Thrombosis in Colorectal Cancer

A thesis submitted to the University of Manchester for the degree of Doctor of Medicine (MD) in the Faculty of Medical and Human Sciences

2016

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School of Medicine
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>AJCC</td>
<td>American joint committee on cancer</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of co-variance</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
</tr>
<tr>
<td>CFE</td>
<td>Colonosphere forming efficiency</td>
</tr>
<tr>
<td>CHAMPion</td>
<td>Cancer-induced Hypercoagulability As a Marker of Prognosis</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
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<td>CTPA</td>
<td>Computed tomography pulmonary angiogram</td>
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<td>DD</td>
<td>D-dimer</td>
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<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
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<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
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<td>DVT</td>
<td>Deep vein thrombosis</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemoluminescence</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>EPCR</td>
<td>Electronic PCR</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>Foetal calf serum</td>
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<td>FDP</td>
<td>Fibrin-degradation products</td>
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<td>GEE</td>
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<td>H&amp;E</td>
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<td>HCL</td>
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<td>HIT</td>
<td>Heparin induced thrombocytopenia</td>
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<tr>
<td>HR</td>
<td>Hazard Ratio</td>
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<td>LGR5</td>
<td>Leucine-rich repeat-coupling G-protein coupled receptor 5</td>
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<td>LMWH</td>
<td>Low molecular weight heparin</td>
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<tr>
<td>LVI</td>
<td>Lymphovascular invasion</td>
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<td>NaCL</td>
<td>Sodium chloride</td>
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<tr>
<td>NAOC</td>
<td>New oral anticoagulant</td>
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<td>NICE</td>
<td>National institute of clinical excellence</td>
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<td>Phosphate buffered saline</td>
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<td>Pulmonary embolism</td>
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<td>PI</td>
<td>Plasma</td>
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<tr>
<td>PS</td>
<td>Penicillin and streptomycin</td>
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<td>sGPV</td>
<td>Soluble glycoprotein V</td>
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<td>siRNA</td>
<td>Synthetic RNA</td>
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<td>TF</td>
<td>Tissue factor</td>
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<td>TMA</td>
<td>Tissue micro-array</td>
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<td>TME</td>
<td>Total mesenteric excision</td>
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<td>TS</td>
<td>Thymidalate synthase</td>
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<td>UK</td>
<td>United Kingdom</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>VQ</td>
<td>Ventilation/perfusion scan</td>
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Abstract

Hamish Clouston. The University of Manchester. Doctor of Medicine (MD). Thrombosis in colorectal cancer. September 2015

Thrombosis and colorectal cancer have a bi-directional relationship. The presence of a colorectal malignancy results in an increased risk of developing a thrombosis and the presence of a thrombosis results in a worse cancer prognosis. The physiology causing this is at present unclear but it is proposed that proteins from the tissue factor (TF) pathway may be the instigator of this bi-directional relationship.

The in-vitro studies have shown that in colorectal cancer TF impairs that action of colorectal cancer stem cells as demonstrated by reduced cancer sphere formation and also lower expression of the stem cell marker ALDH. The ability for a colorectal cell to avoid anoikis is impaired by a reduced TF level. Proliferation is affected by the level of expression of TF with a significant increase in proliferation with additional expression of TF. The increase in proliferation is further increased by the presence of TF’s ligand factor VIIa. Paradoxically reduced expression of TF also increases colorectal cancer expression. The ERK1/2 pathway offers a possible method by which TF and factor VIIa may exert their proliferative effects.

In the prospective clinical cohort study (CHAMPion) abnormal expression of TF pathway proteins (TF, PAR1, PAR2 and thrombin) by both malignant epithelial and cancer associated stromal cells has been demonstrated. The stromal expression was independent of the epithelial expression and was only in stroma in close contact (0.1mm) with epithelial cells suggesting that the TF pathway proteins may have a role in stromal/epithelial communication. There was no link between the expression of TF pathway proteins and clinicopathological markers of a poor prognosis. The plasma expression of markers of TF pathway activation did not demonstrate any role as a biomarker for colorectal cancer or prognosis.

The CHAMPion study has demonstrated that 7% of patients undergoing surgery for colorectal cancer have asymptomatic pre-operative DVTs present. A further 6% who were DVT free pre-operatively developed a DVT in the peri-operative period despite receiving venous thromboprophylaxis in line with current national guidelines. Pre-operative d-dimer may have the potential to identify those patients at risk of a post-operative VTE.

This thesis establishes the role that TF has in promoting proliferation and anoikis resistance. It also confirms the abnormal expression of TF pathway proteins by colorectal cancer epithelial cells and for the first time demonstrates abnormal expression by the cancer associated stroma. The interaction between the stroma and epithelial cells, combined with the cellular effects of TF suggests that targeting this interaction may have a therapeutic role. The incidence of DVTs pre-operatively suggests that screening patients for the asymptomatic presence of a DVT may have an impact on their clinical outcome. The development of DVTs despite prophylaxis suggests that the level of anticoagulation is insufficient and current guidelines need to be revisited.
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Acknowledgements

I would like to acknowledge and give my great thanks to my supervisory team for all the hard work they have put into this MD. Cliona Kirwan for all her corrections and attempts to try and make me write less like a Sun reporter and more like a scientist. Sarah Duff for educating me on the nuances on grammar and also supporting me on my clinical commitments that were undertaken alongside this research. Rebecca Lamb for teaching me and supporting me in the Laboratory.

I would also like to thank the patients for participating in the CHAMPion study, indeed without their selflessness none of this would have been possible. I would also like to thank the research nurses at all the sites where CHAMPion were recruiting as well as the Consultant Surgeons for allowing me to recruit their patients.

I would like to thank everyone from the Cancer Biology Group at the Patterson Institute for all their support and knowledge that guided me on my laboratory research. I would especially like to mention Hud Shaker for his guidance in the laboratory and with CHAMPion, John Castle for his help with the ELISAs, Hannah Gregson for her help with the IHC and Anna Davenport for teaching me how to score TMAs and also co-scoring them with me.

I would like to thank my family for all the help and support that they have offered me over the years. I would like to thank my parents Margaret and Moray for a lifetime of support and backing. I would also like to give great thanks to Kirsty, my wife, who has been there to keep me going, provide a listening ear when things are not working and celebrate when things go well.
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Presentations arising from this project.

International oral presentations


“Expression of the tissue factor thrombin pathway is upregulated in the stroma and epithelium of colorectal cancer”. HW Clouston, A Davenport, S Duff, CC Kirwan ASGBI Manchester June 2015

“Tissue factor promotes colorectal cancer cell proliferation”. HW Clouston, R Lamb, S Duff, CC Kirwan ASGBI. Manchester June 2015


International poster presentations

“Locally advanced colorectal cancers result in a hypercoagulable state: implications for extended post-operative prophylaxis”. HW Clouston, H Shaker, S Duff, CC Kirwan. ICHTIC. Bergamo May 2014
Abstracts submitted for presentation (awaiting response)


The effect of tissue factor expression on colorectal cancer cell proliferation. HW Clouston, R Lamb, S Duff, CC Kirwan. Submitted to ICHTHIC 2016

Expression of proteins of the tissue factor thrombin pathway is upregulated in the stroma and epithelium of colorectal cancer. HW Clouston, H Shaker, A Davenport, S Duff, CC Kirwan. Submitted to ICHTHIC 2016

Patient, tumour and operative factors influencing perioperative hypercoagulability in colorectal cancer. HW Clouston, H Shaker, S Duff, CC Kirwan. Submitted to ICHTHIC 2016

Effect of tissue factor expression by colorectal cancer on cancer stem cell activity. HW Clouston, R Lamb, S Duff, CC Kirwan. Submitted to ICHTHIC 2016

Publications arising from this project

Published abstracts


“Locally advanced colorectal cancers result in a hypercoagulable state: implications for extended post-operative prophylaxis”. HW Clouston, H Shaker, S Duff, CC Kirwan. Colorectal Disease Volume 16, supplement 2


“Expression of the tissue factor thrombin pathway is upregulated in the stroma and epithelium of colorectal cancer”. HW Clouston, A Davenport, S Duff, CC Kirwan. Accepted to BJS awaiting publication.

“Tissue factor promotes colorectal cancer cell proliferation”. HW Clouston, R Lamb, S Duff, CC Kirwan ASGBI. Accepted to BJS awaiting publication.
“Pre-operative and post-operative incidence of Deep Vein Thrombosis in colorectal cancer surgery”. HW Clouston, S Duff, CC Kirwan. Accepted to BJS awaiting publication.

“Tissue factor expression in colorectal cancer cell lines correlates inversely with CSC activity”. HW Clouston, R Lamb, S Duff, CC Kirwan ASGBI. Accepted to BJS awaiting publication.

Abstracts submitted for publication.


The effect of tissue factor expression on colorectal cancer cell proliferation. HW Clouston, R Lamb, S Duff, CC Kirwan. Submitted journal of Thrombosis Research

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1 Introduction

1.1 Colorectal cancer

1.1.1 Epidemiology
Colorectal cancer is the fourth most common cancer in the United Kingdom (UK) with 41,581 new diagnoses in 2011. Worldwide, it is the third most common cancer after lung and breast with an estimated 1.36 million cases diagnosed annually\(^1\). In the UK, colorectal cancer is responsible for 10% of cancer deaths (second only to lung cancer) resulting in 16,187 deaths in 2012\(^2\). The incidence of colorectal cancer has increased 6% over the last decade.

In women, colorectal cancer is the third most common cancer with 18,400 cases diagnosed in 2011 in the UK. In men it is the third most common cancer with 23,200 cases diagnosed in 2011. Approximately 95% of cases of colorectal cancer are diagnosed in people aged 50 and over.

Colorectal cancer occurs throughout the colon and rectum. The distribution of cancers is not uniform with:

- 30% in the right colon
- 10% in the transverse colon
- 15% in the descending colon
- 25% in the sigmoid colon
- 20% in the rectum\(^3\).

1.1.2 The adenoma-carcinoma sequence
Most colorectal malignancies develop following the pathway of adenoma to carcinoma as originally described by Jackman and Mayo in 1951\(^4\). During this process, there is progressive stepwise accumulation of genetic mutations including activation of oncogenes and inactivation of tumour suppressor genes that ultimately result in cancer. These mutations include, among others, the mutation of APC (adenomatous polyposis coli), K-ras and P53 genes\(^5\).

For the purposes of this project the genetic mutations that have relevance are the APC and K-ras mutations.

APC is a multifunctional protein encoded by the tumour-suppressing APC gene on chromosome 5\(^6\). The APC protein interacts with other proteins, in particular β-catenin whose levels it controls. β-catenin is an important cellular protein that
regulates coordination of cell-cell adhesion, gene transcription and is involved in apoptosis. Wild-type APC protein forms a complex with β-catenin which leads to its degradation, the mutated APC protein is unable to form this complex leading to increased β-catenin levels and inhibition of cellular apoptosis. Mutation of the APC gene is strongly linked to colorectal cancer and is the key genetic mutation seen in the inherited condition familial adenomatous polyposis (FAP).

The K-ras gene located on chromosome 12 encodes for the protein p21RAS which is involved in the transduction of mitotic signals and is activated transiently by extracellular stimuli such as growth factors, and cytokines. The control of the activation is via the effector domain which in the mutant gene is disrupted resulting in an activated protein. The K-ras protein controls proliferation and therefore as the mutated gene is abnormally activated this results in unregulated differentiation and proliferation.

1.1.3 Staging
Survival in colorectal cancer at 1 year is 77% for men and 74% for women. The five year survival is 59% and 58% respectively. The staging of patients pre-treatment allows selection of patients for appropriate surgical, neo-adjuvant and palliative treatments. Initial staging involves clinical, histological and radiological techniques. Final pathological staging of disease following complete resection allows prediction of prognosis and selection of patients for adjuvant therapy. Particular pathological characteristics of importance include:

- Stage of the tumour dictated by:
  - Depth of tumour invasion into/through the bowel wall (T stage)
  - Lymph node involvement (N stage)
  - Presence of metastases (M stage)
- Lymphovascular involvement
- Histological differentiation.

Historically, the pathological stage of a colorectal tumour was based on the Dukes’ classification first published in 1932. This has now been replaced in routine clinical practice by the use of the American Joint Committee on Cancer (AJCC) TNM classification. The TNM stage can be determined pre-operatively by cross sectional imaging. Postoperatively TNM is determined histologically using the suffix ‘p’, or ‘yp’ if following neo-adjuvant therapy. The T (tumour) stage is the histological level of tumour penetration through the bowel wall, based on involvement of mucosa, muscularis, serosa or surrounding structures (Figure 1.1). Nodal status (N)
describes lymph node involvement (Table 1.1). Metastatic (M) stage describes the presence (M1) or absence (M0) of metastases. The TNM stage predicts survival (Table 1.2).
Figure 1.1 TNM staging- T stage

- **T1**: Tumour limited to mucosa and submucosa
- **T2**: Tumour extension into but not through muscularis propria
- **T3**: Tumour present on serosa
- **T4**: Tumour invading into surrounding structures

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<td>4 or more local lymph nodes involved</td>
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Table 1.1 TNM staging- N stage
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*Table 1.2 Survival in colorectal cancer by TNM stage. Based on figure from Demols et al¹²*
1.1.3.1 Lymphovascular invasion
Microscopic lymphovascular invasion (LVI) is not included separately in the staging systems described above. However, LVI is associated with poor prognosis, and even without lymph node involvement, results in increased risk of death and recurrence. In patients with TNM stage II (tumour growth through serosa but nodes not involved), the hazard ratio for death and recurrence is increased in the presence of LVI in a large retrospective cohort study (HR 3.11 for death p=0.002 and 3.27 for recurrence p=0.0003)\(^{14}\), which is consistent with other similar studies\(^ {15,16}\).

1.1.3.2 Histological differentiation.
The degree of differentiation of a colorectal cancer is an independent prognostic indicator with more poorly differentiated cancers being associated with a worse prognosis\(^ {17}\). Mucinous tumours are also associated with a poor prognosis.

1.1.3.3 Impact of clinical characteristics
The presence of obstruction or perforation of a tumour both have a negative impact on both the short time survival (with a perioperative mortality of 31%) and 5 year cancer free survival\(^ {18}\). In addition inadvertent perforation of the bowel or tumour during surgery has major implications on disease free survival. In a study of 174 patients with curative resections where spillage occurred distant to the tumour, the five year survival was 29%. In 67 patients where the tumour was perforated, the 5 year survival was 14% in patients with colon cancer and 9.3% in rectal cancer\(^ {19}\).

1.1.4 Cellular impacts on cancer outcome
In addition to the macroscopic tumour, factors at a cellular level affect the prognosis of a tumour. For the purposes of this project the main cellular effects are:

- The presence of cancer stem cells
- The tumour micro-environment.

1.1.5 Cancer stem cells
In normal tissues there are cells which retain the stem cell ability to self-renew and produced differentiated daughter cells. These differentiated daughter cells make up the majority of cells in an organ. These cells possess characteristic surface protein markers that allow their identification and are specific to tissue from which the stem cell originates. Stem cells are involved in tissue repair and replacement. In normal colorectal tissue, stem cells are involved in the constant replacement of the epithelial lining of the bowel\(^ {20}\). They are located in the base of the crypts and produce differentiated epithelial cells that travel up the crypt to replace lost epithelial
cells. In normal colorectal tissue stem cells express the stem cell marker aldehyde dehydrogenase (ALDH)\textsuperscript{20}.

The use of surface markers that are indicative of stem cell activity has led to the identification of stem cells in cancer (cancer stem cells). The presence of cancer stem cells were first described in acute myeloid leukaemia (AML) in 1997 by Bonnet et al\textsuperscript{21}. They found that the majority of AML cells expressed the surface markers CD34+/CD38+. Transplantation of CD34+/CD38+ cells into immunodeficient mice did not cause the development of human AML. However, transplantation of CD34+/CD38- AML cells resulted in development of human AML, suggesting that this small subgroup of cells had the unique ability to develop the leukaemic state.

Equivalent cells have since been found in other cancers including breast\textsuperscript{22,23}, brain\textsuperscript{24}, prostate\textsuperscript{25,26}, lung\textsuperscript{27}, melanoma\textsuperscript{28,29} pancreatic\textsuperscript{30,31} and liver\textsuperscript{32}, with specific cell surface marker indicative of their stem cell status.

Like stem cells in normal tissue, cancer stem cells can self-renew and produce differentiated daughter cells. It is widely held that cancer stem cells are important in treatment resistance and also are unique in their ability to form tumours (tumorogenecity) when introduced into an in-vivo host\textsuperscript{33}. Evidence for their role in treatment resistance in colorectal cancer comes from the finding that these cancer stem cells are selected for by treatment with chemotherapy. In colorectal cancer when mice with xenogeneic tumours are treated with chemotherapy agents, residual tumours have an increased expression of stem cell markers and possess the ability to regenerate tumours\textsuperscript{34}. The tumorigenicity of cancer stem cells can be seen in the ability of cells that possess the characteristic stem cell markers to develop tumours whilst those without cannot. In colorectal cancer, unlike in normal tissues, cancer stem cells are distributed through the tumour\textsuperscript{20}.

1.1.6 **Identification of cancer stem cells**

Cancer stem cells can be identified by:

In vitro:

- Possession of characteristic surface markers
- The ability to self-renew
- The ability to produce differentiated daughter cells
- The ability to resist anoikis (apoptosis in non-adherent conditions).
In vivo:

- Possession of characteristic surface markers
- The ability to self-renew
- The ability to induce tumours.

1.1.6.1 Colorectal cancer stem cell markers

Various surface markers have been identified that appear to be associated with cancer stem cell characteristics in colorectal cells. These stem cell markers are believed to select for a population containing a higher ratio of cancer stem cells. LGR5 is used for immunohistochemistry (IHC). For in-vitro studies (as have been carried out in this thesis) the three most promising markers of colorectal cancer stem cells are: CD133, CD44 and ALDH.

**CD133**

CD133 is a possible stem cell marker in a number of cancers (including brain, liver, pancreas, kidney and lung). Its expression has been demonstrated in colorectal cancer specimens but with variable expression between colorectal cell lines\(^{35,36}\). O’Brian et al\(^{37}\), Ricci-Vitiani et al\(^{38}\) and Todaro et al\(^{39}\) showed that CD133 +ve colorectal cancer cells had the ability to form tumours in immunocompromised mice, whereas CD133 negative (–ve) cells (that formed the majority of the original tumour) did not possess this ability. However, Shmelkov et al\(^{40}\) have cast doubt on the use of CD133 as a stem cell marker as it was expressed on both normal colorectal differentiated epithelial tissue and cancer cells. In their study, both CD133+ve and CD133-ve cells were able to initiate tumours in immunocompromised mice. Kawamoto et al\(^{41}\) reported that although CD133+ve and CD133-ve cells both had the ability to form tumours in immunocompromised mice, the CD133+ve produced larger tumours and CD133+ve cells alone possessed stem cell characteristics, including irradiation and chemotherapy resistance. These findings suggest that the cell surface marker CD133+ve may have a role in detecting colorectal cancer stem cells but this needs to be interpreted with caution. A population of CD133+ve cells is likely to have a higher concentration of stem cells than a CD133 –ve population.

**CD44+**

CD44+ve cells show enhanced tumour forming ability compared to CD44-ve cells in vivo\(^{42-44}\) and demonstrate self-renewal capacity\(^{44}\). As few as 100 CD44+ve colorectal cancer cells are able to form a tumour in a mouse demonstrating
tumorigenicity. CD44+ve and CD133+ve were not co-localised in histological specimens and knockdown of CD44 but not CD133 inhibited both tumour sphere formation (a characteristic of stem cells) and in-vivo tumour growth. A study examining the ability of CD44 to isolate colorectal cancer stem cells from cancer cell lines found that the expression of CD44 was not able to reliably isolate the cancer stem cell population. The stem cell ability was determined by sphere forming ability and tumorigenicity. This research suggests that CD44 has potential as a stem cell marker but it may not be reliable in the cell line population.

**ALDH**

ALDH is a stem cell marker in multiple normal tissues. In colorectal tissue it is expressed at the site where stem cells are present (in the crypt base). It is also well described as a marker of cancer stem cells in breast, head and neck and lung. In colorectal cancer ALDH +ve, unlike ALDH-ve cells have the ability to form tumours in immunocompromised mice. Furthermore, both chemotherapy and radiotherapy select for increasing ALDH expression and the presence of ALDH +ve cells post treatment predicts for recurrence. Thus ALDH +ve cells possess treatment resistance and increased tumorigenicity, highlighting ALDH’s role in colorectal cancer as an effective stem cell marker.

**1.1.6.2 In vitro assays of cancer stem cells**

Cancer stem cells are defined by possession of specific characteristics – the ability to self-renew, produce daughter cells, resist anoikis and induce tumours. The sphere assay can be used to assess all of the stem cell characteristics. The assay involves seeding individual cells at low density into non-adherent media. The differentiated cells undergo anoikis, whereas the stem cells form spheres of daughter cells that are free-floating in the non-adherent media.

The assay is a relatively quick method of demonstrating a cell’s ability to self-renew whilst also demonstrating anoikis resistance. It can be used as a cost and time-effective in-vitro alternative to serial transplantation studies which are time consuming, involve multiple animals and are not suitable for initial research. However, evidence that repeated culture in cell media can induce genetic mutations and allow the selection of atypical clones makes it necessary that positive findings from sphere assays are reproduced in animal serial transplantation models to confirm the findings of cell culture.
The sphere assay was originally used in non-cancer stem cells but it has since been adapted for a number of malignancies including among others breast, lung, prostate and colorectal cancer. In colorectal cancer sphere forming cells express a higher level of stem cell markers than non-sphere cells and cells derived from colonospheres had a 5.5 times higher colonosphere forming ability (ratio of colonospheres formed to cells seeded) than cells from the parent cell line. This demonstrates that the colonosphere assay selects for stem cells and also results in a higher proportion of stem cells in the daughter population. Colonospheres grown from primary colorectal cancer specimens that were then inoculated into immunodeficient mice resulted in tumour formation. This indicates that the cells that made up the colonospheres possess cancer stem cell ability. These findings demonstrate that for colorectal cancer the sphere assay is able to select for cancer stem cells.

