely in the same sample, the mean CXCL 16 expression was 45 pre-operatively and 41 post-operatively, so the decrease of post-operative expression was 4 points. Therefore the expression of this marker was considered as no change.

Not all values decreased post-operatively; for example the mean expression of the normalised values of leptin pre-operatively was 25% while post-operatively it was 42%. In this marker there was an increase of 17 points at post-operative over pre-operative. Therefore, the expression of this marker was considered as increasing post-surgery.

There was no change in the expression of angiostatin/plasminogen, CXCL 16, DPPIV, EGF, endoglin, endostatin/collagen, endothelin-1, IGFB-3, MMP-8, MMP-9, Pentraxin, Platelet factor 4, Serpin E1, TIMP-1, Thrombospondin-1.

Furthermore the expression of angiogenin, angiopoietin-1, IGFBP-1, IGFBP-2, PDGF-AA, PDGF-AB/ PDGF-BB, prolactin and VEGF decreased post-operatively in PC cases. Leptin expression increased post-operatively (Table 6.3).

Table-6.3: Summary of Post-operative changes of angiogenic markers of PC cases.

Decrease	No change	Increase
Angiogenin	Angiostatin/plasminogine	Leptin
Angiopoietin-1	CXCL 16	
IGFB-1	DPPIV	
IGFB-2	EGF	
PDGF-AA	Endoglin	
PDGF-AB/ PDGF-BB	Endostatin/collagen	
Prolactin	Endothelin-1	
VEGF	IGFB-3	
	MMP-8	
	MMP-9	
	Pentraxin	
	Platelet factor 4	
	Serpin E1	
	TIMP-1	
	Thrombospondin-1	

Looking at the changes of PC cases, it was clear that all those which showed a decrease of at least 10% post-operatively were pro-angiogenic (angiogenin, angiopoietin-1, IGFB-1, IGFB-2, PDGF-AA, PDGF-AB/ PDGF-BB, prolactin and VEGF).

6.3.5 VEGF ELISA

This work was designed to measure VEGF concentration of PC cases. This experiment was conducted in 29 cases of group B, 9 cases of group III and 14 cases of group IV as these were the only cases for which serum was available. The second aim was to optimise the angiogenesis array results.

6.3.5.1 Baseline Results

The sample VEGF was measured from a standard curves that performed on the same day of the experiment (Fig. 6.8).

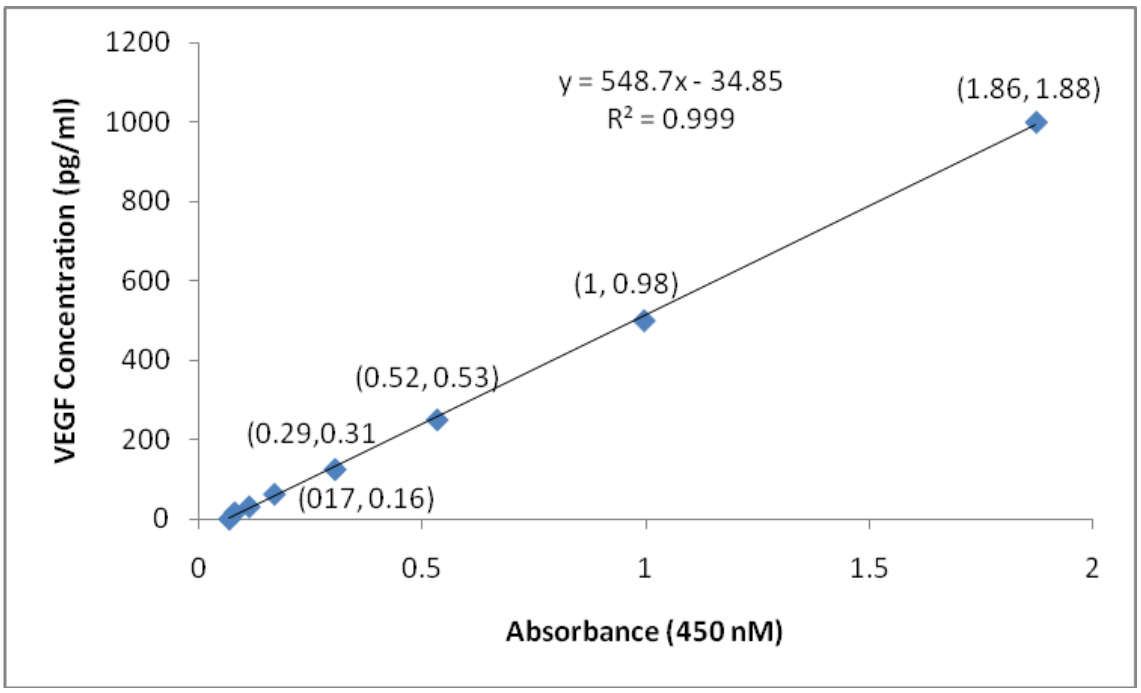


Figure-6.8: Representative standard curve for VEGF measurement that correlates with the average of absorbance value.

The VEGF concentration of group B (all malignant cases) was significantly higher than that of control group IV. Median and interquartile range (IQR) of group B was 840 (464-1477) pg/ml *vs.* 355 (212-433) pg/ml in the controls, p= 0.005. The VEGF concentration of group B was also higher than that of group III but the difference was not significant (Table 6.4).

Table 6.4: VEGF concentration of pancreatic sera compared with the control groups.

Group	No	VEGF Concentration pg/ml	
		Median and IQR	**P Value**
B (all cancer cases)	29*	840 (464-1477)	B *vs.* III P= 0.371
III (pre-cancerous)	9	600 (206-1048)	
IV (cholocystectomies)	14	355 (212-433)	B *vs.* IV p=0.005

Note: Mann Whitney test was used for statistical analysis. 29* (available samples).

6.3.5.2 Pre and post operative data

To investigate the effects of the surgery, VEGF serum level was analysed pre- and post-operatively. The post-operative VEGF concentration of group C was significantly lower than the pre-operative [median and IQR was 409 (218-723) pg/ml *vs.* 840 (611-1846) pg/ml, p=0.013, Table 6.5], while the post operative VEGF of group III and IV did not differ significantly from the pre-operative level {[Median and IQR was 695 (228-1656) pg/ml *vs.* 1048 (371-1053), p= 1], [278 (204-486) *vs.* 373(325-486) pg/ml , p=0.359] respectively, Table 6.5}.

Table 6.5: Pre- and post-operative serum VEGF of PC cases and control groups.

Group	No	VEGF Concentration pg/ml		P value Pre-*vs.* post-operative
		Pre-operative	**Post-operative**	
		Median & IQR	**Median & IQR**	
C	11	840 (611-1846)	409 (218-723)	0.013
III	4*	1048 (371-1053)	695 (228-1656)	1
VI	9	373 (325-486)	278 (204-486)	0.359

Wilcoxon signed rank test was used for statistical analysis. IQR is interquartile range. 4* (The analysis was conducted in 4 out of 5 cases, 1 case was not involved because the entire serum sample had been used previous experiments).

6.3.6 Leptin ELISA

This part of the work was designed to evaluate the results of the angiogenesis array kit. A leptin ELISA kit from the same company as the angiogenesis array (R & D) was performed in available pre- and post-operative PC samples (7), including the six PC samples of angiogenesis array. The methodology was described in section 2.2.12.3. The pre-operative leptin level of PC cases (using ELISA) was lower than the post-operative (5511 ± 4055 pg/ml *vs.* 8890 ± 7686 pg/ml, p= 0.29, Wilcoxon matched-pairs signed rank test). Furthermore the pre-operative normalised optical density of the PC cases (using angiogenesis array) was lower than the post-operative (25 ± 31 *vs.* 42± 43). The post-: pre-operative mean ratio of leptin was 1.6 using ELISA while that for angiogenesis array was 1.7. Therefore, the results of ELISA were comparable to angiogenesis array.

6.4 Discussion

Angiogenesis is a prerequisite for growth and metastasis of solid tumours including PC (Folk man, 2002 and 1997). There is increasing evidence that the disturbance of the balance of pro- and anti-angiogenic factors contributes to the pathogenesis of numerous disorders (Abdollahi *et al.*, 2005, Carmeliet, 2005, Hanahan and Folkman, 1997). A detailed understanding of the mechanism of angiogenesis would probably improve the therapeutic options. Furthermore, measurement of angiogenic markers in a PC patient's serum may provide useful prognostic features. Although the main factor studied in this thesis is TF which "straddles" the thrombosis and angiogenesis pathway, it would be expected that it would not be the only factor driving these complex processes; therefore to gain a greater insight into the potential impact of other angiogenic factors a limited pilot micro-array study was undertaken to try and uncover other angiogenesis related markers that may be affected by the removal of the cancer.

Seven cases were investigated in this pilot including six PC cases and a single pre-malignant lesion. A few previous studies used protein array in PC. One of these, Chang *et al.* (2008), compared biomarkers in PC cases stage using a multiplex assay. The authors found that several tumour markers, such as CA19-9, may have prognostic significance. Furthermore, Fredriksson *et al.* (2008) evaluated 20 angiogenic markers using an angiogenesis array in a series of 18 PC cases with 19 healthy controls plus 19 ovarian cancer with 20 healthy control. Fredriksson noted that CA-125 expression was increased in pancreatic and ovarian cancer serum *vs.* control groups.

The current study is to the best of the author's knowledge the first study to look at an angiogenesis array of 55 markers in PC pre- and post-operatively. It was found that the expression of 30 angiogenic markers (Table 6.1) was undetectable in the serum of the PC cases. This may be due to these angiogenic markers being expressed by PC tumours at very low level and therefore secreted into the circulation at levels below the threshold of detection of the angiogenesis array kit. Furthermore, the undetectable level might be related to biochemical and physiological features of these markers such as their solubility or to the stage of the tumour (none of the cases included had distant metastases at the time of attempted resection).

Ivanovic *et al.* (2006) measured the plasma concentration of TGF-β in 53 breast cancer cases *vs.* 37 healthy controls. The authors reported that the mean plasma concentration of TGF- β of 10 cases of stage I and stage II; (0.94 ng/ml) was significantly lower than that of 43 breast cancer patients with stage III and IV was (2.3 ng/ml). Duranyildiz *et al.* (2009) reported that the TGFβ1 concentration at early stages of PC is not higher than that of healthy controls (0.19 ng/ml *vs.* 0.19 ng/ ml). The level of TGF-β was undetectable in the six PC cases submitted in the current study. In addition to what was mentioned previously, the discrepancy might be related to serum being used in the current study while plasma was used in Ivanovic and Duranyildiz's studies. Furthermore, Ivanovic and Duranyildiz used ELISA.

aFGF, bFGF and FGF 7 were undetectable in the current study, in contrast to other studies (Pistol-Tanase *et al.*, 2008). These authors reported that the concentration of bFGF was significantly higher than that found in healthy controls (28.19 pg/ml *vs.* 11.7 pg/ml) using ELISA in 32 PC cases (21 cases; stages I and II, 11 cases; stages III and IV). Furthermore, it was reported that the serum level of bFGF in 14 out of 21 stage I and II PC cases was < 25 pg/ml, whilst in 10 cases out of 11 stage III and IV PC cases the serum level was 25 pg/ml or greater. There is no immediate explanation for this difference. Given that the microarray is much more sensitive we would have expected to have seen raised levels of bFGF. However, it is possible that the idiotypes of the antibodies used in microarray could be different from those used in ELISA. This difference was found to be significant (Pistol-Tanase *et al.*, 2008). Furthermore, the current study did not show high expression of HGF, although other studies show high levels of EGF and HGF in other types of cancer such as colorectal cancer (Yoon *et al.*, 2006).

Two markers were measured by ELISA (leptin and VEGF) for validation of the angiogenesis results. Both leptin and VEGF ELISA results were comparable to angiogenesis array results. In both techniques, there was increase of leptin and decrease of VEGF post-operatively. The VEGF concentration using ELISA was significantly higher than control group IV (median and IQR of group B was 840 (464-1477 pg/ml) *vs.* 355 (212-433 pg/ml), p= 0.005). This result was in accordance with other studies such as Pistol-Tanase *et al.* (2008). In the latter study, VEGF of 32 PC patients was measured by ELISA and bead-based multiplex assay. They found a significant increase of the mean concentration of VEGF *vs.* 20 healthy controls (349.28 pg/ml *vs.* 115.06 pg/ml). This is consistent with results of other studies (Karayiannakis *et al.*, 2003). In a series of 58 PC cases, the authors observed significant increases of serum VEGF *vs.* 51 healthy

controls [median and IQR; 505 (284–839) pg/ml) *vs.* 193 (104–297) pg/ml]. Furthermore, the authors noted a significant increase of serum VEGF in 40 PC cases with lymph node metastasis *vs.* 18 PC cases without lymph nodes metastasis (609 *vs.* 301 pg/ml). Furthermore, it has been reported that in a series of 92 PC cases *vs.* 60 healthy controls, there was a significant increase of serum VEGF and PIGF. The authors postulated that there was a reverse relationship between serum VEGFR 1 receptor and PC stage and a high VEGF/VEGFR 1 ratio using ELISA was significantly associated with poor prognosis of PC (Chang *et al.*, 2008). This kind of high VEGF expression has been reported for other cancers too. Heer *et al.* (2001) reported that the VEGF serum level of 200 breast cancer cases was significantly higher than that of 80 controls (median with IQR; 306 pg/ml (157-452) *vs.* 168 pg/ml (102-245) using ELISA).

In the current study, multiple analyses were undertaken looking for correlations of expression of these markers. A correlation was found only between VEGF and EGF expression (Fig. 6.8). When 16 serum samples were considered, there was a correlation between expression of VEGF and EGF (R^2=0.64, using linear equation p< 0.002). The result of the current study was in agreement with the results of other studies such as Summy *et al.* (2005) and Tabernero, (2007). Therefore, the suggestion was that the synergistic targeting of VEGF and EGF might have a role in the treatment of PC. The author notes, however, that in the current study, EGF did not change statistically in the post-operative *vs.* pre-operative setting. It is possible that the correlation found could be a statistical event rather than a biological one and the result of the multiple tests undertaken.

This study found there was a significant decrease of angiopoitin-1, angiostatin/plasminogen, PDGF-AA, PDGF-AB/PDGF-BB and VEGF post-operatively (Wilcoxon signed rank test). This result was supported by significant decrease of VEGF concentrations post-operatively when analysed by ELISA. The post-operative VEGF concentration of group C was significantly lower than the pre-operative [The median and IQR was 409 (218-23) pg/ml *vs.* 840(611-1846) pg/ml, p=0.013], while the post operative VEGF of group III and IV did not differ significantly from the pre-operative {[Median and IQR was 695 (228-1656) pg/ml *vs.* 1048(371-1053), p= 1], [278 (204-486) *vs.* 373 (325-486) pg/ml, p=0.359] respectively, Table 6.5, Fig 6.11}.

All of these markers are pro-angiogenic except angiostatin/plasminogen, which acts as an angiogenic inhibitor. Furthermore, there was no change in the expression of angiogenic inhibitor factors such as angiostatin, endostatin, endothelin-1, pentraxin, platelet factor 4, serpin E1, TIMP-1, thrombospondin-1.

However, analysing data using the ≤ 10 cut off point; the current study shows that 15 factors showed no changes, eight of which were angiogenic inhibitors. At the same time, there was an increase in the mean serum expression of leptin post-operatively. Leptin regulates energy intake and expenditure (Brennan & Mantzoros 2006) but does not have a direct effect on angiogenesis and cell invasion. Moreover, the expression of angiogenin, angiopoitin-1, IGFBP-1, IGFBP-2, PDGF-AA, PDGF-AB/PDGF-BB, prolactin and VEGF was decreased post-operatively. Furthermore the summative change of all these pro-angiogenic markers showed a collective reduction post-operatively. Therefore, the re-analysis of data using the cut off point 10 reveals an increase of leptin and a decrease of other interesting factors such as IGFBP-1, IGFBP-2 which did not show significant changes using statistical test, which might be due to the limited tested samples.

Karayiannakis *et al.* (2003) reported that serum VEGF of the 12 PC cases with distant metastasis was significantly higher than that of 46 PC cases without distant metastasis (1142 pg/ml *vs.* 390 pg/ml). In a series of 58 PC cases that underwent surgical removal, there was a significant decrease of serum VEGF levels post-operatively compared with pre-operatively 580 pg/ml *vs.* 512 pg/ml (Karayiannakis *et al.*, 2003). Justinger *et al.* (2008) noted that VEGF and HGF increased post-operatively in PC sera after pancreatic and hepatic resection (1-3 days) thought to be associated with wound healing.

A decrease of VEGF post-operatively was noted with other malignancies, serum VEGF concentration was decreased after surgical removal of breast cancer (Konukoglu *et al.*, 2007, Zhao *et al.*, 2004) , colorectal cancer (Nakayama *et al.*, 2002) and osteosarcoma (Kaya *et al.*, 2004).

Surprisingly, the current study shows a decrease of angiostatin/plasminogen post-operatively. Based on these results the tumour is not only the source of pro-angiogenic markers but also the source of anti-angiogenic markers. Therefore removal of the tumour might decrease both pro-angiogenic and anti-angiogenic factors. This finding is in accordance with the result of other studies such as Peeters *et al.* (2005) who noted a decrease of angiogenic inhibitors including angiostatin and endostatin after surgical removal of colorectal cancer (Peeters *et al.*, 2005). The crucial point is the angiogenic balance which depends on the ratio of the pro- to anti-angiogenic factors and control of the angiogenic switch. Abdollahi *et al.* (2007) proposed that there are different simultaneous genes that could control the angiogenic switch in pancreatic disease, which include "angiogenesis off" as in the normal pancreas, "angiogenesis on" as in PC and somewhere between "on and off" as in chronic pancreatitis. The current study suggests that pancreatectomy significantly decreases angiopoitin-1, PDGF-AA, PDGF-AB/PDGF-BB and VEGF. All of these markers are pro-angiogenic. Furthermore, as hypothesised there was no significant increase of any pro-angiogenic markers. Moreover, there was no change in the expression of key angiogenic inhibitors such as endostatin, endothelin-1, pentraxin, platelet factor 4, serpin E1, TIMP-1, thrombospondin-1. Sequentially, pancreatectomy decreases the ratio of pro-angiogenic factors: angiogenic inhibitors and shifts the angiogenic balance to the angiogenic inhibitor state. Lastly, this study suggested that the tumour could be the source of pro-angiogenic and angiogenic inhibitors as there was a decrease of angiostatin/plasminogen, which is an angiogenic marker.

CHAPTER SEVEN: CHEMOTAXIS AND ANGIOGENIC ACTIVITY OF PANCREATIC CANCER PATIENTS' SERA

7.1 Introduction

7.1.1 Pancreatic Cancer and Invasion

There are a number of factors that influence cancer cell motility. The necessary preconditions and the impact of the coagulation pathway on this process are extensively discussed in section 1.18. There are ,however, other molecular pathways that can contribute to this process in the cancer setting and should be mentioned such as growth factors (EGF, TGF β1, Barrandon and Green, 1987) and cytokines (TNF-α, IL-6, IL-8, Tamm *et al.*, 1994, Orosz *et al.*, 1993, Rosen *et al.*, 1991). In an early study of motility factors secreted from the tumour a molecule termed 'scatter factor' was shown to enhance the movement of cells (Stoker *et al.*, 1987). A subsequent study showed that this factor is identical to hepatocyte growth factor, which induces cell growth, motility and invasion (Naldini *et al.*, 1991). Furthermore, it was reported that geranylgeranyltransferase-I inhibits pancreatic cell invasion *in vitro* (Kusama *et al.*, 2003) by blocking the biosynthesis of farnesylpyrophosphate and geranylgeranylpyrophosphate. Baran *et al.* (2009) reported that tumour infiltrating monocytes and macrophage increase pancreatic cell invasion due to secretion of TNF alpha. Moreover, radiation increases PC invasion by blocking of a matrix metalloproteinase inhibitor (Qian *et al.*, 2002).

7.1.2. Factors Enhancing Angiogenesis Specifically for APC

The first factor found to be important in angiogenesis was basic fibroblast growth factor (bFGF), which was discovered in the 1980s. Fibroblast growth factors (FGF) are heparin-binding proteins involved in angiogenesis, wound healing and embryonic development and cell proliferation (Okada-Ban *et al.*, 2000). FGF-acidic (aFGF), also known as FGF-1 and HBGF-1, is a 17 KDa protein produced by different cell types which has an essential role in cell proliferation, development and angiogenesis (Yamagishi and Okamaoto, 2010). In normal tissue, bFGF is present in basement membranes and in the sub-endothelial extra-cellular matrix of blood vessels (Botta *et al.*, 2000, Okada-Ban *et al.*, 2000).

VEGF is another essential pro-angiogenic factor in the stimulation of angiogenesis. It stimulates vasodilatation of the existing vessels and increases permeability of the vessel. It plays an important role as a general activator of endothelial cell proliferation and capillary formation (Ferrara *et al.*, 2003). VEGF is described in detail in section 1.12.

The angiopoietin group has a role in the survival of normal and malignant endothelial cells (Peters, 1998). Angiopoietin -1 (Ang-1) plays an important role in endothelial survival and inhibits apoptosis by the activation of the PI3K/Akt survival pathway through activation of tyrosine kinase receptor Tie-2 (Papapetropoulos *et al.*, 2000). However, there are some contradictory reports about Ang-1; some have shown that it prevents hepatic growth, vascular permeability and angiogenesis (Stoeltzing *et al.*, 2003).

Transforming growth factor beta (TFG-β) acts as a pro-angiogenic factor in low doses because it up-regulates angiogenic factors and proteinases, while in high doses it acts as an angiogenic inhibitory factor because it prevents endothelial cell growth, stimulates differentiation of smooth muscle cells and enhances formation of the basement membrane (Carmeliet, 2003).

Several interactions among VEGF, Ang and other angiogenic factors have been described by Harvey and Oliver (2004) who identified retinaldehyde dehydrogenase 2, Norrin, Frizzled-4 and Noge-B genes that contribute to these processes. Furthermore, it has been postulated that hedgehog pathway is one of the "core" signalling pathways that undergo somatic alterations in PC. Therefore targeting of this pathway might be helpful in treatment of cancer (Hidalgo & Maitra 2009). Moreover, in mouse experiments, Feldmann (2008) noted that cyclopamine, which blocks hedgehog signalling, prolongs survival in PC. The hedgehog signalling pathway, which has been known to be involved in cancer, is an important regulator of the embryonic development that becomes reactivated in cancer.

Angiogenesis and metastasis are vital aspects of cancer biology and impact on PC treatment. Therefore identifying mechanisms of PC invasion may facilitate the development of more effective treatments that could improve patient outcome. To the best of the author's knowledge this is the first study to evaluate PC serum chemotaxis activity (chemo-taxis; also called

haptotaxis) which is represented *in vitro* by quantitative differences in AsPC1 motility and angiogenic activity as represented by the induction of tubular formation.

7.2 Aims

The purpose of this part of the work was to determine the following:

1. Whether the chemotaxis and angiogenic activity of PC sera is higher than that of control groups.
2. Whether the serum chemo-attractive and angiogenic activity of PC cases' sera are decreased post-operatively.
3. Correlation of cell invasion and angiogenesis with each other and with microparticles levels.

7.3 Methodology

7.3.1 Methodology of Cell Invasion

AsPC1 cells were cultured and harvested to grow in SFM as described in section 2.2.3.1. The Boyden chamber was coated with collagen IV and 10% (v/v) of PC sera was added to the lower compartment as described in section 2.2.7.1. The methylene blue stain method was used to evaluate the invaded cells. The AsPC1 migration is represented by the mean number of migrating AsPC1 counted in five high–powered fields per membrane at 40x objective magnification.

7.3.2 Methodology of Angiogenesis

HDMEC were adapted in SFM (as described in section 2.2.2.2.1) and seeded on the top of collagen type I gel (2.2.6.2). The gel was formed using method B described in section 2.2.6.1.B as this was shown to be the most reliable. HDMEC were treated with PC and control sera (100 µl) pre- and post-operatively. Capillary (tubule) development was observed over a 3-day period and recorded using an Imaging RETIGA 2000 R camera attached to a LeitZ Laborlux S fluorescence microscope as described in section 2.2.6.3. The total length and number in 10 pictures at 40x objective magnification was used as a method for evaluation of the tubule assay and angiogenesis in this study, as detailed previously.

7.4 Results

7.4.1 Cell Invasion Results

7.4.1.1 Baseline Evaluation

Median values of ASPC1 invasion for the cancer groups (A, B, E) were significantly higher than those of control groups III and IV (P= 0.0001, Kruskal-Wallis test). There was no statistical difference in the median values of the AsPC1 cell invasion assay between cancer subgroups (Mann Whitney test). The subgroup comparative analyses to control cases were as follows: the level of AsPC1 cell invasion of group B was significantly higher than that of group III and group IV (Table 7.1; Fig. 7.1). AsPC1 cell invasion of PC cases (groups A); median and IQR; 124 (102-148, mean 126 ± 27) was significantly higher than the pre-operative level of group III (p= 0.05, Mann Whitney test) and was also significantly higher than that of group IV (p= 0.0001, Mann Whitney test, Table 7.1; Fig. 7.1).

The median value for all PC cases (group A) did not differ significantly from that found for group E (median and IQR; 143 (60-131), p= 0.2, Man Whitney test, Figure 7.1). The median of all resected cases group C did not differ from that of the unresected cases (group D, statistics not shown) indicating the homogeneous nature of the 'cancer diagnoses' at baseline.

Table 7.1: The baseline chemo-attractive effect of cancer serum and control group represented by the mean of AsPC1/HPF.

Group	No	Cell Invasion (Cell /HPF)		
		Median and IQR	Mean ± SD	P Value
B (all cancer cases)	32	131 (104-153)	129 ± 26	B *v* III P= 0.04
III (pre-cancerous)	9	79 (55-158)	95±50	
IV (cholecystectomies)	20	54 (48-59)	54±10	B *v* IV p=0.0001

Note: All samples were conducted in duplicate.

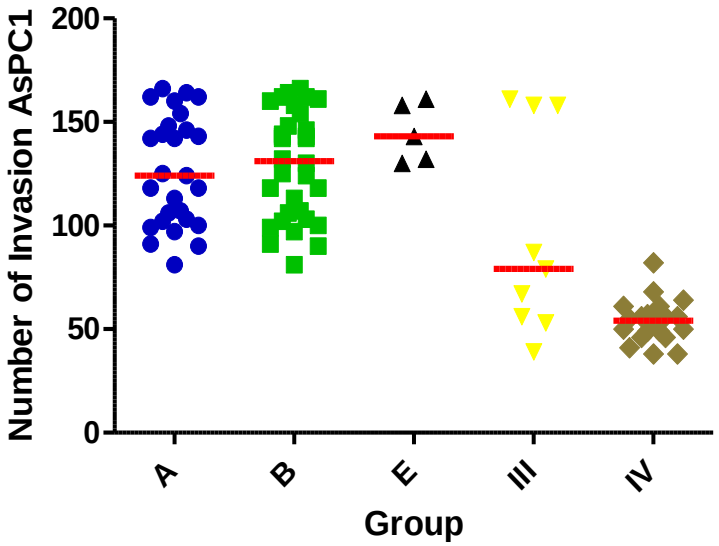


Figure 7.1: The baseline chemo-attractive effect of PC serum and control group that was represented by the mean of AsPC1 /HPF. All samples were conducted in duplicate. The red bar represents the median. Full statistical correlations in the text and salient correlations in table 7.1. This figure shows all cancer cases (Group B) and all pancreatic cases (Group A)-and all cholagiocarcinoma cases Group E for illustrative purposes.

7.4.1.2 Pre- and Post-Resection Data

A comparison of pre-operative and post-operative levels of group C (all the 11 resected cases) revealed that the post-operative level was significantly lower than that of the pre-operative (Wilcoxon signed rank test, Table 7.2, Fig. 7.2-7.4). Comparison of pre-operative and post-operative levels in the nine PC cases of group I, for which both samples were available, revealed that the post-operative median and IQR; 88 (76-100) was significantly lower compared with the pre-operative median; 123 (104-144, p= 0.007; Wilcoxon signed rank test, Fig 7.2-4).

There were no statistically significant differences between the pre-operative and post-operative chemotaxis activity of AsPC1 motility of groups III and IV (P = 0.12, 0.6 respectively, Wilcoxon signed rank test, Table 7.2). There was no statistically significant difference between the pre-operative and post-operative chemo-attractive activity of AsPC1 motility of group D (4 cases, p= 0.6).

Table 7.2: Comparison of the chemo-attractive activities of PC and control sera represented by the mean of AsPC1 cell invasion pre- and post-operatively.