1.1.7 Tumour microenvironment.
A large part of a colorectal cancer is made up of stromal tissue, with an increased tumour-stroma ratio correlating with reduced survival. This suggests that the stroma is able to influence the behaviour of epithelial malignant cells. Non-epithelial cells within the cancer stroma microenvironment include fibroblasts, endothelial cells and macrophages. There is cross talk between the malignant epithelial cells and the stromal cells (largely fibroblasts) with up regulation of 170 of 22,000 genes in fibroblasts that are associated with colorectal metastases compared to skin fibroblasts. This cross talk has a role in the development and maintenance of colorectal cancer. In in-vitro co-culture systems when colorectal cancer cell lines are cultured with the addition of fibroblasts there is up-regulation of multiple pro-cancer pathways in the cancer cell line compared to the cell line cultured alone. Expression of fibroblast activation protein (FAP), expressed by reactive stromal fibroblasts in colorectal cancer samples correlates with advanced disease and reduced survival.

1.1.2 Treatment of colorectal cancer
Treatment of colorectal cancer is undertaken by a multidisciplinary team and can involve one of more of:

- Treatment without curative intent (e.g. stent, palliative chemotherapy, de-functioning etc)
- No treatment
- Surgical resection
• Chemotherapy
• Radiotherapy.

1.1.7.1 Surgical resection
Resection of the primary tumour is the only treatment that offers the possibility of cure for the majority of patients\textsuperscript{64}. In the UK, 32\% of patients with colorectal cancer do not undergo surgical resection largely because of inoperable disease\textsuperscript{65}. In a small subgroup of patients with rectal cancer who undergo long-course chemoradiotherapy a complete pathological response may offer the possibility of cure without surgical resection\textsuperscript{66}.

Surgical resection can be undertaken with curative intent by means of local resection or major resection. In the UK only about 5\% of colorectal malignancies are treated with local resection\textsuperscript{65}. This approach is limited to early tumours where the invasive component is confined to a polyp or the superficial mucosal layers and therefore suitable for resection either endoscopically or trans-anally. The use of local resection is controversial. The Association of Proctology of Great Britain and Ireland (ACPGBI) in their position statement regarding the management of the malignant colorectal polyp highlight the lack of evidence of outcome following the removal of cancers confined to polyps. If the patients do not undergo a major resection then there is no way of identifying either nodal disease or residual malignant cells in the bowel wall\textsuperscript{67}. In early low rectal cancers a review by Tytherleigh et al\textsuperscript{68} recommends the use of local resection in low risk cancers (staged preoperatively at T1). However the lack of evidence regarding the management of T1 rectal cancers has been highlighted by the recent NICE guidance regarding the management of colorectal cancer\textsuperscript{69}.

The majority of colorectal cancers are treated with major surgical resection which involves excising the tumour and its draining lymph node basin and vascular supply. In the most recent data from the UK, 63\% of patients were managed by major resection\textsuperscript{65}. This is increasingly being performed by a laparoscopic approach with approximately 45\% of UK resections been undertaken laparoscopically\textsuperscript{65}.

There are a range of resections that can be undertaken depending on the site of the tumour. The type of resection is dictated by:

• the need for an oncologically sound resection
• the need to for the remainder of the bowel to retain its vascular supply
• the need to retain intestinal continuity
• the desire to avoid long term complications.

1.1.7.2 Chemotherapy
Chemotherapy in colorectal cancer can be adjuvant, neo-adjuvant and palliative therapies. A number of chemotherapy agents are used in colorectal cancer, and these are often used in combination:

• 5-fluorouracil (5-FU), a thymidylate synthase (TS) inhibitor. This is the main and initial chemotherapy agent for colorectal cancer. The inhibition of TS results in DNA strand breaks and cell death when cells are replicating and the effects are therefore potentiated in the replicating cancer cells\textsuperscript{70}
• Oxaliplatin is a platinum based chemotherapy agent that results in caspase-3 activation and therefore apoptosis\textsuperscript{71}
• Capecitabine is a pro-drug that is converted to 5-FU which has the benefit of being an oral preparation\textsuperscript{72}
• Folinic acid is not a chemotherapy agent but is used in addition to 5-FU as it increases the toxicity of 5-FU to cancer cells. The presence of reduced folate (which is derived from folinic acid) increases the ability of 5-FU to inhibit TS\textsuperscript{73}.

The most common regimes are:

• FOLFOX (folinic acid, 5-FU and oxaliplatin
• FOLFIRI (folinic acid, 5-FU and irinotecan
• CAPOX (capecitabine and oxaliplatin).

In addition there are monoclonal antibodies that are used in the metastatic setting:

• Bevacizumab. This targets the Vascular endothelial growth factor (VEGF), the blockading of which results in reduced neo-vascularisation
• Cetuximab. This inhibits the epidermal growth factors (EGFRs) and is therefore anti-proliferative. It does not work if the K-ras mutation is present.

Adjuvant chemotherapy has an established role in the treatment of patients with advanced disease (TNM Stage III), where its use is associated with an absolute survival benefit of 12\%\textsuperscript{74}. Adjuvant chemotherapy is considered selectively for patients with TNM Stage II disease where its survival benefit is lower at approximately 3\%\textsuperscript{75,76}. Selection for treatment in these cases is determined by other pathological markers of poorer prognosis, such as LVI, in combination with patient factors such as age and comorbidity.
Neoadjuvant chemotherapy has a role in metastatic disease when curative surgery may be considered, for example if liver metastases are present that may be down staged to allow resection. In rectal cancer when a curative resection may be compromised by involvement of tissues beyond the mesorectum chemotherapy is routinely offered in combination with radiotherapy as this reduces local recurrence. Studies are ongoing examining the role of neoadjuvant chemotherapy in locally advanced tumours in the colonic cancer setting.

1.1.7.3 Radiotherapy
The use of radiotherapy in colorectal cancers is restricted to the rectum. This is usually as neo-adjuvant therapy as it is associated with a greater reduction in local recurrence rate compared to its use in adjuvant setting. The aim of neo-adjuvant radiotherapy is two-fold: i) down-stage advanced tumours to allow a clear plane of surgical resection and ii) reduce local recurrence. Neo-adjuvant radiotherapy is delivered as ‘short-course’ or ‘long-course’ regimens involving 5 x 5 Gy or 25 x 1.8 Gy radiotherapy fractions. The use of different regimens varies around the world and even across the UK and is still a subject of debate. In general, short-course treatment is used for T3 rectal tumours where the circumferential resection margin (CRM) is not threatened, and for some T2 tumours with radiological features suggesting high risk of recurrence. Long-course radiotherapy is used to downstage disease for tumours where the CRM is at risk during resection. The benefits of short course radiotherapy were demonstrated in the Swedish Rectal Cancer Trial (a randomised controlled study of 1,168 patients who were randomised to undergo surgery alone or preoperative radiotherapy). This showed a benefit in both overall and cancer specific survival (38% vs 30% p=0.008) as well as local recurrence (9% vs 26% p<0.001) in the radiotherapy group. A similar Dutch trial randomised 1861 patients to either short course radiotherapy followed by total mesenteric excision (TME) or TME alone. They demonstrated a reduction in local recurrence rate from 8.2% in the surgery alone group to 2.4% in the radiotherapy and surgery group (p<0.001). The CRO7 trial was a multicentre trial that randomised 1350 patients to short course radiotherapy followed by surgery or surgery followed by selective chemo-radiotherapy. They demonstrated an improvement in disease free survival but no benefit in overall survival in the short course group.
1.2 Venous thrombosis

1.2.1 Definition of venous thrombosis
Venous thrombosis is the abnormal formation of a thrombus in a vein from the constituent products of blood. Venous thromboembolism (VTE) occurs when the thrombus separates and becomes lodged in a distant vessel. The most common site for venous thrombosis is within the deep veins of the legs, termed Deep Vein Thrombosis (DVT) and the most common site of a venous thromboembolism is within the pulmonary artery, called a Pulmonary Embolism (PE).

1.2.2 Clinical presentation and sequelae of deep vein thrombosis (DVT)
Presentation of DVT is highly variable ranging from being completely asymptomatic through simple pain and swelling of the leg to phlegmasia cerulea dolens. In a study of 340 consecutive patients diagnosed with a DVT this was an incidental finding on routine imaging in 28% of cases, all of these incidental diagnoses were clinically silent. Lower limb venous thrombosis can result in post-thrombotic syndrome, which occurs in 20 – 50% of patients within two years of developing a DVT. The syndrome encompasses pain, swelling, stasis dermatitis, lipodermatosclerosis and ulceration in 25 – 33%. Patients with post-thrombotic syndrome have a significantly impaired quality of life. The presence of DVT results in the possibility of a portion of the thrombus becoming dislodged and travelling to the lung forming a pulmonary embolus (PE).

1.2.3 Clinical presentation and sequelae of PE
In a similar manner to DVT, presentation of a PE ranges from an asymptomatic incidental finding on imaging to cardiovascular failure and death. PE is a highly morbid diagnosis. A multicentre prospective cohort study of 1,338 patients with symptomatic, objectively confirmed PE (confirmed either by high probability ventilation-perfusion scan (VQ), indeterminate VQ scan and confirmed lower limb DVT on duplex scan or a intraluminal filling defect on CT scan with PE protocol) demonstrated a 3 month mortality of 10.6% despite treatment.

1.2.4 Risk factors for developing venous thrombosis
Rudolf Virchow, in the 19th century identified three factors (Virchow’s Triad) that are necessary for a venous thrombosis to form:

- Venous stasis
- Injury to blood vessel
- Hypercoagulability.
These factors together contribute to the development of a VTE. For a thrombosis to form it is necessary for there to be activation of the coagulation pathways to allow a clot to develop. There are discussed in the following section.

1.2.5 Physiology of thrombosis

In humans there are two proposed models of coagulation the cascade pathway model and the cell based model.

1.2.5.1 Cascade pathway model

In the cascade pathway model it is proposed that there are two coagulation pathways that are responsible for normal haemostasis. The pathways are cascades of protein interactions responsible for physiological haemostasis. Two convergent pathways exist that result in the formation of a fibrin bound platelet clot:

- Contact activation pathway (intrinsic)
- Tissue factor (TF) pathway (extrinsic)

These both result in the activation of factor X to form factor Xa. Factor Xa then initiates the common pathway.

Contact activation pathway (intrinsic)

The intrinsic pathway is activated when circulating factor XII comes into contact with charged surfaces and becomes activated. This commences a pathway of activation, initially activating factor XI, which activates factor IX. Factor IXa and its co-factor factor VIII activated to factor X, commencing the common pathway94 (Figure 1.2).

The pathway is thought to have a relatively minor role in haemostasis as people who have deficiencies of proteins in this coagulation pathway have near normal clotting abilities.

TF Pathway (extrinsic)

The main pathway in haemostasis is the TF pathway. The TF pathway is initiated by contact between circulating factor VII and TF. TF is a 47 kDA transmembrane bound glycoprotein expressed on the sub-endothelial cells, and is therefore exposed by endothelial damage. The TF and Factor VII form a complex (TF/VIIa) which activates factor X commencing the common pathway (Figure 1.3).

Common Pathway

Factor X that has been activated as part of either the contact activation pathway or TF pathway converts prothrombin to thrombin (factor IIa). Thrombin activates platelets and converts fibrinogen to fibrin forming a stable haemostatic plug95.
Figure 1.2 Intrinsic clotting pathway

Factor XII (XII), Factor XI (XI), Factor IX (IX) Factor X (X), Prothrombin (II), Thrombin (Ila), Antithrombin III (AT), Thrombin antithrombin (TAT), Fibrinogen (Ia), Plasmin (Pl), d-dimer (DD).

Figure 1.3 Extrinsic (TF) pathway

Tissue factor (TF), Factor VII (VII), Factor X (X), Prothrombin (II), Thrombin (Ila), Antithrombin III (AT), Thrombin antithrombin (TAT), Fibrinogen (Ia), Plasmin (Pl), d-dimer (DD).
1.2.5.2 The cell based model

The cell based model is a further development on the cascade based model. This model incorporates the action of the cells within the coagulation pathway, particularly the role that platelets, and their bindings sites for coagulation proteins, have in coagulation. This model allows the interactions between proteins that have been demonstrated in the cascade pathway to function in a dynamic fluid environment of the circulating blood. In the model activated monocytes are a source of TF while the surface of platelets acts as the site of thrombin generation. These cells become lodged at the site of trauma and haemorrhage. This model helps explain the rapid deployment of clot at the site of injury rather than resulting in a disseminated coagulation that would be expected if un-controlled protein to protein interaction took place within the circulating blood.

1.2.5.3 Control of clotting pathway

The clotting cascade is subject to auto-regulation and control.

Amplification of the cascade happens via two methods:

- Factor VIIa activates factor IX, which via the intrinsic pathway increases the conversion of factor X to factor Xa
- Thrombin activates factor VII and XI activating the intrinsic pathway.

Inhibition of the pathway is vitally important to prevent total activation of the clotting cascade causing disseminated intravascular coagulation (DIC). The main control points are:

- Antithrombin III, this protease inhibitor forms a stable thrombin-antithrombin (TAT) complex thus inactivating thrombin. The presence of TAT in the plasma is indicative of the thrombin presence and therefore activation of the coagulation system
- Degradation of fibrin by plasmin which prevents propagation of clot. This forms fibrin degradation products (FDP). One of these products is d-dimer. Presence of d-dimer in plasma is indicative that there is clot breakdown and therefore activation of the coagulation system.

1.2.6 Non-haemostatic functions of TF pathway

In addition to its actions in promoting haemostasis the TF pathway is involved in the inflammation and recovery phase of injury. These roles are activated by a complex
series of interactions between proteins of the TF pathway and other membrane proteins and intracellular signalling pathways (Figure 1.4).

Activation commonly occurs via the cell bound G-protein-coupled protease activated receptors (PARs) PAR1 and PAR2. PAR 1 is activated by the TF-VIIa-Xa complex and PAR2 is activated by the TF-VIIa complex\textsuperscript{100,101}.

PAR1 activation mediates an inflammatory response, with induction of (amongst others) IL-6 and 8, Transforming Growth Factor β, and monocyte chemoattractant protein-1\textsuperscript{102}. It also induces ERK1/2 and AKT activation\textsuperscript{103,104}.

PAR2 activation induces a number of pro-angiogenic factors including vascular endothelial growth factor (VEGF), Cyr61 and VEGF-C. It also induces immune regulators including CXCL1, IL8 and GM-CSF. In addition it can also activate ERK1/2 and AKT\textsuperscript{105,106}.

In addition to the activation of PAR1 and PAR2, TF is able to activate intracellular pathways directly via its cytoplasmic tail, either by TF alone (ERK1/2) or by the TF-VIIa complex (ERK1/2 and AKT)\textsuperscript{107-111}.

The role of these pathways in normal physiology are complex and poorly understood. They are involved in gene regulation and have roles in proliferation, cell survival and apoptosis\textsuperscript{112}. 
Figure 1.4 Non-haemostatic roles of the TF pathway.

TF is able to induce multiple pathways via the action of its cytoplasmic tail, the action of the TF factor VIIa complex, PAR2 and PAR1.
1.2.7 Diagnosis of VTE
In clinical practice it is important to be able to accurately identify patients with venous thrombosis. There are a number of methods that are routinely employed.

1.2.7.1 D-dimer
The presence of a raised plasma d-dimer demonstrates activation of the clotting pathway. This blood test is routinely used in clinical practice, with many commercial assays available. D-dimer quantification allows:

- the diagnosis and monitoring of DIC
- the exclusion of VTE.

Use of d-dimer in risk stratification and exclusion of VTE is well documented. A normal d-dimer had a negative predictive rate of 96.1% (95% CI 93.3 – 98%) for DVT, however its false positive rate is high because d-dimer is raised in many circumstances such as malignancy, post-operatively, post trauma, pregnancy, and in the elderly and over predicts thrombotic disease if used in isolation.

1.2.7.2 Diagnosis of DVT
The diagnosis of a DVT is both clinical (with characteristic features of unilateral swelling and pain) and radiological. The radiological methods that are employed to confirm a diagnosis are:

Venous Duplex

Venous duplex is a non-invasive combined Doppler and ultrasound scan of the deep veins of the limb which produces a 3D representation of the venous system allowing the identification of thrombus. In a small double blinded controlled study of 47 patients, venous duplex was compared to venography with patients undergoing both procedures. This study demonstrated a sensitivity of 95% and specificity of 83% for venous duplex when using the two ultrasonographic criteria of visualised thrombus and absence of spontaneous blood flow. Duplex scan is operator dependent, relying on the expertise of the person undertaking the scan and is a real-time investigation.

Venography

Venography involves injecting intravenous contrast into a distal vein and radiological screening for proximal flow. It is an invasive procedure involving cannulation of a
distal vein and exposes patients to: radiation, risk of reaction to intravenous contrast, contrast induced nephropathy and can even induce a DVT.

Venous duplex has become the standard method for diagnosing lower limb DVTs and is recommended by NICE as the imaging modality in patients with suspected DVTs\textsuperscript{116}. Prospective studies have compared venous duplex with venography and show comparable efficacy in detection of proximal thrombosis but venous duplex is less effective than venography at detecting calf DVTs\textsuperscript{117-119}.

1.2.7.3 Diagnosis of PE

The diagnosis of a PE is clinical (with pleuritic chest pain, respiratory compromise and circulatory collapse). It is confirmed by radiological investigations:

Ventilation perfusion scan (VQ)

A VQ scan is a nuclear medicine scan that uses a radio-isotope to examine ventilation and perfusion in a patient’s lung. The test allows for a ventilation/perfusion discrepancy to be identified that can be a sign of the presence of a PE. It has a sensitivity of 77.4 (95%CI 69.7% – 85.0%) and specificity of 73.5% (95%CI 70.7% – 76.4%)\textsuperscript{120}. Its use is limited to patients without other lung diseases (including infection) that can give rise to false positive results.

CT pulmonary angiography (CTPA)

CTPA uses intravenous CT contrast to detect the presence of PE. Initial studies that compared CTPA with VQ scans demonstrated similar sensitivity (74.1%) and specificity (89.5%)\textsuperscript{121}. A more recent study that has taken advantage of developments in the technologies of CT scans have demonstrated increased sensitivity (81.7%) and specificity (93.4%) of CTPA\textsuperscript{122}. It has the advantage that it can be used in patients with other lung diseases. However, due to the use of intravenous contrast it is contraindicated in a number of circumstances (eg renal failure and contrast allergy).

1.2.8 VTE prophylaxis

Patients in hospital are at increased risk of developing VTE so risk stratification based on known risk factors VTE (Figure 1.5) and bleeding (Figure 1.6) is undertaken. This allows targeted thromboprophylaxis \textsuperscript{123}.

The level of intervention ranges from early mobilisation, use of compression stockings (+/- intermittent compression devises) to anticoagulation.
Risk factors for VTE

- Active cancer or cancer treatment
- Age over 60 years
- Critical care admission
- Dehydration
- Known thrombophilia
- Obesity (Body mass index (BMI) greater than 30kg/m²)
- One or more significant medical comorbidities
- Personal history or first degree relative with a history of VTE
- Use of hormone replacement therapy
- Use of oestrogen-containing contraceptive therapy
- Varicose veins with phlebitis

Figure 1.5 Risk factors for VTE, adapted from NICE guideline (CG92)

Contraindications for LMWH

- Acquired bleeding disorder
- Concurrent use of anticoagulants
- Expected lumbar puncture / epidural or spinal anaesthesia within 12 hours
- Previous lumbar puncture / epidural or spinal anaesthesia within 4 hours
- Acute stroke
- Thrombocytopenia
- Uncontrolled hypertension
- Untreated inherited bleeding disorders

Figure 1.6 Contraindications for LMWH, adapted from NICE guideline (CG92)
1.2.8.1 Mechanical thromboprophylaxis

Mechanical thromboprophylaxis aims to prevent venous stasis. The methods that can be employed include graduated compression stockings and intermittent calf compression devices.

The use of graduated compression stockings is recommended to be routine in patients hospitalised for surgical procedures involving general anaesthetic or regional blocks. A Cochrane meta-analysis confirmed that graduated compression stockings are effective in reducing the risk of DVT in patients hospitalised for a surgical procedure. The CLOTS trial 2 (a randomised controlled trial comparing knee length to thigh length stockings in immobile patients) demonstrated a reduced risk of developing a DVT if patients wearing full length stockings (absolute risk reduction 2.5% 95%CI 0.7 – 4.4). However, stockings are not without risk. In the CLOTS trial 1 (a large (2518 patients) multicentre randomised controlled trial examining the use of compression stockings in stroke patients) there was no significant reduction in the development of DVT with the use of stockings, but there was an increase (5% vs 1%, OR 4.18, 95% CI 2.4 – 7.27) in skin breaks, ulcers and skin necrosis, highlighting the need for caution in some patient populations.

For high risk patients the routine intra-operative use of intermittent calf compression devices is advised, with ongoing use when prolonged immobility is expected, for example in intensive care. The CLOTS trial 3 (a trial of 1438 immobile patients following a stroke where they were randomised to the use of intermittent calf compression stockings or no compression) demonstrated an absolute reduction in the risk of developing a DVT of 3.6% (95%CI 1.4 – 5.8). There was (as in CLOTS trial 1) an increase in the number of patients developing skin breaks in the treated group (3%) compared to the control group (1%, p=0.002). As well as the risk of skin breaks with the use of intermittent calf compression devices there are reports of compartment syndrome following their use in the lithotomy position, however this has not been supported by a study of compartment pressure monitoring in the lithotomy position, which found their use reduced compartment pressure (p<0.05).
1.2.8.2 Pharmacological methods

Low Molecular Weight Heparins (LMWH)

Low Molecular Weight Heparins (LMWH) activate antithrombin III\textsuperscript{132} and are administered by subcutaneous injection without the need for serological monitoring of clotting.

Large recent meta-analyses have demonstrated that LMWH are effective in preventing VTE after non-orthopaedic surgery and are at least as effective as unfractionated heparins in preventing post-operative VTEs\textsuperscript{133,134}.

The use of LMWH can be associated rarely with development of heparin induced thrombocytopenia (HIT), which results paradoxically in an increased risk of thrombosis as well as thrombocytopenia. A cohort study of 598 consecutive patients receiving LMWH for either prophylactic (360 patients) or therapeutic use (238 patients) showed that 0.8% (95%CI 0.1 – 1.6%) developed HIT\textsuperscript{135}.

Vitamin K analogues

Warfarin is a vitamin K analogue. It acts through inhibition of vitamin K epoxide reductase which is required for the reduction of Vitamin K following its use in the production of the clotting factors II, VII, IX and X\textsuperscript{136}. This maintains vitamin K in its oxidised state therefore preventing it from forming further clotting factors.