Group	No	Cell Invasion				P
		Pre-operative		Post-operative		
		Median & IQR	Mean ± SD	Median & IQR	Mean ± SD	
C	11	125 (107-158)	131 ± 23	88 (85-112)	95±27	0.002
III	5	79 (53-158)	100 ± 55	79 (49-110)	79 ± 35	0.12
VI	11	55 (41-61)	54 ± 13	56 (48-58)	54 ± 7	0.6

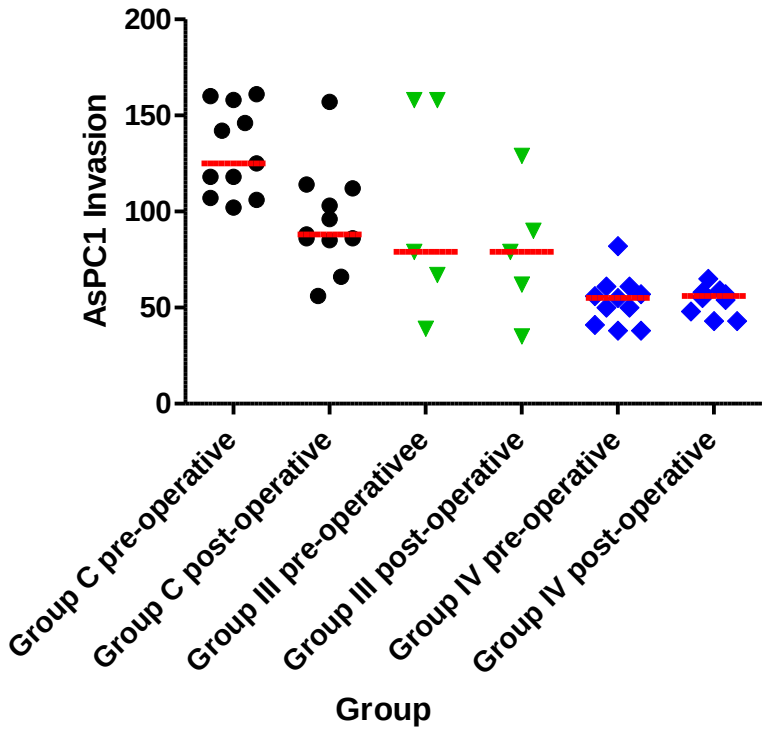


Figure 7.2: A comparison of AsPC1 cell invasion using pre- and post-operative sera. The red bar represents the median.

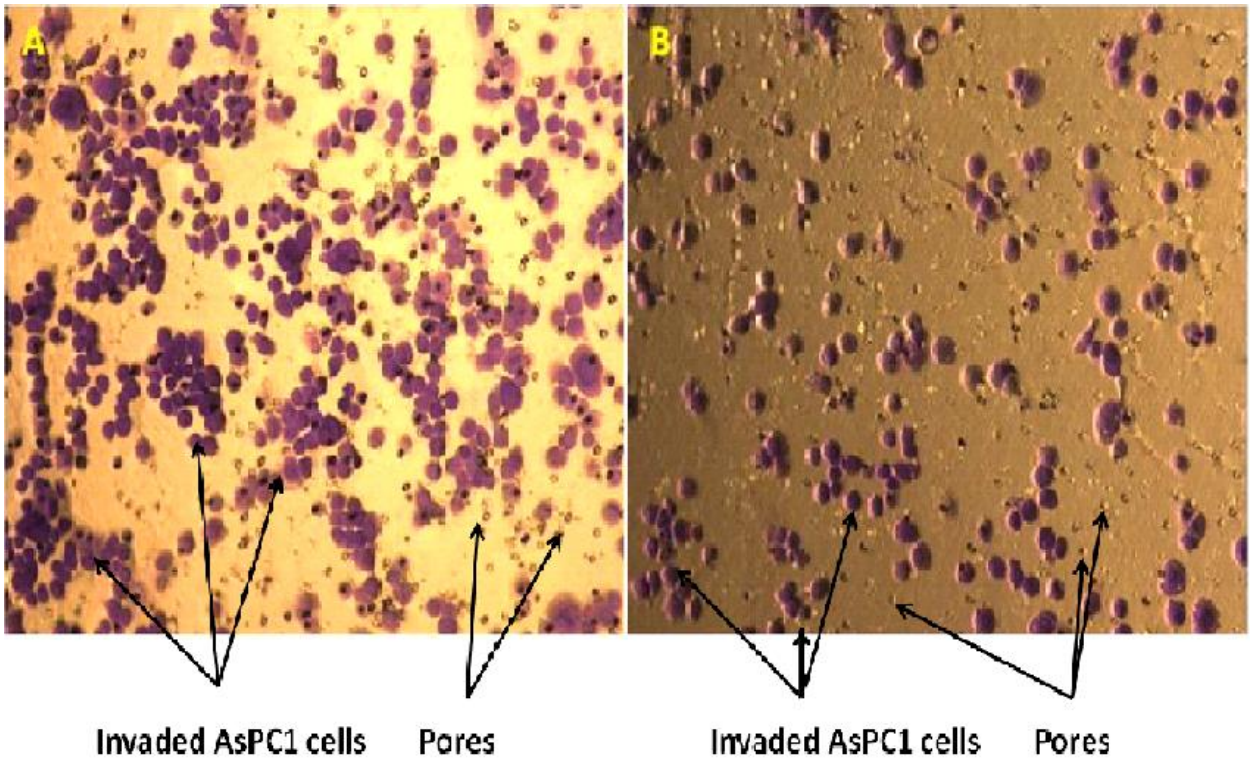


Figure 7.3: Representative example of pre- and post-operative cell invasion. A- Invaded AsPC1 with pre-operative pan. B- Invaded AsPC1 with post-operative pancreatic serum was used as a chemo-attractant (x100)

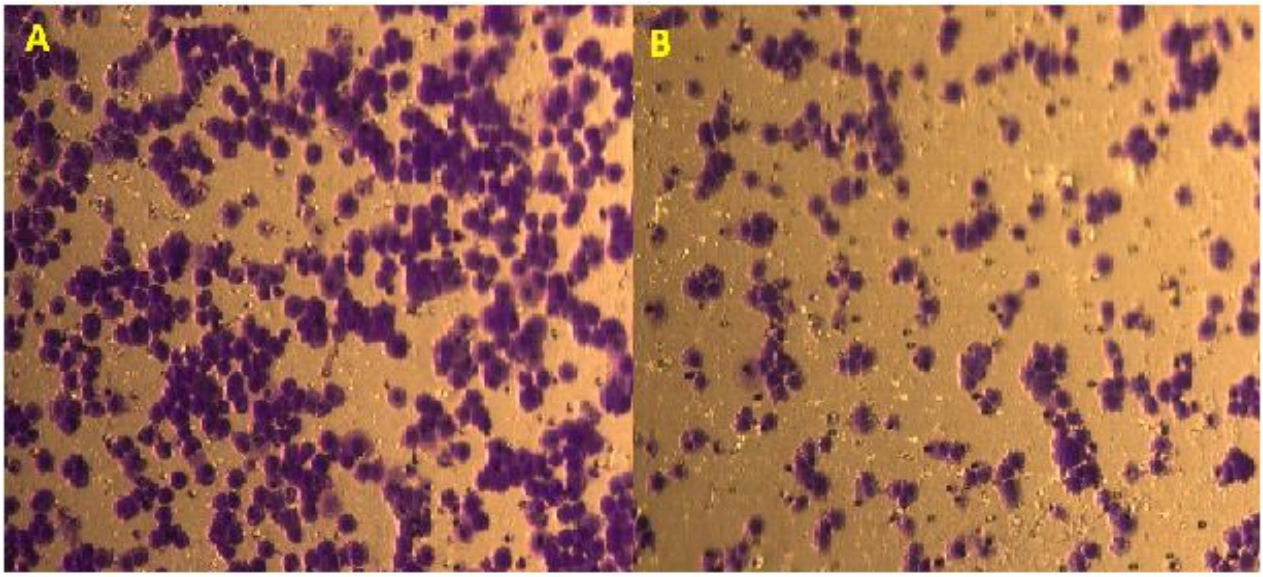


Figure 7.4: Representative example of the effect of serum chemotactic activity on AsPC1 cell migration. A- Pre-operative. B- Post-operative (x100).

7.4.2 Angiogenesis Results

7.4.2.1 Baseline Evaluation

The median values of total length and capillaries number of groups (A, B and E) were significantly higher than those of control groups III and IV (P= 0.0001, Kruskal-Wallis test). There was no statistical difference in the median values of total length and capillaries number between cancer subgroups (Mann Whitney test). The subgroup comparative analyses to control cases were as follows: The HDMEC total length of capillaries of group B was significantly higher than those of control groups III and IV (Mann Whitney test Table 7.3, Fig. 7.5).

Furthermore, the total number of group B cases was significantly higher than those of group III and IV (Mann Whitney, Table 7.3, and Fig. 7.5-7.6). The median and IQR of total length and number for all the PC cases (groups A) did not differ significantly from that found for group E [Total length; 6226(5117-8409) *vs.* 5679 (4518-8406); p= 0.7, total number; 40(33-44) *vs.* 36(32-41); p= 0.3].

143 (131-60), p= 0.2, Mann Whitney test). The median value subgroup of all resected cases group C did not differ from that of the unresected cases (group D, statistics not shown) indicating the homogeneous nature of the 'cancer diagnoses' at baseline.

Table 7.3: The total length and number of capillary formation assay of HDMEC treated with serum.

Group	No	Total length of Capillary/ 10 HPF		Total number of Capillary/ 10 HPF	
		Median and IQR	P value	Median and IQR	P value
B	32	6010 (5014-8373)	B *vs.* III=0.0015	40 (33-44)	B *vs.* III= 0.003
III	9	989 (883-5446)		19 (13-36)	
IV	20	691 (574-1177)	B *vs.* IV=0.0001	15 (9.5-17)	B *vs.* IV=0.0001

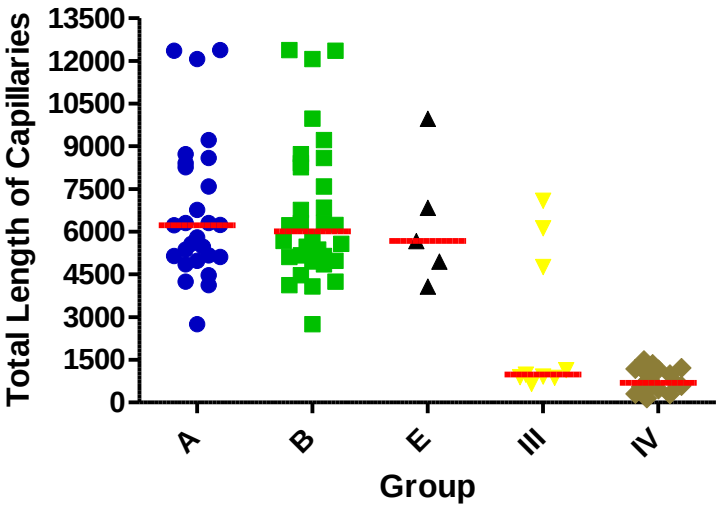


Figure 7.5: The median of total length of capillary formation assay of HDMEC treated with the serum. The red bar represents the median.

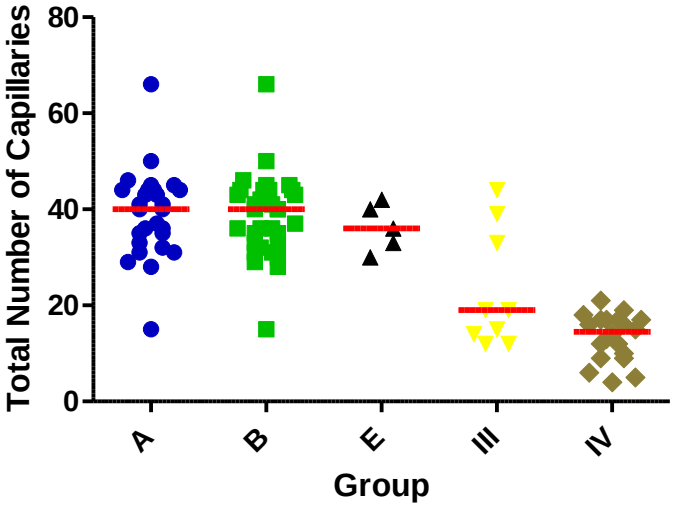


Figure 7.6: The median of total number of tubule formation assay of HDMEC treated with the serum. The red bar represents the median.

7.4.2.2 Pre- and Post-Resection Data

A comparison of pre-operative and post-operative levels of group C (all the 11 resected cases) revealed that the post-operative median total length and number were significantly lower than that of the pre-operative (Wilcoxon matched-pairs signed rank test, Table 7.4, Fig 7.7-7.9).

The pre-operative and post-operative levels in the nine PC cases of group I for which both samples were available revealed that the post-operative median total length and IQR was significantly lower compared with the pre-operative [2174 (1708-3628) *vs.* 8726 (4518-8406), p= 0.0039, Wilcoxon signed rank test]. Furthermore the total median post-operative number of capillaries of group I was significantly lower than that found pre-operatively [24 (16-31) *vs.* 44 (38-48), p= 0.012, Wilcoxon signed rank test]. There were no significant differences in the total length and number of capillaries pre-and post operatively in both groups III and IV (Wilcoxon signed rank test, Table 7.4A - 7.4B). There was no statistical difference between the pre-operative and post-operative total length and number of capillaries of group D (4 cases, p= 0.25 and 0.5 respectively).

Table 7.4A: Total length of tubules pre- and post-operatively.

Group	No	Total length		P values Pre-*vs.* post-operative
		pre-operative median & IQR	post-operative median & IQR	
C	11	7593 (5570-7593)	2488 (1807-4261)	0.001
III	5	897 (761-3560)	980 (766-2567)	1
IV	11	810 (586-1219)	665 (590-1234)	0.2

Table 7.4B: Total numbers of tubules pre- and post-operatively.

Group	Number	Total number		P values Pre-*vs.*Post-operative
		pre-operative median & IQR	post-operative median & IQR	
C	11	43 (36-46)	27 (16-46)	0.02
III	5	15 (12-26)	14 (12-25)	0.78
IV	11	17 (12-18)	15 (13-17),	0.09

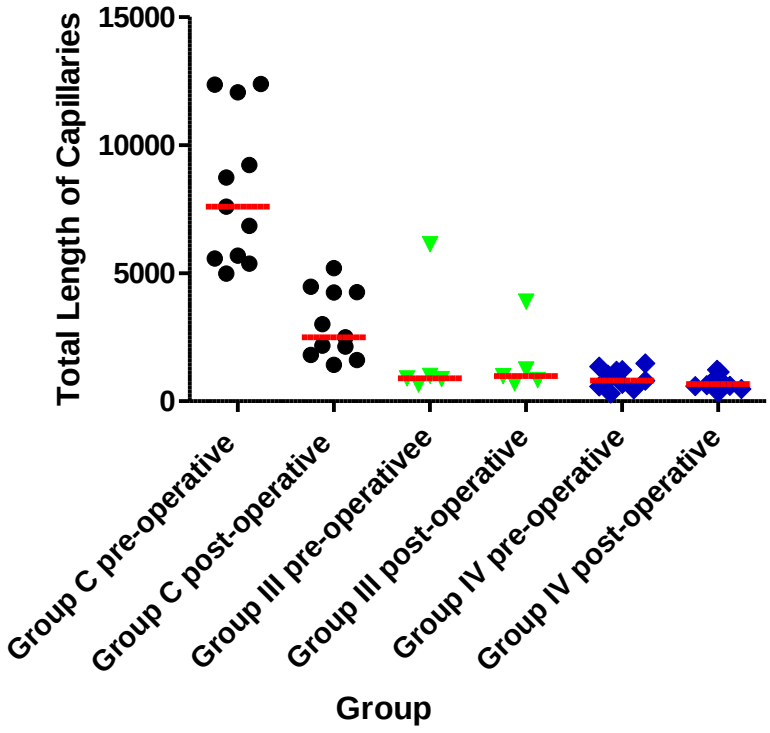


Figure 7.7: Pre- and post-operative total lengths of capillaries

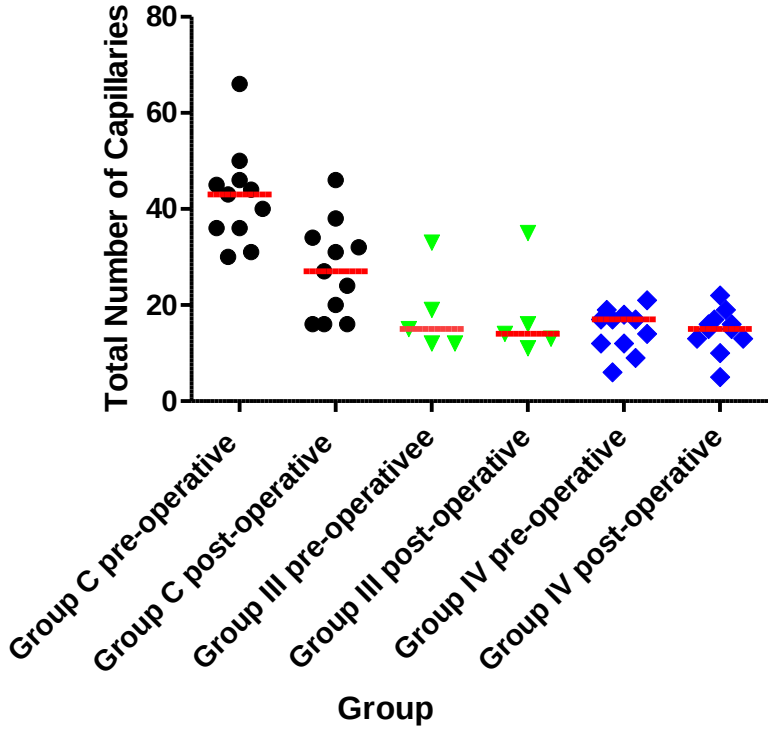


Figure 7.8: Pre-and post-operative total number of capillaries.

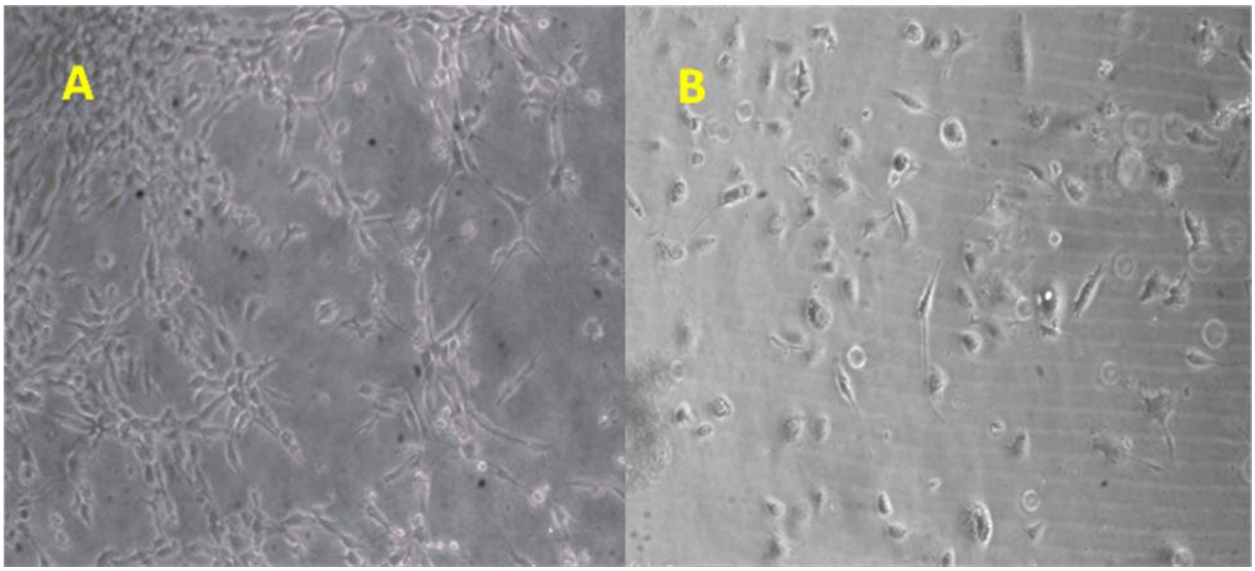


Figure 7.9A: Representative example of capillary formation when HDMEC were treated A- with pre-operative serum from patient with PC. B- with post-operative serum from patient with PC (x100).

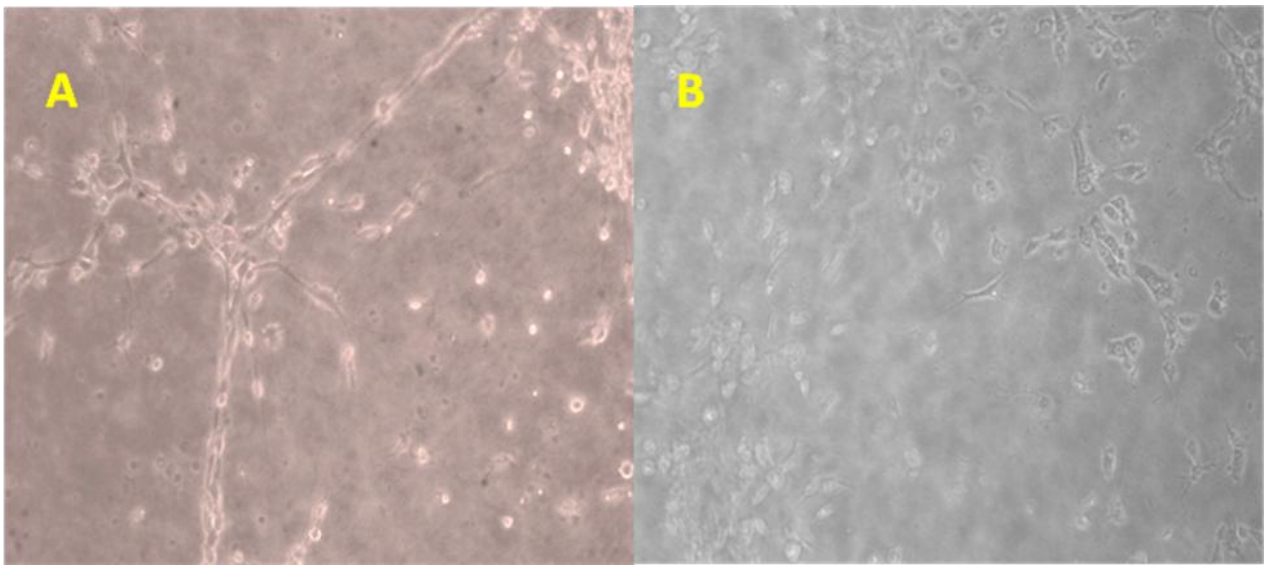


Figure 7.9B: Representative example of capillary formation when HDMEC were treated A- with pre-operative serum from patient with PC. B- with post-operative serum from patient with PC (x400).

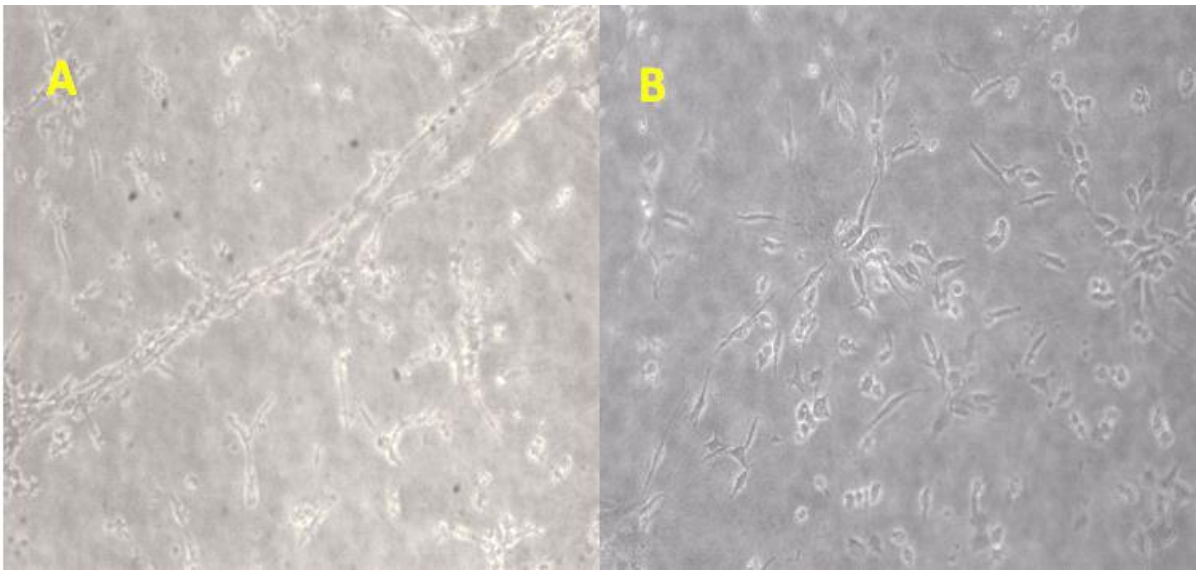


Figure 7.9C: Representative example of capillary formations when HDMEC were treated A- with pre-operative serum from patient with PC. B- with post-operative serum from patient with PC (x400).

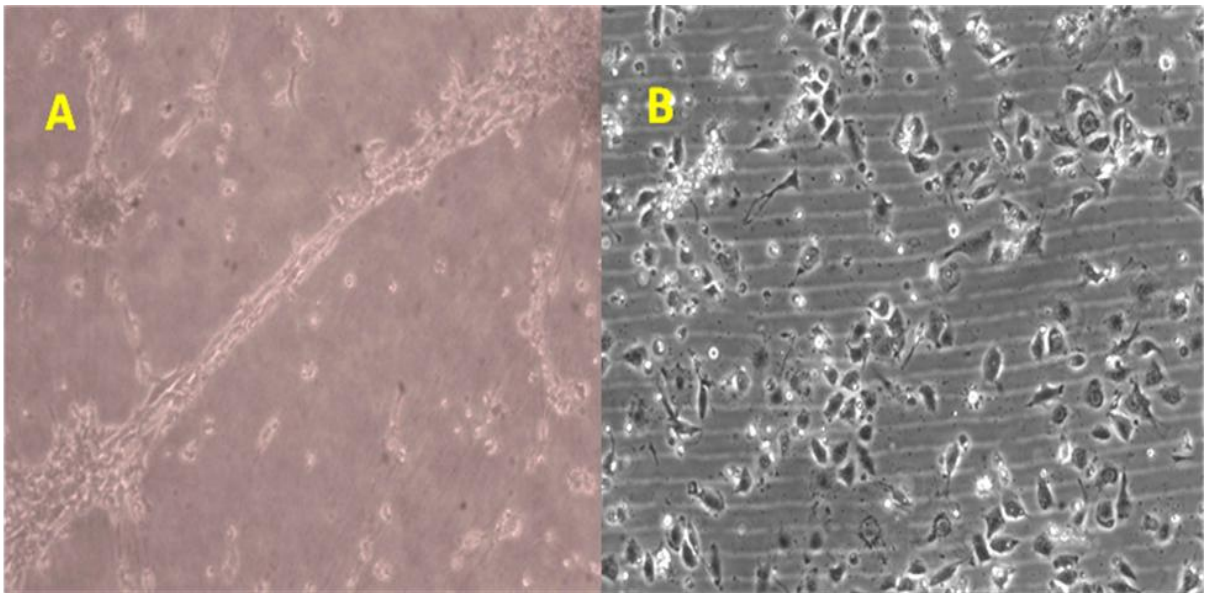


Figure 7.9D: Representative example of capillary formations when HDMEC were treated A- with pre-operative serum from patient with of PC. B- with post-operative serum from patient with of PC (x400).

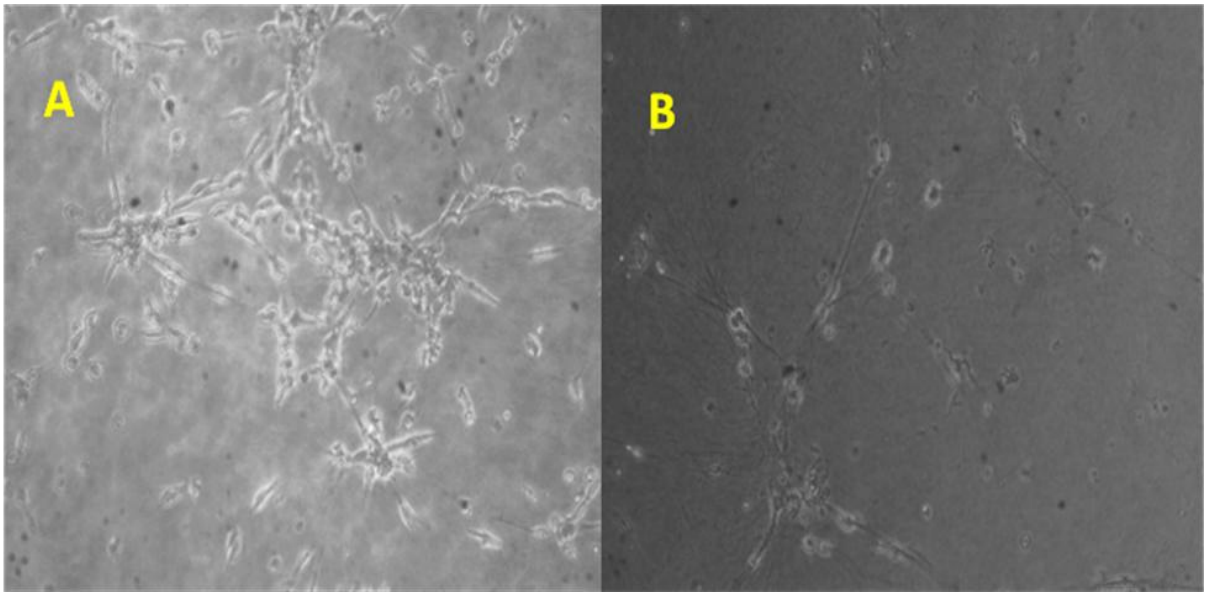


Figure 7.9E: Representative example of capillary formation when HDMEC were treated A- with pre-operative serum from patient with PC. B- with post-operative serum from patient with PC (x400).

7.4.3 Correlation of Cell Invasion with Angiogenesis

There was a significant correlation between base line and post-operative of group B and III cell invasion with total length of capillaries (non parametric Spearman correlation, 0.0001, Fig. 7.10).

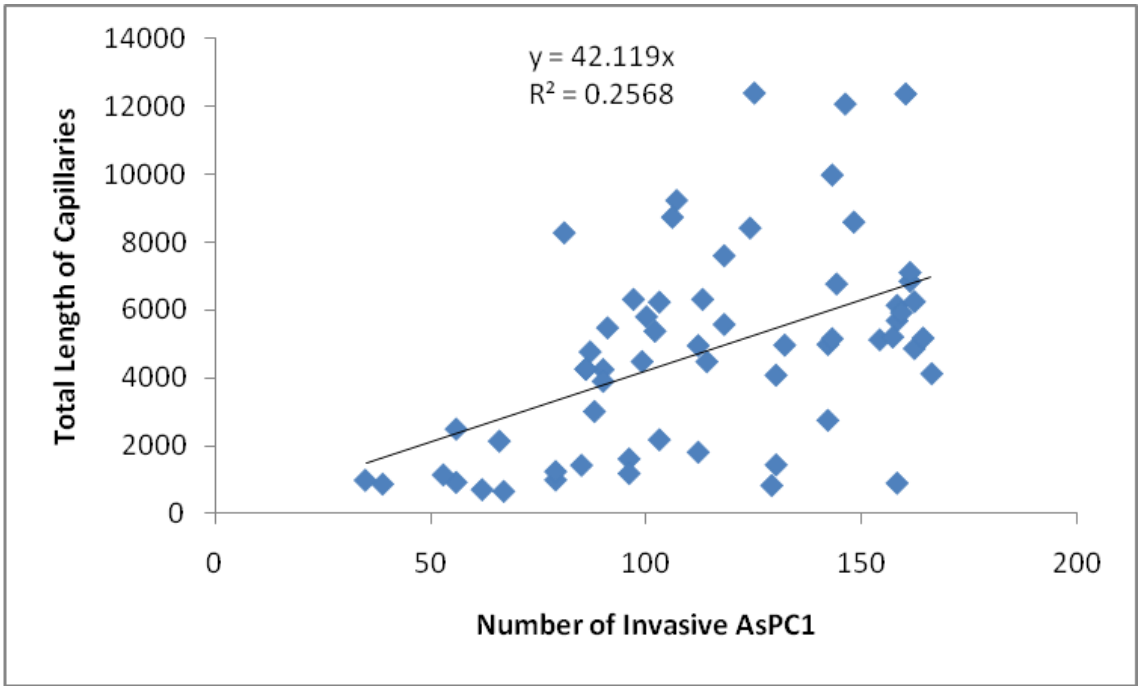


Figure 7.10: The correlation between number of cell invasion and total number of capillaries

7.5 Discussion:

Cancer cell invasion and angiogenesis involve cell migration, proliferation and differentiation. In the *in vitro* experiments of this thesis, it was demonstrated that TF and VEGF increase AsPC1 invasion (section 3.4.4). Furthermore it has been shown that the pro-coagulant activity of PC cells is correlated with cell invasion (section 3.2.5). The blocking of TF on PC cell lines using polyclonal anti-TF antibody also decreases cell invasion (section 3.2.5). Furthermore, it was shown that the maximum tubule formation was achieved when HDMEC were treated with PC sera and 1 U/ml TF. Furthermore, a combination of suramin, anti VEGF and anti TF inhibits angiogenesis (section 4.5). These findings are in accordance with Pradier and Ettelaie (2008). It was first suggested that these assays may have clinical application in monitoring the effects of LMW on the cancer promoting properties of the serum of PC patients with metastatic disease (Maraveyas *et al.*, 2010a). In that work, however, only an invasion assay was used, similar to the one used in this chapter but with a different pancreatic cancer cell line (MIA-PaCa 2). In this chapter it is demonstrated that these optimised *in vitro* assays for invasion may also have clinical significance in the setting where the cancer is removed. The additional contribution of this work is that for the first time it is demonstrated that a further assay developed to study HDMEC locomotion and tubule formation (*in vitro* angiogenesis assay) may also contribute further information. This assay is based on the same principles of studying the capacity of cancer patients' sera to induce locomotion but in this case of a primary non- malignant endothelial cell line (HDMEC). The role of TF on cell invasion was discussed in sections 1.18 and 3.6. The role of TF on angiogenesis was discussed in sections 1.9.1, 1.9.2 and 4.6.

Obviously there can be many other factors in the serum of a cancer patient promote cell invasion and angiogenesis. It was suggested from the angiogenesis array chapter (chapter 6) that the angiogenic balance was shifted to the anti-angiogenic state post-operatively. Therefore this part of the work was designed to investigate whether the change in the angiogenic balance post-operatively has a real impact on decreasing the pancreatic cell invasion and angiogenesis. It was hypothesised that pro-angiogenic and angiogenic inhibitors shift the angiogenesis toward the inhibition of angiogenesis and inhibit chemotaxis activity due to the qualitative and quantitative decrease of angiogenic markers. Furthermore, it was hypothesised that the molecules secreted from the tumour have more than one activity at the same time, as they enhance the migration and differentiation of the cells.

Cancer metastasis involves tumour cells invading the surrounding tissue. Invasive cells must traverse barriers such as basement membrane, composed largely of collagen. Matrix metalloproteinases have a major role in degrading the collagen matrix (Ellenrieder *et al.*, 2000, Mignatti and Rifkin, 1993, Clark, 1979). There is accumulating evidence that growth factors also play major roles as chemo-attractant factors. Farrow *et al.* (2003) suggested that sodium butyrate inhibits AsPC1 cell invasion and reduces integrin expression. EGF was used as a chemo-attractant in these experiments. Wey *et al.* (2005) postulated that VEGFR 1 expression on PC cell lines increased cell invasion and VEGF was used as a chemo-attractant agent. Albo *et al.* (1997) postulated that TGF-β1 and TSP-1 increase AsPC1 cell invasion through up-regulation of the plasminogen/plasmin system. Moreover, glial cell–derived neurotrophic factor (GDNF) is a well known factor that is a major chemo-attractant in epithelial tumours including PC (Pozas and Ibanez, 2005, Tang *et al.*, 1998, Pichel *et al.*, 1996).

It has been noted in the current study that the maximum serum chemotaxis activity (AsPC1 cells motility) and HDMEC angiogenesis (total length and total number) were induced by the baseline serum of the patients with cancer (group B) in the presence of the cancer and was clearly greater than that of the control groups. These findings support the hypothesis that these processes are cancer specific. This suggestion is strengthened by the finding of a significant decrease in the induction of AsPC1 cell motility and HDMEC capillary formation by the post-operative sera compared to the pre-operative sera, of the resected cancer patients (Group C). There was no such effect of resection either in the group III patients (precancerous pancreatic cases, control group) or in the group IV patients (chronic cholecystitis, control group).

The main hypothesis of this thesis is that these processes are promoted by TF through the coagulation pathway and direct signalling; however it is very likely that other pathways may also be involved. The work in chapter 6 points to a significant decrease of four angiogenic markers (angiopoietin-1, angiostatin/plasminogen, PDGF-AA, PDGF-AB/PDGF-BB and VEGF). Therefore the decrease of post-operative cell invasion and angiogenic activity could possibly due to the decrease of several chemotaxis agents and angiogenic markers. In patient serum, it is very likely that the same can be said for the process of invasion for which the ASPC1 assay is a marker. Airaksinen and Saarma (2002), for example, have suggested that the nerve tissue in PC secretes a variety of chemokinetics that facilitate nerve invasion *in vivo*.

As a conclusion, sera from PC patients have higher chemotaxis and angiogenic activity than the control group, which strongly suggests factors made by the tumour itself are responsible. Removal of the tumour decreases chemotaxis and angiogenesis mediated by serum taken from the same patients. Migratory behaviour of PC cells and capillary formation may be correlated with quantitative and qualitative differences in parameters that govern the integration of molecular components.

CHAPTER EIGHT: MICROPARTICLES NUMBER AND ACTIVITY

8.1 Introduction

MP are membrane vesicles of 0.1-1 μm in diameter shed from the plasma membrane of healthy, apoptotic and stimulated cells (Mostefai *et al.*, 2008b). It is well known that TF bearing MP, in addition to their role in coagulation, play a major role in tumour growth, metastasis, angiogenesis and thrombosis (Yu and Rak, 2004, Muller *et al.*, 2003). It was also demonstrated recently that increased levels of MP can be found in patients with various types of cancers and that the highest levels are observed in PC patients (Zwicker *et al.*, 2009, Tesselaar *et al.*, 2007).

Several studies correlate TF-bearing MP numbers in PC with the pro-coagulant activity as will be discussed in detail in section 8.6. To the best of the author's knowledge, no study correlates TF-bearing MP with *in vitro* cell invasion and angiogenesis. Furthermore, to the best of the author's knowledge, the current study is the first study to evaluate TF bearing MP, pro-coagulant activity, cell invasion and angiogenic activity pre- and post-operatively. Therefore the author hypothesised that there is a link among all these indices. Based on this hypothesis, the author suggested that all these indices together are driven by the PC tumour.

Therefore, in the current study, the correlation of TF bearing MP number with the pro-coagulant activity, cell invasion and angiogenesis was investigated to give a comprehensive insight about the thrombotic and aggressiveness course of PC. There are few studies evaluated the association between surgical removal of PC and MP number and/or activity, among these Zwicker *et al.*, 2009 and Tesselaar *et al.*, 2007. Furthermore, there are some limitations including low numbers in these studies. It was shown in chapter 6 that there was a decrease of some angiogenic markers post-operatively. Furthermore, it was shown in chapter 7 that there was a decrease of angiogenic and chemotaxis activity of pancreatic cancer sera.

In this chapter a series of experiments were performed to evaluate the number of different types of MP and coagulation state. TF bearing MP, MoMP, EMP and one stage PT assay were

performed using fresh PPP. Furthermore, frozen PPP were used to measure annexin bearing MP number using flow cytometry, TF, TFPI and MP activity using ELISA.

8.2 MP Count

8.2.1 Optimisation

A flow cytometric study was designed to study total number of MP bearing TF, CD 14, CD 31 CD (for EMP) and 42b (for PMP) / µl in fresh PPP as described in section 2.2.8.2. Annexin bearing MP number was counted in a separate experiment using frozen PPP (section 2.2.8.3). The methodology of MP count was explained fully in section 2.2.8.2. Megamix beads are a mixture of fluorescent beads comprising populations of 0.5; 0.9 and 3 µm in diameter. These beads were used to determine the size of tested MP, which ranged from 0.1-1 µm (Robert *et al.*, 2009, Vince *et al.*, 2009, Fig. 8.1, R1 box). The counting beads allow the setting of the flow cytometery to study MPs within a fixed sized region providing reproducible MP counts.

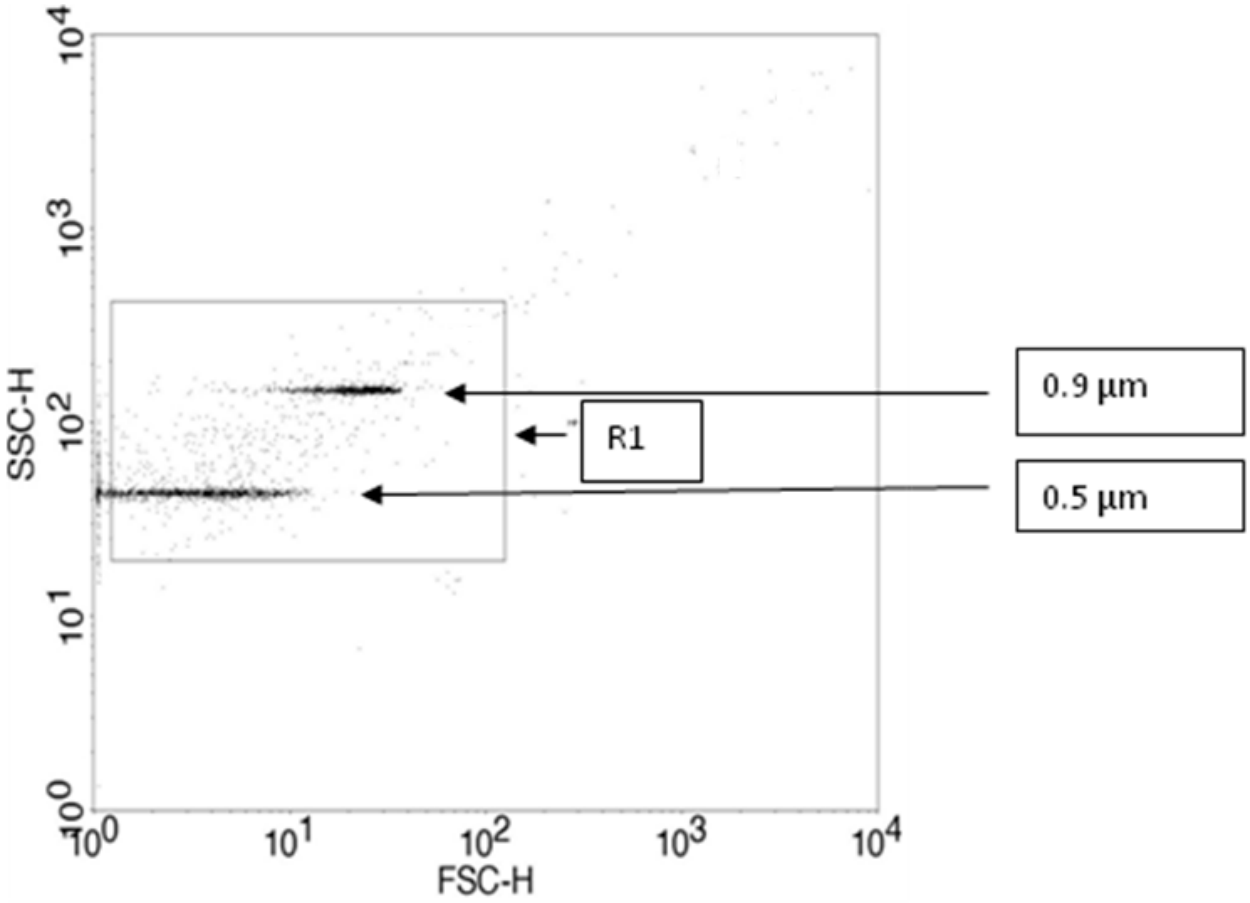


Figure 8.1: A dot plot of Forward scatter (X axis) and Side scatter (Y axis) for a megamix beads sample 0.5 µm and 0.9 µm diameters. MP will be expected within the quadrant box defined as R1.

MPs were identified according to their scatter properties and further according to the specific antibody labelling (TF (FITC), CD 14 (PE), CD 31 (FITC), CD 42b (PE); in a separate experiment annexin bearing MP were counted (Fig. 8.2).

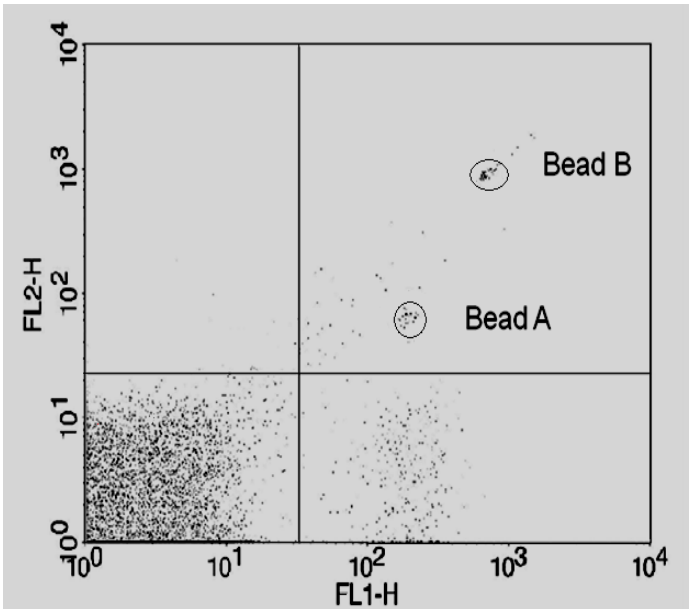


Figure 8.2: A dot plot with CALTAG counting beads was determined. There are two beads in the right upper quadrant, bead A (685) and bead B (667). The negative control is in the lower left quadrant. The expression of FITC marker is in the right lower quadrant. The PE expression is in the upper left quadrant. Dual FITC/PE stained MP will be in the upper right quadrant.

The samples were analysed firstly according to the expression of surface antigen. Negative controls FITC and PE were used to evaluate the specific expression of TF FITC and CD 14 PE, CD 31 FITC and CD 42b (Fig. 8.3).

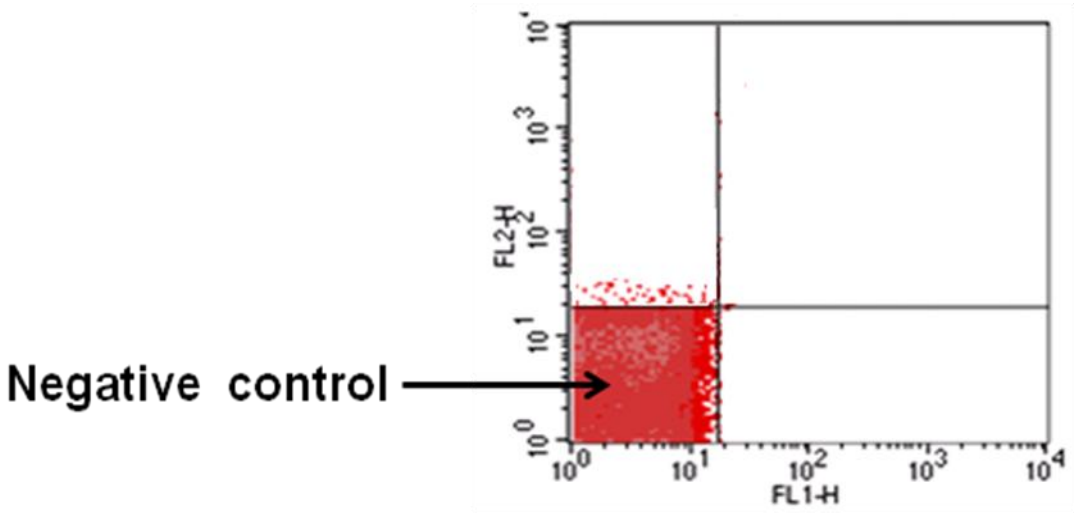


Figure 8.3: Representative example of Mouse IgG negative FITC and PE.

Then the expression of markers was determined according to the expression of negative control (Fig. 8.4, 8.5).

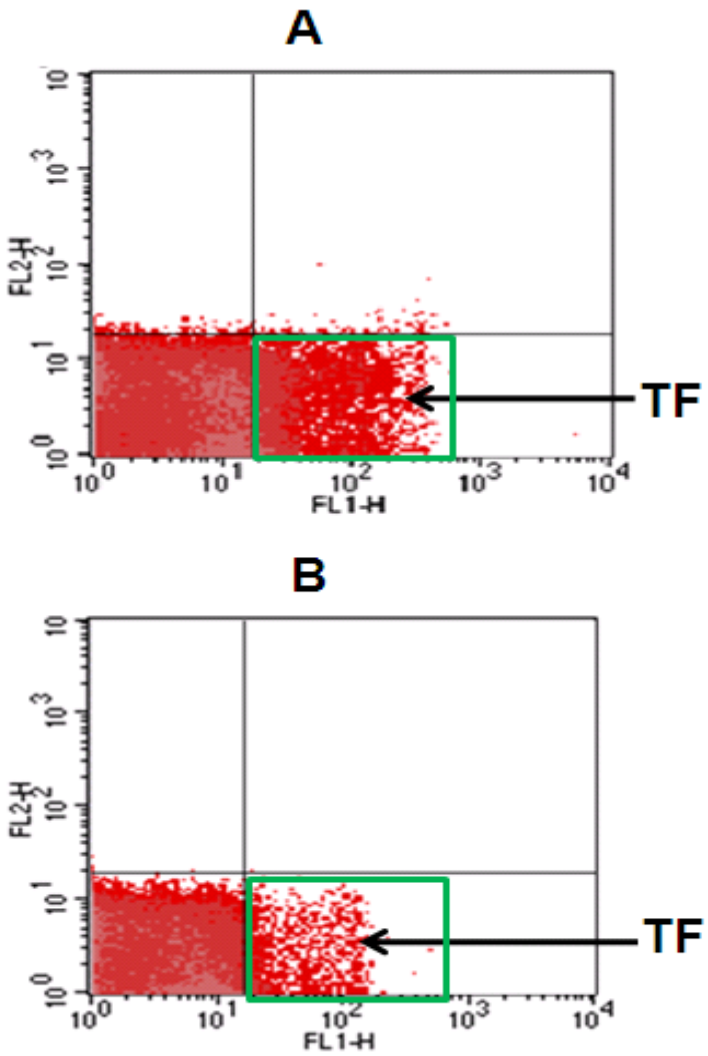


Figure 8.4: Representative example of pre-operative (A) and post-operative (B) plasma TF expression.

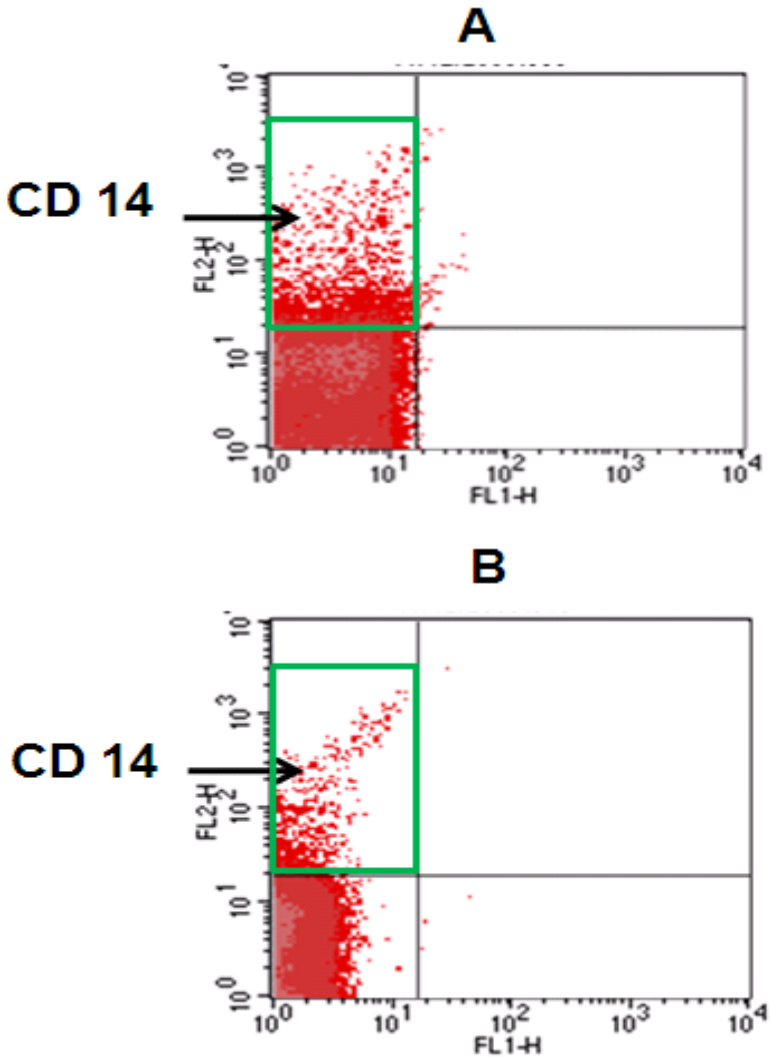


Figure 8.5: Representative example of pre-operative (A) and post-operative (B) plasma CD 14 expression.

The counting bead (A and B) has known number/µl (provided by the supplier) and known number counted from flowcytometry. The plasma MP has known number counted from the flowcytometry. Therefore absolute number of plasma MP/µl was determined by using the following equation.

$$\text{Absolute count} = \frac{\text{Number of MP counted}}{\text{Total number of beads counted (A + B)}} \text{ X Number of beads counted/ µl}$$

For example:

1 TF expressing-MP number was 5928.

2 Number of bead A was 685 and number of bead B was 667. Total bead A and bead B is 1352.

3 The number of beads / µl is 1050. Therefore

$$Absolute\ count = \frac{5928}{1352} \text{ X } 1050$$

$$= 4604$$

8.2.2 Aims

Group IV was not involved in this study and group III control study was used only because the cases of group III are pancreatic lesion and submitted to surgical resection as test cases.

The purposes of this study were to:

1. Investigate whether serum from PC patients contained higher absolute numbers of TF, CD 14 and annexin bearing MP than the serum of the non-tumour control (group III).
2. Investigate the absolute number of TF, CD 14 and annexin bearing MP decreased post-operatively following removal of the tumour.
3. Correlate TF MP number *in vivo* with the *in vitro* pro-coagulant, cell invasion and angiogenic activities.

8.2.3 Results

8.2.3.1 TF Bearing MP

8.2.3.1.1 Baseline Evaluation

The TF bearing MP number of cancer groups (A, B and E) was significantly higher than that of group III (p= 0.0034, Kruskal-Wallis test). There was no statistical difference in the median values of total number of TF-bearing MP between cancer subgroups (Man Whitney test). The subgroup comparative analyses with control cases (Mann Whitney test) were as follows:

The total TF bearing MP number of all malignant cases (group B) was significantly higher than those of control groups III (Table 8.1, Fig. 8.6). The median number and IQR of group A; 4568 (2557-5623) was significantly higher than that of the control group (group III, p= 0.0002) but it did not differ significantly from that of group E; 4387 (2182-32535); (P= 0.8355, Fig. 8.6). The baseline total number of MP of groups I and II differed significantly from control group III (0.004, 0.002 respectively) but it did not differ significantly from that found with group E (0.74, 1 respectively).

Table 8.1: Baseline median number of TF bearing MP/ µl.

Group	NO	Median & IQR	P value *vs.* III
B	32	4476 (2592- 5574)	0.0002
III	9	852 (273-2160)	

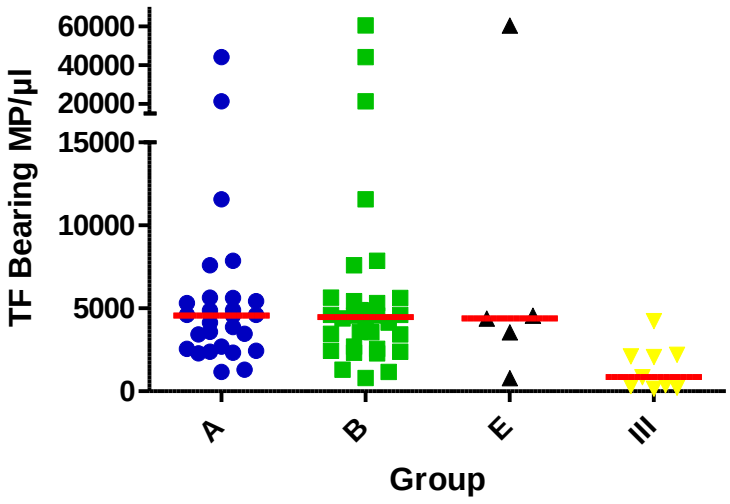


Figure 8.6: Absolute number of TF bearing MP/µl in each group of patients. The red bar represents the median.

8.2.3.1.2 Pre- and Post-Resection Data

The median and IQR post-operative TF bearing MP number of group C; was significantly lower than the pre-operative [1296 (363-4475) *vs.* 4874 (2257-21363), p= 0.042, Wilcoxon matched-pairs signed rank test, Fig. 8.7].

The median post-operative total TF bearing MP median of group I was significantly lower than the pre-operative total TF bearing MP [1296 (467-4983) *vs.* 4874 (3007-14479) respectively, p= 0.03, Wilcoxon matched-pairs signed rank test). The post-operative total TF bearing MP number of group III did not differ significantly from the pre-operative [319 (196-1289) *vs.* 269 (148-870), p= 0.39, Wilcoxon matched-pairs signed rank test]. Furthermore, the post-operative median TF bearing MP of group D did not differ significantly from the pre-operative (p=0.62; Wilcoxon matched-pairs signed rank test).