Warfarin is used for prevention of VTE in high risk patients (including patients who have had previous VTE and with abnormalities in the clotting system that increase the risk of developing a VTE) where long term prophylaxis is needed. Warfarin is cheap and does not involve regular injections but does however involve regular blood monitoring to ensure a therapeutic level of anticoagulation. An important side effect is the risk of haemorrhage which can be serious but this risk is low. The anticoagulation and risk factors in atrial fibrillation study found that there were 170 major haemorrhage events in 15,300 person-years of warfarin therapy compared to 162 major haemorrhages in 15,530 person-years whilst not on warfarin therapy\textsuperscript{137}.

There is no role for warfarin in the acute thromboprophylaxis prophylaxis for surgery.

Direct acting anticoagulants

Newer anticoagulants are available that directly inhibit thrombin or factor Xa. An example of a thrombin inhibitor is dabigatran which reversibly binds to thrombin\textsuperscript{138}. The pro-drug for dabigatran is available in an oral form (dabigatran etexilate) which avoids the need for injections. An example of a factor Xa inhibitor is rivaroxaban.
which directly inhibits factor Xa\(^1\). An advantage of the direct acting anticoagulants over Vitamin K analogues is the lack of requirement for serological monitoring. Dabigatran is as effective as LMWH in prophylaxis against post-operative thrombosis\(^2\). In surgical practice its major problem is the current lack of a reversal agent. The inability to monitor the level of anticoagulation also causes issues as the level of anticoagulation cannot be established. It is not currently used for prophylaxis in the perioperative period.

### 1.2.9 VTE treatment.

The treatment of VTE is largely pharmacological and the aim of treatment in the majority of patients is to prevent propagation or recurrence. Anticoagulation can be undertaken by the use of LMWHs, un-fractionated heparins, vitamin K analogues or NOACs. LMWH are also used at treatment dose for established VTE prior to instigation of long term oral anticoagulation, being as effective as vitamin K analogues with no need for regular blood monitoring\(^3\). Un-fractionated heparins are used in the short term when there is a high risk of bleeding as they (unlike any other method of anticoagulation) can be rapidly reversed. Vitamin K analogues provide the mainstay of long term treatment of VTEs although the use of direct acting anticoagulants has replaced them in some areas of practice. A randomised controlled study of 2539 patients compared dabigatran to warfarin for the treatment of acute VTE demonstrating non inferiority with a similar safety profile\(^4\). Other direct acting anticoagulants that are available and used for treatment of VTE are apixaban\(^5\) and rivaroxaban\(^6\).

Non-pharmacological methods are used to reduce the incidence of complications post VTE. In established DVTs the it has been suggested that the use of compression stockings reduces the risk of developing post-thrombotic syndrome (hazard ratio 0.49 to 0.84, \(p=0.011\))\(^7\) however a randomised controlled study comparing stockings to placebo demonstrated no difference in the rate of post-thrombotic syndrome in the intervention and placebo arms\(^8\). Percutaneous manual aspiration thrombectomy can remove a DVT therefore reducing the risk of developing post thrombotic syndrome. This is an interventional vascular procedure which involves physically removing the clot and in some cases subsequently using catheter-directed thrombolysis. It is generally reserved for the rare acute ilio-femoral DVT. A prospective cohort study of 139 patients with acute or subacute ilio-femoral DVT\(^9\) who underwent thrombectomy demonstrated that 66.2% achieved more than 95% removal of the thrombus, 19% of patients had recurrent thrombosis. There were no serious complications arising from the procedure. Given the serious
consequences of an extensive ilio-femoral DVT this procedure should be considered in appropriate cases.
1.3 Colorectal cancer and thrombosis

1.3.1 Cancer as a risk factor for developing thrombosis

The relationship between cancer and thrombosis was first described by Trousseau in 1865. Cancer is a major risk factor for thrombosis and indeed DVT is not infrequently the initial presentation of an occult malignancy. In a prospective cohort study of 400 consecutive patients with confirmed DVT, 7.3% of the 137 patients with idiopathic DVTs were diagnosed with a malignancy within 6 months. It is likely that the occult malignancy was present at the time of DVT presentation. A further prospective cohort study found that 25% of the 84 patients with an idiopathic DVT were found to have a malignancy during the 2 year follow-up.

The incidence of VTE in the general healthy population is 1.6 per 1000, this being increased considerably by the presence of malignancy. A cohort study by Stein et al. determined VTE rates in cancer and non-cancer patients based on hospital discharge codes (40,787,000 patients). The overall VTE rate in cancer patients was twice that of patients without cancer. A Dutch population case controlled study (n = 3220) demonstrated that patients with malignancy had a 7 fold increase risk of VTE when compared to the general population, and patients with distant metastasis had a further increased risk (adjusted OR, 19.8; 95% CI, 2.6-149) compared to patients with local disease. The incidence of diagnosed venous thrombosis in patients with cancer appears to be increasing. A population study by Khorana et al. showed that between 1995 and 2003 the incidence of VTE in patients with malignancy demonstrated a relative increase of 28% (p<0.0001). In patients receiving chemotherapy the relative increase was 47% (P<0.0001). Possible explanations for the observed increase include the introduction of newer, more thrombogenic chemotherapy regimens, increased awareness of the relationship between malignancy and thrombosis resulting in higher diagnostic vigilance and more intensive staging and follow up using CT scans resulting in the detection of more incidental VTEs.

1.3.2 Colorectal cancer is a risk factor for developing a VTE

Stein et al. found that the incidence of VTE in patients with colorectal cancer was 2.1 cases / 100 hospitalisations, these hospitalisations being for any reason and the data being acquired from hospital discharge codes. Blom et al. demonstrated that the odds ratio of developing a VTE with colorectal cancer compared to non-cancer controls is 16.8 (95% CI 4.1 – 60.1). The incidence of thrombosis in colorectal cancer is 13.4 (95% CI 10.8 – 16.6) per thousand patients per year compared to
the whole population incidence of 1.83 (95% CI 1.69 – 1.98) per thousand population per year\textsuperscript{159}. In subgroup analysis of a retrospective cohort study, patients undergoing colonic (n=12946) and rectal (n=2258) cancer resection had a 30 day post-operative risk of VTE of 2.0% and 2.3% respectively\textsuperscript{160}. This study was undertaken at a time when only in-hospital VTE prophylaxis was routine. The data was only available for the first 30 days post-operatively and probably underestimates the incidence of VTE as patients may have developed a VTE after this time and many may have been asymptomatic. There was no screening undertaken.

1.3.3 Pathophysiology of colorectal cancer associated VTE
As with non-cancer associated VTE, Vichows Triad underlies the pathophysiology of cancer associated VTE. The triad is:

- Venous stasis
- Injury to blood vessel
- Hypercoagulability.

The presence of all these factors is demonstrated in patients with colorectal cancer:

1.3.3.1 Venous stasis
Large pelvic tumours and inguinal or pelvic lymphadenopathy may compress the pelvic veins resulting in chronic venous stasis. Perioperative factors contribute to venous stasis. Anaesthesia for abdominal surgery requires intermittent positive pressure ventilation, which results in venous stasis when compared to spontaneous breathing\textsuperscript{161}. Pneumoperitoneum and patient positioning in the reverse Trendelenburg position for laparoscopic surgery reduces venous return and increases venous stasis\textsuperscript{162,163}. Other factors include volume overload with intravenous infusions\textsuperscript{164}, and post-operative immobility from pain reduces calf muscle pump induced venous return.

1.3.3.2 Injury to blood vessels
The act of undertaking surgery results in the damage of multiple small blood vessels. This damage results in activation of the clotting pathway. The use of indwelling venous catheters in the perioperative and postoperative period significantly increases the risk of VTE due to both localised damage to the blood vessel and venous stasis\textsuperscript{165-167}. Furthermore, chemotherapy agents are damaging to the vascular endothelial cells resulting in activation of the clotting pathway. The site
of the VTE is related to the position of the line and is therefore generally in the upper limbs or superior vena cava.

1.3.3.3 Colorectal cancer associated hypercoagulability
Colorectal cancer associated hypercoagulability is demonstrated in patients with colorectal cancer. This hypercoagulability results from activation of the coagulation system. The activation of the coagulation system is demonstrated as patients with colorectal cancer have raised d-dimer and thrombin-anti-thrombin (TAT) levels. A study by Iverson et al\textsuperscript{168} compared 93 patients with colorectal cancer undergoing resection with 30 age-matched control patients undergoing resection for benign disease. Cancer patients had raised d-dimer and TAT compared to controls. D-dimer and TAT were further elevated postoperatively in patients who had an incomplete resection compared to those where curative resection was achieved, suggesting that the residual tumour was activating the clotting system. The relationship between cancer and the presence of d-dimer and TAT has also been seen in breast cancer\textsuperscript{169}.

The physiology by which cancers result in a hypercoagulable state may be explained by the finding that cancers abnormally express TF. TF is not normally expressed by cells except in the sub-endothelial cells of the vasculature. TF is the instigator of the clotting pathway (section 1.2.5.1). The level of expression of TF is not universal across all cancers and cancers that express high levels of TF (e.g. brain and pancreatic) have a higher risk of developing VTE than low expressing cancers (e.g. breast)\textsuperscript{170}. This suggests that the TF expressed by the cancer may have a role in promoting systemic hypercoagulability. This relationship between the presence of TF and hypercoagulability is not only demonstrated by differences between cancer types. It is also demonstrated within cancer types. In a prospective cohort study of pancreatic cancers, VTE was more common in patients with high TF expressing tumours than those with low TF expressing tumours (P=0.04)\textsuperscript{171}. Similar findings are seen in ovarian cancers, with TF expression being an independent predictor for VTE development (P<0.05)\textsuperscript{172}.

In addition to the aberrant expression of TF on the cancers themselves cancers also release circulating micro particles that express TF. These are cell membrane derived vesicles released when the cell is activated or on apoptosis\textsuperscript{173,174}. Their presence has been demonstrated in wide range of cancers\textsuperscript{175}. In colorectal cancer the number of TF positive micro particles is increased two-fold in cancer patients (n=20) compared to age and sex matched controls (n=20) (p=0.007)\textsuperscript{176}. In the same
study the presence of the TF expressing micro particles appears to activate the coagulation system with their presence being positively correlated with d-dimer levels (p = 0.002). This relationship between the presence of TF positive micro particles and systemic hypercoagulability has been demonstrated in pancreatic cancer. In a case controlled study of pancreatic cancer patients with VTE were compared to pancreatic cancer patients without VTE. Patients diagnosed with a VTE had elevated levels of TF micro particles compared to patients without a VTE (p = 0.01). Of the control group (without VTE at presentation) at 1 year 35% of TF micro particle positive patients compared with 0% of TF micro particle negative patients developed VTE (p = 0.002).

It is also proposed that other TF pathway proteins that are abnormally expressed by the cancer cells may promote aberrant haemostasis resulting in increased risk of VTE development. These proteins that have been identified as been expressed by cancers include factor VIIa and factor X. There is to date no evidence establishing a causative link between the expression of these proteins and the development of VTEs.

In addition to the role the TF pathway proteins have in promoting thrombosis there are a number of other possible factors that may result in cancers inducing thrombosis. One possibility is the effect of the cancer in inducing inflammation that has been linked to a prothrombotic state. The non-TF pathway mediated effects on thrombosis are beyond the scope of this work.

1.3.3.4 Colorectal cancer treatment associated hypercoagulability

Surgical, chemotherapy and radiotherapy treatment of cancer further activates the coagulation system. The trauma of surgery activates the coagulation system as part of the normal physiological response for haemostasis. This activation is seen in both benign and malignant conditions. However, the duration and level of activation of the coagulation system is increased in patients undergoing surgery for cancer compared to benign indications. A more prolonged activation of the coagulation is also seen when tumours are incompletely excised compared to patients with similar operation where a curative resection had been achieved. These findings suggest that the cancer itself has a role in activating the coagulation system in addition to the activation resulting from surgery.

Chemotherapy results in a hypercoagulable state. A study of 49 breast cancer patients undergoing chemotherapy showed a reduction in thrombin time and partial prothrombin time, representing a hypercoagulable state. This hypercoagulability
develops early, within 24 hours of chemotherapy administration\textsuperscript{185}. In a population based study chemotherapy was associated with a 50% increase in VTE risk compared to cancer patients not receiving chemotherapy (4.1\% (CI 1.9 – 8.5) to 6.5\% (CI 2.1 – 20.2)) \textsuperscript{186,187}.

Patients with rectal cancer frequently undergo radiotherapy prior to resection. In gynaecological cancers, pelvic irradiation increases the risk of a DVT\textsuperscript{188}. Although no studies have looked at the risk of DVT following pelvic radiotherapy for rectal cancer it can be postulated that this group of patients are also at increased risk.

1.3.4 Impact of developing a VTE on mortality in cancer

Patients who develop postoperative VTE have an increased mortality. In a retrospective cohort study of 44,656 patients undergoing elective surgery for a range of cancers there was a 6-fold increase in 30 day mortality in patients with VTE compared to those without\textsuperscript{160}. Although it was not possible to determine the cause of death in these patients, given the endpoint of only 30 days it is reasonable to assume that a large proportion of this increased mortality is secondary to the presence of the VTE. A prospective, observational study of 4466 consecutive patients undergoing chemotherapy for various cancers revealed that 9.2\% of the 141 patients who died during the period of the study (median period 75 days) died as a result of arterial or venous thrombosis. This was the second highest cause of death after cancer, and the same rate as infection\textsuperscript{189}.

In an autopsy based study, 20\% of the colorectal cancer patients who underwent autopsy had died from PE\textsuperscript{190}. Of these, 10\% had local or regional disease and 50\% had limited metastatic disease, thus 60\% had disease that had potentially curative disease.

1.3.5 Special considerations regarding venous thromboprophylaxis and treatment of VTE in cancer

1.3.5.1 Recommendations for thromboprophylaxis in cancer

International consensus documents\textsuperscript{191,192} and meta-analysis\textsuperscript{193} have recommended the routine use of prophylactic LMWH in hospitalised patients with cancer. It is also recommended that patients receive prophylactic LMWH pre-operatively. The use of extended prophylaxis (up to 4 weeks post major cancer surgery) has weaker evidence but is recommended by the consensus documents\textsuperscript{191,192}. NICE guidance in the UK recommends the use of both in hospital and extended course venous thromboprophylaxis in patients undergoing abdominal or pelvic surgery for
cancer. This recommendation results from the finding that a large number of DVTs develop following discharge from hospital. The study by Merkow et al. found that 33.9% patients undergoing colonic resection who developed a VTE did so after discharge from hospital.

1.3.5.2 Treatment of incidental VTE in cancer
The incidental finding of a VTE in patients with cancer is common due to frequent CT imaging of these patients. In a retrospective study of 1,921 patients receiving chemotherapy 3.2% were diagnosed with incidental VTEs. A retrospective cohort study of patients with cancer diagnosed with an incidental VTE (n=51) and symptomatic VTE (n=144) demonstrated no difference in the recurrence rate between the symptomatic and incidental VTEs when both were treated with anticoagulation. A study in pancreatic cancer demonstrated that patients with asymptomatic cancer associated VTE has a similar prognosis to patients with a symptomatic presentation. The finding that incidentally diagnosed VTE have a similar prognosis to symptomatic VTE has led to recommendations that incidental VTE should be managed the same way as those that are symptomatic.

1.3.5.3 Treatment of below knee DVT in patients with cancer
Healthy patients who do not have risk factors for further propagation of thrombosis (of which active cancer is a risk factor) do not need to be anticoagulated as the risk of propagation in these patients is low. The American College of Chest Physicians in their 2012 guidelines and the National Comprehensive Cancer Centre Network recommend that patients with below knee DVTs and cancer should be anticoagulated for at least 3 months with consideration of longer anticoagulation.

1.3.5.4 Selection of treatment of VTE in cancer
The selection of treatment of VTE in cancers requires special consideration. This is because patients with cancer have an increased risk of VTE recurrence compared to patients without cancer. In a study of 842 patients of which 181 had cancer followed up for 12 months 20.7% (95%CI 15.6 – 25.8) of the patients with cancer compared to 6.8% (95%CI 3.9 – 9.7) of the patients without cancer developed recurrent VTE. Patients with cancer are also at increased risk of developing bleeding complications as a result of their anticoagulation. It has been shown that patients with cancer treated with LMWH have a reduced risk of recurrence of their VTE than patients treated with warfarin. This has therefore led to guidelines that patients with an active cancer and a VTE should be maintained on LMWH rather than being commenced on oral anticoagulation.
1.3.1 Pre-operative biomarkers for prediction of postoperative VTE
Given that VTE has such a significant burden for patients and health care in terms of increased morbidity and mortality and that tumour factors are so important in the development of VTE, research has been undertaken to identify pre-operative serum biomarkers for the development of VTE. A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological process, pathogenic process or pharmacologic responses to a therapeutic intervention”\(^{206}\). A biomarker that quantifies risk of post-operative VTE would allow the rationalisation of thromboprophylaxis. Possible candidates that have been investigated include:

1.3.1.1 TF
As a serum biomarker there is limited evidence. One small study in pancreatic cancer measured TF levels during chemotherapy and found that there was a significant correlation between TF levels and the risk of developing a DVT\(^{207}\). This has also been demonstrated in breast cancer\(^{185}\). There has been no study regarding serum TF levels and the risk of developing a DVT in colorectal cancer.

1.3.1.2 D-dimer
The Vienna Cancer and Thrombosis study demonstrated that a raised pre-operative d-dimer predicts for post-operative VTE\(^{208}\). This was a prospective observational study including patients with a wide range of malignancies who were observed for 2 years for symptomatic or clinically relevant asymptomatic VTE or death. DVT was confirmed by venous duplex or venography, PE by CTPA or VQ scans. Baseline plasma d-dimer and pro-thrombin fragment 1 + 2 were performed. The results showed patients with a two times higher d-dimer had a 1.3 fold (95%CI 1.1 – 1.5 \(p<0.001\)) increase in the hazard ratio for developing a VTE. This was independent of other risk factors for raised d-dimer.

In a study of 93 colorectal cancer patients and 30 non-cancer controls undergoing abdominal surgery, 23% of cancer patients who went on to develop a postoperative VTE had elevated pre-operative fibrin degradation products when compared to those without VTE\(^{168}\). This study used robust methodology with patients screened for development of a DVT using venography post-surgery.

1.3.1.3 Thrombin-antithrombin (TAT)
Plasma TAT predicted increased risk of developing post-operative VTE in patients undergoing abdominal cancer surgery\(^{209}\). In colorectal cancer specifically, no
difference was seen in plasma TAT in patients who developed post-operative VTE compared to those who did not\textsuperscript{168}.

1.3.1.4 P-selectin

P-selectin is a marker of platelet activation by thrombin\textsuperscript{210} and is therefore indicative of thrombin generation and activity of the coagulation system. In the Vienna Cancer and Thrombosis study an elevated P-selectin level identified patients at increased risk of cancer associated VTE\textsuperscript{211}. There was no subgroup analysis for colorectal cancer.

1.3.1.5 sGPV

sGPV (like P-selectin) is a marker of thrombin generation as it is released from the platelet surface by the action of thrombin\textsuperscript{212,213}. It has been demonstrated as a marker of thrombosis in ischaemic strokes\textsuperscript{214}. No research has been undertaken regarding using it to predict VTE in cancer patients.

1.3.2 The link between VTE and cancer prognosis

The relationship between thrombosis and aggression of cancer has been suggested in a number of studies. In gynaecological cancers, a retrospective cohort study found that patients with a DVT in addition to their malignancy had a twofold greater risk of dying from all causes than patients with matched cancers without a DVT\textsuperscript{215}.

The large population studies of cancer and thrombosis (Danish Cancer Registry study\textsuperscript{216} and the California Cancer Registry study\textsuperscript{217}) both showed a negative impact on survival in patients who had cancer associated VTE. The Danish Cancer Registry study\textsuperscript{216} compared 668 patients with cancer and DVT with 5,271 matched cancer patients without a DVT. They demonstrated a reduction of 1 year survival from 36% in the control group to 12% in the DVT positive group (p<0.001). The increase in mortality ratio in the DVT positive group for the whole period of follow up was 2.20 (95% CI 2.05 – 2.40). These findings could not be explained by the type or stage of cancer, age or gender of the patients. The California Cancer Registry trial\textsuperscript{217} incorporated 235,149 patients with cancer. Of these patients 3775 were diagnosed with a VTE within 2 years of the cancer diagnosis. The patients with a VTE had decreased survival during the first year for all cancer types. In a subgroup analysis for colorectal cancer patients, the hazard ratio (95%CI) for the risk of death within 1 year of diagnosis in patients with VTE compared to those without was 3.2 (1.8 – 5.5) in local disease, 2.2 (1.7 – 3.0) in regional disease and 2.0 (1.7 – 2.4) in remote disease (all achieved p<0.001). Due to the nature of these studies (with the data extracted from hospital codes) the mortality figures are all cause mortality making it
difficult to determine if the cause of death is related to the cancer or is as a direct result of the VTE. The death rate is higher than would be expected if only resulting from VTE induced deaths and also the timing of the deaths (most deaths caused by a VTE happen in the acute setting) suggests that patients with a DVT and cancer are more likely to die from their cancer than patients without a DVT. One possible explanation for this is that the cancers that express components of the TF pathway, not only result in a hypercoagulable state but also leading to an aggressive tumour biology.

1.3.3 TF pathway protein expression and cancer prognosis
As previously mentioned in cancer there is aberrant expression of TF and the TF pathway proteins. The development of this aberrant expression develops during the transition from adenoma to carcinoma. During the adenoma-carcinoma sequence increasing TF expression is seen. The expression of correlates with the K-ras and p53 mutation which are intrinsic to the adenoma-carcinoma sequence. It has been shown that in colorectal cancer cell TF expression is under the influence of K-ras with mutated K-ras up-regulating the expression of TF. Many of the effects of TF on tumour biology are similar to the effects of the K-ras mutation and it has been suggested that the putative effects of K-ras may be partially mediated through the TF pathway. The clinical significance of the expression of the TF pathway proteins have been partially demonstrated in clinical studies.