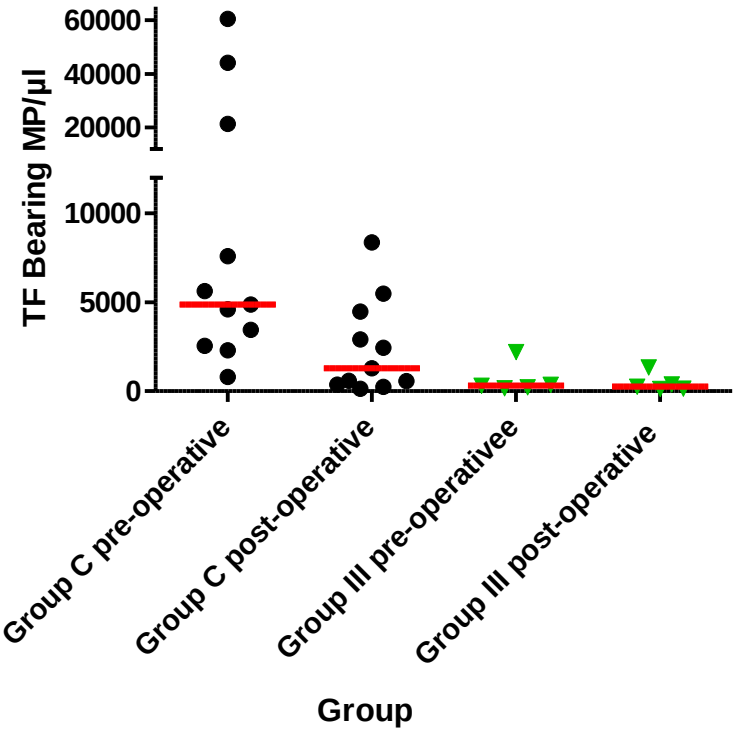


Figure 8.7: Pre-and post-operative TF bearing MP. The red bar represents the median.

8.2.3.2 CD 14 MP – Baseline

8.2.3.2.1. Baseline CD 14 Bearing MP

The CD-14 bearing MP of groups A, B and E were significantly higher than those of group III (p= 0.05, Kruskal-Wallis test). The study shows that the median and IQR pre-operative level of CD 14 bearing MP/ µl of group B was significantly higher than that found in group III [2179 (1349-3799) *vs.* 264 (170-1991), Mann Whitney test, Table 8.2, Fig. 8.8]. There was no statistical difference in the median values of total number of CD-14-bearing MP between cancer subgroups (Mann Whitney test). The subgroup comparative analyses to control cases (Mann Whitney test) were as follows:

The median number and IQR of CD-14 bearing MP of group A differed significantly from control group III [median and IQR; 2167 (1017-9363), p= 0.02] but did not differ significantly from those of group E [median and IQR; 3646 (2403-8255), p= 0.07]. Furthermore, there was no significant difference between the median CD 14 bearing MP of group I and that of group II (0.34, Mann Whitney test).

Table 8.2: Total number of CD 14 bearing MP/µl of cases involved in the study.

Group	NO	Median & IQR	P value
B	32	2179 (1349-3799)	B *vs.* III= 0.02
III	9	264 (170-1991)	

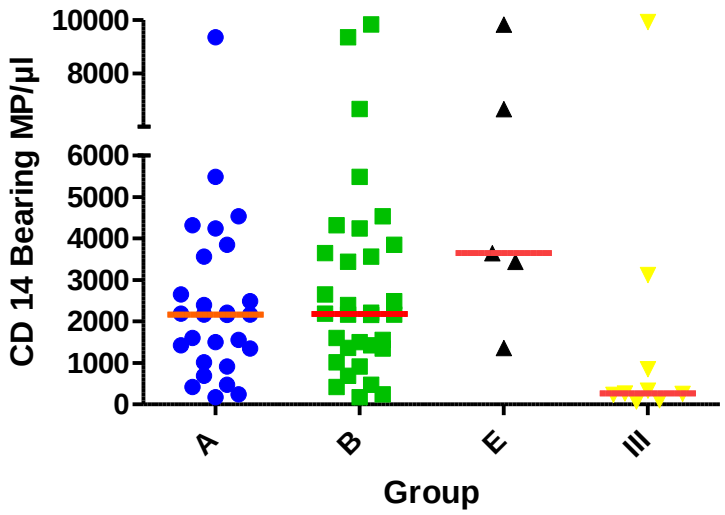


Figure 8.8: Absolute number of CD 14 bearing MP. The red bar represents the median.

8.2.3.2.2 Pre- and Post- Resection Data

The post-operative CD 14 bearing MP number of group C was significantly lower of the pre-operative. Median and IQR was 349 (37-1530) *vs.* 3444 (1505-3850), p= 0.02, Wilcoxon matched-pairs signed rank test, Fig. 8.9. The median post-operative total CD 14 bearing MP median of group I was significantly lower than the pre-operative total TF bearing MP (post-operative median; 349 (50-2702) *vs.* 2394 (1089-4049) , p= 0.03, Wilcoxon matched-pairs signed rank test. The post-operative total TF bearing MP number of group III did not differ significantly from the pre-operative [median and IQR; 183 (128-2646) *vs.* 234 (89-560), p= 0.39, Wilcoxon matched-pairs signed rank test]. The post-operative CD 14 bearing MP median

of group D did not differ significantly from the pre-operative (Wilcoxon matched-pairs signed rank test, p= 0.12).

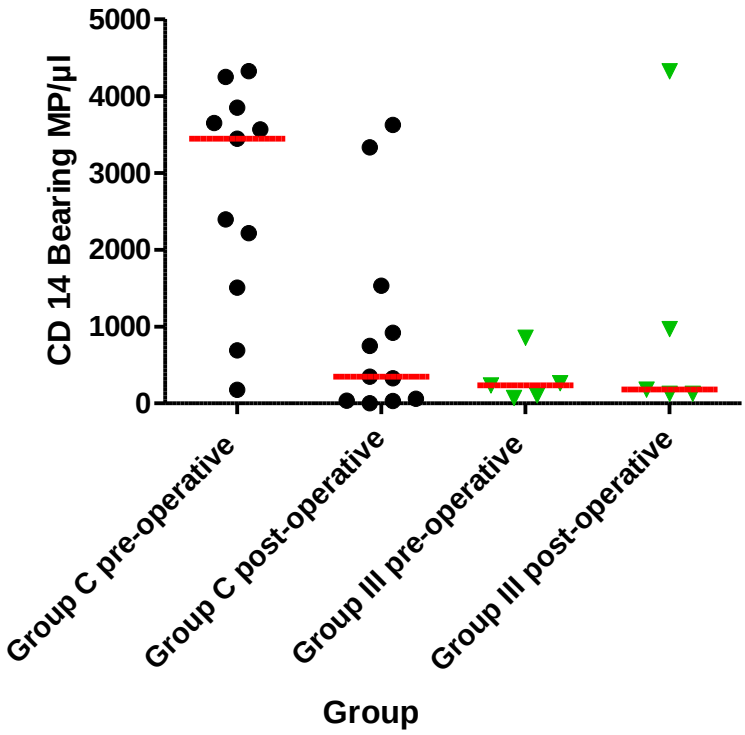


Figure 8.9: Pre-and post-operative CD 14 bearing MP of all malignant cases including pancreatic adenocarcinoma. The red bar represents the median.

Other MP group subsets (CD 31, CD42b and annexin) were studied and did not show differences between groups at baseline, nor did surgery have any influence on their levels.

8.3 Pro-coagulant Activity

One stage PT and TF MP activity ELISA were used to evaluate the pro-coagulant activity of PC samples.

8.3.1 Methods and Principles

8.3.1.1 One Stage Prothrombin Time

One stage PT evaluates the extrinsic pathway of coagulation. Clinically, PT is used to monitor the coagulation state of patients with vitamin K deficiency and during treatment with vitamin K antagonists such as warfarin (Bogdanov *et al.*, 2003). PT depends on six factors [I, II, III (TF), V, VII, X], one of which is TF. Therefore, one stage PT can be considered as a general indicator of coagulant status. Using the principles of one stage PT on fresh PPP (within first four hours of venapuncture) could give a good insight into coagulation activity of TF bearing MP shed from the tumour. Blood was taken by venesection and was collected in sodium citrate tubes to inactivate Ca^{2+} by chelation. Sample collection and preparation was described in section 5.2. Methodology of PT was described in section 2.2.9.

After breaking the annonymisation code, it was noted that five patients were treated with prophylaxis dalteparin (5000 U); two patients were from group B; two were from group E and one from group C.

8.3.1.2 Microparticles Activity

The pro-coagulant activity of MPs was evaluated using the ZYMUPHEN MP-Activity kit. Frozen PPP was used to evaluate the MP activity as explained in section 2.2.11. The principle of this kit was to evaluate the pro-coagulant activity with the limiting factor being phosphatidyl serine (PS) of MP. Therefore the results represent the concentration of PS on MP. The plasma, controls and calibrator were diluted with sample diluents, supplemented with Factor Xa inhibitors and prothrombin inhibitor added to the microplate wells previously coated with streptavidin and biotinylated annexin V and incubated for one hour. Following washing, the Factor Xa-Va mixture containing calcium and finally purified pro-thrombin were added.

MP binds to annexin V and exposed causes PL to be on the MP surface, thus allowing FXa-FVa, in the presence of calcium, to activate prothrombin into thrombin. There is a direct relationship between the PS concentration and the amount of thrombin generation, which is measured via its activity on the thrombin substrate (Fig. 8.10).

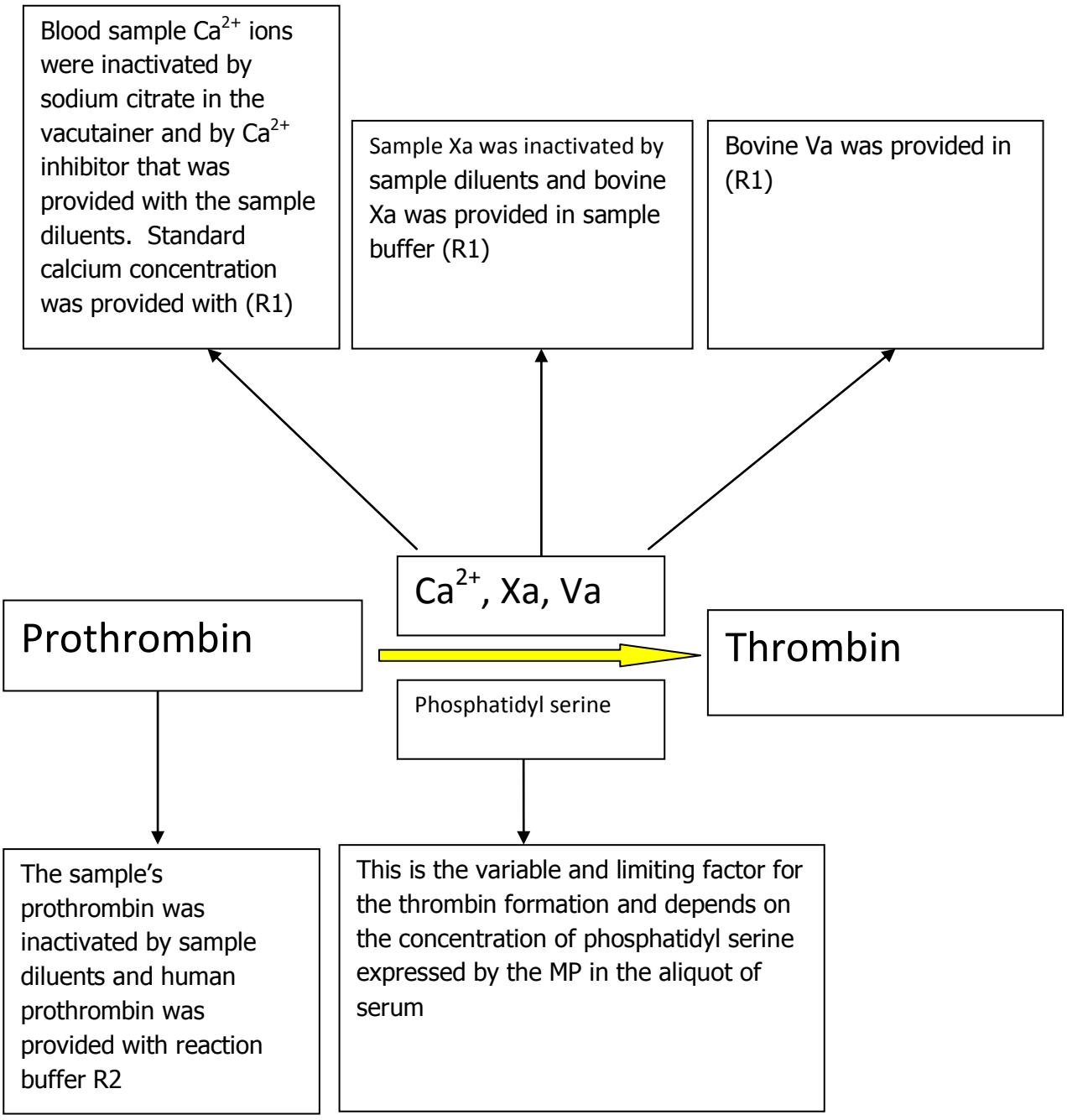


Figure 8.10: Diagrammatic representation of the mechanism of zymuphen MP activity. The limiting factor was the PL which represents the MP activity. R1: Bovine FXa-FVa mixture also containing calcium. R2: Human pro-thrombin.

8.3.2 Aims

This part of the study aimed to find out:

1. Whether the baseline pro-coagulant activity in PC patients is higher than that of the control (group III).

2. Whether the pro-coagulant activity of the cancer patient decreased post-operatively.

8.3.3 Results

8.3.3.1 Prothrombin Time

8.3.3.1.1 Baseline Evaluation

The PT of groups A, B and E was significantly shorter than that of group III (P. 0.019, Kruskal-Wallis test). The current study shows the baseline PT (S) of group B was significantly shorter than that of group III (Mann Whitney test, Table 8.3, Fig. 8.11).

There was no statistical difference in the median values of PT between cancer subgroups (Man Whitney test). The subgroup comparative analyses to control cases (Mann Whitney test) were as follows:

PT of PC cases [group A, median and IQR; 35 (33-37, mean; 34.7± 2.9)] was significantly shorter than that of group III (p= 0.02, Mann Whitney test) but it did not differ significantly from that of group E (median and IQR; 35 (32-37), mean; 34.6 ± 3.2, p= 0.85).

Table 8.3: Baseline prothrombin time.

Group	No	Median IQR (seconds)	Mean ± SD (seconds)	P value
B	32	35 (33-36.7)	34.6 ±2.9	B *vs.* III = 0.02
III	9	40 (35-41)	38.11± 3.25	

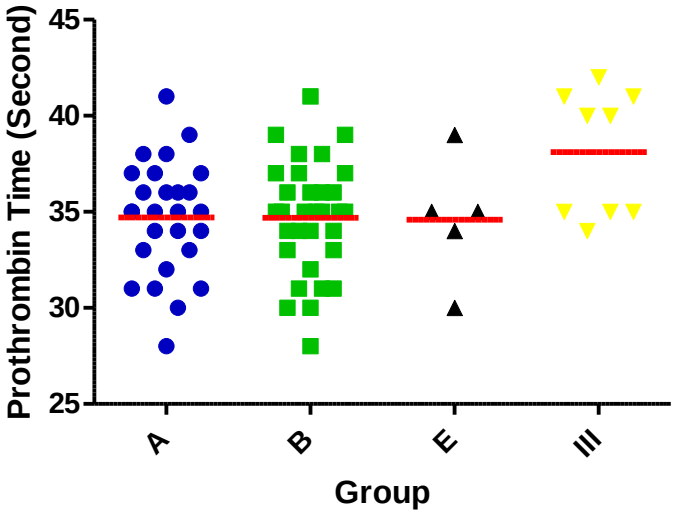


Figure 8.11: Median PT of PC cases and control group. Red bar represents the median.

8.3.3.1.2 Pre- and Post- Resection Data

The post-operative PT (S) of group C (11) was significantly longer than the pre-operative [median and IQR; 37 S (37- 40), mean; 37.9 ±2.9 *vs.* 34 (31-35), mean; 33.5 ±2.1, p= 0.008, Wilcoxon matched-pairs signed rank test (Fig. 8.12)]. Comparison of pre-operative and post-operative levels of group I cases revealed that the post-operative PT (S) was significantly longer than pre-operative PT [median and IQR; 37 S, (36-40) *vs.* 33 (31-35), p= 0.02, Wilcoxon matched-pairs signed rank test].

The pre-operative median PT (S) of group III did not differ significantly from the post-operative [median and IQR; 40 (37-41.5) *vs.* 41 (38-41.5), p= 0.34, Wilcoxon matched-pairs signed rank test]. The pre-operative PT median of group D did not differ significantly from post-operative (Wilcoxon matched-pairs signed rank test, p= 0.12).

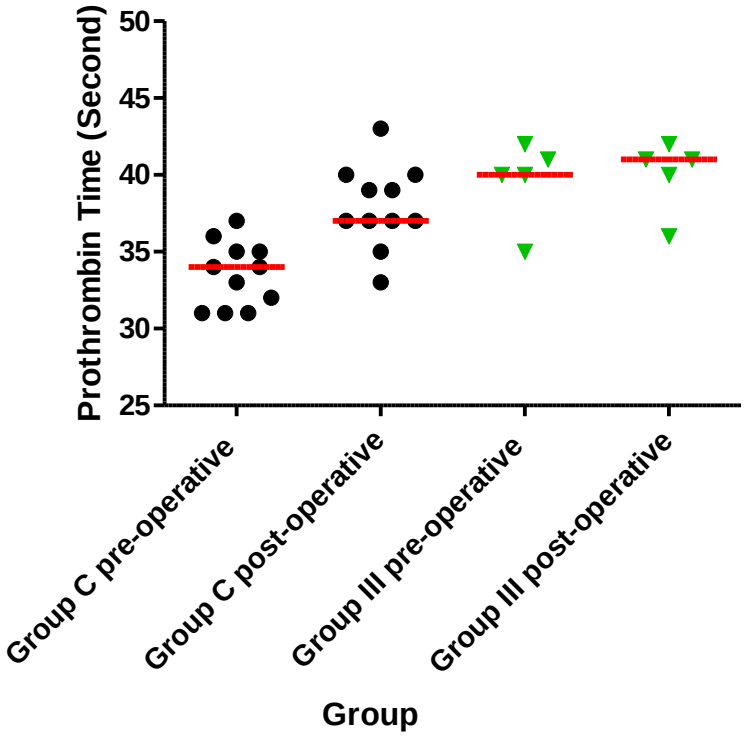


Figure 8.12: Pre-and post-post-operative prothrombin time. Red bar represents median.

8.3.3.2 MP Activity

The PS concentration of tested MP was calculated from the standard curve generated as part of the experiment (Fig. 8.13).

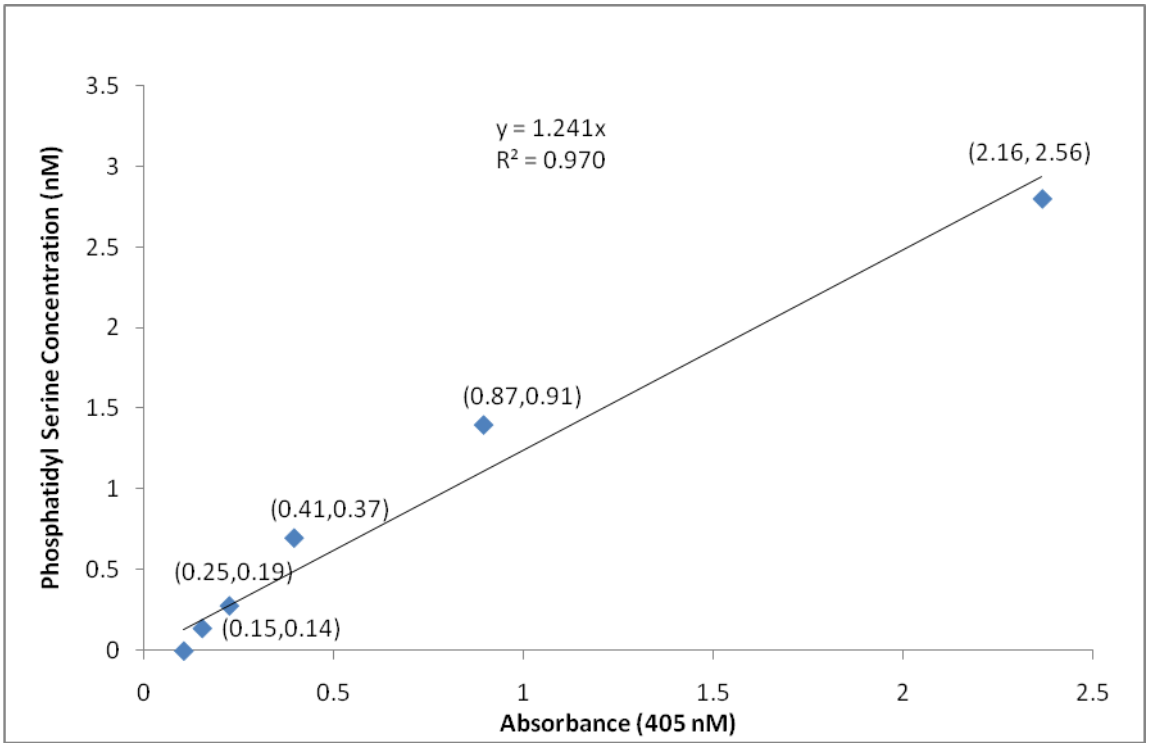


Figure 8.13: Representative standard curve of the phosphatidyl serine concentration using Zymphen assay.

MP activity ELISA was not performed in all cases as it emerged during this work that this test was unreliable and this was also borne out in our experience from the few 'pilot' cases we studied, which demonstrated pre-operative PS concentration (nM) of group B (conducted in 12, median and IQR was 9.4 (6.6-16) nM, mean; 10.4 ± 4.9 nM) did not differ significantly from that of group III [four cases, median and IQR was 6.4 (5.4-7.3) nM, mean; 6.4 ± 1 nM, p= 0.13; Mann Whitney test]. In seven cases (six group A, and one group E), post-operative PS did not differ significantly from pre-operative [median and IQR (7.1 nm (4.6-8.2) nM *vs.* (5.7 (5.8-18.4) nM, p= 0.2; Mann Whitney test]. Recently, several authors have disappointing results (see 8.6.1).

8.4 TF and TFPI ELISA

Frozen PPP samples were used to evaluate the level of TFPI and TF. The methodology was described in sections 2.2.12.2 and 2.2.12.3 respectively.

8.4.1 Results

The TFPI and TF (pg/ml) levels were measured from respective standard curves generated as part of each experiment (Fig. 8.14 – 8.15 respectively).

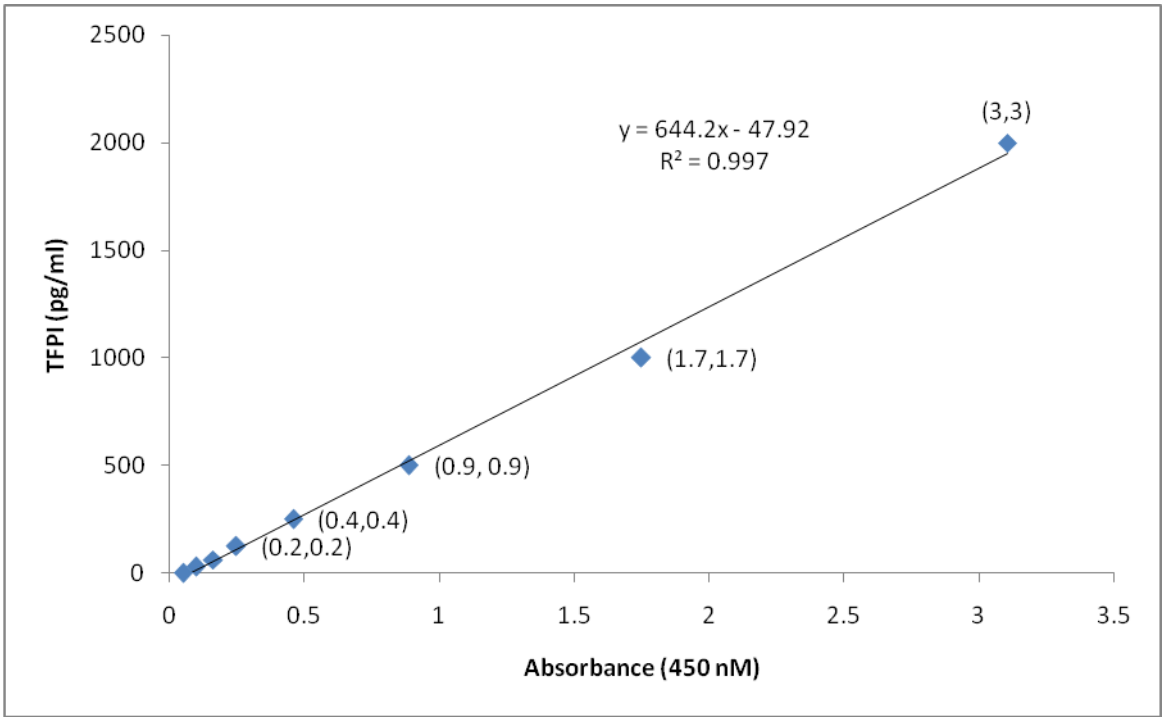


Figure 8.14: Standard curve of TFPI

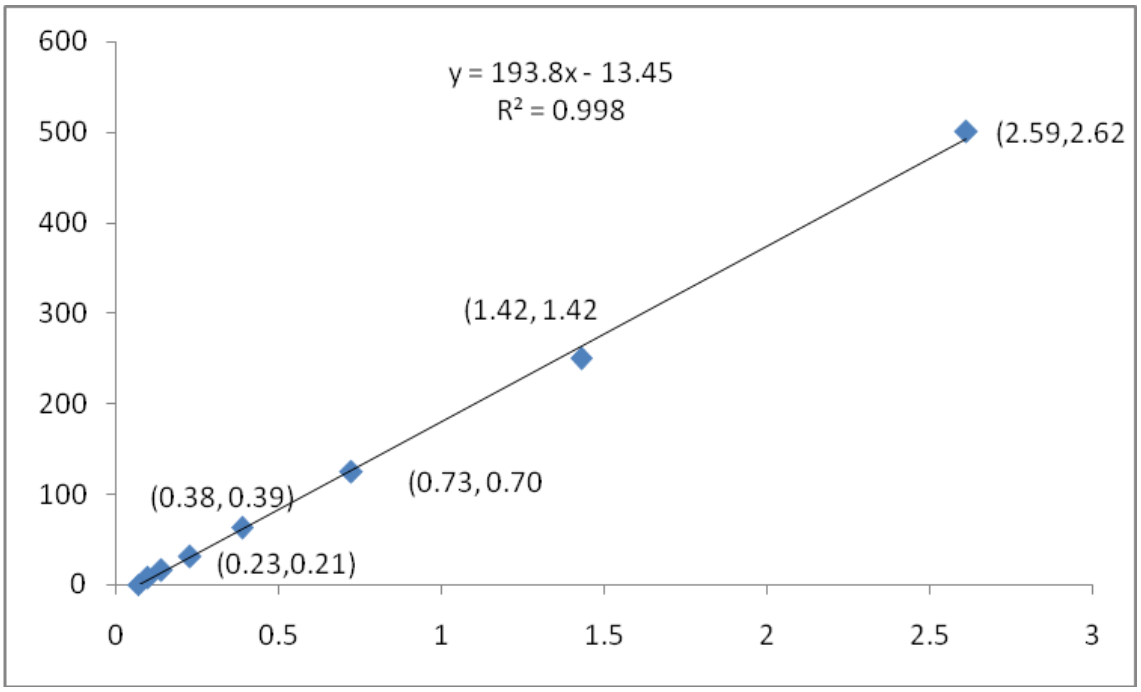


Figure 8.15: Standard curve of TF

A few PPP samples had run out; therefore TF and TFPI were conducted in available PPP plasma samples, which included 23 samples from group B, 6 samples from group III and 21 samples from group IV.

8.4.1.1 Baseline Evaluation

In 23 cases of group B, the concentration of TFPI (pg/ml) was significantly higher than that found in control group III (Mann-Whitney test, Table 8.4, Fig. 8.20) and significantly higher than TFPI concentration of control group IV as well (Mann-Whitney test, Table 8.4, Fig. 8.16).

Table 8.4 Pre-operative TFPI concentration of PC cases and control groups.