1.3.3.1 TF
TF is not expressed in normal tissues but its expression has been detected in colorectal and other cancers including pancreatic, renal cell, ovarian, breast, prostate, hepatocellular and lung. Tumour expression of TF by colorectal cancer correlates with increasing tumour TNM (p=0.02) and Dukes (p=0.01) stage and increasing metastatic potential. There is increased TF expression in colorectal cancer tumour tissue from resected metastatic liver sites (p = 0.01) than in primary tumours and in advanced compared to early tumours (Dukes’ C and D compared with Dukes’ A and B, p=0.03). This suggests that TF level is related to the metastatic potential of colorectal cancer. Seto et al showed that TF expression in the primary tumour was a significant (p=0.0001) and independent risk factor for hepatic metastases and that the overall survival of patients without synchronous metastases was significantly worse in patients whose tumour expressed TF than those whose did not (p=0.0001).
In breast cancer, TF expression is by both epithelial and stromal cells\textsuperscript{223} and it is stromal rather than epithelial expression of TF that correlates with progression from carcinoma in situ (DCIS) to invasive cancer\textsuperscript{227,228}. In breast cancer the localisation of TF expression by stromal cells correlates with the expression of Smooth Muscle Actin (SMA)\textsuperscript{227}. SMA in breast cancer is indicative of stromal activation and its expression by stromal cells is associated with a poor prognosis\textsuperscript{229} highlighting the important role of a procoagulant tumour micro-environment in breast cancer. In colorectal cancer no studies have yet determined whether expression of TF is by epithelial or stromal cells.

1.3.3.2 Thrombin

In prostate cancer thrombin expression has been demonstrated in both epithelial and stromal tissues whilst in normal tissue the expression was limited to the stroma\textsuperscript{230}. In cerebral metastases from clear-cell renal tumour increased expression compared to normal brain tissue has been demonstrated\textsuperscript{231}. Previous research has not demonstrated a prognostic link with thrombin expression in cancer. In colorectal cancer minimal expression has been demonstrated\textsuperscript{232}.

1.3.3.3 PAR1

PAR1 expression has been demonstrated in colorectal cancer but not in normal colonic tissue\textsuperscript{233}. The expression of PAR1 in cancer has also been demonstrated in prostate, endometrial and in squamous cell carcinoma\textsuperscript{103,234-236}. In prostate cancer, the expression of PAR1 by endothelial cells in the stroma correlates with PSA level (a marker of recurrence risk) and advanced disease stage\textsuperscript{237}. No studies have determined the clinical significance of PAR1 expression in colorectal cancer.

1.3.3.4 PAR2

A study of 115 colorectal cancer specimens and adjacent normal tissue demonstrated PAR 2 expression in 47% of the cancer specimens but not normal epithelium\textsuperscript{238}. PAR 2 expression has been also been demonstrated in oesophageal squamous cell carcinoma and gastric cancer\textsuperscript{239,240}. In colorectal cancer PAR2 expression correlates with the presence of distant metastasis, higher TNM stage, presence of LVI and poor differentiation\textsuperscript{238}. In gastric cancer it is associated with mucosal invasion\textsuperscript{239}. The previous studies have not determined whether this expression is epithelial or stromal.
1.3.4 Clinical significance of serological expression of markers of TF pathway activation

Following the identification of TF as a marker of aggressive cancer and the association of VTE with poor outcome cancers, markers of the different components of the TF pathway have been investigated as potential biomarkers for cancer. To be successful as a biomarker for cancer a biomarker must be measurable in a reproducible fashion and must achieve at least one of the following:

- Differentiate patients with a cancer from those without
- Differentiate early from advanced disease
- Demonstrate a change with recurrence of disease.

1.3.4.1 TF

Plasma TF is elevated in cancer compared to non-cancer controls in ovarian, non-small lung, breast and bladder cancers. In ovarian cancer, plasma TF correlates with reduced cancer-specific survival and overall survival, highlighting TF's potential as a cancer biomarker. There is no published work regarding plasma TF in colorectal cancer.

1.3.4.2 Thrombin-antithrombin (TAT)

Plasma TAT is elevated in cancers in comparison to non-cancer controls in many malignancies, including breast, ovarian, uterine and cervical cancers. In lung cancer, plasma TAT is increased with increasing disease burden. Pre-operative TAT is significantly elevated in patients with colorectal cancer compared with non-cancer controls, and is further elevated in patients with metastases. In patients undergoing curative resection for colorectal cancer, pre-operative levels of TAT did not correlate with prognosis. However, TAT is significantly increased with the development of tumour recurrence, suggesting that TAT may be useful as a biomarker in colorectal cancer.

1.3.4.3 D-dimer

The Vienna Cancer and Thrombosis Study was a retrospective cohort study which incorporated 1178 patients with a variety of cancers (including solid and haematological tumours) and found that d-dimer correlated with mortality with a hazard ratio of 1.5 (95%CI 1.4 – 1.6, p<0.001) per doubling of d-dimer value across all the cancers in the trial. In a subgroup analysis for colorectal cancer the hazard ratio was 1.7 (95%CI 1.4 – 2.2, p<0.001).
Specific to colorectal cancer, the association of increasing d-dimer with cancer stage is illustrated in a case-controlled study of 96 colorectal cancer patients and 40 non-cancer surgical controls. Plasma d-dimer correlated with poor prognosis tumour in terms of tumour size \( (p=0.013) \), serosa penetration \( (p=0.006) \), TNM stage \( (p=0.13) \), lymphatic invasion \( (p=0.05) \) and reduced overall survival \( (p=0.004) \). Similar findings have been seen in other cancers including lung, breast, ovarian and gastric. The relationship of d-dimer to prognostic indicators suggests that it may have a role as a biomarker.

1.3.4.4 Fibrinogen

Elevated fibrinogen levels are associated with a poor prognosis in cancers including lung, endometrial and ovarian. In colorectal cancer, fasting pre-operative fibrinogen levels correlated with adverse clinicopathological factors including advanced tumour stage, LVI and lymph node involvement. Its ability to differentiate patients with or without colorectal cancer has not been investigated.

1.3.4.5 P-selectin

P-selectin is released by platelets on activation by thrombin therefore providing an indication that thrombin is present in the plasma and therefore that activation of the coagulation system has taken place. Elevated serum P-selectin levels were seen in colorectal compared to benign controls, with a further increase in patients with metastatic disease. Pre-operative levels were associated with recurrent disease and increased mortality. In breast and haematological cancers, P-selectin was higher in patients with cancer compared to healthy controls but there was no relationship with clinicopathological factors.

1.3.4.6 Glycoprotein V

Soluble glycoprotein V (sGPV) like P-selectin in released by platelets on activation by thrombin. It has been shown to have normal distribution in healthy subjects. Given the demonstration of P-selectin as a possible biomarker in cancer sGPV has also been proposed as a potential candidate. Its use as a biomarker in any cancer has not yet been evaluated.

1.4 Oncogenic roles of TF pathway proteins

The finding that cancers that express TF pathway proteins (TF and PAR2) are associated with a more aggressive cancer phenotype have led to research into possible oncogenic effects of the TF pathway.
1.4.1 The role of TF in cancer growth
In vivo, TF inhibition results in smaller, slower growing melanoma, breast, lung and colorectal cancer tumours. In melanoma mouse models, the use of the TF inhibitor ixolkaris resulted in a slower growth in both primary and metastatic tumours\(^{265,266}\). Similar inhibition of growth was seen with a factor VIIa inhibitor but not by a factor Xa inhibitor suggesting that the effect was exerted through a mechanism separate from the haemostatic pathway. In a murine breast cancer model, tumour growth was inhibited by a TF antibody (Mab-10H10) which blocks the non-haemostatic functions of TF. In addition, mice lacking PAR2 demonstrated reduced tumour growth, suggesting that TF promotes tumour growth via the PAR2 pathway\(^{267}\). Lung and colorectal cancer cell lines transfected with TF siRNA had a reduced rate of tumour growth in-vivo, in vitro proliferation was inhibited in lung but not in colorectal cancer\(^{268,269}\).

In colorectal cancer SW620 cell line the addition of factor VIIa and up regulation of the expression of PAR2 increased proliferation. This effect is reduced by TF antibody\(^{270}\). This is mediated by the ERK1/2 pathway with the factor VIIa pro-proliferative effects inhibited by both PAR2 and ERK1/2 inhibitors\(^{105}\).

1.4.2 The role of TF in cancer angiogenesis
TF has an important role in cancer angiogenesis\(^{271,272}\) which is also mediated via the PAR2 receptor. In mouse models, TF overexpressing sarcoma cancer cells produced more vascular, larger tumours with higher levels of VEGF than TF under-expressing and control cells\(^{273}\). Use of warfarin did not affect the development of cancer, suggesting that the TF effects on angiogenesis are mediated via a separate pathway from the normal coagulation pathway. The link between TF and VEGF has also been demonstrated in colorectal cancer with high TF expressing colorectal cancers being associated with high levels of VEGF and higher micro-vessel density in the cancer specimen (P<0.001)\(^{221}\).

1.4.3 The role of TF in metastasis
Inhibition of the coagulation pathways reduces metastases in murine tumour models of both melanoma and pancreatic cancer. Esumie et al\(^{274}\) used recombinant desulfatohirudin, a thrombin inhibitor, and showed inhibition of melanoma metastasis to the lungs. This anti-metastatic effect has also been demonstrated with TF, factor Xa and thrombin antibodies\(^{275-277}\). Similarly, TF antibody inhibited the ability of pancreatic cancer cells to metastasise to the liver\(^{278}\). This suggests that
TF, acting through the coagulation pathway, may have a stimulatory role in the development of metastases.

TF may promote metastasis via activation of platelets and the formation of tumour emboli. The formation of a clot around tumour cells provides a protective niche for malignant cells in circulation. The platelet aggregate around circulating malignant cells protect them from attack by natural killer cells and increases their metastatic potential\(^{279}\). Gorelik et al\(^{280}\) investigated the anti-metastatic effect of heparin on normal mice and mice with depressed natural killer cells. Normal mice cleared the tumour cells quicker in the presence of heparin but not in mice with depressed natural killer cells. This could be explained by the reduction of platelet aggregation and fibrin surrounding the circulating cells in heparin treated mice therefore removing protection from natural killer cells.

Both PAR1 and PAR2 are involved in the control of inflammation and immune response. The abnormal activation by TF of PAR1 and PAR2 result in an immune-privileged environment preventing the immune system from undertaking its normal role and attacking damaged cells\(^{281,282}\).

### 1.4.4 The role of TF in anoikis resistance

Normal epithelial cells undergo apoptosis if there is loss of contact with the basement membrane. This ensures that cells that are not in the correct location undergo cell death and that cells that detach from the basement membrane are not able to survive or grow in another location. This apoptosis on detachment from the basement membrane has been called anoikis\(^{283}\). For cancers to be able to metastasise they have to acquire resistance to anoikis\(^{284}\).

Factor VIIa has a TF dependant role in preventing apoptosis\(^{107}\) and anoikis \(^{285}\). This ability of TF pathway proteins to prevent anoikis and therefore enhance metastatic potential may partially explain the role of TF in the development of metastasis. Sorensen et al\(^{107}\) demonstrated a factor VIIa dependent reduction in anoikis in baby hamster kidney cells grown in non-adherent conditions in serum free media. This effect was blocked by anti-TF antibodies but was independent of factor Xa.

The anti-anoikis effects appears to result from activation of the ERK1/2 (p44/42 MAPK) and AKT pathways with selective inhibition of the ERK1/2 resulting in a small reduction in the factor VIIa/TF anti-apoptotic effect. Inhibition of the AKT pathway by inhibition of its mediator (PI3K) fully inhibited the factor VIIa/TF anti-apoptotic effect\(^{107}\). This suggests that the anti-apoptotic effect of VIIa is induced via the
FVIIa/TF complex induction of phosphorylation of AKT via PI3K. In breast cancer cells anoikis was not affected by thrombin inhibition, implying it is thrombin independent. Inhibition of ERK1/2 and the AKT pathways when undertaken separately prevented anoikis resistance, suggesting that both pathways are involved in VIIa induced anoikis resistance. These findings suggest that both pathways have an anti-apoptotic effect but that the AKT pathway effect is more pronounced.

1.4.5 TF may promote cancer stem cells
There is limited evidence that TF promotes cancer stem cell activity, with conflicting reports in the literature. The effect of TF on stem cell in colorectal cancer has not been investigated.

Increased sphere formation and increased TF expression is seen in CD133+ve compared to CD133-ve vulval squamous cell carcinoma. TF antibody significantly delayed in vivo tumour formation following mouse tail injection of these CD133+ve cells. This suggests TF is necessary for tumour initiation and it is tempting to consider that this might be acting via cancer stem cells. It is also possible that the TF antibody delayed tumour growth by interrupting other non-stem cell related oncogenic TF effects. In breast cancer one researcher has demonstrated that high TF expressing cells have more stem cell activity in-vitro with increasing sphere forming ability and higher expression of stem cell markers. This is contradicted by research in the breast cancer cell line MDA-MB-231 which has demonstrated that cells expressing TF are differentiated and are not the same population as cells expressing EPCR (a breast cancer stem cell marker). This suggests that TF expressing cells are not stem cells.

There has been no published research directly investigating the relationship between stem cells and TF in colorectal cancer but inferences can be made from work on the K-ras mutation and stem cells. It has been shown that the presence of the K-ras mutation increases the expression of TF in colorectal cancer. In a study of the effect of K-ras on stem cell activity in-vitro it has been shown that the presence of the K-ras mutation does not affect the stem cell population. It would be expected that if TF promoted the activity of cancer stem cells then so would the K-ras mutation (given the control that K-ras has on TF expression). Another study demonstrated that K-ras can increase the stem cell population but only in cancer cells with an additional APC mutation. This suggests that the APC mutation may be required for K-ras (and therefore possibly TF) to increase the stem cell population. TF has been shown to induce the ERK1/2 signalling cascade which has
been shown to trigger the transition from pluripotent embryonic stem cells from self-renewal to differentiated cell type in both normal\textsuperscript{292-294} and cancer cell lines\textsuperscript{295}. This suggests that TF may have a role in transforming cells away from stem cells to the differentiated cell type. The research to date is equivocal with some research suggesting a role of TF in promoting cancer stem cells and other research suggesting that it has a role in inhibiting stem cells.

1.5 **Anticoagulants as anti-cancer treatment**

Several studies have investigated the potential anti-cancer effects of existing anticoagulants.

**1.5.1.1 Warfarin**

Evidence for a possible anti-cancer effect of warfarin is found in a prospective randomised trial studying of 902 patients diagnosed with VTE\textsuperscript{296}. Patients were randomised to 6 weeks or 6 months of warfarin then annual follow up. A higher risk of developing cancer was seen in the study group than in the general population, indicating a link between VTE and cancer, but over the follow-up period (mean follow-up 8.1 years) patients who were randomised to 6 months of warfarin had a reduced frequency of cancer diagnosis (odds ratio 1.6 (95% CI 1.1 – 2.4)) which could not be explained by gender, age or whether the VTE was idiopathic or not.

The anti-cancer effect of warfarin may be specific to types of cancer and lung cancer in particular seems to be a case in point. A randomised controlled study undertaken in 1981 in which age, cancer stage and WHO stage matched non-small cell lung cancer were randomised to receive standard treatment or standard treatment with the addition of warfarin\textsuperscript{297}. This study showed that the addition of warfarin to standard treatment more than doubled median survival from 24 weeks for the control group (25 patients) to 50 weeks in the warfarin group (25 patients). The number of patients in the study was small. However, a further similar study\textsuperscript{298} undertaken by the same research group, randomised patients with colorectal, head and neck and prostate cancer as well as non-small cell cancer to receive or not to receive warfarin. This again showed a survival benefit in non-small cell lung cancer but demonstrated no improvement in the other cancers.

These two isolated studies have shown some benefit from warfarin in non-small cell cancer however they have not been repeated and the benefit has not been seen in other cancers.
1.5.1.2 Heparins

Some evidence regarding the use of LMWH as an anti-cancer treatment came from analysis of trials into treatment of VTE with LMWH compared to unfractionated heparins.

A meta-analysis of trials comparing LMWH and unfractionated heparins undertaken for VTE treatment included subgroup analysis in cancer patients. Cancer patients receiving treatment for VTE with LMWH compared to unfractionated heparin showed a 40% improved mortality at 3 months. This difference persists after correction for other prognostic indicators. The cause of mortality for these patients was not established and it is conceivable that the increase in survival is not as an effect of any anti-cancer properties but merely that LMWH are better at preventing thromboembolic death than unfractionated heparins.

A randomised controlled trial (n=385) of LMWH versus placebo for a year in patients with locally advanced or metastatic malignancy (arising from multiple different sites) had a primary end point of 1 year overall survival. Overall, there was no significant survival benefit seen with LMWH treatment (p=0.19). A subset of longer term survivors (beyond 17 months) were analysed separately as they were felt to have a more indolent disease than the rest of the patients, many of whom died shortly after randomisation. There was a benefit in survival of 60% vs 36% at 3 years (p = 0.03) in those treated with LMWH in this subgroup. However, subgroup analysis is problematic as there were possibly other confounding factors (for example different tumour biology) and prospective identification of a longer-term survival cohort is impossible (as this group was selected on retrospective survival data not on prognostic factors) making targeted LMWH treatment impractical.

A randomised controlled study was undertaken where 244 patients with non-small cell lung cancer, hormone refractory prostate cancer or locally advanced pancreatic cancer were randomised to LMWH or no treatment in addition to their normal treatment. The median survival was 13.1 months in the treatment arm and 11.9 months in the control arm (HR 0.94 95%CI 0.75 – 1.18) demonstrating no significant survival benefit. A cohort study by Kucukoner et al also failed to demonstrate survival benefit in LMWH treated, compared to no LMWH in inoperable small cell lung cancer patients (n=82) but this trial was fraught with methodological problems, including selection bias in allocation of treatment.

A more recent randomised controlled phase III study (FRAGMATIC) has been presented at the International Conference of Thrombosis and Haemostasis. Patients
with lung cancer were randomised to LMWH in addition to standard treatment for 24 weeks, the primary end point of this study being overall survival. Formal publication is still awaited but early results show no survival benefit in the LMWH treated arm\textsuperscript{304}. These studies when taken together suggest that LMWH does not have a role as an anti-cancer treatment.

1.6 Summary and Hypotheses

This thesis aims to examine, using in-vitro techniques and a prospective clinical study, the role of the TF pathway in colorectal cancer. Specifically, it aims to determine:

- The role of TF in promoting cancer stem cells, anoikis resistance and proliferation in colorectal cancer cell lines
- The correlation between a pro-coagulant tumour phenotype and clinicopathological factors in colorectal cancer
- The relationship between TF pathway proteins expressed by colorectal cancer and the development of post-operative VTE and the use of biomarkers to predict VTE development.

1.6.1 Hypothesis 1. TF increases colorectal cancer stem cell activity, anoikis resistance and proliferation in vitro

1.6.1.1 Summary of current knowledge

Cancer stem cells represent a population of cells that are resistant to treatment and therefore inhibition of cancer stem cells may reduce treatment resistance. There is limited evidence regarding the role TF has in their activity in other cell lines but no research has been undertaken in colorectal cancer\textsuperscript{287 228 288}. In addition TF, and its ligand Factor VIIa, via P13K/AKT and ERK1/2 pathways, increases cells ability to avoid anoikis, which is necessary for formation of metastases\textsuperscript{107 285}. The P13K/AKT and ERK1/2 pathways are activated directly by TF and indirectly by TF / thrombin activation of PAR1 and PAR2. Both the P13K/AKT and ERK1/2 are known to increase proliferation. In several cancers, including colorectal, TF inhibition reduces proliferation and results in smaller tumour formation in-vivo \textsuperscript{105 267 269 270 53 54}. These findings are supported clinically with high TF expressing colorectal cancers being more locally aggressive and also forming metastases earlier\textsuperscript{222 226}. It has not been demonstrated previously that TF over expression results in increased proliferation in colorectal cancer cell lines.
1.6.2 Hypothesis 2. A colorectal cancer with prothrombotic phenotype correlates with poor prognosis clinicopathological features and early recurrence

1.6.2.1 Summary of current knowledge

In colorectal cancer TF and PAR2 expression is associated with advanced disease\textsuperscript{221, 238} whilst in prostate cancer PAR1 expression is related to increased PSA\textsuperscript{237}. Very little research has been undertaken into the expression of thrombin. The clinical significance of PAR1 and thrombin in colorectal cancer has not been researched. There is also no research into stromal expression of TF in colorectal cancer, although in breast cancer stromal TF expression is associated with the development of invasive disease\textsuperscript{35, 36}.

Systemic clotting pathway activation in colorectal cancer is associated with a poor prognosis. D-dimer is elevated in more advanced cancers\textsuperscript{250}. In lung, TAT is correlated with advanced disease. However in colorectal cancer this has not been demonstrated\textsuperscript{248}. Other promising procoagulant biomarkers that may have a role as prognostic biomarker in cancer are P-selectin and sGPV.

1.6.3 Hypothesis 3. TF pathway biomarkers are predictive biomarkers of VTE

1.6.3.1 Summary of current knowledge

VTE is common in patients undergoing surgery for colorectal cancer and is a leading cause of morbidity and mortality\textsuperscript{153, 154, 189}. Thromboprophylaxis has risk and cost implications. Various serological biomarkers have been suggested that may predict the development of a post-operative VTE allowing the rationalisation of treatment. The proposed markers include d-dimer\textsuperscript{168} and P-selectin\textsuperscript{211} but their appropriateness in colorectal cancer needs further clarification. Other possibilities include TF, TAT and sGPV.

1.7 Aims

1.7.1 TF’s role in colorectal cancer stem cell, anoikis resistance and proliferation

Determine the effect of TF on cancer stem cell activity, anoikis resistance and proliferation by comparing:

1. Intrinsically high TF expressing colorectal cancer cell lines to intrinsically low TF expressing colorectal cancer cell lines
2. Colorectal cell lines transfected to over express TF and negative controls
3. Colorectal cell lines transfected to knock down TF compared to negative controls

Explore the effect of TF expression and VIIa activity on known down-stream pathways resulting from TF activation:

1. AKT
2. MAPK.

1.7.2 Relationship between a prothrombotic phenotype and a poor cancer prognosis

In a prospective cohort study of patients with non-metastatic colorectal cancer:

- Determine epithelial and stromal expression of TF, Thrombin, PAR1 and PAR2 in cancer compared to normal colorectal tissue
- Determine if epithelial and stromal expression of TF, thrombin, PAR1 and PAR2 correlate with and predict for clinicopathological factors: T stage, Node involvement, TNM stage, Dukes stage, LVI and differentiation
- Determine if epithelial and stromal expression of TF, thrombin, PAR1 and PAR2 correlate with and predict for early disease recurrence
- Determine if systemic markers of the TF pathway are elevated in cancer compared to benign control patients
- Determine if systemic markers of the TF pathway correlate with T stage, Node involvement, TNM stage, Dukes stage, LVI and differentiation.

1.7.3 Biomarkers for development of VTE in patients with colorectal cancer

In a prospective cohort study:

- Determine the rates of screen detected and symptomatic VTE in patients with colorectal cancer and benign controls
- Determine if a prothrombotic cancer phenotype is associated with development a VTE
- Determine if systemic markers of activation of the TF pathway are predictive biomarkers for postoperative VTE.
2 Materials and Methods

2.1 In vitro studies

The in vitro studies were undertaken to determine the effect of TF on:

- Colorectal cancer stem cells
- Anoikis resistance in colorectal cancer cells
- Proliferation in colorectal cancer cells.

2.1.1 Cell lines

Eight colorectal cancer cell lines were purchased from the American Type Culture Collection (ATCC). Details of their origin and ATCC catalogue number are given in Table 2.1. All cell lines were authenticated by multiplex PCR assay using the AmpT/STR system (Applied Biosystems) and subjected to regular mycoplasma testing.

2.1.2 Materials used

Details of reagents used are given in Table 2.2. Details of kits used are given in Table 2.3. Details of antibodies used are given in Table 2.4. Details of buffers used are given in Table 2.5. Details of cell culture media composition are given in Table 2.6.
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<th>Origin</th>
<th>ATCC catalogue number</th>
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<td>CCL 227</td>
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<tr>
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<td>SW480</td>
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Table 2.1 Origin of cell lines.

Details of colorectal cancer cell lines used.
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<th>Reagent</th>
<th>Company</th>
<th>Catalogue number</th>
</tr>
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<tbody>
<tr>
<td>Alamar Blue</td>
<td>Invitrogen</td>
<td>DAL1025</td>
</tr>
<tr>
<td>Aldefluor Buffer</td>
<td>Stem Cell Technologies</td>
<td>01701</td>
</tr>
<tr>
<td>B27 Supplement</td>
<td>Gibco</td>
<td>17504-044</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>Sigma</td>
<td>A9418</td>
</tr>
<tr>
<td>Dimethyl-sulfide</td>
<td>Sigma</td>
<td>D5879</td>
</tr>
<tr>
<td>DMEM media</td>
<td>Sigma</td>
<td>D6546</td>
</tr>
<tr>
<td>DMEMF-12 Media</td>
<td>Gibco</td>
<td>31331-028</td>
</tr>
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<td>Foetal Bovine Serum</td>
<td>Sigma</td>
<td>F7524</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>Sigma</td>
<td>G7513</td>
</tr>
<tr>
<td>Lipofectamine</td>
<td>Invitrogen</td>
<td>1168-027</td>
</tr>
<tr>
<td>McCoys 5A Media</td>
<td>Gibco</td>
<td>26600-23</td>
</tr>
<tr>
<td>Penicillin-streptomycin</td>
<td>Sigma</td>
<td>SLBB9306</td>
</tr>
<tr>
<td>Poly(2-hydroxyethylmethacrylate)</td>
<td>Sigma</td>
<td>P3932</td>
</tr>
<tr>
<td>(polyhema)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polybrene</td>
<td>Santa Cruz</td>
<td>SC-134220</td>
</tr>
<tr>
<td>Precision Plus Protein Standards</td>
<td>BioRad</td>
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<td>Puromycin</td>
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<td>Recombinant Vlla</td>
<td>Enzyme Research</td>
<td>HFVII</td>
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<tr>
<td>Laboratoritories</td>
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<td></td>
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<tr>
<td>RPMI-1640 Media</td>
<td>Sigma</td>
<td>R0883</td>
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<tr>
<td>Trizma base</td>
<td>Sigma</td>
<td>T1503</td>
</tr>
<tr>
<td>Trypan Blue</td>
<td>Sigma</td>
<td>T8154</td>
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<tr>
<td>Trypsin-SDTA</td>
<td>Sigma</td>
<td>SLBH5917</td>
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<td>Tween 20</td>
<td>Sigma</td>
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<tr>
<td>Western Block Reagent</td>
<td>Roche</td>
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Table 2.2 Reagents.

Details of reagents used.
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<tr>
<td>Aldefluor</td>
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</tr>
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<td>Pierce-BCA protein assay kit</td>
<td>Thermo scientific</td>
<td>23225</td>
</tr>
<tr>
<td>P-selectin ELISA kit</td>
<td>Life Technologies</td>
<td>KHS2021</td>
</tr>
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<td>sGPV ELISA kit</td>
<td>Stago</td>
<td>00276</td>
</tr>
<tr>
<td>Thrombin-Antithrombin complex ELISA</td>
<td>Sysmex</td>
<td>OWMG 15</td>
</tr>
<tr>
<td>Tissue Factor quantikine ELISA</td>
<td>R&amp;D systems</td>
<td>DCF300</td>
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Table 2.3 Commercial kits.

Details of commercial kits used.
<table>
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<tr>
<th>Antibody</th>
<th>Company</th>
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<th>Conjugation</th>
<th>Catalogue number</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD133</td>
<td>Miltenyi Biotec</td>
<td>Mouse</td>
<td>PE</td>
<td>293C3</td>
<td>FACS</td>
</tr>
<tr>
<td>CD44</td>
<td>Beckman Coulter</td>
<td>Mouse</td>
<td>FITC</td>
<td>IMI219U</td>
<td>FACS</td>
</tr>
<tr>
<td>Goat anti-mouse</td>
<td>Invitrogen</td>
<td>Goat</td>
<td>APC</td>
<td>A865</td>
<td>FACS</td>
</tr>
<tr>
<td>PAR1</td>
<td>Santa Cruz</td>
<td>Mouse</td>
<td>NA</td>
<td>SC-13503</td>
<td>WB / IHC</td>
</tr>
<tr>
<td>PAR2</td>
<td>Santa Cruz</td>
<td>Mouse</td>
<td>NA</td>
<td>SC-13504</td>
<td>WB / IHC</td>
</tr>
<tr>
<td>Phospho-AKT</td>
<td>Cell signalling</td>
<td>Rabbit</td>
<td>NA</td>
<td>4051</td>
<td>WB</td>
</tr>
<tr>
<td>Phospho-ERK1/2</td>
<td>Cell signalling</td>
<td>Rabbit</td>
<td>NA</td>
<td>9101</td>
<td>WB</td>
</tr>
<tr>
<td>Rabbit anti-goat</td>
<td>Dako</td>
<td>Rabbit</td>
<td>HRP</td>
<td>P0449</td>
<td>WB</td>
</tr>
<tr>
<td>Sheep anti-mouse</td>
<td>GE Healthcare</td>
<td>Sheep</td>
<td>HRP</td>
<td>NA931V</td>
<td>WB</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>NA</td>
<td>AB83981</td>
<td>IHC</td>
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<tr>
<td>Tissue Factor</td>
<td>Sekisui</td>
<td>Mouse</td>
<td>NA</td>
<td>ADG4508</td>
<td>WB / FACS</td>
</tr>
<tr>
<td>Total AKT</td>
<td>Cell signalling</td>
<td>Rabbit</td>
<td>NA</td>
<td>9272</td>
<td>WB</td>
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<tr>
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<td>4695</td>
<td>WB</td>
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<td>β-Actin</td>
<td>Santa Cruz</td>
<td>Goat</td>
<td>NA</td>
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<td>7AAD</td>
<td>Life technologies</td>
<td>NA</td>
<td>NA</td>
<td>A1310</td>
<td>FACS</td>
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</tbody>
</table>

Table 2.4 Antibodies

Details of antibodies used. (WB= western blot, FACS= fluorescence activated cell sorting, IHC=immunohistochemistry)
<table>
<thead>
<tr>
<th>Buffer</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading</td>
<td>40% Glycerol, 8% SDS, 0.8% bromophenol blue, 20% β-mercaptoethanol</td>
</tr>
<tr>
<td>Lysis</td>
<td>10 ml RIPA Buffer (Sigma-Aldrich), 1 tablet PhosStop (Roche), 1 tablet Complete ultra tablets mini (Roche)</td>
</tr>
<tr>
<td>Running</td>
<td>3% Tris HCL, 14.2% Glycine, 1% SDS</td>
</tr>
<tr>
<td>TBS-Tween</td>
<td>10mM Tris HCL, 150 mM NaCl, 0.05 (v/v) Tween 20</td>
</tr>
<tr>
<td>Transfer</td>
<td>1.4% Glycine 0.3% Tris HCL, 20% methanol</td>
</tr>
</tbody>
</table>

*Table 2.5 Buffer formulations.*

*Compositions of buffers used.*

<table>
<thead>
<tr>
<th>Media</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonosphere</td>
<td>DMEM/F12, B27 supplement, 20 mg/ml rEGF</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium (DMEM), 10% Foetal Calf Serum (FCS), 2mM L-Glutamine (LG), 50ug/ml Penicillin / Streptomycin (PS)</td>
</tr>
<tr>
<td>McCoys</td>
<td>McCoy’s 5A Medium , 10% FCS, 50 ug/ml PS</td>
</tr>
<tr>
<td>RPMI</td>
<td>RPMI-1640 Medium, 10% FCS, 2mL LG, 50 ug/ml PS</td>
</tr>
</tbody>
</table>

*Table 2.6 Cell culture media composition.*

*Compositions of cell culture media used.*
2.1.3 **Creation of a stable transfection**

Stable transfections were made to allow a constant over-expression and knock-down of tissue factor in the cells.

Stable transfections were made for the over-expression of tissue factor (GeneCopepia) and tissue factor knock-down (Santa-Cruz). The cells lines DLD-1 and SW620 were transfected with the active gene and relevant controls (negative control and Green Fluorescent Protein (GFP)).

2.1.3.1 **Constructing Tissue factor over-expression lentiviral particles**

Lentiviral particles were constructed for tissue factor over-expression using EX-Q0306-Lv105-10 (GeneCopoeia), a negative control of EX-NEG-Lv105 (GeneCopoeia) and a GFP control of CS-HPRM25711-LvPF02 (GeneCopoeia) cDNA expression plasmids.

1.5x10⁶ 293 Ta cells (GeneCopoeia) were plated in 10 cm diameter dish and incubated for 2 days with DMEM media supplemented with 10% heat inactivated FBS at 37°C with 5% (v/v) CO₂/air. After 2 days, the DNA/EndoFectin lenti complex was prepared. 2.5 μg of cDNA expression plasmid was mixed in 200 μl of Opti-MEM (Invitrogen) with 5 μl of Lenti-pack HIV. Separately, 15 μl of Endofectin Lenti was mixed with 200 μl of opti-MEM which was added to the expression plasmid mix. This was incubated at room temperature for 20 minutes. The DNA/EndoFectin mix was added to the dishes containing the 293 Ta cells and incubated overnight at 37°C at 5% v/v CO₂/air. After 10 hours the media was replaced with fresh DMEM media supplemented with 5% heat inactivated FBS. TitreBoost was added at a ratio of 1:500 to the media. The cells were cultured at 37°C at 5% v/v CO₂/air for a further 48 hours at which point the lentiviral particles were harvested by removing the media (which contained the lentiviral particles produced by the 293 Ta cells) and centrifuging at 500g for 10 minutes to remove cell debris. The supernatant was filtered through 0.45 μm polyethersulfone low protein binding filters. The lentiviral particles were frozen at –80°C in 1 ml aliquots until needed.

2.1.3.2 **Transfection of colorectal cell lines with lentiviral particles**

The cell lines DLD-1 and SW620 were transfected to over express and knock down TF.

**Transfecting cell lines to over express TF**

The DLD-1 and SW620 cells were cultured with 250,000 cells in a 6 well plate. For each cell line 5 wells were cultured (for active gene, negative control, GFP,
untransfected with puromycin and untransfected without puromycin). After 24 hours, the media was changed to 1 ml normal culture media supplemented with 5% heat inactivated FBS, L-glutamine and polybrene (10 µl per 20 ml media). 1 ml of the relevant lentiviral particles (active gene, negative control and GFP) were added with normal media alone for the untransfected controls. These were cultured at 37°C 5%v/v CO₂/air for 8 hours. The media was removed and replaced with cell media supplemented with 10% heat inactivated FBS and L-glutamine. After 72 hours the cells were split and placed in 10 cm plates. After 120 hours, media supplemented with puromycin was added to all wells except to the untransfected control without puromycin. The puromycin was used to select for cells that had received the plasmid as the plasmid contains a gene that renders the cells resistant to puromycin that would normally be toxic. To ensure that an adequate dose of puromycin was used untransfected cells were treated with the same dose of puromycin to ensure that it resulted in complete cell death.

**Transflecting the cell lines to knock down TF**

Cultures of 25,000 DLD-1 and SW620 cells were grown in 12 well plates. Each cell line had 5 wells cultured. After 24 hours, the media was changed to 1 ml normal culture media supplemented with 5% heat inactivated FBS, L-glutamine and polybrene (10 µl per 20 ml media). For the knock-down and negative control 20 µl of lentiviral particles were added. These were cultured at 37°C 5%v/v CO₂/air for 8 hours. The media was removed and replaced with cell media supplemented with 10% heat inactivated FBS and L-glutamine. After 72 hours the cells were split and placed into 6 well plates. After 120 hours media supplemented with puromycin was added to all wells except to the untransfected control without puromycin to select for the successfully transfected cells.

2.1.4 **Cell culture**

2.1.4.1 **Monolayer culture**

Monolayers of the human colorectal cells lines DLD1, SW620, Colo205, HCT116, LS174T HT29, LOVO and SW480 were cultured in their relevant media with 10% FBS and L-glutamine (Table 2.7). The cells were maintained at 37°C in humidified 5%v/v CO₂/air. Cells were passaged at approximately 80% confluence at a ratio of 1:5. Cell lines were not cultured beyond 20 passages.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colo205</td>
<td>RPMI</td>
</tr>
<tr>
<td>DLD-1</td>
<td>McCoys5A</td>
</tr>
<tr>
<td>HCT116</td>
<td>McCoys5A</td>
</tr>
<tr>
<td>HT29</td>
<td>RPMI</td>
</tr>
<tr>
<td>LOVO</td>
<td>DMEM</td>
</tr>
<tr>
<td>LS174T</td>
<td>RPMI</td>
</tr>
<tr>
<td>SW480</td>
<td>DMEM</td>
</tr>
<tr>
<td>SW620</td>
<td>DMEM</td>
</tr>
</tbody>
</table>

Table 2.7 Cell culture media
**Cell passage**
Monolayer cells were suspended by incubation with 1% trypsin at 37°C until complete loss of adherence. The trypsin was inactivated by the addition of complete media at a ratio of 1:3 trypsin:media. A cell pellet was formed by centrifuging at 800g for 2 minutes. The pellet was re-suspended in complete media.

2.1.4.2 Colonosphere Culture.
Colonosphere culture was undertaken to determine the proportion of cells having stem cell properties. This is determined by the proportion of cells that form spheres when grown at low densities in non-adherent conditions.

**Creation of flasks for culture in non-adherent conditions**
Flasks for non-adherent conditions were prepared by coating the relevant flasks with 1.2% (w/v) with polyHEMA (poly-2-hydroxyethyl methacrylate) dissolved in 95% ethanol. These were baked at 60°C until dry. Cells were grown in colonosphere media.

**Colonosphere culture method**
A single-cell suspension in PBS was made by enzymatic (1% trypsin) and mechanical (using 25 gauge needle with 10 passes) separation. The cells were seeded at a density of 300 cells per cm² in a 6 well non-adherent plate.

To determine the colonosphere forming potential, the total number of colonospheres with a diameter greater than 50µm were counted using a microscope fitted with a reticle at 4x magnification and the number of cells seeded was divided by the number of colonospheres. For the colonosphere assay, three wells were used for each variable in each experiment and the experiments were replicated in triplicate.

2.1.5 Anoikis resistance
Anoikis resistance experiments were undertaken to determine the proportion of cells that possess the ability to resist anoikis when seeded at low density in non-adherent conditions.

Cells were grown in non-adherent plates in colonosphere media at a density of 300 cells/ cm². At 0, 2, 4, 6, 10, 24 and 30 hours the media containing the cells was collected and centrifuged at 800g for 10 minutes. The cell pellet was resuspended in 20 µl of PBS and 20 µl of trypan blue was added. Alive / dead counting was carried out by using an Automated Cell Counter (BioRad) in triplicate for each experiment. The experiment was repeated three times.
2.1.6 Proliferation assay
The Alamar blue proliferation assay was used to determine the rate of cellular proliferation and the effect of variable manipulation of the proliferation rate. The Alamar blue assay functions as metabolically active cells convert the active ingredient resazurin to the fluorescent molecule, resorufin. The level of fluorescence is proportional to the number of metabolically active cells. By measuring the change in fluorescence at different time points this indicates the rate of proliferation. The amount of fluorescence was measured by the level of absorption measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm on a FLUOstar Omega software V1.20 (BMG Labtech).

Cells were plated at 10,000 cells per 200 µl of media in a 96 well plate. 6 wells were used per cell density. Cells were cultured at 37°C in 5% v/v CO₂/air. At 6, 24, 48, 72 and 96 hours 2 µl of Alamar blue was added to each well and incubated for 60 minutes at 37°C in 5% v/v CO₂/air. The media was removed and the plate washed with PBS before 200 µl of fresh media was added. Six wells were used for each variable in each experiment. The experiment was repeated in triplicate.

2.1.7 Flow cytometry
Flow cytometry was used to determine the expression of cell surface markers on individual cells by using a fluorescent antibody to highlight the cell surface markers. This allows the presence and amount of expression to be quantified.

Antibody staining was undertaken as optimised (see below). Flow cytometry was carried out using a Becton Dickins FACS calibur with BD CellQuest Pro version 6 and analysed using FlowJo 5.0 software. Relevant unstained samples were used for controls.

2.1.7.1 Tissue factor
300,000 cells were suspended in 100 µl of PBS and incubated with tissue factor antibody (Sekisui) at a concentration of 1:25 (v/v) for 20 minutes at room temperature in the dark. Cells were centrifuged at 800g for 2 minutes before being washed with PBS twice and re-suspended in 200 µl of PBS. The secondary antibody (APC) (Invitrogen A865) was added at a concentration of 1:100 before being incubated at room temperature for 20 minutes in the dark. Following centrifuging at 800g for 2 minutes this was washed twice with PBS and re-suspended in 400µl of PBS and 5 µl of 7AAD (Life Technologies) added as a cell viability marker to allow dead cells to be excluded from the analysis.
2.1.7.2 CD44 / CD133
300,000 cells were suspended in 100µl of PBS. 10 µl of conjugated antibody CD44 (FITC Beckman Coulter) or CD133 (PE Milteyi biotec) was added and incubated for 20 minutes at 37ºC before being centrifuged at 800g for 2 minutes and washed twice with PBS. They were re-suspended in 400 µl of PBS and 5 µl of 7AAD added.

2.1.7.3 Aldefluor assay
The aldefluor assay was undertaken using the Aldefluor Assay Kit (Stem Cell Technologies). 1,000,000 cells were suspended in 1 ml of assay buffer and 5 µl of aldefluor reagent was added, 500 µl was immediately transferred to a control tube containing DEAB, which is a potent inhibitor of ALDH1. Both samples were incubated at 37ºC for 30 minutes before being centrifuged at 800g for 2 minutes and the cell pellet re-suspended in 500 µl of assay buffer. 5 µl of 7AAD added to both the assay and control samples.

2.1.7.4 Gating strategy
Gating allowed the cells that express a surface marker to be identified. The presence of the antibody to the surface maker with either its conjugated fluorescent marker or a secondary conjugated antibody was detected by the use of the FACS Calibre. To determine the presence of a fluorescent marker a specific laser was used in a channel specific to the colour of the fluorescence (the channels were FL1 to FL4). To set the gate cells without the antibody were used and the gating set to exclude the natural level of fluorescence of the cells. The gate was applied on a plot of the level of the fluorescence in the relevant channel (FL1 – 4) vs side scatter (SCC). This gate was applied to the cells that had had the antibody added and the cells with the relevant surface antibody had a higher level of fluorescence than the cells without the antibody.

Gating for live cells. All experiments were undertaken with 7-AAD (Life Technologies) which is a cell viability indicator. A FL3 (7-AAD)/SCC plot was produced. This produced two distinct populations and allowed the dead (fluorescent) cells to be excluded from subsequent analysis by negative gating.

Tissue factor (APC). Once dead cells had been excluded with 7-AAD, a FL4 (APC) channel /SSC plot of the unstained control cells were produced. A gated region incorporating 0.5% of the highest fluorescing cells was created. This was applied to the stained cells and cells within this area were considered TF positive cells. The mean fluorescence was calculated from the TF positive cells.
**CD44 (FITC).** Once dead cells had been excluded with 7-AAD a FL1 (APC) channel /SSC plot of the unstained control cell was produced. A gated region incorporating 0.5% of the highest fluorescing cells was created. This was applied to the stained cells and cells within this area were considered CD44 positive cells. The mean fluorescence was calculated from the CD44 positive cells.

**CD133 (PE).** Once dead cells had been excluded with 7-AAD a FL2 (PE) channel /SSC plot of the unstained control cell was produced. A gated region incorporating 0.5% of the highest fluorescing cells was created. This was applied to the stained cells and cells within this area were considered CD133 positive cells. The mean fluorescence was calculated from the CD133 positive cells.

**Aldefluour assay.** Once dead cells had been excluded with 7-AAD and FL1 (aldefluour is detected on the FL1 channel)/SSC plot of the ALDH control (treated with DEAB to inhibit the action of ALDH). A gate was applied to select for the higher 0.2% of fluorescent cells. This gate was the applied to the cells treated without DEAB. The cells that were within this area were considered ALDH positive.

### 2.1.8 Western blotting

#### 2.1.8.1 Protein lysis

Cells were cultured in 10cm diameter plates. The cells were washed twice with ice cold PBS and 200 µl of RIPA lysis buffer with protease and phosphatase inhibitors added. The cells were incubated on ice for 10 minutes. A cell scraper was used to remove the cells into a centrifuge tube and they were immediately centrifuged at 4ºC for 10 minutes at 12,000g before the supernatant was removed and stored at -20ºC. Prior to use the protein content was established using the BCA protein quantification assay (Thermo-Scientific) which was quantified using a FLUOstar Omega plate reader. The protein was diluted in lysis buffer to a concentration 2 µg / µl.

#### 2.1.8.2 Western blot protocol

16 µl of protein was boiled with 4 µl of SDS sample buffer for 5 minutes at 100ºC and loaded into a precast gel (BioRad) along with Precision Plus protein duel colour standards ladder (BioRad). This was run at 120v with running buffer. The protein was transferred to a nitrocellulose membrane using a trans-blot turbo transfer system (BioRad). The quality of transfer was assessed using Ponceau S solution. The membranes were blocked using Magic Block for 1 hour at room temperature. The primary antibody was incubated in Magic Block overnight at 4ºC on a rocker. Following incubation, the membrane was washed for 1 hour with TBS-Tween changing the wash every 10 minutes. The membrane was incubated with the
secondary HRP-conjugated antibody on a rocker for 1 hour at room temperature. The membrane was washed with TBS-tween for 1 hour changing the wash every 10 minutes. ECL was added for 5 minutes before exposing the photographic film. Membranes were stripped using ReBlot strong (Millipore) prior to re-probing with further antibodies. A β-actin control was undertaken to ensure equal loading of proteins. For analysis of phosphorylation of ERK and AKT the analysis for both was undertaken on the same membrane.