Group	NO	Media and IQR (pg/ml)	P value
Group B	23	357 (253-417)	B *vs.* III = 0.02
Group III	6	250 (154-298)	
Group IV	21	204 (177-242)	B *vs.* IV = 0.0001

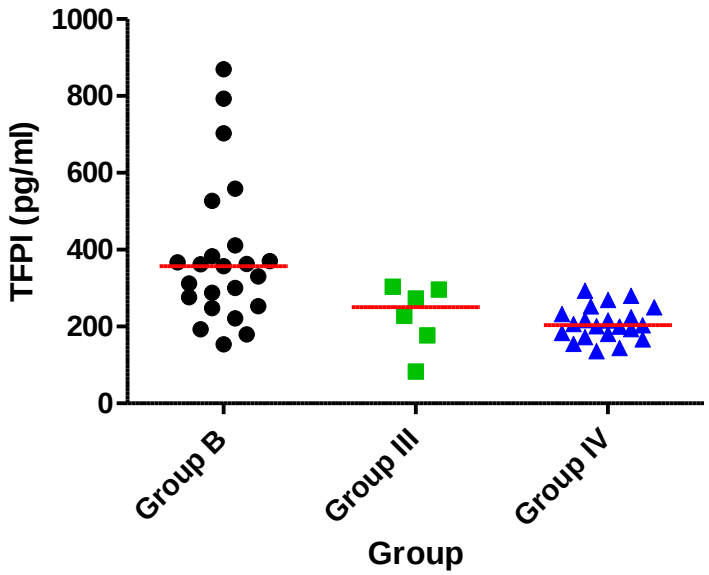


Figure 8.16: Pre-operative TFPI concentration of PC cases and control group.

The TF (pg/ml) concentration of group B did not show a significantly higher level than control groups III and IV. [The median and IQR, 20.4 pg/ml (17.8-25.7), mean, 22 pg/ml ± 4.5 *vs.* 21.8 pg/ml (14.3-25), (20.4 pg/ml ± 5.1) of group III, and [(27.5 pg/ml (24.9-30.2), 28.2 pg/ml ± 4.6 of group IV].

8.4.1.2 Pre-and Post-Resection Data

The pre- and post-operative evaluation was conducted in 10 cases from group B and 4 cases from control group III.

The pre-operative TFPI of group C did not differ significantly from the post-operative. [The median and IQR; 300 (214-374), mean; 333 ± 179 *vs.* 360 (259-447); 356 ± 112, p= 0.31, Wilcoxon signed rank test]. The pre-operative TFPI of group III did not differ significantly from the post-operative as well [The median and IQR; 262 (119-302), mean; 228 ± 102 *vs.* 291 (155-323); 356 ± 97, p= 0.48, Wilcoxon signed rank test].

There was no significant difference between the pre-operative and post-operative TF concentration of group B [the median pre-operative TF; 21.4 pg/ml (15.9-25.7), mean; 21.2 pg/ml ± 5.4 *vs.* 23.3 pg/ml (19.7-32.1), 26.6 pg/ml ± 9.2, p= 0.4, Wilcoxon signed rank test]. Furthermore, there was no significant difference between pre- and post-operative TF concentration of group III (The median pre-operative TF; 18.9 pg/ml (14.1-24.6), mean; 19.2 pg/ml± 5.7 *vs.* 14.3 (5.4-25.9), 15.2 pg/ml ± 7, p= 0.4, Wilcoxon signed rank test).

There was a significant correlation between the concentration of TF and TFPI pg/ml of all samples (p= 0.0005, Spearman correlation).

8.5 Correlations of TF Bearing Microparticles with other Indices

There was a significant correlation between baseline and post-operative of group B and III)] TF bearing MP levels and PT (non parametric Spearman correlation, 0.0001). Furthermore, there was a significant correlation between TF bearing MP and cell invasion [(All samples (baseline and post-operative)], non parametric Spearman correlation, 0.004, spearman. Moreover, there was a significant correlation between [all samples (base line and post-operative samples)] TF bearing MP and total length of capillaries (non parametric Spearman correlation, 0.0001, Spearman).

8.6 Discussion

The number of MP differs from one study to another depending on the methodology used and the type of MP. Some authors reported that the number of TF-bearing MP ranged from 240/µl to 1550/µl in PC and breast cancer (Tesselaar *et al.*, 2007) and endothelial MP from 148-448 /µl in small vessel vasculitis (Erdbruegger *et al.*, 2008), while other studies showed the range varied from 70,000/µl - 2,300,000/µl in healthy cases, PC, breast cancer, lung, colorectal and ovarian cancers (Zwicker *et al.*, 2009). In the current study the median number of TF bearing MP was 4568/µl in PC cases, 852/µl in pre-malignant pancreatic cases, 4387/ µl in cholangiocarcinoma cases. The median CD 14 MP number was 2167 in PC cases, 264/ µl in pre-malignant pancreatic cases and 3646/µl in cholangiocarcinoma cases. Therefore, it is necessary to standardise the methodology of detection of MP and this is in accordance with the recommendation of the 5th International Society on Thrombosis and Haemostasis (ISTH) 2011 that was held in Kyoto in Japan 23-28-July 2011.

8.6.1 The Significance of MP in Thrombosis

The current study shows that the TF-bearing MP and the pro-coagulant activity of PC patients were significantly higher than those of the control. In this regard the results of the current study were consistent with the results of other studies (Tesselaar *et al.*, 2009, Zwicker *et al.*, 2009, Tesselaar *et al.*, 2007). It has been reported that an increased level of MP could be found in patients with a number of cancers but the highest levels of TF activity were those in PC patients. In a series of 37 cancer-free controls, 23 advanced PC and 17 advanced breast cancer, the upper limit of TF activity in the normal range was 273 fM Xa min^{-1}(Tesselaar *et al.*, 2007). Thirty-four percent of PC patients and 29% of advanced breast cancer cases involved in the latter study had TF activity above the upper limit of the normal range. Interestingly, it has been reported that there was no correlation between the absolute number of MP and the reported TF activity. Furthermore, Tesselaar and co-workers used the same upper limit for normal range of TF activity, and also reported that the TF activity associated with TF + MP was significantly higher in cancer patients than non-cancer patients. For 51 cancer patients with thrombosis the mean TF activity was 1125 fM Xa min−1 (19–12,333 fM Xa min^{-1}).

This group consisted of patients with the following types of tumour: 14 colorectal, 10 pancreatic, six testicular, four renal, three ovarian, two oesophageal, two prostatic, two bones, two laryngeal, two breasts, two respiratory tracts, one bile duct and one adrenal cancer. Of these, the highest noted MP associated average TF activity was 2,080 fM Xa min^{-1} (510–12,333) in PC. TF activity was also relatively high (55–1,578 fM Xa min^{-1}) in colorectal cancer. The range of other tumours studied showed a TF activity between 80–603 fM Xa min^{-1} (Tesselaar *et al.*, 2009). The mean TF + MP activity in 49 cancer patients without thrombosis was 162 fM Xa min−1 (23–535 fM Xa min^{-1}). Moreover, in a series of nine patients with lung cancer, three with breast cancer, one with PC, one with renal, one with sarcoma and 23 healthy controls, Tilley *et al.* (2008) reported that PC had the highest TF activity (48.3 pM) of all malignancies tested within the study. Zwicker *et al.* (2007) observed that there was no statistical difference in TF + MP numbers in healthy controls and non-small cell lung cancer, while TF + MP were significantly higher in PC (32 of 47 PC cases). It was reported that the highest incidence of patients with TF + MP above a lower detectable limit was observed in PC (25 of 39) followed by colorectal carcinoma (7 of 12), compared with non-cancer controls (6 of 31) and that the number of TF + MP in PC and colorectal cancer was significantly higher than in non-cancer controls (Zwicker *et al.*, 2009). However, the difference in the number of TF + MP compared with non-cancer controls was insignificant in lung cancer (5 of 28), breast cancer (4 of 9) and ovarian cancer (5 of 8) suggesting that TF + MP have an important role in the pro-coagulant phenotype and may be a contributing factor related to the high incidence of TE associated with PC patients. Furthermore, Zwicker *et al.* (2009) reported that two thirds of PC patients have a high number of TF-bearing MPs in plasma. The number and the activity of TF of advanced colorectal, pancreatic and breast cancer were higher than those of healthy normal controls but this was not true for small cell cancer of the lung were (Hron *et al.*, 2007, Khorana *et al.*, 2007, Tesselaar *et al.*, 2007, Zwicker *et al.*, 2007). Khorana *et al.* (2007) noted that the level of TF antigen increased in PC and it is associated with a high incidence of thrombosis. Similarly, Horn (2007) noted the same finding but this study used D-Dimer as an indicator of clotting. In a series of 20 advanced colorectal cancers and in 20 age-sex matched healthy controls, the median (inter-quartile range) number of TF bearing MP using flow cytometry (25.9 (15.4-42.0) x 10^3/ml) was significantly higher than the control group (medium and inter-quartile range (IQR); 13.1 (11.9 - 19.7) x 10^3/ml plasma, p = 0.007. This high TF bearing MP was significantly associated with high concentration of D-Dimer. D-dimer is a fibrin degradation product that is present in blood after a blood clot gets degraded by the process of fibrinolysis (Hron *et al.*, 2007).

The work presented here shows there was significant increase in the number of TF bearing MP in PC cases compared with the control group III. This change was associated with high pro-coagulant activity (shorter PT) of PC cases compared with control. However, the MP activity (using ELISA) did not show a significant difference from that of the control. This was not unexpected; the recently presented data at 5th ISTH 2011 is casting doubt on the discriminating ability of these markers (MP activity kit). Lin et al. (2011), reported that MP activity using Zymuphen MP test kit did not correlated with the incidence of DVT (conf reference 2). Kleinjan *et al.* (2011) (conf reference 3) reported that the hypercoagulable condition in cancer patients was not associated directly with the PL MP dependent activity. Furthermore, flowcytometry has been recently acknowledge as a more sensitive and precise method than ELISA (conf reference 1, conf reference 2, Vignali 2000). The authors noted that five cancer patients (12%) that developed venous thromboembolism had comparable levels of phospholipid dependent MP pro-coagulant activity. Therefore, the assay was not used in the current study after the analysis of a small number of cases. Furthermore, in the current study the number of annexin bearing MP/µl of PC cases did not differ from that of the control group. However, MP activity and annexin MP results might be affected by freezing as frozen PPP was used for these assays. At the same time there was no auto annexin antibody against the annexin. That indicates that the pro-coagulant activity of PC depends mainly on the TF. Therefore, pro-coagulant activity of PC depends mainly on the TF bearing MP as the freezing affect the number of MP. This finding was supported by, TF (measured by ELISA) of PC cases in the current study was not higher than that of control groups III and IV and this indicates that not only the TF activity but also the TF antigen is affected by long time freezing.

Therefore it is suggested that the ELISA might not be a sensitive method for measurement of TF or that due to long time freezing of PPP, the TF bearing MP was completely degraded. This supports the suggestion that the measurement of TF bearing MP in fresh PPP using flow cytometry is more sensitive than ELISA. Furthermore, the current study showed that the post-operative TF (measured by ELISA) did not differ significantly from the pre-operative level.

In the current study one stage prothrombin time was used to evaluate the pro-coagulant activity in the PPP of PC cases. Although it is not specific for TF activity, it provides an idea about the pro-coagulant activity. However it was noted after breaking the code that 5 out of 41 patients in the clinical trial were treated with prophylaxis 5000 U of dalteparin. Two were from group II,

two from group E and one patient from group III. It is postulated that that dalteparin has no effect on PT (Stief, 2006) . Furthermore Vargo *et al.* (2009) postulated that 100 U/ Kg twice daily has no effect on PT and activated partial Thromboplastin (APTT) because the main function of dalteparin is to allow the body's natural clot lysis mechanisms to work normally to break down clots that have already formed. Dalteparin binds to and accelerates the activity of anti-thrombin III. This is why PT and APTT are not helpful in monitoring coagulation state when dalteparin is used, particularly when dalteparin is used prophylactically (web reference 5, web reference 6). Moreover it has been noted in feline experiments that 1000 U dalteparin daily or divided into two doses did not affect PT, APTT and platelet factor 4 (web reference 7). Therefore deltaparin has no effect on one stage PT.

Two out of 5 cases (which were treated with dalteparin) were from group E, PT was 35, 30. The median of PT of group was 40 and the mean 38.1 S. PT of both cases was shorter than the mean of group E. This indicated that the prophylactic dalteparin did not prolong PT more than the mean of the PT of group E. Therefore, dalteparin did not decrease the pro-coagulant activity more than the mean of the group. Two cases were from group II. PT was (39 and 35). The median PT of group B was 36 and the mean 36.2 S. This indicated that the prophylactic dalteparin did not prolong the PT of one case and might have had an effect on the other case. Therefore, the current study was in agreement with previous findings that suggest prophylactic doses of dalteparin have no effect on PT.

Another interesting result of the current study was that there was a significant decrease of TF bearing MP number and pro-coagulant activity (represented by PT) after surgical removal of the PC but annexin bearing MP did not decrease post-operatively. In regard to the decrease of MP post-operatively, the result of the current study is comparable to the result of others (Zwicker *et al.*, 2009, Tesselaar *et al.*, 2007,), although only three patients were included in the Zwicker study and it is also comparable to the results of other cancer studies such as Haubold *et al.* (2009), who found a significant decrease of TF MP two weeks after surgical removal of prostatic cancer using automated thrombography that was used to monitor thrombin formation. It can be suggested that the result of this study supports the hypothesis that tumour cells are the main source of TF-bearing MPs. Moreover, Francis *et al.* (2010) found a decrease of TF MP activity after surgical removal of glioblastoma multiforme. Therefore the significant decrease of TF MP and pro-coagulant MP activity may have an important role in the decrease of the PC

aggressiveness and metastasis. Therefore the origin and the type of the tumour might have an effect on coagulant activity. The pro-coagulant activity of PC might be related mainly to the TF bearing MP. In this regard our results support the prevalent theory that TF bearing MP accounts for a major fraction of circulating TF activity and supports the suggestion that TF-bearing MPs are the main source of TF activity produced by cancer cells (Yu and Rak, 2004).

The findings of the current study indicate that the majority of patients with PC will have high levels of MPs and pro-coagulant activity. These findings have prompted the commencement of a trial (Micro-TEC). The MicroTEC study is investigating enoxaparin in patients with pancreatic, lung, and colorectal cancer with elevated plasma TF microparticles. Patients with high levels of circulating MP activity are randomised to treat with anticoagulation *vs.* no anticoagulation (Web reference 8).

8.6.2 Circulating TF- and the Metastatic Process

As shown previously, the majority of the existing work has emphasised the potential correlation of increased levels of total TF bearing MPs with thrombosis as shown in Zwicker *et al.* (2007) and Teselaar et al. (2007) and other studies. The current study is in agreement with other studies (described in section 8.6.1) that showed significant increase of TF bearing MP and procoagulant activity compared with the control. However, to the best of the author's knowledge the current study was the first study to correlate the TF bearing MP with the aggressiveness of the tumour as there was significant correlation between TF bearing MP and tumour cell invasion ($p=0.004$) and on HDMEC angiogenesis assay ($p=0.003$). Therefore, the importance of TF bearing MP is not limited to the coagulation state through the extrinsic pathway but might play a major role in enhancement of cell invasion and angiogenesis that has been demonstrated in section 8.2.3.1. Furthermore, the decrease of TF bearing MP and the pro-coagulant activity post-operatively might be a causative factor in the decrease of cell invasion metastasis, angiogenesis post-operatively. The main author was blinded and therefore he couldn't correlate the finding with the clinical outcome.

CD 14 bearing MP behave like the TF bearing MP. The CD 14 bearing MP of PC cases was significantly higher than that of the pre-cancerous control group. The absolute number of CD 14

bearing MP was decreased post-operatively. This is evidence that could support the hypothesis that removal of the tumour decreases many factors, including TF and CD 14.

As the current study suggested that TF has an important role in thrombosis, cell invasion and angiogenesis, targeting of TF might therefore have a role in the controlling of the aggressiveness of PC. This result and suggestion supports the use of anti TF therapy in combination with chemotherapy in the treatment of PC.

It has been postulated that recombinant nematode (hookworm Ancylostoma caninum) anticoagulant protein c2 (rNAPc2) is a specific inhibitor of tissue factor (TF)/factor VIIa complex with novel antithrombotic activity (Zhao *et al.* 2009). In addition to its anticoagulant activity it inhibited angiogenesis, primary and metastatic tumour growth in mice (Hembrough *et al.* 2003). Furthermore a randomised phase II clinical trial of gemcitabine with and without PCI-27483 (1.2 mg/kg two times daily, which act as TF and FVIIa inhibitors) in patients with advanced PC is ongoing (Ramanathan *et al.* 2011).

Based on this data, MP appear to have an important role in aggressiveness and thrombotic conditions of PC. Therefore control of MP might have an important role in the prognosis of PC. This could be achieved through removal of MP from the blood by dialysis, enhancement of anti-tumour immune response and or through inhibition release of MP. In this regard, in a clinical trial, the asciticfluid MP have been captured by specific antibodies and then removed completely by dialysis using Hemopurifier^{TM} [(Ichim *et al.*, 2008). Furthermore, it has been reported that dendritic cells promote initiation and amplification of anti-tumour immune response (Chaput *et al.*, 2004a, Wolfers *et al.*, 2001). This process has been manipulated to create an anti-tumour vaccine. Chaput *et al.* (2004b) proposed two distinct methods for treating melanoma and ovarian cancer. Two clinical trials aim to amplify the anti-tumour cytotoxic T cell response. "Vaccination" consists of injecting the patient with dendritic cell-derived MP loaded with tumour peptides. An alternative method of "vaccination" starts with the isolation of ascites-derived MP from a patient with ovarian carcinoma. The MP were supplemented with adjuvant and re-injected into the patient. The MP are injected into the subcutaneous tissues at different sites, which provokes a strong and targeted anti-tumour T cell response (Liu *et al.*, 2006). The third method which might control MP is inhibition of MP formation and release. Taxol and vinca alkaloids, as conventional chemotherapeutic medicaments not only inhibit cell division, but

additionally decrease MP release. Using proton pump inhibitors, exosome formation can be blocked in lack of gradually decreasing acidic milieu (Iero *et al.*, 2008). At the same time ceramide inhibition leads to exosome release block (Trajkovic *et al.*, 2008). Additional proteins, such as clathrin, sorting nexins and more have also been described to be over expressed in different tumours (Wright, 2008, Cheng *et al.*, 2005, Jordens *et al.*, 2005). Control of these might have an important role in the inhibition of release of MP.

Conclusion

It has been observed that there was a significant correlation between MP number and PT and this could explain the short PT in PC patients and decrease of PT after surgical removal was mainly due to TF that related to MP. The other interesting correlation is the significant correlation between the MP and angiogenic and cell invasion activity and this could indicate that the tumour could be responsible for different malignant mechanisms such as high procoagulant activity, cell invasion and angiogenesis and all synergistically might be responsible for the aggressiveness of PC. Therefore the suggestion is that controlling these factors might affect the aggressiveness of PC cases.

CHAPTER NINE: HISTOPATHOLOGY

9.1 Introduction

This section involved the evaluation of the expression of TF, VEGF, VEGFR 1, VEGFR 2, EGF, EGFR 1 and CD 34 by immunohistochemistry on PC tissue and non cancerous lesions from the patients involved in the clinical study. Correlation was also studied between the level of expression of these markers with each other and with the serological/plasma findings from the invasion, angiogenesis, pro-coagulant activity and MP assay results previously described in chapters 7 and 8.

9.2 Methodology

9.2.1 H & E Staining

All cases shown in Fig. 9.1 (24 cases) were stained with H & E. For the rest of cases histopathological evaluation, including grading was taken from the existing histopathological reports. Therefore, analysis of histopathologial grading was conducted in all malignant cases of group B (32 cases) shown in Fig. 5.3.

9.2.2 Immunohistochemistry Staining

The methodology and the optimization necessary for each of the markers have been described in section 2.214. Immunohistochemistry staining was conducted for 24 cases, of which 10 were from group I, 8 from II, 3 from group E and 3 from group III. Therefore a total of 21 malignant cases were available for the immunohistochemistry study (Fig. 9.1). The sections were stained in single batches at for each specific marker.

All cases were reviewed and scored separately by two specialist pathologists (Dr Hussein Echrish and Dr Justin Cooke) in an anonymised manner. The two pathologists then examined and discussed the sections together and agreed a score that was allocated to each sample as the definitive value. Both investigators were 'blinded' to the results of the serology and plasma assays. The purpose of immunohistochemistry is to visualise and localise the

expression of a particular protein in the tissue. The histopathological sections of PC cases were classified as positive or negative for each factor. Interpretation of positive versus negative expression is relatively subjective and due to this each immunohistochemically stained protein was allocated a scoring system based on relevant and validated methodologies published in the literature (Smith *et al.*, 2010, Pryczynicz *et al.*, 2009, Pryczynicz *et al.*, 2008, Vermeulen *et al.,* Khorana *et al.*, 2007, Kajita *et al.*, 2001, Weidner *et al.*, 1991).

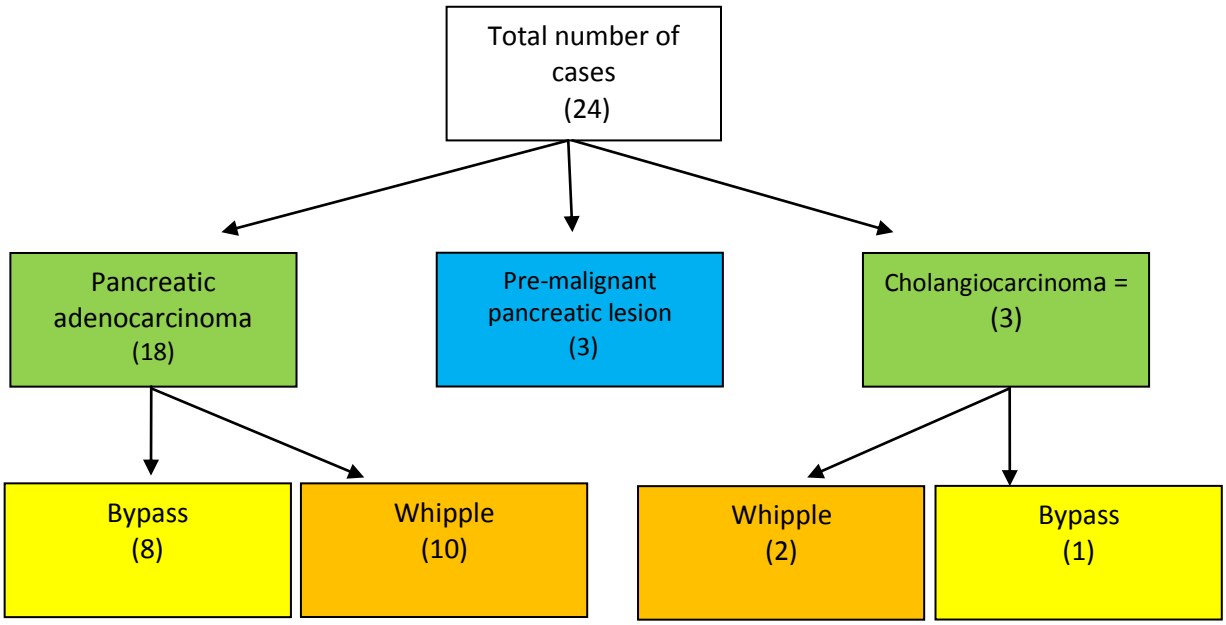


Figure 9.1: Consort diagram sample studied by immunohistochemical analysis.

The Islets of Langerhans give strong signals for many markers such as VEGF (Christofori *et al.*, 1995, Kuroda *et al.*, 1995) TF and EGFR (Feng *et al.*, 2002). Therefore in this study the Langerhans cells were used as an internal positive control. Similarly, large blood vessels were used as an internal quality control for CD 34 staining. In this project, only the cancer areas were scored and normal areas, if present, were disregarded. Also, staining around the periphery of the section was often more intense; this is widely recognised as edge-artefact and these areas were also disregarded. In the assessment of the positivity of the staining, the intensity of the stain as well as the coverage of the stain were both taken into consideration.

There are differing degrees of positivity that were categorised and optimised for each marker to conform to the relevant published data. In all cases the scoring system adopted was based on the existing literature.

9.3 Results.

9.3.1 H & E Stain (whole cohort)

All sections were stained with H&E. For the 24 cases used in the correlative immunohistochemistry study H&E sections were reviewed and re-examined by two pathologists (Dr Hussein Echrish and Dr Justin Cook) and the grading according to cell differentiation agreed. For the cases that were not available for studying in this thesis grading based on the existing pathological reports was used. The histological categories were correlated on the grounds of differentiation grade with the serological data generated from these patients (Chapters 7 and 8). For statistical analysis, well and moderately differentiated cases were grouped together.

The current study revealed that out of 32 cases in group B (all malignant cases), 20 (64.5%) were poorly differentiated. This included 8 cases out of 16 of resectable malignant cases (group C and 12 cases out of 16 uresectable malignant cases (group D). There was no statistical difference in grading between groups C and D (Chi square; p= 0.17).

TF bearing MP in patients with poorly differentiated pancreatic cancer were significantly higher than those with well and moderately differentiated cancers [Median and IQR was 4604 (3580-7596) *vs.* 2696 (2342-3922), p= 0.011, Mann Whitney test]. There was no other significant correlation between any of the other measured variables (PT, chemotaxis activity and angiogenic activity) with the histological grade of the cancers.

No correlations between the stage of the resected cancers and the peripheral markers were undertaken due to the relatively small number of resected patients, which would make the statistical analysis unreliable (advice from Dr. Victoria Allgar, biostatistician).

9.3.2 Immunohistochemical Correlation Study (Fig.9.1)

Twenty four patients had complete immunohistochemical evaluation of the relevant tumour marker expression and distribution on the pancreatic cancer, the results of which were correlated

with circulating factors of biological activity of angiogenesis and invasion as described in the previous chapters (7 and 8).

9.3.2.1 TF Expression

The first marker to be evaluated was TF using a polyclonal rabbit anti human antibody. For TF scoring, intensity was divided into 4 degrees: 0 when there was no staining, 1 when the staining was weak, 2 when the staining was moderate and 3 when the staining was of strong intensity. TF staining was considered "positive" when the staining intensity was moderate or strong and according to the proportion of expression in this group staining was sub-divided into 3 grades, grade I when the expression was 1-33%, grade II when the expression 34-66% and grade III when the expression was 67-100% (Khorana *et al.*, 2007). The total number of group B cases (PC and cholangiocarcinoma) was 21, of which 13 (62%) were positive for TF expression. Five of these cases (38%) were grade I, 5 cases (38%) were grade II and 3 cases (23%) were grade III. All 3 cases in control group III were TF negative. The results of TF staining are summarised in Table 9.1, and typical examples of staining shown in Fig. 9.2.

Table -9.1: TF expression.

Group	Positive	Grade of Staining			Negative	Total
		Grade I	Grade II	Grade III		
B	13	5	5	3	8	21
III	0	0	0	0	3	3

Note: Group B represents all malignant cases. Group III represents control group.

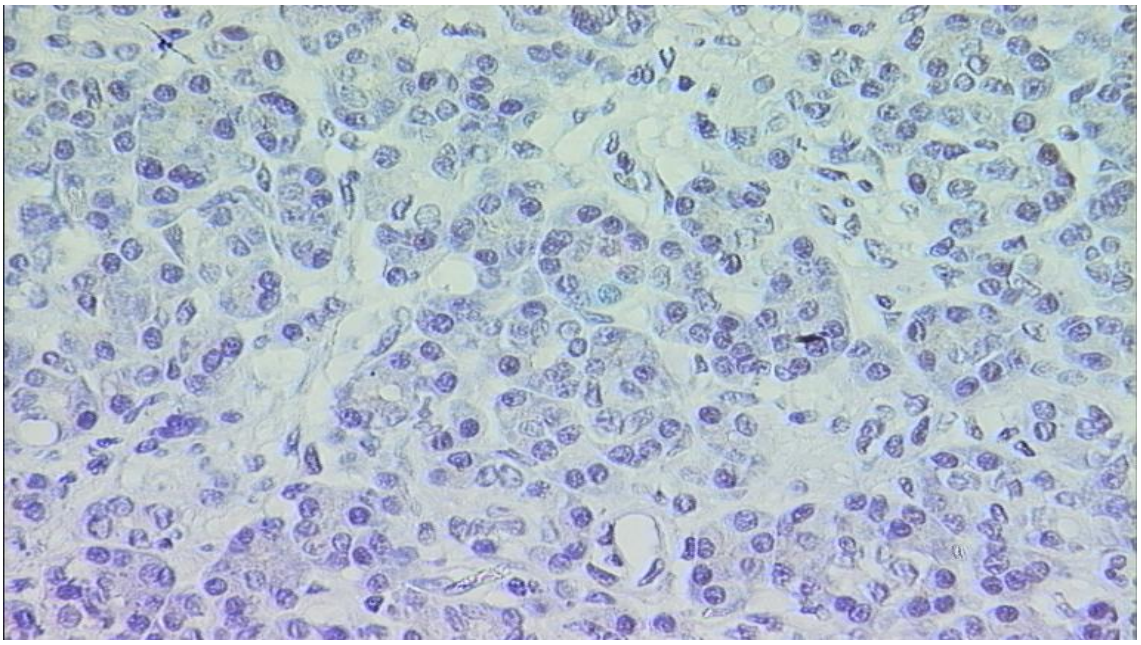


Figure 9.2A: Representative example of PC case typifying 'negative TF expression (x400). This is a poorly differentiated tumour. No staining is observed.