2.1.9 Effect of TF and VIIa on AKT and ERK1/2 signalling
Transfected cells and their negative controls were plated at 1x10⁶ cells in a 10 cm plate in complete media. After 6 hours, the media was changed to serum free media for 48 hours at 37°C in 5% v/v CO₂/air. The media was changed for fresh serum free media for 1 hour to remove any accumulated growth factors secreted by the cells themselves. Following this, the media was changed for media with varying levels of factor VIIa at levels above and below physiological concentrations. Following incubation at 37°C in 5% v/v CO₂/air for 10 minutes the media was removed and the protein lysed as in section 2.1.8.1 and a western blot undertaken as in section 2.1.8.2.

2.1.10 Microscopy
An Olympus CKX41 microscope was used for all microscopic assessments.

2.1.11 Statistical Analysis
Throughout this project, laboratory experiments were undertaken in triplicate unless otherwise stated. Data is presented as mean +/- SEM. Statistical significance has been measured using parametric tests assuming equal variance. A standard t-test has been used for samples to assess difference between the test and control samples. Statistical analysis was undertaken using Excel version 15 (Microsoft 2013).

2.2 Clinical Study

2.2.1 Background
The clinical study CHAMPion (Cancer-induced Hypercoagulability As a Marker of Prognosis) is a prospective clinical cohort study to investigate the role of systemic and tissue markers of coagulation as a prognostic marker in colorectal cancer. CHAMPion has ethical approval from Oldham Research Ethics Committee (REC reference number 09/H1011/47). It is also registered on the United Kingdom Clinical Research Network Study Portfolio (UK CRN study ID 8685).
CHAMPion had been opened previously at the University Hospital of South Manchester NHS Foundation Trust. To aid recruitment, I have opened the study in 4 additional centres:

- Central Manchester Foundation Trust
- Salford Royal NHS Foundation Trust
- East Cheshire NHS Foundation Trust
- Stockport NHS Foundation Trust.

### 2.2.2 Inclusion and exclusion criteria

**Inclusion criteria:**

- Patients undergoing curative surgery for abdominal malignancy
- Patients undergoing major abdominal surgery matched for length of operating time / inpatient stay to the cancer patients
- Written informed consent
- Age over 18.

**Exclusion criteria:**

- History of VTE or coagulopathy
- Anticoagulation (not including prophylactic LMWH)
- Patients having taken oestrogens or anti-oestrogens in the last 2 weeks
- Patients having received neo-adjuvant chemotherapy
- Patients with WHO performance status 3 – 4
- Previous cancer treatment within 5 years (excluding non-melanoma skin cancers).

Patients taking aspirin were not excluded as there is no evidence that the use of aspirin reduces the incidence of VTEs. As it is commonly used in the population undergoing surgery for colorectal cancers due to co-morbid conditions its adoption as an exclusion criteria would result in a significant reduction of patients suitable for inclusion in the study.

### 2.2.3 Power calculation

The study has been powered to identify a difference in the rate of VTE in abdominal cancer patients undergoing surgery based on their pre-operative d-dimer. Stender et al\(^\text{305}\) reported DVT rates of 20% in colorectal cancer patients with elevated d-dimer, and 5% in patients with normal d-dimer. With 150 abdominal cancer patients, the study will have 80% power to detect a difference in DVT rates of 15% or more between those with and without raised d-dimer pre-operatively (ie 20% vs 5%).
assuming 43% of patients have a raised d-dimer. With an overall cancer recurrence rate of 30% at two years, the study will also have 80% power to detect differences in recurrence rates of 22% or more by two years (eg 42% vs 20%) using Kaplan Meier analysis.

The above calculations assumed the use of a simple chi-square test with the conventional 5% significance level.

Increasing age has been shown to correlate with d-dimer levels, therefore the sample size has been adjusted to account for age (increased by 10%). The power calculations are based upon simple analysis, but analysis adjusting for race and cardiovascular risk factors (possible confounders for activation of haemostasis) will be carried out.

Based on the power calculation, the number of patients to be recruited is 165 with cancer and 20 benign controls.

2.2.4 Data collection
Data was collected prospectively and from the patient’s notes by myself (UHSM) and research nurses at the additional sites using a standardised data collection form.

2.2.5 Plasma sample collection
Samples were taken at set time points:

- Pre-operatively
- Day 1 post-operatively
- Day 14 post-operatively
- 6 weeks post-operatively.

In total, 20 mls of blood were taken via venepuncture in evacuated tubes. This was separated as follows:

- 1 EDTA tube (sent for full blood count)
- 1 citrate tube for d-dimer and fibrinogen
- 1 CTAD tube
- 3 citrate tubes
- 1 serum gel.

2.2.6 Plasma Sample analysis
The EDTA sample was analysed for full blood count in the local hospital. For patients recruited at UHSM, a serum citrate sample was analysed immediately for d-
dimer and fibrinogen by the Anticoagulation and Haematology Research Unit at UHSM. For all other sites, the sample was centrifuged (as below) and stored at -80°C prior to transfer to UHSM for analysis. The method of analysis at UHSM was changed during the course of the study. Initial samples were analysed using miniVIDAS (BioMerieux Clinical Diagnostics, Marcy L’Etoilem France). This was changed to TOP500 (Instrumentation Laboratory, Bedford, MA USA) during the study. The effect of this change has also been studied in the unit and has demonstrated that both methods produce consistent results\textsuperscript{228}. The analysis also confirmed that frozen samples produced consistent results with fresh samples.

The remaining samples were centrifuged at 2500g for 20 minutes before the supernatant was transferred to a further tube for further centrifugation at 2500g for 20 minutes. The supernatant was aliquoted into 0.5 ml aliquots and stored at -80°C until batched ELISAs were undertaken for tissue factor and thrombin anti-thrombin complexes, sGPV and P-selectin.

2.2.6.1 ELISA methods

ELISA was undertaken to determine the level of expression in the serum of the proteins:

- TF
- TAT
- sGPV
- P-selectin.

The analysis was undertaken on frozen aliquots of serum. TF, TAT and P-selectin analysis was undertaken in serum in the presence of citrate and sGPV in the presence of CTAD.

Tissue factor

Expression of TF in the serum was measured by use of the Tissue Factor ELISA (R&D systems) according to the manufacturer’s instructions. Calibration was with samples of concentration from 0 – 5000 pg/ml. Control samples (QC) were undertaken with high, medium and low QCs which were positioned in duplicate at the start, middle and end of the 96-well plate. Serum samples were centrifuged at 2000g for 5 minutes. In duplicate, 100µL of serum was added to each well and diluted with 100µL of assay diluant. The plate was incubated at room temperature on a shaker at 500rpm for 2 hours. The plate was washed 5 times with 400µL of wash buffer. 200µL of substrate solution was added to each well and the plate
incubated in the dark at room temperature for 30 minutes. 50µL of stop solution was added and mixed on a plate shaker at 600 rpm until even colour was achieved. The plate was read with a MRXe plate reader (Dynex, Chantilly, USA) within 30 minutes.

**Thrombin-anti-thrombin (TAT)**

Expression of TAT in the serum was measured by use of the TAT Micro Thrombin antithrombin ELISA (Sysmex) according to the manufacturer’s instructions. Calibration was with samples of concentration from 0 – 60 ng/ml. Control samples (QC) were undertaken with mid-concentration QCs which were positioned in duplicate at the start, middle and end of the 96-well plate. Serum samples were centrifuged at 2000g for 5 minutes. 50µL of sample buffer was added to each well and in duplicate 50µL of serum was added to each well. The plate was mixed at 600rpm on a plate shaker for 10 seconds. The plate was incubated at 37ºC on a shaker for 15 minutes. The plate was washed 3 times with 300µL of wash buffer. 100µL of working conjugate solution was added to each well and the plate incubated at 37ºC on a shaker for 15 minutes. The plate was washed 3 times with 300µL of wash buffer and 100µL of Chromogen solution added. The plate was incubated in the dark at room temperature for 30 minutes. 100µL of stop solution was added and mixed on a plate shaker at 600 rpm until even colour was achieved. The plate was read with a MRXe plate reader (Dynex, Chantilly, USA) within 1 hour.

**sGPV**

Expression of sGPV in the serum was measured by use of the soluble Asserachrom sGPV immunoassay ELISA (Stago) according to the manufacturer’s instructions. Calibration was with samples of concentration from 0 – 195 ng/ml. A high and low concentration QC was positioned in duplicate at the start, middle and end of the 96-well plate. Serum samples were centrifuged at 2000g for 5 minutes. In duplicate, 200µL of diluted serum (diluted by adding 1000µL of sample dilatant to 50µL of serum) was added to each well and the plate was incubated at room temperature for 2 hours. The plate was washed 5 times with 300µL of wash buffer. 200µL of Anti-sGPV-peroxidase was added to each well and the plate incubated at room temperature for 2 hours. The plate was washed 5 times with 300µL of wash buffer. 200µL of tetramethybenzidine (TMB) substrate was added and incubated in the dark at room temperature for 5 minutes. 50µL of stop solution was added and mixed. The plate was read with a FLUOstar Omega (BMG Labtech, Ortenberg, Germany) plate reader after 15 minutes.
P-selectin

Expression of P-selectin in the serum was measured by use of the soluble P-selectin immunoassay ELISA (R&D systems) according to the manufacturer’s instructions. Calibration was with samples of concentration from 0 – 55 ng/ml. A mid-concentration QC was positioned in duplicate at the start, middle and end of the 96-well plate. Serum samples were centrifuged at 2000g for 5 minutes. In duplicate, 100µL of diluted serum (diluted by adding 285 µL of sample dilutant to 15 µL of serum) was added to each well and 100µL of P-selectin conjugate added to each well and mixed. The plate was incubated at room temperature for 1 hour. The plate was washed 3 times with 300µL of wash buffer. 100µL of substrate solution was added to each well and the plate incubated in the dark at room temperature for 15 minutes. 100µL of stop solution was added and mixed. The plate was read with a FLUOstar Omega (BMG Labtech, Ortenberg, Germany) plate reader within 30 minutes.

2.2.7 Clinical tests

Participants underwent full length duplex imaging of both legs to detect incidental DVTs at the following time points:

- Pre-operatively
- 6 weeks post operatively.

Duplex scanning was undertaken by Independent Vascular Services using IU22 scanners (Philips).

2.2.8 Histology investigations

Surgical specimens were formalin fixed, paraffin embedded and stained with haematoxylin and eosin (H&E) as in normal NHS procedures. These specimens were subject to analysis by a consultant histopathologist according to normal NHS procedures to produce a histological report achieving the minimum cancer dataset.

2.2.9 Tissue microarray (TMA) construction of tumour and normal samples

Two relevant paraffin blocks containing an area of cancer and a distant normal area were selected by a pathologist and the H&E side marked to select a representative area of malignant and normal epithelium.

Using the marked areas on the H&E slide as a guide, a TMA was created by a pathology technician (Ms Hannah Gregson) using a Manual Tissue Arrayer (MTA-01
Beacher Inc). Two 1mm cores of invasive and normal epithelium were extracted from the relevant donor block. These were placed in a recipient block with each subject having two cores for malignant and two for normal tissue mounted on separate TMAs.

2.2.10 Immunohistochemistry (IHC)

4µm sections of the TMA blocks were prepared at the Paterson histology facility. These then underwent IHC performed by Ms Hannah Gregson. The TMAs were stained for:

- TF
- PAR1
- PAR2
- Fibrinogen.

The IHC was undertaken on the Leica Bond using the Leica Bond Polymer Refine detection kit. The protocols for the IHC are given in Table 2.8.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Protocol</th>
<th>Antibody dilution</th>
<th>Isotype control</th>
<th>Antigen retrieval</th>
<th>Additional block</th>
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</thead>
<tbody>
<tr>
<td>TF</td>
<td>Protocol F with casein</td>
<td>1:100</td>
<td>IgG</td>
<td>pH9, 20 mins</td>
<td>10 min 10% casein</td>
</tr>
<tr>
<td>PAR1</td>
<td>Protocol F with casein</td>
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<td>IgG1</td>
<td>pH9, 20 mins</td>
<td>10 min 10% casein</td>
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<tr>
<td>PAR2</td>
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<td>IgG2a</td>
<td>pH6, 20 mins</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>Protocol F with casein</td>
<td>1:200</td>
<td>IgG</td>
<td>pH6 20 mins</td>
<td>10 min 10% casein</td>
</tr>
</tbody>
</table>

Table 2.8 IHC protocols
2.2.11 Evaluation of IHC staining

Stained slides were simultaneously scored by myself and a NHS consultant pathologist (Dr Anna Davenport). Prior to scoring, the H&E stained TMA slides were evaluated to ensure that a representative section of malignant or normal epithelium had been captured.

Evaluation of the intensity of IHC staining was undertaken separately for epithelial and stromal cells in both benign and malignant tissues.

There was heterogeneity evident in IHC staining therefore a semiquantitative method was undertaken. The use of the H score allows the overall expression of a protein to be calculated\textsuperscript{307}. The score is calculated by assessing individual cells level of expression into absent (score 0), low (score 1), medium (score 2) and high (score 3). The percentage of the total number of cells for each of these intensities is established. The H score is calculated by multiplying the percentage that each level of expression has by its score and adding these to give a total (maximum score 300). The formula for the calculation is $H = (\% \text{ score 1}) + (\% \text{score 2} \times 2) + (\% \text{ score 3} \times 3)$.

2.2.12 Data management, analysis and statistical methodology

2.2.12.1 Software used and presentation of data.

Data was entered directly into SPSS 22.0 for windows (IBM Corporation released 2013) which was used for all statistical analysis. Statistical analysis was undertaken under the guidance of Dr Julie Morris (Honorary Reader in Medical statistics).

Skewed continuous data (plasma; TF, TAT, d-dimer, sGPV and P-selectin, Tissue H score; TF, PAR1, PAR2 and thrombin) were logarithmically converted to normalise the data and allow parametric statistical analysis to be undertaken. Following analysis the values were reverse log converted. The reverse log converted data is presented as geometric mean +/- 95% confidence interval (CI). For log converting H score an arbitrary figure of 1 was added to all the scores (as 0 existed as a score and cannot be log converted). Once the analysis was undertaken and the values reverse log converted the arbitrary value of 1 was removed.

2.2.12.2 Univariate analysis

Students’ t-test and analysis of variance (ANOVA) were used for continuous data. Post-hoc analysis of ANOVA was undertaken by using Tukey test. Relationship between categorical data was compared using Fishers Exact test. Pearson’s Rank correlation was used to analysis correlations between normalised variables.
2.2.12.3 **Analysis of co-variance (ANCOVA)**

ANCOVA was used to determine the relationship between a dependent and independent variable whilst controlling for co-factors that had been shown to show a strong correlation with the dependant variable. The co-factors that were controlled for were:

- D-dimer- age
- TF- age
- Fibrinogen- presence of hypertension.

2.2.12.4 **Generalised estimating equations (GEE)**

GEE is a statistical method that compares differences in repeated measurements in a subject. It takes into account the longitudinal nature of the measurements (in time) and allows analysis of whether an individual factor determines the rate of change of the level of the measurements over time. It also allows compensation for the initial (pre-operative) measurement and any confounders.

2.2.12.5 **Correction for multiple analysis.**

As multiple analyses were undertaken the level of significance was reduced to 1% therefore a p value of 0.01 was taken as significant. Statistical analysis that reached this level of significance will require further analysis in a clinical study designed for this endpoint.
3 The role of Tissue Factor in colorectal cancer cell line stem cell activity, anoikis resistance and proliferation

3.1 Introduction

Tissue factor (TF) is a transmembrane protein that in normal tissues is present in the sub endothelial cells in the vascular system. TF, with its ligand FVIIa, initiates haemostasis via the tissue factor-thrombin pathway resulting in stable clot formation. In addition TF induces non-haemostatic pathways involved in inflammation and wound healing via PAR2.

Abnormal expression of tissue factor occurs in many cancers including colorectal cancer. In colorectal cancer TF expression correlates with poor prognostic factors including advanced Dukes' and TNM stage as well as an enhanced ability to form metastases.

In in-vivo models using lung, sarcoma and melanoma cancer cell lines TF have been shown to promote tumour growth, metastasis and angiogenesis. Research has suggested that in vulval squamous cell carcinoma and breast cancer TF promotes cancer stem cells. This is contradicted by other research in breast cancer that suggests that TF is a marker of differentiation. Cancer stem cells demonstrate treatment resistance and are a source of recurrence of cancers. No research has been carried out into the effect of TF on colorectal cancer stem cells.

In baby hamster kidney (BHK) and breast cancer cell lines, TF is necessary for anoikis resistance (a requirement for metastasis to take place). Anoikis is also a property of cancer stem cells. No research has been undertaken regarding the effect of TF on anoikis resistance in colorectal cancer.

Colorectal cancer cells when TF was inhibited with siRNA resulted in smaller tumours in vivo, a finding also seen in melanoma. This decrease in growth was explained by a lower micro-vessel density due to TF promoting angiogenesis. A direct effect of TF on proliferation has been seen in lung cancer where inhibition of TF reduces proliferation in-vivo and in-vitro. In colorectal cancer the addition of factor VIIa and up-regulation of PAR2 increases proliferation in vitro. This increase in proliferation is reduced by the use of TF antibody, PAR2 and ERK1/2 inhibitors.
The direct effect of TF on proliferation has not been investigated in colorectal cancer.

The objective of this chapter is to determine the effect of tissue factor expression on colorectal cancer cell line function. Specifically, I hypothesise that:

- increased TF expression promotes cancer stem cell activity
- increased TF expression promotes anoikis resistance
- increased TF expression promotes proliferation.

### 3.2 Colorectal cancer cell lines.

I aimed to investigate cell line function (stem cell activity, anoikis resistance and proliferation) in colorectal cancer cells with intrinsic high TF and intrinsic low TF expression. To select the most appropriate cell lines the level of tissue factor protein expression in eight colorectal cancer cell lines was determined by Western Blot (see section 2.1.8 for method) and Fluorescence-activated Cell Sorting (FACS) (see section 2.1.7 for method) (Figure 3.1).

DLD-1 was shown to have a high tissue factor expression and SW620 low expression so these two cell lines were chosen for subsequent experiments.
TF expression by wild type colorectal cell lines was confirmed by western blot and FACS. Lysates from the eight cell lines underwent western blot analysis for tissue factor (47kDa) expression (top panel). β-actin (42kDa) was used for approximate loading control. FACS was used to determine surface expression of tissue factor in the wild type cell line. The cell line DLD-1 expressed high levels of TF and SW620 low levels of TF expression. There was a 10x fold increase in level of TF expression in DLD-1 compared to SW620.
3.2.1 TF overexpression and TF knockdown in selected CRC cell lines

To determine the effect of TF on cell function the selected cell lines, DLD-1 and SW620 were transfected to overexpress and knock down TF.

To determine the effect of TF over expression on cell function, DLD-1 and SW620 were transfected using lentiviral particles containing TF cDNA. Lentiviral particles containing an empty vector were used as a negative control (GeneCopeia) (see section 2.1.3.2 for method).

To determine the effect of TF knockdown on cell function, DLD-1 and SW620 were transfected with shRNA lentiviral particles containing two target-specific constructs that encode for the shRNA that inhibits TF gene expression or non-targeting vector as a control (SantaCruz) (see section 2.1.3.2 for method).

To allow monitoring of a stable transfection, a separate control cell line underwent the same transfection methods with plasmids containing Green Fluorescent Protein (GFP) and visualised with a fluorescent light microscope. This was undertaken for DLD-1 (Figure 3.2) and SW620 (Figure 3.3).
GFP was used as a transfection control for the transfection of cDNA to over express and shRNA to knock down TF. The cells were transfected with the relevant GFP control and then subjected to the same conditions as the TF over expressing and knock down cells. This allowed the transfection to be visualised under a fluorescent light source microscope. The presence of GFP (seen as green in fluorescence source view) demonstrated that GFP has been successfully transfected into the cell. This confirmed the conditions for the transfections were suitable for successful transfection.
Figure 3.3 Verification of GFP transfection in SW620

GFP was used as a transfection control for the transfection of cDNA to over express and shRNA to knock down TF. The cells were transfected with the relevant GFP control and then subjected to the same conditions as the TF over expressing and knock down cells. This allowed the transfection to be visualised under a fluorescent light source microscope. The presence of GFP demonstrated that GFP has been successfully transfected into the cell. This confirmed the conditions for the transfections were suitable for successful transfection.
Following transfection the protein expression was confirmed by Fluorescence-activated cell sorting (FACS). The FACS analysis quantifies the amount of TF that is present on the cell surface. The results are expressed as a fold change compared to the negative control (Figure 3.4).

The cell lines transfected to over express TF demonstrated an increase in TF expression compared to their relevant negative controls. The fold change in TF expression compared to their negative control was; (DLD-1 TF+ve 2.85 (SE 0.12), p=0.001, SW620 TF+ve 4.61 (SE 0.79), p=0.01).

The cell lines transfected to knock down TF demonstrated a decrease in TF expression compared to their relevant negative control. The fold change in TF expression compared to their negative control was; DLD-1 TF-ve 0.73 (SE 0.02), p=0.01, SW620 TF-ve 0.79 (SE 0.04), p=0.01.

To verify the stability of the transfection over time FACS analyses of tissue factor expression were undertaken over multiple passages. For the TF over expressing cell lines it was monitored over 14 months and the TF knock down over 5 months, and in addition after freezing and thawing cells. Although there were variations in the fold change between the transfected cell line and the negative control the relevant increase or decrease in expression was preserved. This confirms that a stable transfection was achieved (Figure 3.5).
Figure 3.4 Confirmation of transfection of colorectal cell lines DLD-1 and SW620 to over express and knock down TF.

Transfection was confirmed by FACS analysis for each of the transfected cell lines. A. DLD-1 transfected to over express TF. B. DLD-1 transfected to knock down TF. C. SW620 transfected to over express TF. D SW620 transfected to knock down TF. The FACS analysis was the result of a minimum 3 repeats of the experiment. FACS displayed as mean florescence normalised to the negative control. Displayed with standard error of the mean (SEM). Student T-test used for statistical analysis.
**Figure 3.5** Confirmation of transfection over multiple passages in DLD-1 and SW620.