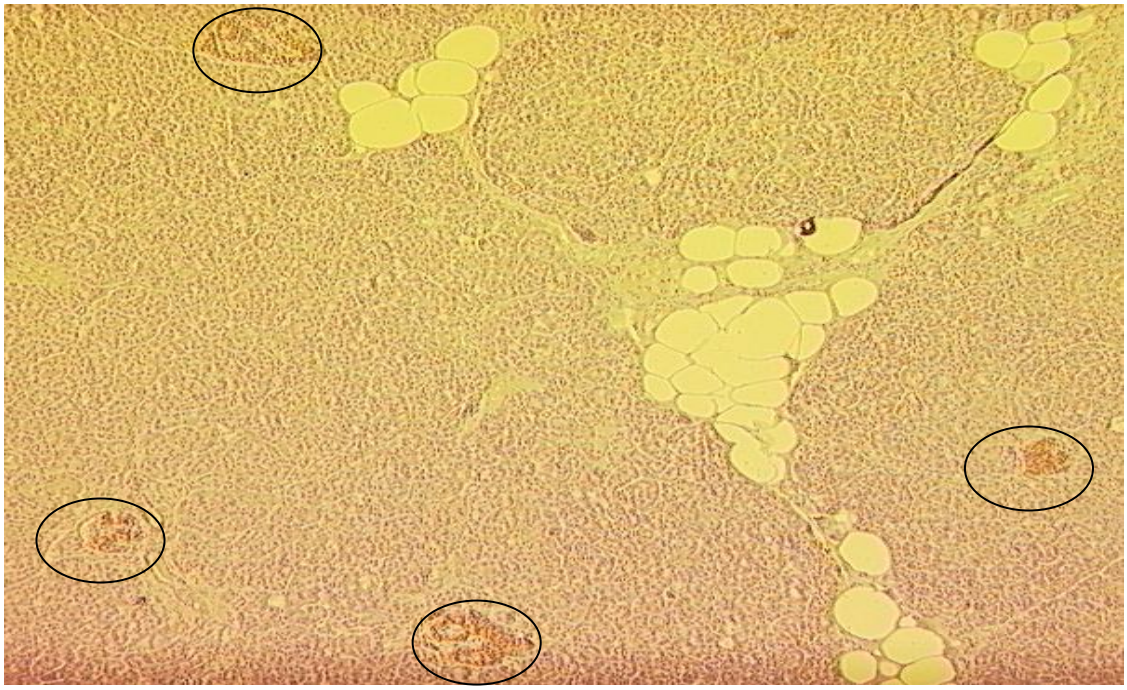


Figure 9.2B: Representative low-power section (x40) typifies internal control process. Islets of Langerhans showing positive TF expression (black circles) were used as a positive control.

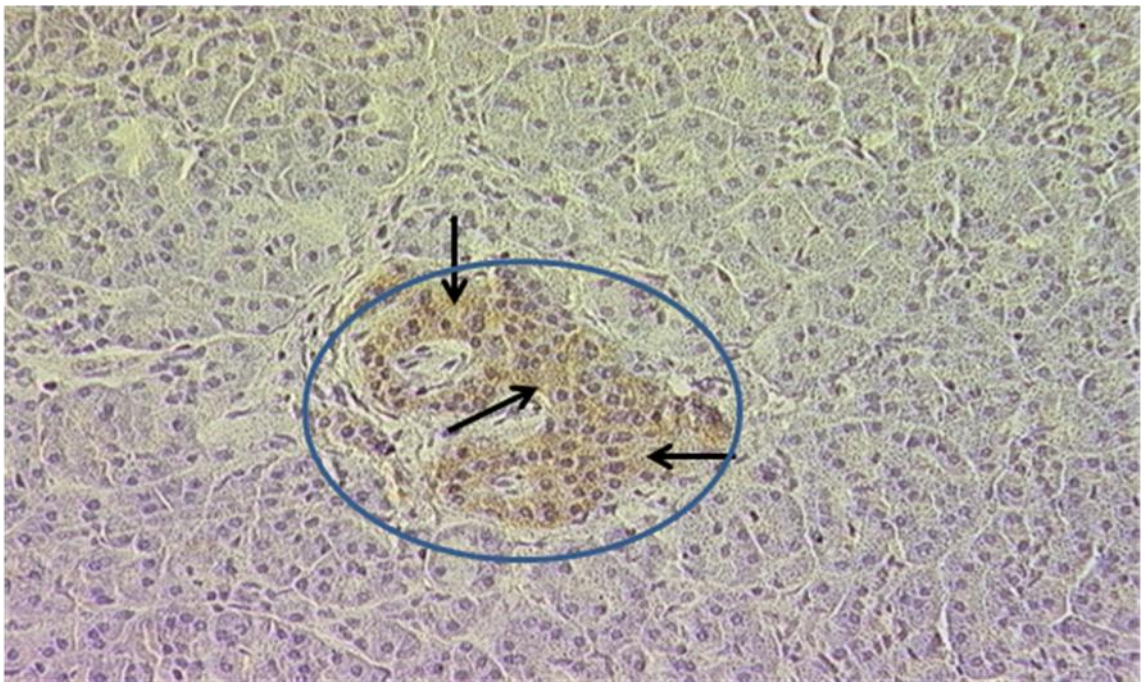


Figure 9.2C: Representative example of high magnification (x100). As can be seen immunohistochemistry staining of pancreatic tissue. Islet of Langerhans (circle) with positive cytoplasmic TF expression (arrows).

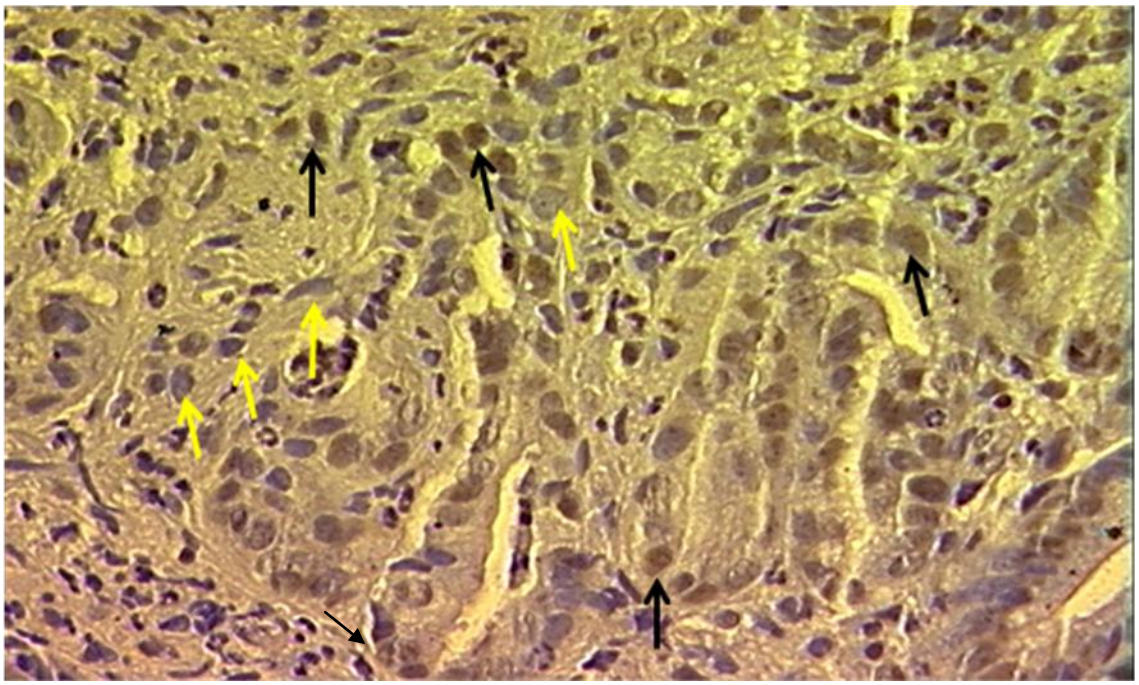


Figure 9.2D: Representative section typifying 'positive cytoplasmic' TF expression in a PC specimen (x400). Black arrows represent examples of positive expression. Yellow arrows represent examples of negative expression. This was a moderately-poorly differentiated pancreatic adenocarcinoma.

9.3.2.2 VEGF Expression

VEGF was the second marker evaluated on the PC tissue in this study using a polyclonal rabbit anti-human antibody. For VEGF, the intensity was divided into four groups (0-4) similar to the TF. Positive staining (medium and strong staining and the proportion of expression more than 5%) was graded according to the proportion of staining into three grades: grade I when the proportion of the staining was 6-36%; grade II when the proportion of the stain was 37-68% and grade III when the proportion of the staining was 69-100% (Khorana *et al.* 2007). The current study showed that 20 cases (95%) out of 21 of group B expressed VEGF. All cases of group III were VEGF negative. Table 9.2 and typical examples shown in Figure 9.3 demonstrate the staining findings for VEGF.

Table 9.2: VEGF expression.

Group	**Positive**	**Grade**			**Negative**	**Total**
		Grade I	**Grade II**	**Grade III**		
B	20	4	10	6	1	21
III	0	0	0	0	3	3

Note: Group B represents malignant cases. Group III represents pre-cancerous pancreatic lesions.

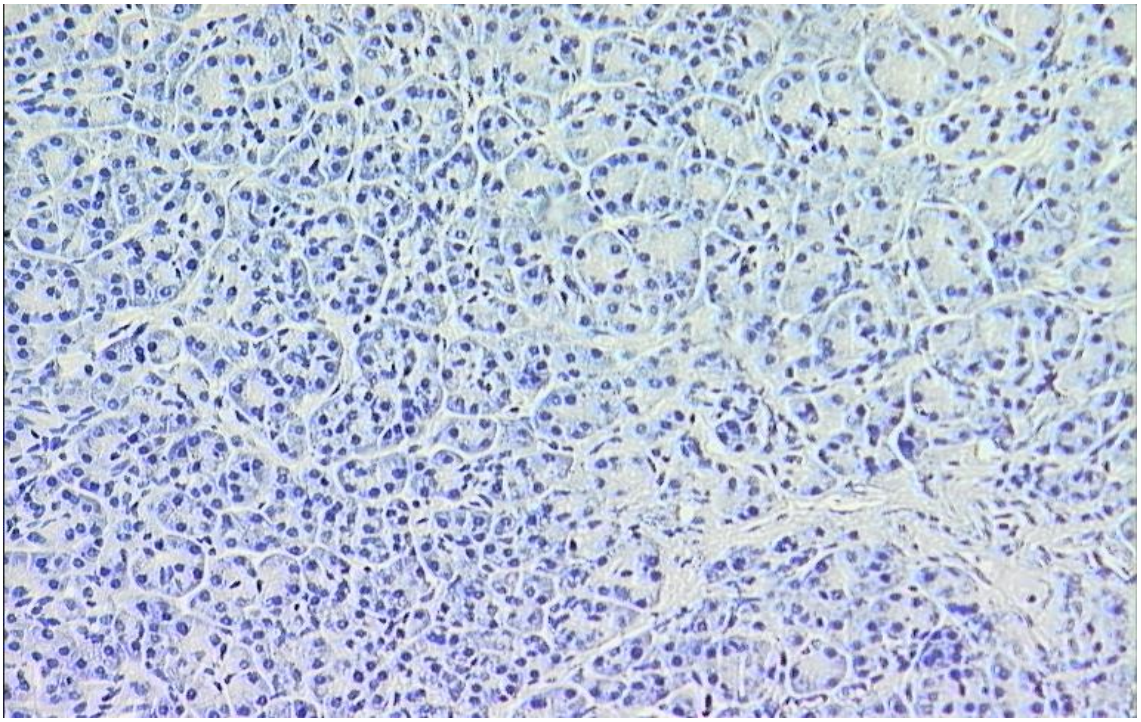


Figure 9.3A: Representative example of negative VEGF Expression (x100). No staining is observed.

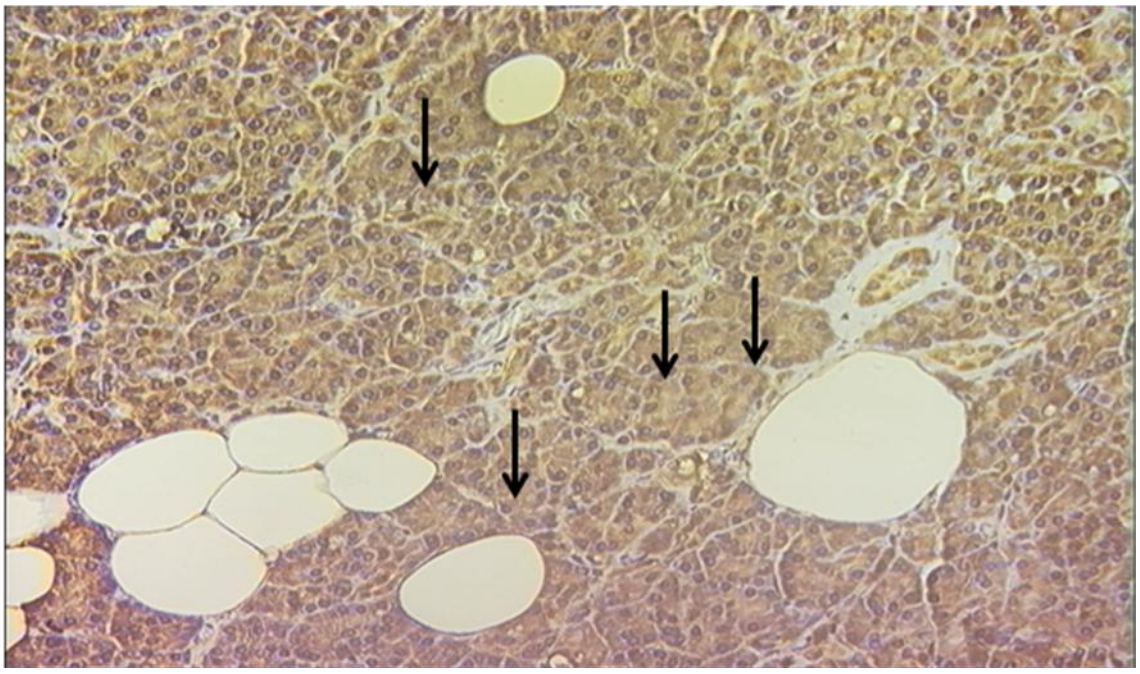


Figure 9.3B: Representative section of cytoplasmic VEGF positive expression (black arrows) in a poorly differentiated PC (x100).

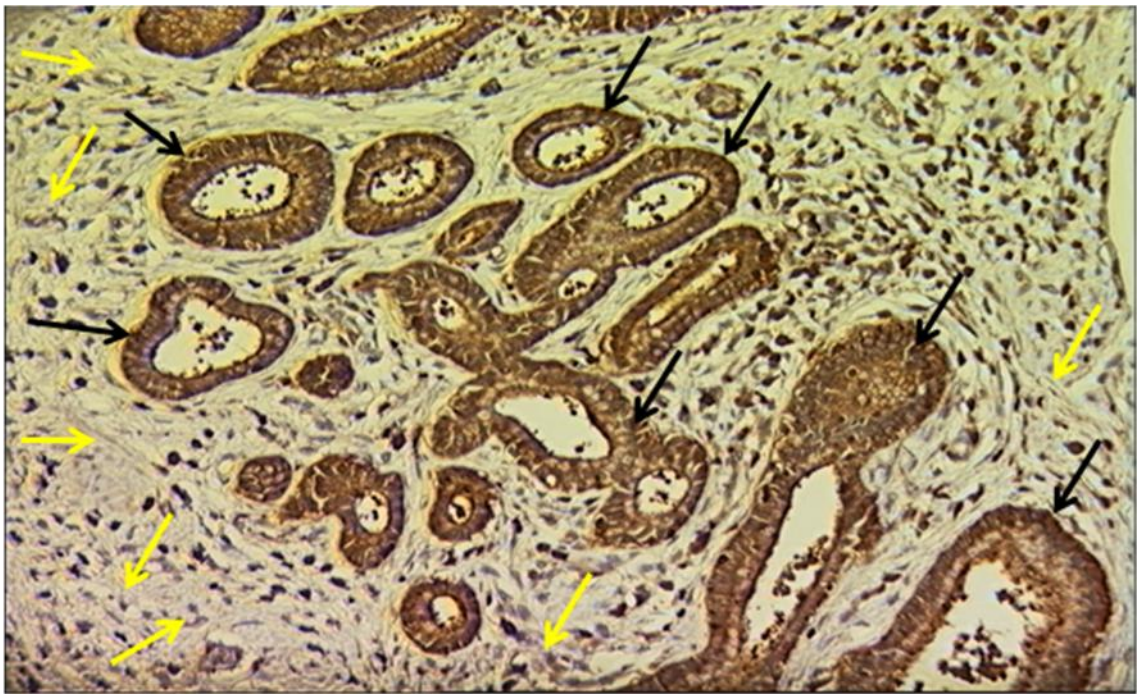


Figure 9.3C: Representative example of well differentiated PC with positive VEGF expression (x400). Black arrows represent examples for positive expression. Yellow arrows represent examples of negative expression.

9.3.2.3. VEGFR 1 Expression

VEGFR1 was evaluated in the current study using a polyclonal goat anti-human antibody. Five per cent expression and less was considered negative. The intensity of expression was again divided into four groups: none, weak, medium and high. Weak and no staining were considered as negative in agreement with Smith *et al.* (2010) and Kajita *et al.* (2001). The grades of scoring were then subdivided according to the proportion of the expression of VEGFR in a method similar to that used with VEGF (Khorana *et al.*, 2007). Medium and strong intensity were considered as positive. The current study showed that sixteen cases (76%) out of 21 cases of group B expressed VEGFR 1, of which 10 cases (48%) were grade I and six cases (29%) were grade II. Five cases (24%) were considered as negative. Two cases out of three of control group III were positive. Table 9.3 and typical examples shown in Fig. 9.4A-9.4B demonstrate the staining obtained with VEGFR1.

Table-9.3: VEGFR 1 expression by PC cases.

Group	Positive	Grade			Negative	Total
		Grade I	Grade II	Grade III		
B	16	10	6	0	5	21
III	2	0	2	0	1	3

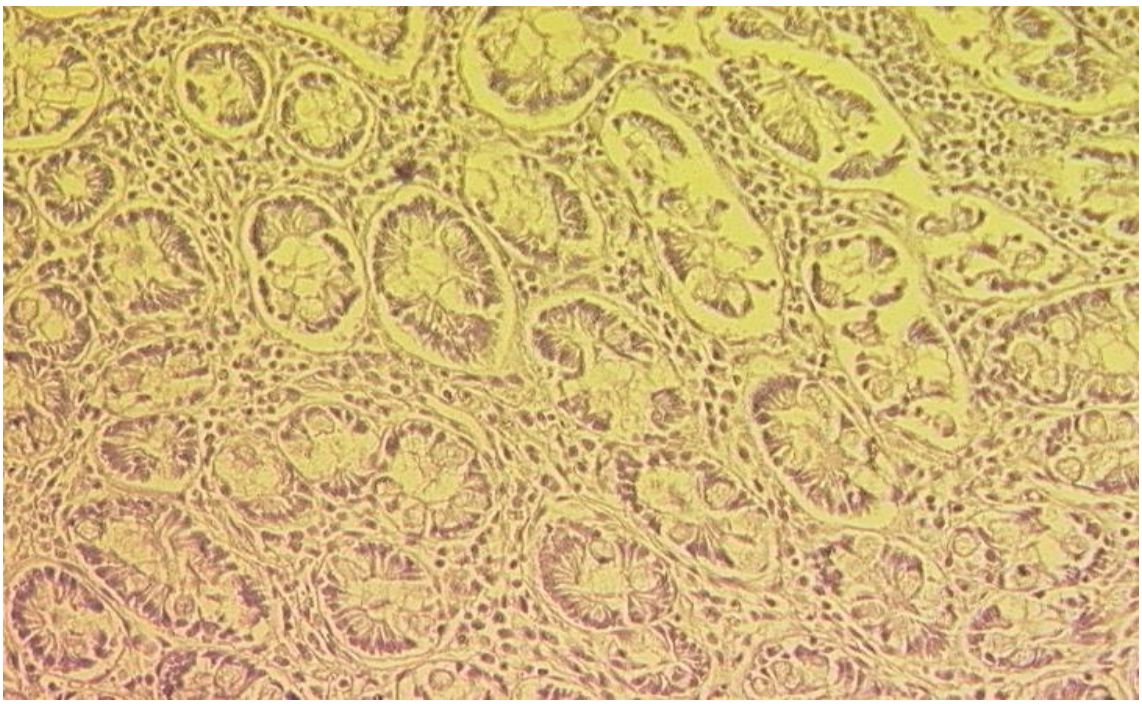


Figure 9.4A: Representative section typifying negative cytoplasmic and nuclear VEGFR1 expression of normal pancreatic tissue (x100).

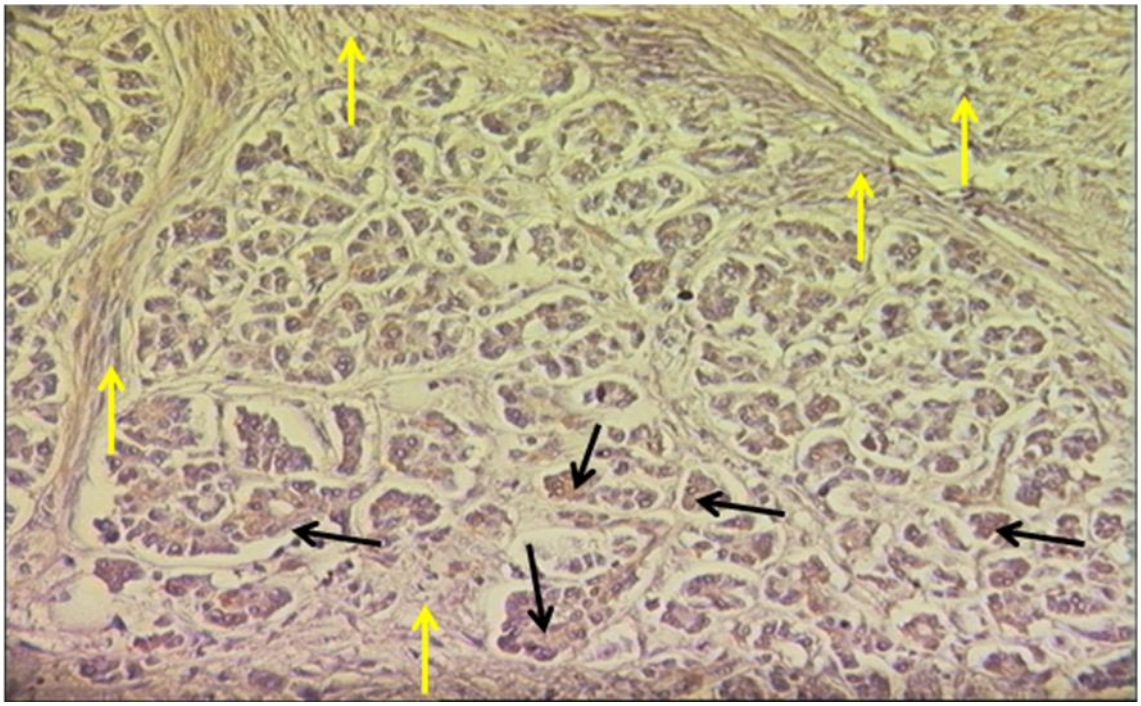


Figure 9.4B: Representative section typifying 'positive cytoplasmic' VEGFR1 expression in a poorly differentiated PC specimen (x100). Black arrows represent examples of positive expression. Yellow arrows represent examples of negative expression.

9.3.2.4 VEGFR 2 Expression

The VEGFR2 expression of PC cases was evaluated using a goat polyclonal anti-human antibody. The scoring was the same as in VEGFR1 (section 9.3.3). The current study showed that out of 21 cases of group B, 13 (62%) expressed VEGFR 2; of which eight cases (62%) were grade I and five cases (38%) were grade II. Out of the 10 cases in group I, five (50%) showed positive expression. Six cases out of the eight (75%) in group II had positive expression of VEGFR 2. All cases of the control group were negative. Table 9.4 and typical examples shown in Fig. 9.5 demonstrate the staining findings for VEGFR2.

Table 9.4: VEGFR 2 expressions of cases submitted to immunohistochemistry

Group	Positive	Grade			Negative	Total
		Grade I	Grade II	Grade III		
B	13	8	5	0	8	21
III	0	0	0	0	3	3

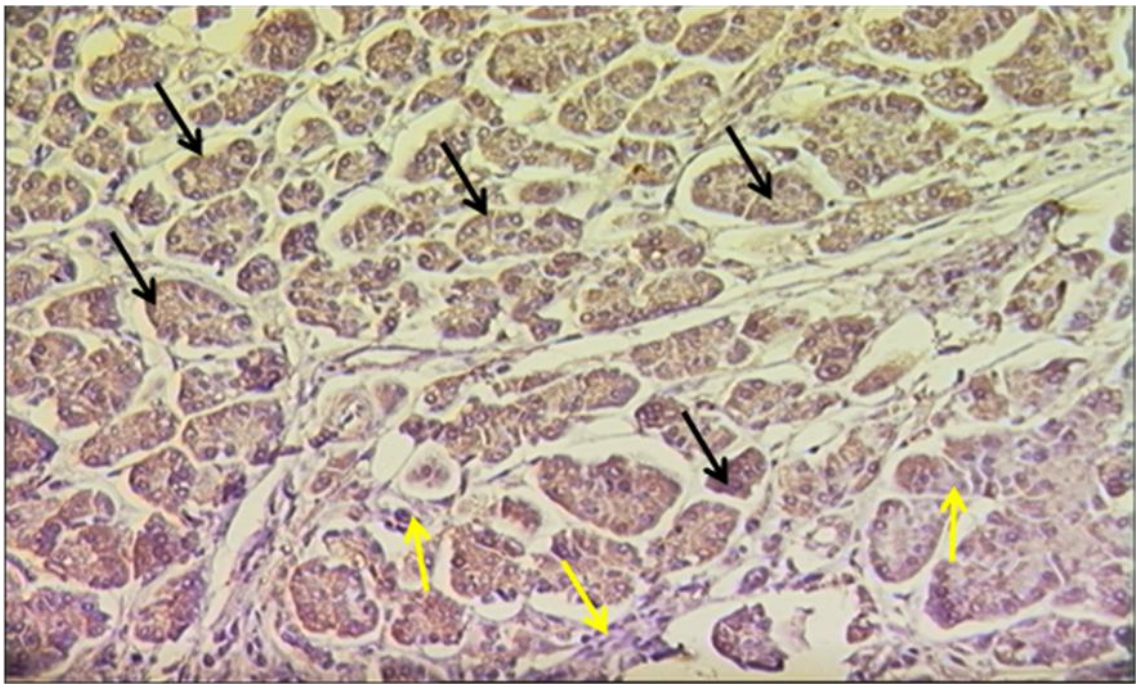


Figure 9.5: Representative section typifying 'positive cytoplasmic' VEGFR2 expression in a poorly differentiated PC specimen (x100). Black circles represent examples of positive cytoplasmic expression. Red arrows represent examples of negative expression.

9.3.2.5 EGFR 1 Expression

EGFR1 expression of PC cases were evaluated using a polyclonal rabbit anti-human EGFR1 antibody. The scoring of EGFR 1 and EGF (9.3.2.6) was divided simply into positive and negative. The result was considered negative if there was no staining or the intensity was weak and/or the proportion was less than 30%. Expression was considered as positive if the intensity of the staining was moderate to strong and the proportion of expressing cells was ≥ 30% (Pryczynicz *et al.*, 2009, Pryczynicz *et al.*, 2008). In the current study, out of 21 cases of group B, 12 (57%) were EGFR 1 positive. Table 9.5 and typical examples shown in Fig. 9.6 demonstrate the staining findings for EGFR1.

Table 9.5: EGFR 1 expression of cases that submitted to immunohistochemistry study.