FACS analysis was undertaken over an extended time course to ensure that the transfection was stable. Successful prolonged over expression or knock down of TF was seen in A. DLD-1 TF+ve, B. DLD-1 TF-ve, C. SW620 TF+ve and D. SW620 TF-ve. Data is displayed as fold change in TF expression normalised to the relevant negative control.
3.3 Does TF expression promote colorectal cancer stem cell activity?

Stem cells possess the ability to self-renew and produce differentiated daughter cells. Stem cells are present in both normal and malignant tissue. In normal colonic and rectal tissue they are responsible for the constant renewal of the epithelium. They primarily reside in the base of the crypts and produce differentiated cells that migrate up the crypts to the epithelial surface. Cancer stem cells are less differentiated than the majority of cancer cells. In colorectal cancer, cancer stem cells are distributed throughout the cancer. They are thought to have resistance to chemotherapy and radiotherapy and are therefore responsible for tumour recurrence and treatment resistance.

As TF expression correlates with poor clinical prognosis in several cancers including colorectal cancer, and increased cancer stem cell number and activity also correlate with cancer outcome, the in-vitro effect of TF on cancer stem cell activity is now being investigated. In vulval squamous cell carcinoma and breast cancer, stem cell markers correlate with TF expression with high tissue factor expressing cells having increased stem cell like behaviour, although in breast cancer this is contradicted by other research. In vulval squamous cell carcinoma TF inhibition reduces tumorigenicity. No research has been undertaken into the role of tissue factor on cancer stem cells in colorectal cancer.

Stem cell activity can be estimated by laboratory assays including cancer sphere formation and the expression of stem cell markers. For a detailed discussion of these methods see section 1.1.5. In colorectal cancer the use of stem cell markers is controversial, however the most established in-vitro stem cell marker is Aldehyde dehydrogenase (ALDH). CD133 and CD44 are additional colorectal cancer stem cell markers, however the evidence base for their reliability is not as sound as for ALDH (see section 1.1.6.1).

3.3.1 Stem cell activity assays

3.3.1.1 Optimisation of the Colonosphere assay

Although the principle of the cancer sphere assay is well established in colorectal cancer there have been many different methods of undertaking the assay. For example different number of cells are plated and also different times are used to count the resultant spheres, by different authors.
The first aim of this work was to develop a reliable methodology for the colonosphere forming assay with the selected cell lines (DLD-1 and SW620).

The aim of a reliable colonosphere assay is to provide sufficient sphere formation to achieve the ability to detect statistical significance, without the formation of aggregates of spheres, which may occur when excessive sphere formation is allowed, as this makes the counting unreliable. For optimisation the protocol was based on that published by Shaw et al.\textsuperscript{51}

For a detailed methodology see section 2.1.4.2 but briefly single cells were plated in non-adherent, serum free conditions in cancer sphere media. After culturing the cells at 37\(^\circ\)C for 3 days the number of spheres that are present with a diameter of greater than 50\(\mu\)m are counted. This number is reflective of the proportion of undifferentiated cancer stem cells in the cells that were plated out. These undifferentiated cells have survived anoikis (programmed cell death on loss of contact with the basement membrane) and have reproduced forming a sphere of differentiated daughter cells.

The number of spheres that are present are then used to calculate the Colonosphere Forming Efficiency (CFE). CFE is the number of spheres present divided by the number of cells seeded expressed as a percentage.

To optimise the assay, six commonly studied colorectal cancer cell lines were used so as to allow for a range of sphere forming abilities to be accommodated into the assay. This is to ensure that the assay can be used in all of these cell lines. Each cell line was plated in a 6-well plate at varying densities from 200 – 500 cells/cm\(^2\). After culturing for 3 days the resulting spheres were examined and counted. There were variations between the cell lines in the number of spheres produced. At the higher densities there was marked sphere aggregation making counting difficult and unreliable. At the 300 cells/cm\(^2\) density, approximately 30 – 90 spheres were formed allowing sufficient sphere formation without significant aggregation. This density was therefore chosen for all cancer sphere assays.

The CFE for each cell line at 300 cells/cm\(^2\) are shown in Figure 3.6. The CFE for the cell lines ranged from 1.3 – 3\%. Representative examples for the spheres formed for each cell line are shown in Figure 3.7.
Figure 3.6 Colonosphere forming efficiency in six different colorectal cancer cell lines.

Cells were seeded at a density of 300 cells/cm² in non-adherent conditions. The number of spheres greater than 50µm that were present at day 3 were then counted. The CFE was calculated by dividing the number of cells seeded by the number of spheres and is expressed as a percentage. Presented as mean of 3 biological repeats with SEM.
Figure 3.7 Representative examples of cancer spheres

The spheres that formed by colorectal cancer cell lines after 3 days growth in non-adherent conditions. The cells were plated at a density of 300 cells per cm². After 3 days the spheres formed were visualised and photographed. The cancer spheres are discrete spheres with a diameter of greater than 50µm imaged using an Olympus CKX41 microscope fitted with a reticle.
3.3.2 Stem cell activity in wild type colorectal cancer cells

3.3.2.1 Sphere assay

The CFE of a high TF expressing cell line (DLD-1) and a low TF expressing cell line (SW620) was determined to establish if TF expression correlated with CFE. The cells were plated out at densities of 300 cells/cm² in 6 well plates. The experiment was performed in triplicate with three independent biological repeats. The resulting spheres were counted at day 3 (detailed methodology in section 2.1.4.2). The higher tissue factor expressing cell line (DLD-1) produced 10.52 fold (SE 4.10) fewer spheres than the lower tissue factor expressing cell line (SW620) (colonsphere forming efficiency of 0.70% (SE 0.13) vs 1.38% (SE 0.23), p=0.01 (Figure 3.8).
Figure 3.8 CFE in wild type high TF expressing cell line (DLD-1) compared to wild type low TF expressing cell line (SW620).

A. CFE of wild type colorectal cancer cells DLD-1 and SW620. 300 cells/cm² were seeded in non-adherent conditions. The resulting spheres were counted at day 3 and CFE calculated. B. The tissue factor expression by the respective cell lines as determined by FACS expressed as a fold change compared to SW620. Results displayed as mean from at least three biological repeats with SEM. Statistical analysis with student t-test
3.3.2.2 ALDH assay

ALDH expression was determined in the high tissue factor cell line (DLD-1) and low tissue factor expressing cell line (SW620) to determine if ALDH expression, a known stem cell marker, correlated with TF expression.

The ALDH expression was determined using the Aldefluor assay (StemCell Technologies). Detailed methodology is given in section 2.1.7.3 but briefly cells were incubated with aldefluor reagent (ALDH substrate) in the presence or absence of DEAB (a potent ALDH inhibitor). Following re-suspension in aldefluor buffer, the fluorescence indicating ALDH expression, was measured by use of a FACSCalibur (BD Biosciences) and analysed using Version 5.0 FlowJo software (FlowJo). The viability dye 7AAD was added to all the samples to allow the exclusion of dead cells from subsequent FACS analysis.

Examples of the gating used for one of the three repeat experiments are given in Figure 3.9. The gating enables cells with or without fluorescence to be selected when compared to an unstained control. Similar gating was used for the other replicates.

The percentage of cells that expressed ALDH was significantly higher in the low tissue factor expressing cell line (SW620) when compared to the high tissue factor cell line (DLD-1) (15.3% (SE 0.9) vs 26.9% (SE 1.4,) p=0.02). This represents a 1.8 times fold increase in the expression of ALDH by SW620 compared to DLD-1. (Figure 3.10). This is consistent with the finding that the low tissue factor expressing cell line (SW620) produces more spheres than the high tissue factor expressing cell line (DLD-1) and therefore suggests that SW620 has more intrinsic stem cell activity.
Cells were stained with 7AAD to allow alive/dead sorting with fluorescent cells being dead. The dead cells were excluded by gating for the non-fluorescent cells (A and C). The cells that had been selected as being alive were then gated using the FL1 laser. The gating strategy was applied to the control cells (that had been treated with DEAB) to select the area that did not contain fluorescence (B) and then transferred to the non-control group (D) to determine the percentage of cells that express ALDH.
Figure 3.10 ALDH expression in high and low TF wild type cells.

ALDH expression was determined using FACS as above and presented as the percentage of cells expressing ALDH. This demonstrates that in the lower tissue factor expressing cell line (SW620) a significantly higher percentage of cells express ALDH compared to the high tissue factor expressing cell line (DLD-1). Results are means from 3 biological repeats and are shown with SEM. Student T-test was used for statistical analysis.
3.3.3 **Stem cell activity in colorectal cell lines with altered TF expression.**

The finding that in wild type cell lines the higher tissue factor expressing cell line (DLD-1) appears to have less stem cell activity than the lower tissue factor cell line (SW620) contradicts the hypothesis that TF increases stem cell activity.

To determine if the effect seen in the wild type cell lines was secondary to their TF expression levels, stem cell activity in the wild type cells that had been transfected to over express and knock down tissue factor were examined.

3.3.3.1 **Colonosphere assay**

The CFE of DLD-1 and SW620 transfected to over express and knock down TF was determined. Each experiment was undertaken in triplicate and a minimum of three biological repeats were undertaken (see section 2.1.4.2 for method).

DLD-1 transfected to over express TF had a significant reduction in the CFE when compared to its negative control (0.33% (SE 0.05) vs 0.66% (SE 0.14), p=0.006) (Figure 3.11). The reduction in CFE for SW620 transfected to over express tissue factor compared to its negative control was not significant (1.21 (SD 0.23) vs 1.29 (SE 0.17), p=0.4) (Figure 3.12).

In both DLD-1 and SW620 when transfected to knock down tissue factor there was a significant increase in the CFE compared to their relevant negative control: DLD-1: 0.63% (SE 0.09) vs 0.41% (SE 0.12), p=0.01 (Figure 3.13); SW620: 2.03% (SE 0.29) vs 1.21% (SE 0.23), p=0.01) (Figure 3.14).
Figure 3.11 Effect of transfecting DLD1 to over express tissue factor on CFE.

A. CFE for the DLD-1 TF +ve and its negative control.  B. Level of tissue factor expression as demonstrated by FACS as a fold change for the DLD-1 TF +ve compared to its negative control. The CFE is significantly lower in the DLD-1 TF +ve cells line compared to its negative control. Results presented are means from 3 biological repeats given with SEM. Student T-test for statistical analysis.
Figure 3.12 Effect of transfecting SW620 to over express TF on CFE

A CFE for the SW620 TF +ve and its negative control. B. Level of tissue factor expression as demonstrated by FACS as a fold change for the SW620 TF +ve compared to its negative control. There is no significant difference between the SW620 TF +ve cell line and its negative control. Results presented are means from 3 biological repeats given with SEM.
Figure 3.13 Effect of transfecting DLD-1 to knock down TF on CFE.

A. CFE for DLD-1 TF -ve and its negative control. B. Level of tissue factor expression as demonstrated by FACS presented as a fold change for the DLD-1 TF -ve compared to its negative control. The CFE is significantly higher in the DLD-1 TF -ve cell line compared to its negative control. Results displayed are means from 3 biological repeats given with SEM. Student T-test for statistical analysis.
Figure 3.14 Effect of transfecting SW620 to knock down TF on CFE.

A. CFE for the SW620 TF-ve and its negative control.  B. Level of tissue factor expression demonstrated by FACS is expressed as a fold change for the SW620 TF-ve compared to its negative control.  The CFE is significantly higher in the SW620 TF-ve cell line compared to its negative control.  Results displayed are means 3 biological repeats given with SEM.  Student T-test for statistical analysis.
3.3.3.2 Stem cell markers

The effect of transfecting the wild type cell lines to over express and knock down TF on cancer stem cell markers was determined. The expression of ALDH was undertaken as in the wild type cells (for method see section 2.1.7.3). CD133 and CD44 expression was determined by FACS (for method see section 2.1.7.2). The cells were stained with conjugated CD133 and CD44, and 7AAD was used as viability marker. An example of the gating used for one of the biological repeats for CD133 and CD44 is shown in Figure 3.15. Similar gating was used for the other repeats.

The levels of expression of the stem cell markers CD44 and CD133 was established in the untransfected cell lines. (Figure 3.17). CD44 was expressed by 79% (SE 9) of cells in DLD-1 and 43% (SE10) of cells in SW620. CD133 was expressed by 9% (SE4) of cells in DLD-1 and 67% (SE 10) of cells in SW620. This lack of consistency between the expression of the possible stem cell markers is consistent with the lack of ability for these makers to accurately identify colorectal cancer stem cells. The high levels of expression also suggests that these markers are not able to select for stem cells as the proportion of cells expressing the marker are much higher than the proportion of cells that would be expected to possess stem cell activity.

When DLD-1 was transfected to over express TF (DLD-1 TF +ve) the number of cells expressing ALDH was reduced in the DLD-1 TF +ve cell line compared to its negative control, representing a 0.72 (SE 0.04) fold change when normalised to the negative control (p=0.001). There was no difference in the expression of CD133 or CD44 in DLD-1 TF+ve compared to DLD-1 negative control (fold change: CD133 1.22 (SE 0.66), p= 0.4; CD44 0.97 (SE 0.12), p= 0.4 (Figure 3.18).

When SW620 was transfected to over express TF (SW620 TF +ve) the reduction in the number of cells expressing ALDH compared to its negative control was not significant, with a fold change of 0.81 (SD 1.00), p=0.4. There was also no difference in the number of cells expressing CD133 or CD 44 (fold change: CD133 1.03 (SE 0.01), p= 0.2; CD44 1.07 (SE 0.09), p= 0.4 (Figure 3.19).
The cells were sorted for alive / dead with 7AAD (not shown) and the dead cells excluded. The gating was set on the unstained control (shown in A for CD44 and C for CD133). The gating was then applied to the stained cells (shown in B for CD44 and D for CD133). This demonstrates very high levels of expression of CD133 and especially CD44.
The cells were sorted for alive / dead with 7AAD (not shown) and the dead cells excluded. The gating was set on the unstained control (shown in A for CD44 and C for CD133). The gating was then applied to the stained cells (shown in B for CD44 and D for CD133). This demonstrates very high levels of expression of CD133 and CD44.
Figure 3.17 Level of expression of CD44 and CD133 in untransfected DLD-1 and SW620

The level of CD44 and CD133 expression by untransfected cell lines was determined in both cell lines using FACS. The data presented is the mean of three biological repeats with SEM.
Expression of stem cell markers and TF in DLD-1 TF +ve. A. The number of cells expressing ALDH in DLD-1 TF+ve as a fold change compared to DLD-1 negative control. B. The number of cells expressing CD133 in DLD-1 TF+ve as a fold change compared to DLD-1 negative control. C. The number of cells expressing CD44 in DLD-1 TF+ve as a fold change compared to DLD-1 negative control. There was a significant decrease in the number of cells expressing ALDH when DLD-1 was transfected to over express TF. There was no significant difference in the number of cells expressing CD133 or CD44. Results displayed are means of 3 biological repeats displayed with SEM. Students T-test for statistical analysis.
Figure 3.19 Effect of transfecting SW620 to over express TF on stem cell markers.

Expression of stem cell markers and TF in SW620 TF +ve. A. The number of cells expressing ALDH in SW620 TF+ve as a fold change compared to SW620 negative control. B. The number of cells expressing CD133 in SW620 TF+ve as a fold change compared to SW620 negative control. C. The number of cells expressing CD44 in SW620 TF+ve as a fold change compared to SW620 negative control. There was no significant change in the number of cells expressing ALDH when SW620 was transfected to over express TF. There was no significant difference the number of cells expressing CD133 or CD44. Results displayed are means of 3 biological repeats displayed with SEM. Student T-test for statistical analysis.
In DLD-1 transfected to knock down the expression of TF (DLD-1 TF-ve), there was an increase in the number of cells that expressed ALDH with a 1.63 (SE 0.14) fold change compared to DLD-1 negative control (p=0.04). There was no difference in the fold change expression of CD133 and CD44 by DLD-1 TF-ve compared to DLD-1 negative control; CD133 1.33 (SE 0.22), p= 0.1; CD44 1.36 (SE 0.34), p= 0.2) (Figure 3.20).

In SW620 transfected to knock down TF (SW620 TF-ve), there was a 1.31 (SE 0.04) fold increase in the number of cells expressing ALDH compared to SW620 negative control (p<0.001). There was no difference in the number of cells expressing CD133 or CD44 in SW620 transfected to knock down TF compared to its negative control (Fold change in CD133 and CD44 expression by SW620 TF-compared to SW620 negative control: CD133 1.37 (SE 0.27), p= 0.1; CD44 0.98 (SE 0.19), p= 0.9) (Figure 3.21).
Expression of stem cell markers and TF in DLD-1 TF-ve. A. The number of cells expressing ALDH in DLD-1 TF-ve as a fold change compared to its negative control. B. The number of cells expressing CD133 in DLD-1 TF-ve as a fold change compared to DLD-1 negative control. C. The number of cells expressing CD44 in DLD-1 TF-ve as a fold change compared to DLD-1 negative control. There was a significant increase in the number of cells expressing ALDH when DLD-1 was transfected to knock down TF. There was no significant difference in the number of cells expressing CD133 or CD44. Results displayed are means of 3 biological repeats displayed with SEM. Student T-test for statistical analysis.
Figure 3.21 Effect of transfecting SW620 to knock down TF on stem cell markers.

Expression of stem cell markers and TF in SW620 TF-ve. A. The number of cells expressing ALDH in SW620 TF-ve as a fold change compared to its negative control. B. The number of cells expressing CD133 in SW620 TF-ve as a fold change compared to SW620 negative control. C. The number of cells expressing CD44 in SW620 TF-ve as a fold change compared to SW620 negative control. There was a significant increase in the number of cells expressing ALDH when SW620 was transfected to knock down TF. There was no significant difference in the number of cells expressing CD133 or CD44. Results displayed are means of 3 biological repeats displayed with SEM. Student T-test for statistical analysis
3.3.4 Summary of effect of TF on stem cell activity.

The hypothesis that TF increases stem cell activity was based on the previously published results that in vulval squamous cell carcinoma and in breast cancer TF increased stem cell activity.

The experiments undertaken have demonstrated that in wild type colorectal cancer cell lines, stem cell activity, as demonstrated by colonosphere forming ability and ALDH expression, does not increase with TF activity.

When the wild type cells were transfected to over express tissue factor in DLD-1, there was a decrease in colonosphere forming ability and a consistent decrease in the expression of ALDH. There was no significant effect in SW620 of transfecting the cell line to over express tissue factor in either colonosphere forming ability or ALDH expression.

Consistent with the above findings, in both DLD-1 and SW620 transfected to knock down TF there was an increase in stem cell activity as demonstrated by an increased ability to form cancer spheres and also increased expression of ALDH.

This suggests that, contrary to the hypothesis, in colorectal cancer cell lines TF inhibits cancer stem cell activity.
3.4 Does TF promote colorectal cancer cell anoikis resistance?

Anoikis resistance is the ability of a cell to resist apoptosis after loss of contact with the basement membrane and it is a requirement for the formation of metastasis\textsuperscript{283, 284}. In \textit{in-vivo} studies of pancreatic and melanoma cell lines TF inhibition reduces the development of metastases\textsuperscript{277, 278}. In BHK cells and breast cancer cells transfected to over express TF, the addition of factor VIIa (TF co-factor) at physiological levels reduced the number of cells undergoing anoikis. This effect was dependent on tissue factor as it was blocked by use of a tissue factor inhibitor\textsuperscript{107, 285, 286}. In colorectal cancer, clinical studies have demonstrated that TF expression by the primary tumour is an independent risk fact for the development of metastases\textsuperscript{222, 277}. I therefore hypothesised that in colorectal cancer TF expression may promote anoikis resistance.

3.4.1 Effect of tissue factor expression on anoikis resistance

An anoikis assay using the colorectal cell line DLD-1 which had been transfected to over express (DLD-1 TF+ve) and knock down (DLD-1 TF-ve) tissue factor was undertaken.

For a detailed method see section 2.1.5 but briefly cells were plated at low density in non-adherent conditions in serum free media and no added VIIa. The proportion of cells alive at set time points over 24 hours was determined using trypan blue and an automated cell counter in triplicate for each experiment. The experiment was repeated three times. Those cells that did not die from being cultured in non-adherent conditions possessed anoikis resistance.

There was no difference at any time points in the proportion of alive cells between DLD-1 TF+ve and its negative control. At 24 hours the proportion of alive cells in DLD-1TF+ve vs negative control was 52.1% (SE 2.2) vs 49.7% (SE 2.5), $p=0.2$.

In DLD-1 TF-ve cell lines there was a reduction in the number of cells alive at 24 hours compared to their negative control (49.4% (SE 2.4) vs 57.3% (SE 2.9), $p=0.04$ (Figure 3.22). This suggests that tissue factor may protect against cells undergoing anoikis although this does need to be interpreted with caution as this result has only been demonstrated in one cell line and the difference in the number of cells alive at 24 hours is only a small decrease in the TF-ve cell line.
Cells were plated at 300 cells/cm² in non-adherent conditions. The proportion of cells that were alive were determined at each time point using trypan blue and an automated cell counter. A. Effect of transfecting DLD-1 to over express TF on anoikis resistance. B. Effect of transfecting DLD-1 to knock down TF on anoikis resistance. The results show that when TF expression is reduced there was an impaired ability to resist anoikis. Results displayed are means of 3 biological repeats displayed with SEM. Student T-test for statistical analysis.

Figure 3.22 Effect of TF over expression and knock down on anoikis resistance in DLD-1
3.5 Does TF promote colorectal cancer cell proliferation?

In colorectal cancer TF expression is associated with more advanced stage of disease\textsuperscript{226}. In animal models, high tissue factor expressing melanoma and colorectal cancer cell lines produce faster growing tumours than low TF expressing cell lines and when the TF was inhibited this effect was reversed\textsuperscript{265} \textsuperscript{266} \textsuperscript{269}. In lung cancer, TF inhibition reduces proliferation in vivo and in vitro\textsuperscript{268}. In colorectal cancer the TF agonist VIIa increases proliferation indicating that TF pathway activation may promote proliferation. The pathways by which VIIa increase proliferation is via PAR2 and ERK1/2 activation\textsuperscript{105} \textsuperscript{270}. This gives rise to the hypothesis that tissue factor expression by colorectal cancer cells may increase cell proliferation.