Group	Positive	Negative	Total
B	12	9	21
III	1	2	3

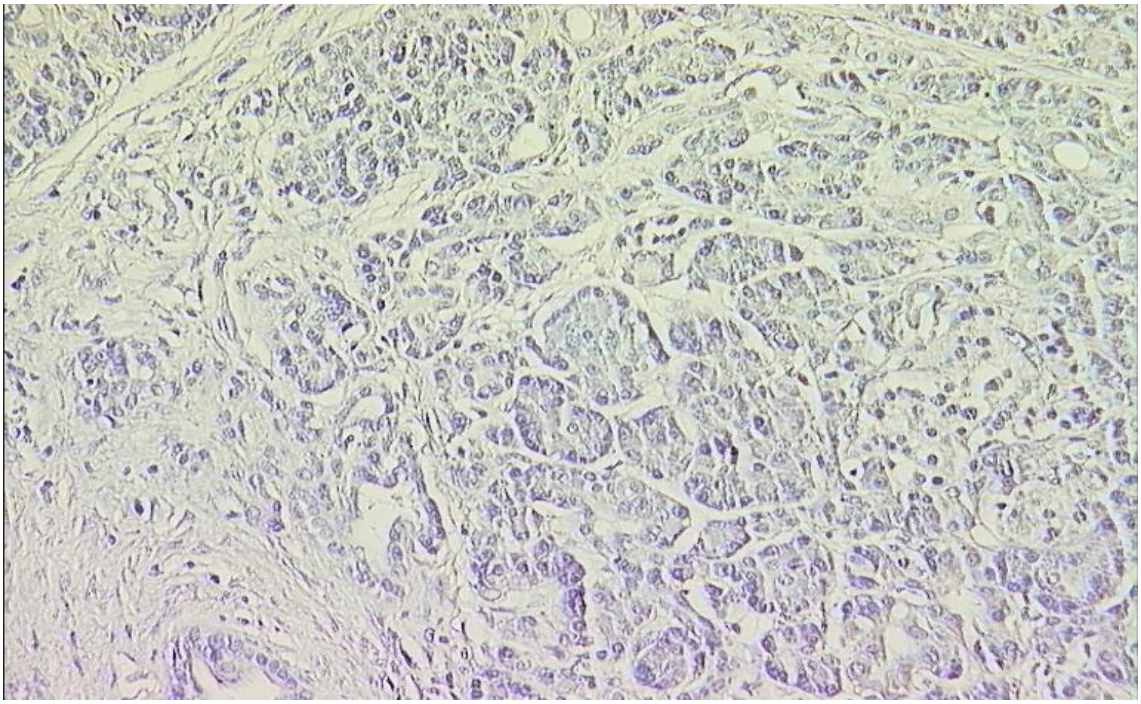


Figure 9.6A: Representative section typifying negative cytoplasmic and nuclear EGFR1 expression of in a poorly differentiated PC specimen (x100).

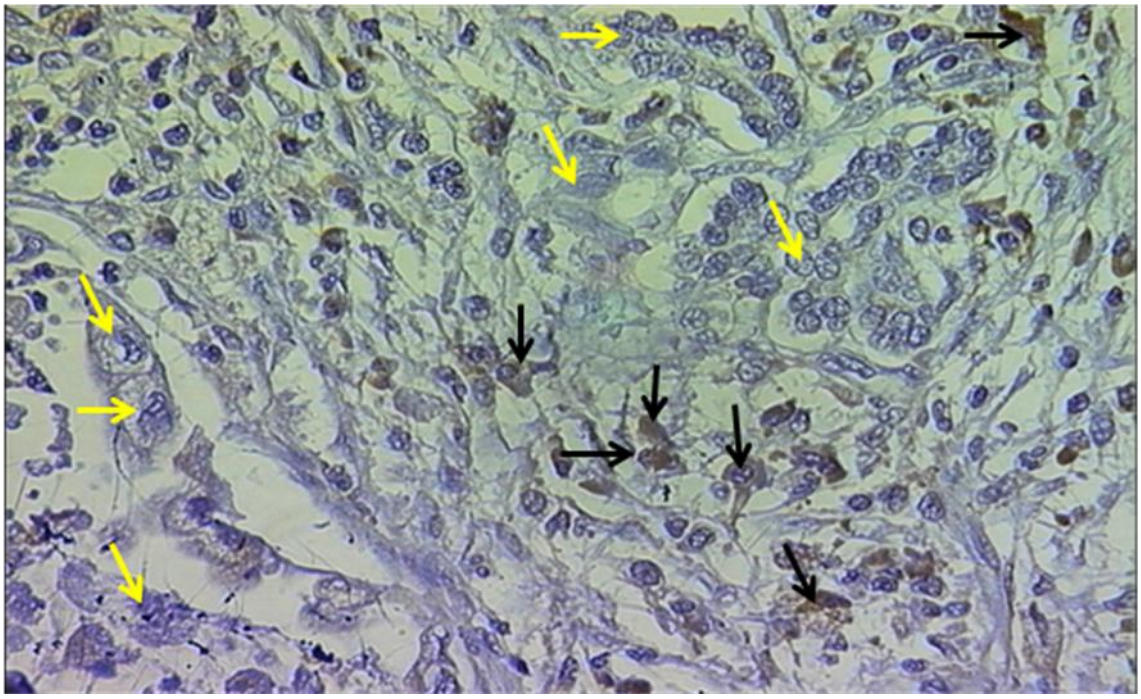


Figure 9.6B: Representative section typifying 'positive cytoplasmic' EGFR1 expression in a poorly differentiated PC specimen (x400). Black circles represent examples of positive cytoplasmic expression. Yellow arrows represent examples of negative expression.

9.3.2.6 EGF Expression

EGF on PC tissue was evaluated using polyclonal rabbit antihuman antibody. Scoring was the same as that used for EGFR (section 9.3.2.6). Seven cases out of 21 malignant cases of group B were EGF positive (33%). All cases of control group III (3) were negative. Typical examples (Fig. 9.7) demonstrate the staining findings for EGFR.

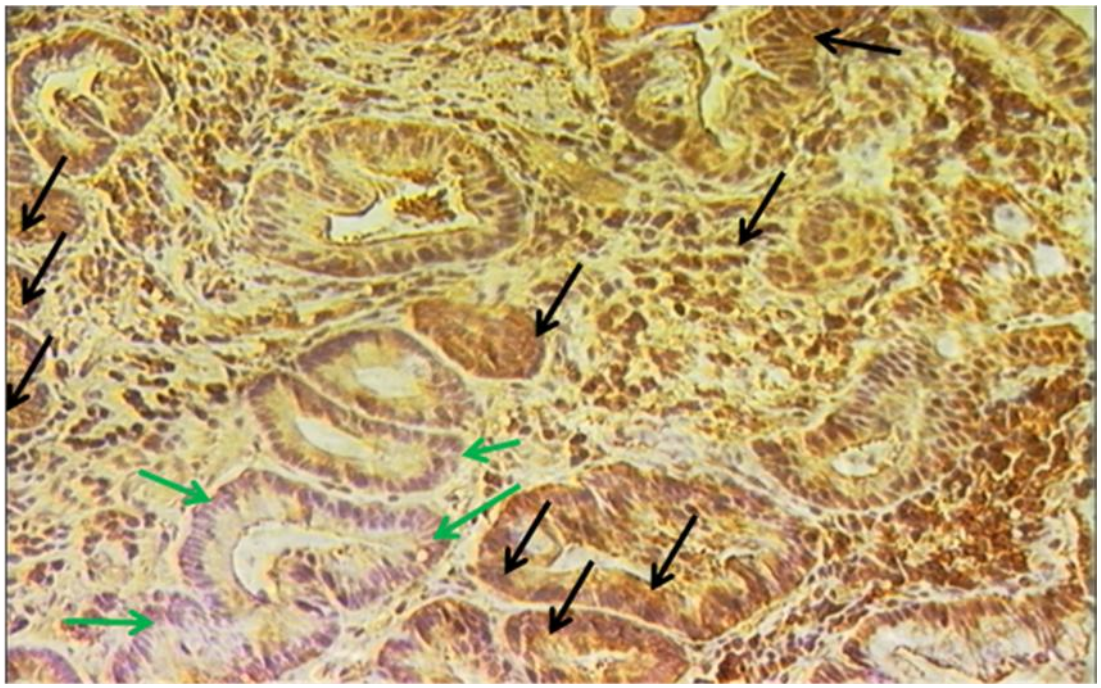


Figure 9.7: Representative section typifying 'positive cytoplasmic' EGF expression of a poorly differentiated PC specimen (x400). Black circles represent examples of positive cytoplasmic expression. Green arrows represent examples of negative expression.

9.3.2.7 Microvascular Density

MVD of PC cases was evaluated using a CD 34 polyclonal rabbit antihuman antibody. The highest 3 neovascularised areas (discrete micro-vascular staining for CD 34) were identified under low power (100X). The number of micro-vessels was counted in 400x magnification (Khorana *et al.*, 2007, Vermeulen *et al.*, 2002, Weidner *et al.*, 1991). The mean number of capillaries in three high power fields (PC cores) was considered as microvascular intensity. The current study showed that the mean number of new capillaries/ HPF (400x) of group B cases was 47±11 while that for control group III (four cases) was 17±6. Typical examples (Fig. 9.8) demonstrate the staining results for CD 34.

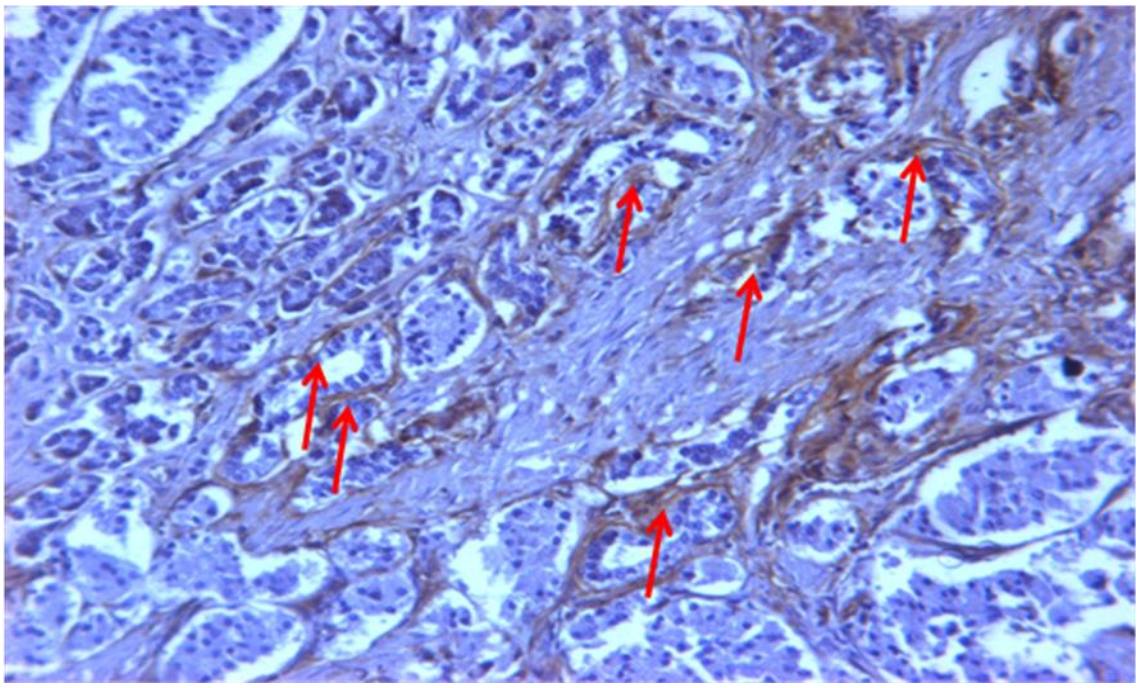


Figure 9.8: Representative section typifying 'positive cytoplasmic' CD 34 expression of a poorly differentiated PC specimen (x400). Red arrows represent examples of positive cytoplasmic expression.

9.3.3 Correlation

This part of the thesis undertook the chemotaxis activity, angiogenic activity (chapter 7), TF bearing MP number and pro-coagulant activity according to the positive or negative expression (chapter 8) of these tissue markers on the histopathological sections. As the values were not normally distributed the non-parametric Mann Whitney test was used for statistical analysis.

9.3.3.1 TF

Firstly the number of TF bearing MP, pro-coagulant activity, chemotaxis activity and angiogenic activity were correlated with the positive or negative TF expression on the histological sections.

9.3.3.1.1. Correlation of TF Expression with TF Bearing MP:

The number of TF bearing MP in immunohistochemically TF positive cases did not significantly differ from that of TF negative cases [median with IQR, 4585 (3479-8787) *vs.* (2251-5008, p 0.065)]. Although it was statistically non significant, the p value was 0.06, which is tending towards the significance threshold (Fig. 9.9).

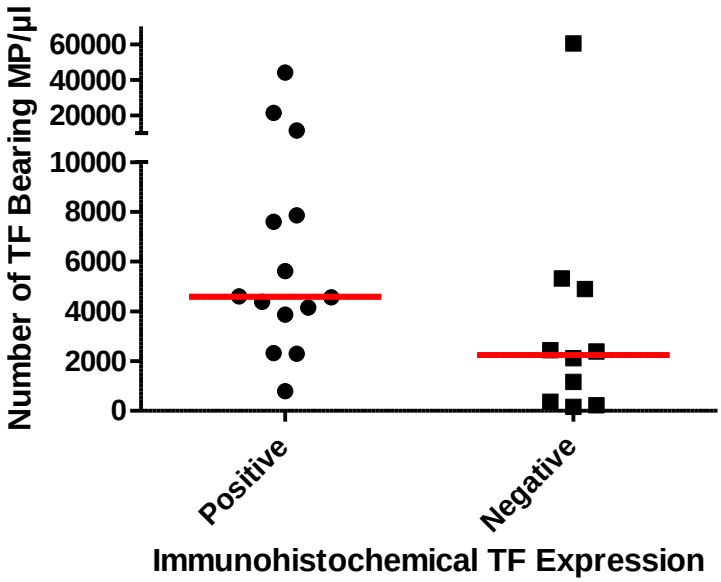


Figure 9.9: The relation between TF expression and TF bearing MP/ µl. The red bar represents the median.

9.3.3.1.2. Correlation of TF Expression with PT

The pro-coagulant activity was evaluated according to positive or negative TF expression on the histological sections. The current study showed that the median pro-coagulant activity of immunohistochemistry TF positive cases was significantly higher (low PT) than that of negative TF cases [median PT and IQR; 33 (33-37.5) S] *vs.* [36.5 (34.7-40) S, p= 0.029, Fig. 9.10)].

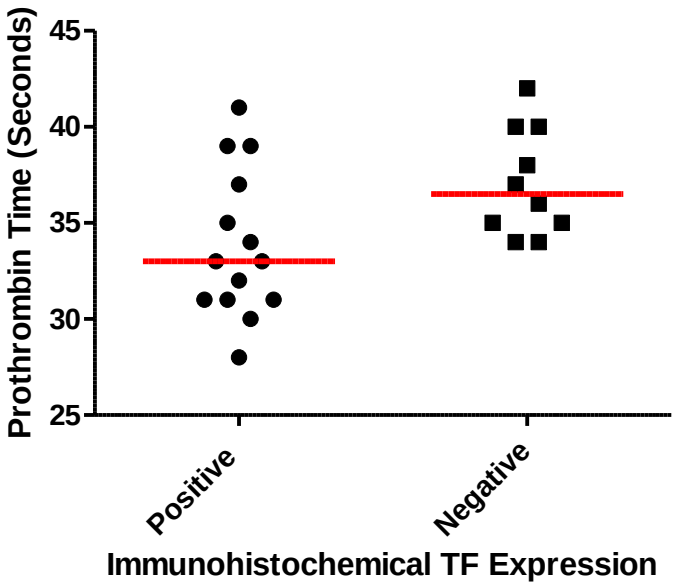


Figure 9.10: The relation between TF expression and PT. The red bar represents the median

9.3.3.1.3. Correlation of TF Expression with Cell Invasion

The chemotaxis activity of sera was evaluated according to the positive or negative TF expression on the histopathological sections. The level of invaded AsPC1 cells from immunohistochemistry TF positive cases [median and IQR; 136 (115-147) did not differ significantly from the TF negative cases [136 (74-163), p= 0.9].

9.3.3.1.4. Correlation of TF Expression with Angiogenesis

The angiogenic activity (total length and number of gel capillaries and MVD) of sera was evaluated according to the positive or negative TF expression on the histological sections. The current study showed that the total length and number of gel capillaries of immunohistochemistry TF positive cases were significantly higher than those found in negative cases [median and IQR, 8001 (4437-9407) and 40 (32-44) *vs.* 4489 (915-5678) and 32(15-36), (p= 0.01, 0.02) respectively] (Fig. 9.11-9.12).

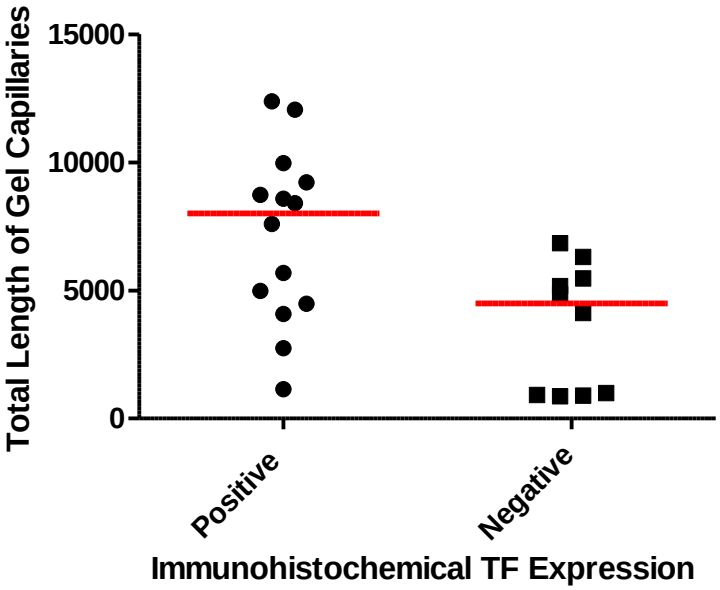


Figure 9.11: The relation between TF expression and total length of capillaries. The red bar represents the median.

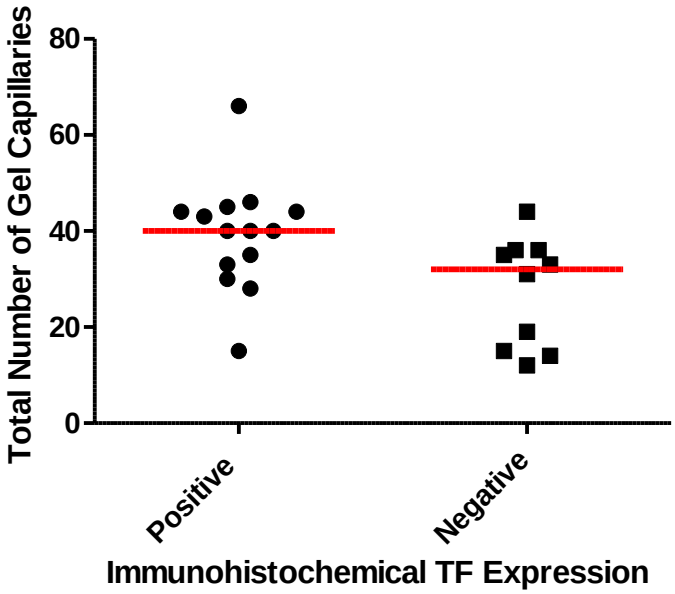


Figure 9.12: The relation between TF expression and total number of capillaries. The red bar represents the median

The microvascular density of TF positive cases was higher than that of negative cases but the result was not significant [median and IQR (45 (17-47) *vs*. 37 (27-52), p= 0.07)].

Finally the correlation between the TF expression and VEGFR2 (angiogenic receptor) was evaluated. Fourteen cases showed positive TF expression, of which 11 (79%) showed positive VEGFR 2 and 10 cases showed negative TF expression, of which three (30%) showed positive VEGFR 2 expression. The difference was statistically significant (p= 0.01, Chi square, Fig. 9.13).

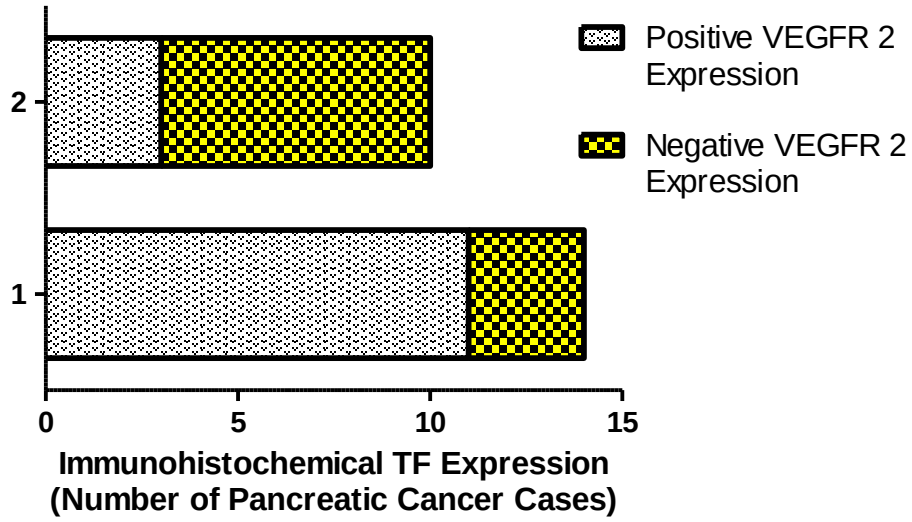


Figure 9.13: The relation between TF and VEGFR 2 expression. 1- Positive TF expression. 2- Negative expression of TF.

As a conclusion, correlation data appear to show that TF factor expression of the tissue is significantly correlated with the procoagulant activity of the plasma, angiogenic activity of the serum and the tissue.

9.3.3.2. VEGF

In the same manner as for TF, these parameters were correlated with the positive or negative expression of VEGF on the histopathological sections. However as mentioned in section 9.3.22, 95% of PC cases showed positive VEGF expression; therefore further analysis of TF bearing MP, pro-coagulant activity, chemotaxis and angiogenic activity according to the grade of VEGF expression was added. VEGF expression was simply divided into high VEGF expression (Grades II and III) and low VEGF expression (Grade I and negative expression).

9.3.3.2.1. The Correlation of VEGF Expression on PC Tissue with TF Bearing MP and PT.

The current study showed that the number of TF bearing MP and procoagulant activity of immunohistochemistry VEGF positive cases did not significantly differ from that of VEGF negative cases {[median number and IQR of TF MP/ µl was 4476 (2336-7300) *vs.* 1171 (181-6226), p= 0.09], [median PT and IQR was [35 (31.5-38)] *vs.* [37 (32.5-40), p= 0.5)] respectively}. Furthermore, the TF bearing MP and PT of high VEGF expression cases did not significantly differ from those of negative VEGF expression cases.

9.3.3.2.2. The Correlation of VEGF Expression on PC Tissue with Cell Invasion

There were no significant changes in cell invasion, either between VEGF positive and negative cases or between high and low VEGF expression. The median cell invasion number and IQR of positive VEGF was 136 (109-157) *vs.* 113 (62-155) of negative VEGF expression cases, p= 0.4). Furthermore, the median cell invasion number and IQR of high VEGF expression was 134 (107-106) *vs.* 138 (90-57) of those with low VEGF expression, p= 0.8.

9.3.3.2.3. The Correlation of VEGF Expression on PC Tissue with Angiogenesis

There were no significant changes in angiogenic activity between VEGF positive and negative cases. However, when cases divided into high and low grade of VEGF expression, the current study showed that there was a significant relation between the number of gel capillaries formed and MVD (tissue capillaries detected by CD 34) with the grade of VEGF expression. The total number of capillaries (gel capillaries) of high VEGF expression cases was significantly higher than that of those with low expression [median and IQR, 40 (34-44) *vs.* 30 (14-35), p= 0.02, Fig

9.14]. Furthermore, MVD of VEGF positive expression cases were significantly higher than that of low expression cases [median number and IQR of new blood vessels detected by CD 34 was 53 (46-59) *vs.* (16-37), p= 0.0009, Fig 9.15)].

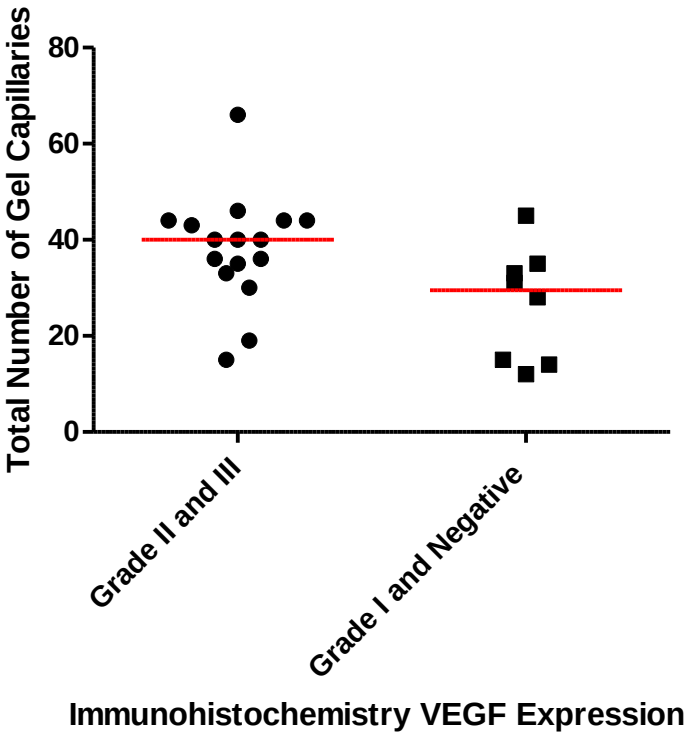


Figure 9.14: The relation between VEGF expression and total number of gel capillaries. The bar represents the median.

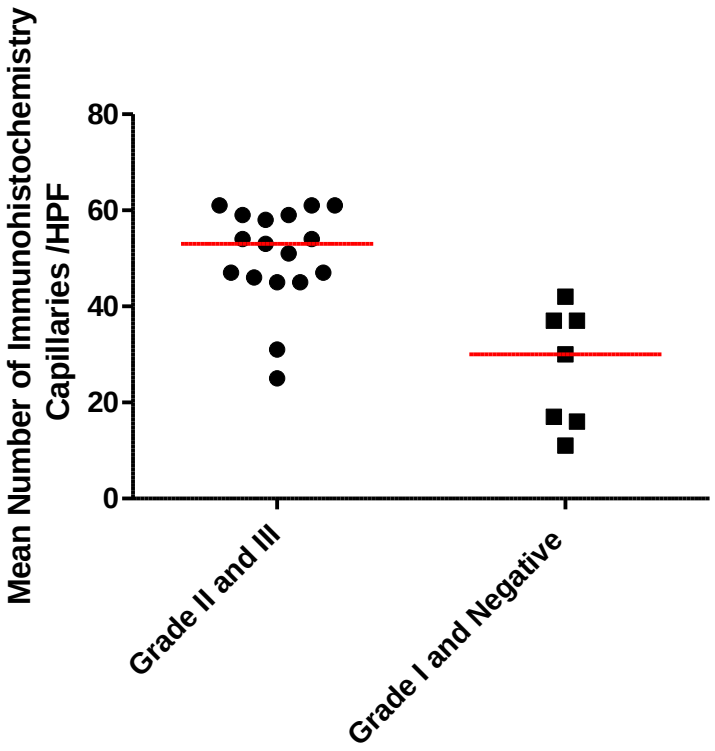


Figure 9.15 The relation between VEGF expression and number of new blood vessels on tissue. The bar represents the mean.

The pro-coagulant activity of positive VEGFR2 cases was significantly higher than those of negative cases [median; 33.5 (31-36.2) *vs.* 36.5 (34-40.2), p= 0.05].

There was no significant relation between TF bearing MP, chemotaxis activity and angiogenic with the positive and negative cases or high and low expression of VEGFR 1, VEGFR 2, EGFR and EGF on the histopathological section. There were no significant differences in the procoagulant activities of positive and negative cases or between high and low TF, EGF and EGFR immunohistochemical markers.

Table 9.6: Summary of immunohistochemical correlation with other indices.

TF expression on the tumour	Significant , p value	VEGF expression on the tumour	Significant, p value
TF-bearing MP	No , p= 0.065	TF-bearing MP	No, p=0.09
Procoagulant acttivity	Yes, p=0.029	Procoagulant activity	No, p=0.5
Cell invasion	NO, p=0.9	Cell invasion	No, p=0.4
Total number of capillaries	Yes, p=0.01	Total number of capillaries	Yes, p=0.02
Total length of capillaries	Yes, p=0.02	Total length of capillaries	No, 0.06
VEGFR2 on the tissue	Yes, p=0.01	VEGFR2 on the tissue	No, p=0.1
MVD (CD 34)	No, p=0.07	MVD (CD 34)	Yes, p=0.0009

9.4. Discussion

This study was designed to evaluate the correlation between TF bearing MP, procoagulant activity, chemotaxis activity and angiogenic activity with relevant immunohistochemical markers such as TF, VEGF (and receptors VEGFR1, VEGFR2) and EGF. It was originally planned to include all cases as per the consort diagram (Fig 5.3). However, it was only possible to study 24 cases (Fig. 9.1) because of time constraints in obtaining the tissue from Pathology archives.

Several studies correlate the TF expression on PC tissue with procoagulant activity; other studies correlate the TF expression with the MVD on the tissue. To the best of the author's knowledge this study is the first that correlates TF expression on the tissue with the serum and tissue angiogenic activity, plasma TF bearing MP and procoagulant activity.

It is postulated that there is no TF expression on the exocrine part of the normal pancreas (Kakkar *et al.*, 1995). In the current study, Islets of Langerhans expressed TF and other markers in accordance with literature references (Feng *et al.*, 2002, Kuroda *et al.* 1995, Christophori *et al.* 1999). Therefore these Islets were used as an internal positive quality control.

It has been reported previously that the high TF expression on PC (tissue) is associated with accelerated growth of the primary PC (Kakkar *et al.*, 1999). Several studies have shown positive expression of TF on PC tissue; some have shown a high percentage of PC cases expressing TF, such as the study of Khorana *et al.*(2007), who of 130 PC cases studied noted positive TF expression in 89%. The cases in the latter study were preserved in 10% formalin and after processing were embedded in paraffin similar to the cases in our study. Furthermore the scoring system was similar to that used in our study. Moreover, Nitori *et al.* (2005) on paraffin embedded PC tissues, found that in a series of 113 PC cases, 12% showed negative TF expression, 42% showed weak TF expression and 42% showed strong expression of TF. In the latter study the TF expression was considered as negative when the expression was 0%, weak when the TF expression was 1-24% and strong when the expression was ≥ 25. Among these studies, the current study is the smallest but it yielded a high level of TF expression (62%).

Several studies (Khorana *et al.* 2007, Nitori *et al.* 2005, Kakkar *et al.* 1999) have been used to support the role of TF (on the tissue) in the hypercoagulable condition of PC cases and certainly in the current study the procoagulant activity of immunohistochemistry TF positive cases was significantly higher than that of TF negative cases [the median and IQR PT was 33 (33-37.5) *vs.* median was 36.5 (34.7-40), p= 0.029] corroborating the effect of cancer related TF on the procoagulant status of the host. This result is supported by the finding of TF factor bearing MP of TF positive cases being higher than that of TF negative cases (p value was 0.06 which is tending towards significance).

The aim of this work, however, has been much more than just to demonstrate the procoagulant impact of tissue derived from the cancer. Following previous observation from co-investigators from our group (Maraveyas *et al.* 2010a) the serum of patients with PC, apart from having high levels of circulating TF, also demonstrated high levels of invasion activity. A correlation was found between high TF and high invasion activity, it was hypothesised that TF could be a major factor driving this process, inhibition of which may have a therapeutic effect on pancreatic cancer. Several studies postulated the relation between the TF expression on the tissue and the intratumour MVD as quantified by different endothelial markers in different studies e.g. CD34, CD31, von Willebrand factor or the correlation of TF expression with tumour angiogenic markers such as VEGF or VEGF 2. Khorana *et al.* (2007) for example noted that 80% of 122 cases that showed TF also expressed VEGF. Furthermore the authors in the latter study observed that the MVD of cases with high TF was higher than in those with low TF expression. Nakasaki *et al.*, (2002) reported that there was a correlation between TF expression and VEGF expression with MVD. Furthermore, TF expression was correlated with the immunohistochemical distribution of von Willebrand factor (Guan *et al.*, 2002). However none of these and other studies correlated tumour TF with the serum angiogenic activity.

In the current study, several lines of evidence are presented to show that TF expression on the tissue is significantly correlated with high angiogenic activity of sera and the tissue. These include the significant correlation between tumour TF expression on tissue and the angiogenic activity of the serum [total length and number of capillaries] when HDMEC was treated with the serum. Furthermore, out of 14 positive TF expressing PC cases, 13 of these (93%) showed positive expression of VEGF, and 79 % showed positive staining for VEGFR 2. The VEGFR 2 expression on positive TF cases was significantly higher than that of TF negative cases (p=0.01).

Furthermore, MVD of immunohistochemistry positive TF expressing cases was higher than that of negative TF expressing cases. However the relationship only tended towards significance (0.07), possibly due to a relatively small cohort.

Based on the result of the current study we provide support to the theory that tumour TF expression may be enhancing angiogenesis and the coagulable state either by its extracellular domains through clotting dependent mechanism (section 1.9.1) or through other indirect (clotting independent) mechanisms -such as induction of VEGF but that TF , the source of which based on the current study (Chapter 7 and 8) is most likely from the tumour, should be viewed as a major link between the hypercoagulable state and angiogenesis.

One of the hypotheses of the present study was that TF contributed to the serum induced invasiveness as measured by the ASPC1 assay optimised earlier in this work. Although in the previous part of this work it was shown that circulating TF MP changes before and after surgery was closely correlated to the changes in the invasion assay, in the smaller sample of cases submitted to immunohistochemistry analysis, no difference in serum invasion properties was found between positive TF expression cases (in tissue) compared to negative TF expression. If it had been possible to analyse the full cohort, some correlation may have been found. A further explanation may be the necessity to use serum for the invasion assay. In this work it has been shown that most TF antigen dependant activity relates to TF bearing MP, which remains in this state only in fresh plasma and therefore some of the effect on invasion could be from small amounts of soluble TF. The lack of correlation between histological TF and invasion raises the hypothesis that it is possible that the effects seen on the serum assay may be driven by other soluble factors not studied in this thesis.

Another marker is tumour VEGF, which has shown different results from one study to another in the literature, possibly depending on the variety methodology and the scoring systems used. In a series of 140 PC cases investigated using immunohistochemistry, 94% of cases showed positive VEGF expression and this expression was associated with MVD (Seo *et al.*, 2000). It has been reported in another study that in a series of 70 PC cases, 81% were VEGF positive. Niedergethmann *et al.* (2002) observed that VEGF expression is associated with MVD in PC. Ikeda *et al.*, (1999) reported that in a series of 40 PC, 27 (68%) were immunohistochemistry

positive. Furthermore, in a series of 124 cases of PC tissue studied by immunohistochemistry, the expression of VEGF was observed in 70 cases (56%) (Khorana *et al.*, 2005). Moreover, Khorana et al (2007) noted that in PC with high tissue TF, VEGF expression was higher than in cases with low TF expression (80% *vs.* 27%).

Among these studies, although the number of cases submitted in the current study is the smallest, high expression of VEGF (95%) was noted as well. Furthermore, in a total of 21 immunohistochemical VEGF positive cases, 13 expressed TF (62%), 13 cases expressed VEGFR 2 (62%), 16 (76%) expressed VEGFR 1 and 12 cases (57%) expressed EGFR. The VEGF expression associated with microvascular density as the mean intra-tumour microvascular density of cases of high VEGF expression cases (grade II and III) VEGF was significantly higher than that of low VEGF expression cases (grade I and negative expression). Furthermore, the total number of HDMEC capillaries (gel capillaries) from high VEGF expressing cases was significantly higher than in those with low expression. This result was in accordance with the results of other studies such as that of Niedergethmann *et al.* (2002) and Seo *et al.* (2000). Furthermore, it has been reported in section 6.3.5 of the current study that there are several angiogenic molecules driving the PC; one of these is VEGF. This result has been supported by the VEGF ELISA result as VEGF concentration of PC sera was significantly higher than that of control. Furthermore, post-operative VEGF concentration was significantly lower than pre-operative. Therefore we also conclude that VEGF plays an essential role in angiogenesis in PC. This result is also supported by the *in vitro* results in chapter 3 as the maximum AsPC1 invasion was achieved when PC sera were supplemented with extra TF and VEGF. Moreover, the maximal angiogenesis inhibition was achieved when HDMEC were treated with anti-VEGF, anti TF and suramin. Therefore, there is strong evidence that VEGF on PC tissue and soluble serum VEGF play an essential role in the aggressiveness and pathogenesis of PC and the combined targeting of these molecules might provide the basis for a synergistic antiangiogenic strategy for the treatment of PC.

Out of 21 malignant cases of group B that submitted to immunohistochemistry study, 12 (57%) were EGFR positive and 7 (33%) were EGF positive. For EGFR1, the result of this study is in agreement with the results of other studies such as Lozano *et al.* (2011) and Pryczynicz *et al.*(2008).

Lozano *et al.* (2011) noted that in a series of 50 PC cases, 52% of them were positive for EGFR1 1 2. Pryczynicz *et al.* (2008) noted that in a series of 40 PC cases, 50% of them were positive to EGFR1. In a series of 87 PC tissues, Yamanaka *et al.* (1993) noted that the expression of EGFR was 43% and the expression of EGF was 46 %. Furthermore Pryczynicz *et al.*(2008) noted that in a series of 40 PC cases, 50% of them were positive to EGF. EGF expression in this study was not in accordance with the results of other studies might be due to small number of cases involved in this study.

Furthermore, it has been noted in the current study that immunohistochemistry VEGFR 2 positive cases were significantly correlated with pro-coagulant activity (PT). This correlation might be a real correlation and therefore further study is indicated to prove that or it could be indirect correlation, because it has been noted that there was a significant correlation between the immunohistochemistry expression of TF and VEGFR2.

In summary, this study has shown that high TF and VEGF expression in PC tumours are associated with high new capillary formation and MVD. These results lead to the conclusion that these molecules and their pathways are both equally important in driving angiogenesis in pancreatic cancer and interrelated, raising the possibility of a dual targeting strategy of the angiogenesis pathway.

CHAPTER TEN: GENERAL DISCUSSION

PC has the poorest prognosis of all adenocarcinoma in humans (Jemal *et al.*, 2003) and it is one of the malignancies with the highest prevalence and incidence of TE in the western world (Khorana *et al.*, 2007, Chew, *et al.*, 2006). There is accumulating evidence of a correlation between TF activity expressed by MP and (TE) in cancers (Tilley *et al.*, 2008). The suggestion that the tumour may be the main source of TF bearing MP comes from the demonstration that the number and activity of these parameters reduce substantially after successful surgery to remove a tumour (Zwicker *et al.*, 2009, Tesselaar *et al.*, 2007) and the fact that there is a correlation between the level of TF bearing MP activity and incidence of thrombosis in cancer (Khorana *et al.*, 2007). Recently, Lomberk (2010) also showed that rapid tumour growth was positively correlated with increased angiogenesis of the tissue. Therefore, it has been suggested that the very poor prognosis of PC might be related to the enhanced procoagulant status of these patients strongly promoting the metastatic process (angiogenesis and cell invasion activity). However, the biological link(s) between PC cancer, haemostasis, cell invasion and angiogenesis remain unclear. These three indices may be driven by PC cells directly or may be related to the cancer's effect on the microenvironment of the host tissues or the macroenvironment of the host itself or a combination of one or more of these. Our hypothesis was that factors affecting the coagulation pathway (e.g. TF) directly attributed to the cancer also promote the mechanisms underpinning cancer invasion and metastasis and that the removal of the tumour should result in reversal of these abnormalities.

The initial experiments were designed to optimise the methodology that would be used in the clinical study. It was demonstrated that TF and VEGF increase AsPC1 invasion (section 3.4.4). Furthermore it was shown that the pro-coagulant activity of PC cells was correlated with cell invasion (section 3.2.5). The blocking of TF on PC cell lines or in the serum, using a polyclonal anti-TF antibody, also decreased cell invasion (section 3.2.5). In another series of experiments, it was shown that weight adjusted dalteparin is strongly associated with the reduction in the capacity of patient's sera to stimulate cancer cell invasion *in vitro*, a key phenotypic feature of cancer progression and metastasis. Therefor, it has been suggested that LMWH could ha a role prevention of tumour progression. Based on these two findings the suggestion was that the coagulation and cell invasion activities in PC are driven by the tumour and similarly the trigger of the thrombosis relates to the biological properties of the underlying malignancy. Furthermore, in

a complementary series of experiments it was shown that TF also enhanced angiogenesis (total length and number of capillaries on gel). The *in vitro* evidence of the efficacy of the combination of anti-VEGF, anti TF and suramin to inhibit angiogenesis completely (section 4.5), needs to be supported by clinical research as this could represent a novel form of treatment following additional experimentation.

In the clinical part of the study, 55 angiogenic markers were evaluated in the serum of a small series of PC cases pre- and post-operatively using an angiogenesis array kit. This revealed that angiopoietin-1, PDGF-AA, PDGF-AB/PDGF-BB and VEGF were significantly decreased post-operatively. The result was supported by a significant decrease of VEGF concentrations of a cohort of 29 PC cases post-operatively when analysed by ELISA. These data suggest that the processes are cancer specific and that pancreatectomy decreases the ratio of pro- to anti-angiogenic factors and shifts the angiogenic switch towards "off". The validity of this hypothesis is further strengthened by the significant impact of pancreatectomy on the assays of invasion, angiogenesis, TF bearing MP, procoagulant activity and CD 14 bearing MP. It was noted that the maximum serum chemotactic activity (AsPC1 cells motility) and HDMEC angiogenesis (total length and total number) was induced by the baseline serum of the patients with cancer (group B) and was significantly greater than that of the control groups. Following resection there was a significant decrease in the induction of AsPC1 cell motility and HDMEC capillary formation by the post-operative sera in the cancer patients (Group C). There was no such effect of resection either in the group III patients (precancerous pancreatic cases, control group) or in the group IV patients (chronic cholecystitis, control group).

Flow cytometry was used to evaluate TF bearing MP (which might has a role in the pro-coagulant, cell invasion and angiogenic activities) and CD 14 bearing MP (which might has a role in cell invasion activity). A one stage PT assay was used to evaluate the procoagulant activity. The work presented here shows that there was significant increase in the number of TF bearing MP, number of CD 14 bearing MP and procoagulant activity in PC cases compared with the control group III (Precancerous pancreatic lesions). Furthermore there was significant decrease of TF bearing MP, procoagulant activity and CD 14 bearing MP post-operatively. This novel finding supports the author's hypothesis that cell invasion, angiogenesis and haemostatic condition in PC patients are cancer specific, driven by the cancer and that removal of the cancer reverses them towards normal. Although it seems that these three indices have different

signalling pathways, the current study suggests that there is a clinical correlation between them. The potential biological links between the coagulation pathways and molecules driving the cancer process have been discussed in the introduction and published recently (Echrish *et al.* 2011). Furthermore, it seems to be the tumour enhance incidence of the thrombosis and the hypercoagulability drives tumour progression.

In the few cases studied, ELISA did not show a significant difference in MP activity from that of the control. Furthermore, TF (measured by ELISA) of PC cases in the current study did not differ significantly from that of control group III and IV, and this indicates that not only TF activity but also TF antigen may also be affected by frozen storage. MP activity is generally considered to be a good indicator of procoagulant activity, however in this thesis the MP activity ELISA was not used, as it emerged during this work that this test was unreliable. The other possibility is that ELISA is an insensitive technique to measure TF antigen and MP activity. As mentioned previously in section 8.6.1, this suggestion was supported recently by data presented at the 5th ISTH 2011, casting doubt on the discriminating ability of the ELISA technique, and it was reported that flow cytometry is both a more sensitive and precise method (conf reference 2 and 3).

Several studies postulated the relationship between the TF expression on the tissue and the intra-tumour MVD representing endothelial markers (Khorana *et al.*, 2007 , Guan *et al.*, 2002, Nakasaki *et al.*, 2002). However none of the published studies have correlated the tumour TF with the serum angiogenic activity or correlated tumour TF expression with all three biological indices (haemostasis, angiogenesis and cell invasion). In the current study, 62% of group B (cancer patients) showed positive TF expression which was positively correlated with the procoagulant activity; furthermore the number of TF bearing MP of TF positive cases was higher than negative cases (p= 0.06). This result tended towards significance and is hypothesised that these would become significant if further cases are studied. Moreover there were several lines of evidence to show that TF expression on the tissue is significantly correlated with high angiogenic activity. These include the significant correlation between tumour TF expression and the total length and number of capillaries when HDMEC were treated with serum. Furthermore, the VEGFR 2 expression on positive TF cases was significantly higher than that of TF negative cases (p=0.01), and the MVD of positive TF expression cases was higher than that of negative TF expression cases; the latter relationship was tendeding towards significance (p= 0.07).

Furthermore, the current study revealed that TF expression by tissue was significantly associated with angiogenesis and procoagulant activity measured in serum and plasma respectively. Therefore the high PC procoagulant activity and angiogenesis activity described previously, could in large part be due to TF expression on the tumour with TF potentially representing the link between them.

It has been reported previously that tumour VEGF expression was significantly associated with MVD (Khorana *et al.,* 2007, Khorana *et al.*, 2005, Niedergethmann *et al.*, 2002, Seo *et al.*, 2000, Ikeda *et al.*, 1999). The current study revealed that VEGF was expressed in 20 malignant cases out of 21 (95%). In a total of 21 immunohistochemical VEGF positive cases, 62% expressed TF, 62% expressed VEGFR 2. The VEGF expression associated with MVD of cases of high VEGF expression cases (grade II and III) VEGF was significantly higher than that of low VEGF expression cases (grade I and negative expression). Furthermore, the total number of HDMEC capillaries (gel capillaries) of high VEGF expression cases was significantly higher than those with low expression. To summarise the results: haemostasis, cell invasion, angiogenesis are cancer specific and the removal of the PC reverses them toward the normal. These indices are significantly associated with each other and the link between them could be in part through the TF on the tumour; both TF and VEGF seem to be correlated with angiogenesis.

As most soluble TF is MP dependent, targeting of MP might play an important role in the control of the tumour. It has been reported that some conventional treatments (chemotherapy) can inhibit MP formation; i.e. both taxol and other vinca alkaloids, conventional chemotherapeutic medicaments not only inhibit cell division, but additionally will decrease MP release (Iero *et al.*, 2008, Wright 2008, Cheng *et al.*, 2005, Jordens *et al.*, 2005). Interestingly these agents have been classified as non specific anti-angiogenic molecules (Kerbel and Kamen 2004). Furthermore, the correlation between TF-MP and the procoagulant state have led to the commencement of a randomised trial. In this trial the usefulness of prophylactic enoxaparin (a type of low molecular weight heparin) was investigated in patients with pancreatic, lung, and colorectal cancer with elevated plasma TF microparticles. Patients with high levels of circulating MP activity are randomised to anti-coagulation *vs.* no anti-coagulation (web reference 8). Moreover, it has been postulated that recombinant nematode (*Ancylostoma caninum*) anticoagulant protein c2 (rNAPc2) is a specific inhibitor of tissue factor TF/FVIIa complex with novel anti-thrombotic activity (Zhao *et al.* 2009). In addition to its anticoagulant activity it also

inhibits angiogenesis, primary and metastatic tumour growth in mice (Hembrough *et al.*, 2003). Furthermore, in a randomised phase II trial, chemotherapy with and without PCI-27483 (FVIIa/TF inhibitor, 1.2 mg/kg two times daily) was investigated. The study is ongoing (Ramanathan *et al.* 2011).

Work in this thesis has strengthened the concept of TF having an important role in angiogenesis together with VEGF. There are numerous targeting strategies of the VEGF pathway but the possibility is raised that dual anti-TF and anti VEGF strategies, in combination with chemotherapy, will have a more potent role in the treatment of PC; this hypothesis would need to be tested in a clinical trial. Furthermore the results support the suggestion that the tumour might increase the hypercoagulability and the hypercoagulabilty might increase the tumour progression.

Future Work

The microarray work of the current study has demonstrated further molecules that may be involved in PC angiogenesis, such as angiopoitin-1, PDGF-AA, PDGF-AB/PDGF-BB and VEGF. One strategy would be to elucidate whether invasion and angiogenesis of the serum is prevented by blocking of these soluble factors using the *in vitro* experiments described herein. Furthermore, although the result of the current study strengthened the data on the correlation of TF on angiogenesis and the VEGF/VEGFR pathway, it raises the idea that there may other molecules that also effect invasion. For this reason there may be more work to be done on other factors such as Ephrin, E-cadherin, IL-6 and IL-8 all of whom have shown to have potential activity in cell invasion and angiogenesis. Following further testing *in vitro* it would then necessary to move to *in vivo* testing having correlated the effects of the combination of tumour attributes (invasion, angiogenesis and promotion of procoagulant activity) with specific markers. These can be targeted specifically *in vivo*.

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Appendix-A

Patient Information Sheet for Pancreatic Cancer Patients Undergoing Whipple's operation

A Study of the Effect of Resection of Localised Pancreatic Cancer on Tissue-Factor Promoted Pathways of Thrombosis and Angiogenesis Markers

Introduction

You have been invited to take part in a research study. Before you decide, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Consumers for Ethics in Research (CERES) has published a leaflet called Medical Research and You. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy can be obtained from a doctor or nurse inviting you to take part or from CERES, PO Box 1365, London N16 OBW.

What is the Purpose of the Trial?

You have been diagnosed as having cancer of the pancreas, which is localised and can hopefully be removed. If this is successful you may be offered more treatment called chemotherapy. This will depend on the stage of the tumour and will only be accurately known after the pathologist has had a chance to study the tumour under the microscope. Chemotherapy may be offered after surgery. The aim of chemotherapy is to improve symptoms and to prevent or delay further complications of the cancer in your gut such as blockages. Apart from the common symptoms of blockage of the gut and the bile system that this cancer causes, and which you may be experiencing, it can also have effects on the blood. We and others have found that it increases the capacity of the blood to clot and to provide nutrients for cancer blood vessels. All these effects promote the growth of secondary cancer and are collectively called 'cancer promoting factors'. To prove this we have designed a clinical study of these 'cancer promoting effects' before and after surgery and during and after chemotherapy. We are studying two major groups of pancreatic cancer patients, those in which the surgeon can remove the tumour and those in which he cannot.

What will happen to me During the Trial?

We would want to study the concentration of these 'cancer promoting factors' in your blood before and after surgery, and during and after chemotherapy if it is offered to you. We therefore would like to ask your permission for some of your blood and for a small portion of the tumour to be used for research purposes.

Before the operation, blood will be drawn for regular blood tests and at this point we would like to draw some more (about two-three tablespoons) for the study. The surgeon will proceed with the operation as planned.

About 4-6 weeks after the operation, by which time we think that healing is complete, and during a regular follow-up visit, we would like a further sample of blood of similar quantity as before the research. If you are not offered chemotherapy this will be the end of the study for you.

If you are offered chemotherapy a further two or three blood tests will be taken, depending how you are responding to treatment. One blood test will be taken during chemotherapy, at around 8-12 weeks when you would also be due a CT scan to assess the response of the cancer. If your doctor decides to continue for a further 12 weeks a further blood test will be taken at 24 weeks of treatment at which point usually a break in the chemotherapy will follow. About eight weeks into the break a further blood test will be required. At this point, your involvement with the study finishes. In the case, therefore, of you going on to chemotherapy, a total of five blood samples are required.

Are There any Side Effects Associated with These Treatments?

There should be no extra risks or side effects from the procedures as they will be part of the regularly planned tests that your doctors need before the surgery and to monitor your progress after the surgery and during and after chemotherapy.

Are There any Other Extra Procedures?

No extra procedures are planned. The tissue will be taken from the tumour that has already been removed.

What are the Possible Benefits of Taking Part?

No direct benefits to you are expected from this research. The information we get from this study may help us to improve the future treatment of patients with pancreatic cancer.

What if Something Goes Wrong?

If you were harmed by taking part in this study, no special compensation arrangements exist. However, if you were harmed due to someone's negligence, then you would have grounds for legal action. Regardless of this, if you have any cause to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

What if New Information Becomes Available?

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Will my Taking Part in This Study be Kept Confidential?

All information which is collected about you during the course of this research will be kept strictly confidential. With your permission we will inform your GP of your participation in the study. Other than this, any information about you that leaves the hospital will have your name and address removed so you cannot be identified from it.

Who is Organising and Funding the Research?

This is a trial sponsored and funded by the NHS (HEYNHST).

What if I do not wish to Take Part or Change my Mind?

You do not have to take part in this trial if you do not wish to do so. If you decide to take part you are free to withdraw at any time. In either case you do not have to give a reason for your decision and this will have no influence over your future medical care. If you do decide to take part in this study you will be asked to sign a consent form.

We would like to thank you for your attention so far and hopefully for your participation in this study. Please feel free to ask your doctor any questions about the study or about any of the treatment described above.

Please contact:

Dr Anthony Maraveyas - Princes Royal Hospital.

Telephone Number: 01482 676703.

Dr Hussein Echrish – Biomedical Science Department, Hull University.

Mobile Number: 07592271016.

Please feel free to ask your doctors any questions about the study or about any of the treatments described above.

Note: There is a similar Patient Information Sheet for Pancreatic Cancer Patients Undergoing Bypass Operation with some modification in the paragraph on purpose of the trial (You have been diagnosed as having cancer of the pancreas, which is not localised and cannot be removed. Your surgeon therefore has offered you bypass surgery to relieve the symptoms. Chemotherapy may be offered after surgery. The aim of chemotherapy is to improve symptoms and to prevent or delay further complications of the cancer in your gut such as blockages. Apart from the common symptoms of blockage of the gut and the bile system that this cancer causes, and which you may be experiencing, it can also have effects on the blood. We and others have found that it increases the capacity of the blood to clot and to provide nutrients for cancer blood vessels. All these effects promote the growth of secondary cancer and are collectively called 'cancer promoting factors'. To prove this we have designed a clinical study of these 'cancer promoting effects' before and after surgery and during and after chemotherapy. We are studying two major groups of pancreatic cancer patients, those in which the surgeon can remove the tumour and those in whom he cannot).

There is a similar Patient Information Sheet for Patients Undergoing Laproscopic-Cholecystectomy with some modification in the introductory paragraph and the purpose of the trial. Introductory paragraph was (We would like to invite you to take part in a research study as a patient volunteer who does not suffer from cancer but has inflammation in the gall bladder or bile duct. Before you decide, it would be much appreciated if you could read the following information to understand why the research is being done and what it involves. We are happy to explain anything that you do not understand. Please take as much time as you need to read the information and make a decision).

what is the purpose of the trial (The actual study is focused on a type of cancer of the gut called pancreatic cancer and we are studying changes in the patient's blood that may cause the cancer to spread or to cause clots in the veins and the other vessels. We are trying to evaluate these changes in patients with pancreatic cancer before and after surgery and before and after chemotherapy. In order to establish the significance of these measurements it is essential that we also study patients that do not suffer from cancer but suffer from inflammation of the same organs that can be affected by this cancer and also individuals that are healthy and do not suffer from any inflammation. This will allow us to compare the values and trends of the blood tests we are studying).

Appendix-B

Consent Form for Patients Undergoing Whipple's operation

Title of project: A study of the effect of resection of localised pancreatic cancer on tissue–factor promoted pathways of thrombosis and angiogenesis markers.

Name of the researcher: Hussein Echrish

1. I confirm that I have read and understood the information sheet (dated.....version .....) for the above study. I have had the opportunity to consider the information, ask questions and have these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that if I decide later to withdraw from the study, the blood samples taken from me and any analysis results will be destroyed and not included in the study.

4. I understand that relevant sections of my medical notes and data will be collected during the study from regulatory authorities or from the NHS Trust, where they are relevant to my taking part in this research. I give permission for these individuals to have access to my record.

5. I agree to take part in the above study and give part of my tissue which is already resected by the surgeon.

6. I agree to take part in the above study and give my blood for the purpose of this research.

Signature of participant: ……..

Full name of participant: ……..

Date: ……..

Signature of person taking data consent: ……

Full name: ……..

Date: ……..

Note: There is a similar consent form for patients Undergoing By-Pass Surgery, for patients Undergoing Laproscopic Cholecystectomy and for patients Undergoing Laproscopic Cholecystectomy.

Appendix-C

Data Collection Form

Patient Initials ☐ Trial Number ☐ Hospital Number ☐

Pancreatectomy and Tissue Factor
Data Collection Form

Pre-Operative

Gender: M ☐ F ☐ Date of Birth: ☐☐☐

Date of Consent: ☐☐☐ (Please attach a copy of consent form)

Present Clinical History:

Cardiac System

Hypertension Y ☐ N ☐

Myocardial Infarct Y ☐ N ☐

If yes, give Date of Infarct

DVT/VTE Y ☐ N ☐

Date(s) of VTE ☐ ☐

Type of VTE ☐

Recurrent VTE Y ☐ N ☐

Alimentary System:

History of diabetes mellitus Y ☐ N ☐

History of Jaundice Y ☐ N ☐

Alcohol consumption (per week) .....
Smoking (per day) .....

Are there any signs of infection? Y ☐ N ☐

Is the patient receiving thromboprophylaxis? Y ☐ N ☐

Is answer Yes; What type? _____

When stopped? ☐

Patient Initials | Trial Number | Hospital Number

Concurrent Medication Including Anti-coagulant and Dose:

Haematological Test:

Date:

Hb:

WBC:

Platelet:

Neutrophis:

Biochemical Test:

Date:

CRP:

Bilirubin:

ALT:

Alkaline Phosphatase:

Total Serum Protein:

Albumin:

Surgery:

Type of Surgery:

Date of Surgery:

Date of Venesections:

Pre-Surgery:

Patient Initials | Trial Number | Hospital Number

Pathological Staging and Margins (for the patients that have resected tumour)

Histopathology:

Specimen made available: Y ☐ N ☐

Date:

Assays done: (please detail with dates)

BB-TF:

Angiogenesis:

Invasion:

TFPI:

MP-FACS:

MP-ELISA:

Investigator's Signature: _____ Date: _____