3.5.1 Proliferation assay

Proliferation was determined using the Alamar Blue assay (Life Technologies). Alamar blue is a cell viability indicator. Metabolically active cells convert its non-fluorescent form to a fluorescent form. The florescence (read as absorption on a plate reader) is proportional to the number of viable cells. Its use is well established in colorectal cell lines including DLD-1 and SW620\textsuperscript{313}. The method for undertaking the Alamar blue proliferation assay is given in section 2.1.6.

Alamar blue has a linear relationship with the number of live cells. This allows the level of absorption over time to indicate proliferation. To ensure that a linear relationship was present in the colorectal cancer cells a verification assay was undertaken. DLD-1 cells were plated at 2500, 5000 and 10,000 cells per well in a 96 well plate. The absorption was measured after 6 hours. The linear nature of absorption (arbitrary units (u)) is demonstrated in Figure 3.23. The correlation coefficient (Spearman’s) was 0.84 with a p value of <0.001.
Figure 3.23 Confirmation of the linear nature of Alamar blue absorption and the number of cells.

Different densities of cells were plated out and subjected to an Alamar blue assay after 6 hours. This confirms that in DLD-1 (the cell line used for the assay) the absorption had a linear relationship with the number of cells present. Statistical analysis undertaken with spearman's correlation.
3.5.2 The effect of TF over expression on proliferation
DLD-1 and SW620 transfected to over express tissue factor (TF+ve) and their negative controls underwent proliferation assays using Alamar blue.

The Alamar blue assay was undertaken at 6 hours and at 24 hour intervals over 4 days.

DLD-1 TF+ve proliferated more rapidly than its negative control. By 18 hours, the level of absorption by DLD-1 TF+ve was higher than DLD-1 negative control reflecting an increase in the number of viable cells (4131u (SE 501u) vs 2218u (SE 375u), p<0.001). The increase in proliferation continued and was demonstrated at all subsequent time points (absorption at 42 hours: 5455u (SE 718u) vs 2246u (SE 310u), p<0.001; 66 hours: 13132u (SE 2288u) vs 5494u (SE 950u), p<0.001; 92 hours: 13300u (SE 1731u) vs 7606u (SE 848u), p<0.001). While there was still a difference in the absorption at 92 hours between DLD-1 TF+ve and DLD-1 negative control the proliferation appeared to have slowed. This is likely to reflect the fact that the DLD-1 TF+ve were fully confluent by this time (unlike its negative control) impairing further proliferation (Figure 3.24).

The increase in proliferation with TF over expression was also demonstrated in SW620. At 18 hours, although there was an increase in absorption (and therefore number of cells) in the SW620 TF+ve cell line compared to SW620 negative control, this was not significant (485u (SE 80u) vs 360u (SE 65u), p=0.2). By 42 hours the difference in absorption between SW620 TF+ve compared to SW620 negative control was significant (414u (SE 27.6u) vs 286u (SE 42u), p=0.02). The increasing proliferation was seen in the SW620 TF+ve cell line with an increase at 66 hours (726u (SE 139u) vs 221u (SE 67u,) p=0.01). Whilst the difference between the TF+ve cell line and its negative control continued to 92 hours (789u (SE 139u) vs 476u (SE 99u), p=0.02) the growth of the SW620 TF+ve cell line was again limited by the cells becoming confluent (Figure 3.24).

It is not possible to comment on the difference in absorption between the two cell lines as the Alamar blue is a marker of metabolism and different cell lines metabolise at different rates.
Figure 3.24 Effect of over expressing TF in colorectal cancer cell lines on proliferation.

Alamar blue assay was undertaken to determine the number of cells alive over time and therefore indicate proliferation. A. Alamar blue assay of DLD-1 TF+ve cells and its negative control. B. Alamar blue assay of SW620 TF+ve cells and its negative control. The assay demonstrated increased proliferation the TF over expressing cell line in both DLD-1 and SW620. * = p<0.05, ** P<0.001. Data presented is the result of 3 biological repeats and presented with SEM. Student T-test used for statistical analysis.
3.5.3 The effect of TF knock down on proliferation

DLD-1 and SW620 transfected to knock down tissue factor and their negative controls underwent proliferation assays using Alamar blue.

In DLD-1 the DLD-1 TF-ve demonstrated no difference in absorption compared to DLD-1 negative control at 18 hours (3360u (SE 378u) vs 3023u (SE 549u), p= 0.6) and 42 hours (5004u (SE 792u) vs 2943u (SE 1360u), p=0.06). At 66 hours, there was an increase in the absorption, reflecting an increase in the number of cells, in the DLD-1 TF-ve compared to DLD-1 negative control (9602u (SE 1270u) vs 5218u (SE 810u) p=0.001). The difference persisted at 92 hours (14182 (SE 1053u) vs 10566u (SE 1077u), p<0.05) (Figure 3.25).

In SW620 there was no difference in the absorption between SW620 TF –ve compared to SW620 negative control at 18 hours (384 (SE 44u) vs 324 (SE 95u), p=0.3). At 42 hours there was an increase in absorption in the tissue factor knock down compared to its negative control (223u (SE 25u) vs 149u (SE 26u), p=0.05). This increase in absorption continued. At 66 hours the absorption was 540u (SE 197u) vs 336 (SE 27), p=0.003, at 92 hours 731u (SE 105u) vs 450u (SE 49u), p= 0.02 (Figure 3.25).
Figure 3.25 Effect of knocking down TF in colorectal cancer cell lines on proliferation.

Alamar blue assay was undertaken to determine the number of cells alive over time and therefore indicate proliferation. A. Alamar blue assay of DLD-1 TF-ve cells and DLD-1 negative control. B. Alamar blue assay of SW620 TF-ve cells and SW620 negative control. The assay demonstrated increased proliferation at 66 hours in the TF knock down cell line in both DLD-1 and SW620. *=p<0.05, **=p<0.001. Data presented is the result of 3 biological repeats and presented with SEM. Student t-test for statistical analysis.
3.5.4 The effect of factor VIIa on proliferation

Factor VIIa is the ligand of tissue factor. To determine if interaction between TF and VIIa increased proliferation the Alamar blue proliferation assay was undertaken in the presence of increasing concentrations of exogenous FVIIa. Using DLD-1 TF+ve and SW620 TF+ve and their relevant negative controls 10,000 cells were plated out in a 96-well plate in complete media. After 6 hours an initial assay with Alamar blue was undertaken. The media was then removed and replaced with media without FBS but with additional factor VIIa added in a concentration ranging from absent to 50nM. It is known that 0.1nM represents normal physiological levels. The Alamar blue assay was repeated at 24 hours to determine any effect on proliferation with the addition of factor VIIa (for method see section 8.2.1.6). The results are expressed as a fold change in absorption at 24 hours from the baseline assay undertaken at 6 hours when the VIIa was added.

The experiment demonstrated that in both DLD-1 and SW620 there was a significant increase in proliferation with the addition of VIIa up to normal physiological levels (0.1nM) in a dose dependent manner. This increase was seen in both the TF over expressing cell lines and their negative controls.

Without factor VIIa, the DLD-1 TF+ve and SW620 TF+ve cell line proliferated more rapidly than their relevant negative control as demonstrated by increased absorption (TF+ve vs negative control; DLD-1 4.21 (SE 0.31) vs 3.21 (SE 0.25), p=0.004, SW620 2.91 (SE 0.31) vs 2.33 (SE 0.18), p= 0.02). This is consistent with the findings from the previous experiment undertaken in complete media (section 3.5.2). This demonstrates that the previous findings are reproducible and are not dependent on the factors in the media.

Addition of factor VIIa to the DLD-1 TF+ve cell line resulted in an increase in absorption, indicating increased proliferation. This increase was seen up to 0.1nM concentration of VIIa (0nM VIIa vs 0.1nM VIIa; 4.21 (SE 0.31) vs 7.05 (SE 0.87), p=0.04). The increase in absorption indicating proliferation was also seen in the DLD-1 negative control (0nM VIIa vs 0.1nM VIIa; 3.21 (SE 0.25) vs 6.17 (SE 0.78), p= 0.03).

Addition of factor VIIa to the SW620 TF+ve cell line showed an increase in the absorption up to 0.1nM VIIa ,however, it was not significant (2.91 (SE 0.31) vs 4.24 (SE 0.87), p= 0.1). In the SW620 negative control there was a significant increase in the absorption between 0nm of VIIa and 0.1nM of VIIa (2.33 (SE 0.18) vs 4.69
(SE 0.64), p<0.01). This lack of increase in absorption in the SW620 TF +ve cell line may reflect that it is already at maximum proliferation rate.

In both DLD-1 and SW620 cells further increases in VIIa concentration beyond physiological concentration (0.1nM) resulted in a reduction in proliferation (Figure 3.26). This suggests that beyond physiological levels VIIa may become toxic to the cells or result in over activation of pathways.
Figure 3.26 Effect of factor VIIa on proliferation in colorectal cancer cells.

Alamar blue assay to determine the effect on proliferation of factor VIIa in colorectal cancer cells. The Results are displayed as a fold change in absorption at 24 hours from the baseline at 6 hours. Cells were cultured in varying concentrations of VIIa during this time. A. Effect of VIIa on proliferation in DLD-1 TF+ve and its negative control. B. Effect of VIIa on proliferation in SW620 TF+ve and its negative control. Increasing doses of VIIa enhanced proliferation up to the normal physiological concentration (0.1nM). Results of 3 biological repeats presented with SEM. Student T-test for statistical analysis.
3.5.5 Summary of effect of TF on proliferation.

TF over expression increased proliferation in both DLD-1 and SW620 in media containing serum. There was also a paradoxical increase in proliferation with the TF knock down. The increase in proliferation with TF over expression was also demonstrated in both cell lines when there was no serum or factor VIIa present.

In DLD-1 the presence of VIIa increased proliferation in both the TF +ve cell line and also DLD-1 negative control. In SW620 the increase was seen in the negative control but, although there was an increase in proliferation in the TF+ve cell line, it was not significant. In both cell lines beyond physiological levels of VIIa there was a reduction in proliferation.

3.5.6 The role of PAR2, ERK1/2 and AKT pathway activation in TF induced cell proliferation

Tissue factor and the tissue factor/VIIa complex induce multiple pathways that may increase proliferation in colorectal cancer including the PAR2, ERK1/2 and AKT pathways\textsuperscript{105,315-317}.

TF can activate these pathways by a number of different interactions. Tissue factor can activate intracellular pathways (via its cytoplasmic domain) without the presence of its ligand VIIa\textsuperscript{318} including activation of the ERK1/2 pathway\textsuperscript{319}. The tissue-factor VIIa complex activates PAR 2 \textsuperscript{100,101}, which in turn activates the ERK1/2 and AKT pathways increasing survival, proliferation, differentiation and anoikis resistance\textsuperscript{320-322}. In addition, the TF/VIIa complex can induce ERK1/2 pathway activation independently of PAR2\textsuperscript{108} via the TF cytoplasmic domain.

To determine which of these pathways promote the proliferation seen with high TF expression and increasing VIIa concentration, western blots of the expression of PAR2 and the phosphorylation of ERK1/2 and AKT were undertaken. The activation of these pathways was correlated with the proliferation of DLD-1 and SW620 transfected with TF and with additional VIIa added.

For a detailed method of how the protein lysates were prepared see section 2.1.9 in methods but briefly transfected cells and their negative controls were plated separately in complete media. After 6 hours the media was changed to serum free media. Following incubation for 48 hours the media was changed for fresh serum free media for 1 hour to remove any accumulated growth factors secreted by the cells themselves. The media was again replaced with fresh serum free media with
varying concentrations of VIIa or Foetal Calf Serum (FCS) as a control. After 10 minutes incubation the cells were lysed with RIPA cell lysis buffer with protease and phosphatase inhibitors.

3.5.6.1 The effect of high TF expression and increasing exogenous FVII on ERK1/2 phosphorylation

Protein lysate was subjected to a western blot (for detailed method see section 2.1.8.2). The expression of phosphorylated ERK1/2 was determined using a phospho-ERK1/2 antibody (Cell Signalling). The same membrane was stripped using reBlot strong (Millipore) and the level of expression of total ERK1/2 determined using an ERK1/2 antibody (Cell Signalling). The membrane was stripped again with reBlot strong and the level of actin determined as a loading control with a β-actin antibody (Sigma).

In DLD-1, there was an increase in the phosphorylation of ERK1/2 in the DLD-1 TF+ve cell line compared to its negative control. There was also a further increase in phosphorylation with the addition of factor VIIa which was seen in both the DLD-1 TF+ve cell line and its negative control (Figure 3.27).

In SW620, there was an increase in the phosphorylation of ERK1/2 in the SW620 TF+ve cell line compared to its negative control. In the negative control there was an increase in the phosphorylation with increasing doses of VIIa. This response to VIIa was not seen in the SW620 TF+ve cell line (Figure 3.28).

The changes seen in the phosphorylation of ERK1/2 are consistent with the changes in proliferation seen in both DLD-1 and SW620. The effect on proliferation of transfecting the cell lines to over express TF and the addition of VIIa is mirrored in the phosphorylation of ERK1/2 in both DLD-1 (Figure 3.29) and SW620 (Figure 3.30).

In DLD-1 the proliferation assay demonstrated a significant increase in proliferation in the DLD-1 TF+ve cell line compared to the negative control consistent with the increased phosphorylation of ERK1/2. In both the DLD-1 TF+ve and its negative control there was a dose response to VIIa increasing proliferation and also in increased phosphorylation of ERK1/2.

In SW620 there was an increase in the phosphorylation of ERK1/2 in SW620 TF+ve compared to its negative control which is again consistent with the increase in proliferation in SW620 TF+ve compared to its negative control. The increase in phosphorylation in the negative control cell line with the addition of VIIa is consistent
with the increase in proliferation in this cell line with VIIa. In the SW620 TF+ve cell
line the increased dose of VIIa did not increase the phosphorylation and in the
proliferation assay there was no significant increase in proliferation with further
increases of VIIa. It may be that in the SW620 cell line the maximum level of
phosphorylation of ERK1/2 and proliferation was reached by TF over expression
alone therefore further increases with the addition of VIIa are not possible.
Figure 3.27 Phosphorylation of ERK1/2 with TF and VIIa in DLD-1.

A western blot of ERK1/2 phosphorylation of DLD-1 TF+ve compared to its negative control with increasing doses of VIIa. Cells cultured with FCS was used a positive control. The DLD-1 TF+ve cell lysate and the lysate from its negative control were run on the same gel and transferred to the same membrane. The phosphorylated ERK1/2 was probed, the membrane then stripped and then re-probed for total ERK1/2 before been stripped and probed for Actin as a loading control. It demonstrated increased phosphorylation with increased TF expression and also a dose response to VIIa.
A western blot of ERK1/2 phosphorylation of SW620 TF+ve compared to its negative control with increasing doses of VIIa. Cells cultured with FCS was used a positive control. The SW620 TF+ve cell lysate and the lysate from its negative control were run on the same gel and transferred to the same membrane. The phosphorylated ERK1/2 was probed, the membrane then stripped and then re-probed for total ERK1/2 before been stripped and probed for Actin as a loading control. It demonstrated increased phosphorylation with increased TF expression when no VIIa was introduced. In the negative control there was also a dose response to VIIa.
Figure 3.29 Correlation between proliferation and phosphorylation of ERK1/2 in DLD-1 with TF over expression and addition of factor VIIa.

The level of phosphorylation of ERK1/2 is consistent with the level of proliferation in DLD-1. The phosphorylation of ERK1/2 is increased with over expression of TF. There is a further increase with the addition of factor VIIa. The proliferation of DLD-1 is increased with the over expression of TF and further increased with the addition of factor VIIa in increasing concentration up to physiological levels (0.1nM).
Figure 3.30 Correlation between proliferation and phosphorylation of ERK1/2 in SW620 with TF over expression and addition of factor VIIa.

The level of phosphorylation of ERK1/2 is consistent with the level of proliferation in SW620. The phosphorylation of ERK1/2 is increased with over expression of TF. In the negative control for TF expression in SW620 there is a further increase in proliferation with the addition of factor VIIa. There is no increase in phosphorylation in the TF over expressing cell line with the addition of factor VIIa, however in the TF negative control cell line the phosphorylation was further increased by addition of factor VIIa. The level of phosphorylation of ERK1/2 mirrors the effect on proliferation of TF and factor VIIa.
3.5.6.2 The effect of high TF expression and increasing exogenous FVII on AKT phosphorylation

Protein lysate was prepared as described in section 2.1.8.1 and a western blot run as described in section 2.1.8.2. The level of phosphorylation of AKT was determined using a phospho-AKT antibody (Cell Signalling). The membrane was stripped using reBlot strong (milipore) and re-probed using a total AKT antibody (Cell Signalling). Following stripping again with reBlot strong the membrane was probed for expression of actin using a β-actin antibody (Sigma) as a loading control.

In DLD-1, there was no difference in the phosphorylation of AKT between the DLD-1 TF+ve compared to their negative controls. There was no difference in phosphorylation of AKT with the addition of factor VIIa. The cell lines that were treated with the addition of FCS demonstrated an increase in phosphorylation compared to the cells that were serum deprived (Figure 3.31).

In SW620, there was a small increase in the phosphorylation of AKT in the SW620 TF+ve cell line compared to its negative control. There was no increase in phosphorylation with increasing doses of VIIa however there was a marked increase with the addition of FCS as was seen with DLD-1 (Figure 3.32).
Figure 3.31 Phosphorylation of AKT with TF and VIIa in DLD-1.

A western blot of AKT phosphorylation of DLD-1 TF+ve compared to its negative control with increasing doses of VIIa. Cells cultured with FCS were used as a positive control. The DLD-1 TF+ve cell lysate and the lysate from its negative control were run on the same gel and transferred to the same membrane. The phosphorylated AKT was probed, the membrane then stripped and then re-probed for total AKT before been stripped and probed for β-Actin as a loading control. Arrow marks the band for phosphorylation of AKT. It demonstrated no change in phosphorylation with either increased TF expression or increased dose of VIIa.
Figure 3.32 phosphorylation of AKT with TF and VIIa in SW620.

A western blot of AKT phosphorylation of SW620 TF+ve compared to its negative control with increasing doses of VIIa. Cells cultured with FCS were used as a positive control. The SW620 TF+ve cell lysate and the lysate from its negative control were run on the same gel and transferred to the same membrane. The phosphorylated AKT was probed, the membrane then stripped and then re-probed for total AKT before being stripped and probed for β-Actin as a loading control. Arrow marks the band of phosphorylated AKT. It demonstrated increased phosphorylation of AKT in SW620 TF+ve compared to the negative control but with no response to increasing factor VIIa concentrations.
3.5.6.3 The effect of high TF expression and increasing exogenous FVII on PAR2 expression

Protein lysates from DLD-1 and SW620 transfected to over express TF and their relevant negative controls treated with varying concentrations of FVIIa were prepared as described in section 2.1.9. The lysates were subjected to a western blot to determine the level of expression of PAR2 using an anti-PAR2 antibody (Cell Signalling). The membrane was stripped using reBlot strong (Millipore) and the level of actin was established using a β-actin antibody (Sigma) as a loading control.

In the DLD-1 cell line there was no difference in the expression of PAR2 in the DLD-1 TF+ve cell line compared to its negative control. There was also no increase in the expression of PAR2 with increasing doses of factor VIIa or the presence of FCS (Figure 3.33).

The same findings were seen in the SW620 cell line with no increase in expression of PAR2 in the SW620 TF+ve compared to its negative control or with the addition of factor VIIa or FCS (Figure 3.34).
Figure 3.33 PAR2 expression with TF and VIIa in DLD1.

A western blot of PAR2 expression by DLD-1 TF+ve compared to its negative control with increasing doses of VIIa. Cells cultured with FCS were used as a positive control. The DLD-1 TF+ve cell lysate and the lysate from its negative control were run on the same gel and transferred to the same membrane. The PAR2 expression was probed, the membrane then stripped and probed for β-actin as a loading control. It demonstrated no change in PAR2 expression with increased TF expression or VIIa concentration.
A western blot of PAR2 expression by SW620 TF+ve compared to its negative control with increasing doses of VIIa. Cells cultured with FCS were used as a positive control. The SW620 TF+ve cell lysate and the lysate from its negative control were run on the same gel and transferred to the same membrane. The PAR2 expression was probed, the membrane then stripped and probed for β-actin as a loading control. It demonstrated no change in PAR2 expression with increased TF expression or VIIa concentration.

Figure 3.34 PAR 2 expression with TF and VIIa in SW620.
3.6 Discussion.

3.6.1 Tissue factor suppresses stem cell activity in colorectal cells

Colorectal cancer stem cells are thought to be responsible for treatment resistance in colorectal cancer. In mouse models of colorectal cancer, chemotherapy increases the proportion of tumour cells that have stem cell properties, with the ability to form tumours and expression of stem cell markers. In squamous cell carcinoma and breast cancer it has been shown that TF increases stem cell activity. I therefore hypothesised that the same finding would be seen in colorectal cancer.

The results of the experiments into the effect of TF and stem cell activity have demonstrated that in wild type cell lines the higher tissue factor cell line (DLD-1) produced less cancer spheres and expressed lower levels of the stem cell marker ALDH than the lower tissue factor cell line SW620. Whilst it is recognised that there are multiple factors between cell lines, for example different mutations and signalling pathways driving the cancer, that could explain this difference in stem cell activity, the results suggest that tissue factor may not promote cancer stem cells in colorectal cancer. To control for potential differences between the cell lines that may affect stem cell activity the wild type cell lines were transfected to over express and knock down TF.

TF knockdown increased colonosphere formation and ALDH expression in DLD-1 and SW620. Consistent with this was the finding that TF over expression in DLD-1 decreased colonosphere formation and ALDH expression. Whilst there was a reduction in both sphere formation and ALDH expression when SW620 was transfected to over express TF this did not reach statistical significance. Whilst this could be because TF over expression has no effect on SW620 it seems more likely that this is a type II error and that the reduction in sphere formation and ALDH expression was there but failed to reach statistical significance.

The two other stem cell markers (CD133 and CD44) did not demonstrate any statistically significant difference when the cell lines were transfected to over express or knock down tissue factor. The levels of expression of the stem cell markers is high in the untransfected cells. This high level of expression of the two stem cell markers that I have demonstrated is consistent with the literature. The level of expression of these proposed stem cell markers suggests that neither may be able to identify stem cell populations as the proportion of cells that express these markers are much higher than the proportion of stem cells that would be
expected to be present. My research has also not demonstrated any difference in expression of CD133 or CD44 with increasing stem cell activity as demonstrated by colonosphere formation or ALDH expression. These findings suggest that neither CD133 nor CD44 are reliable stem cell markers which is consistent with previous research that has cast doubt on their role as a stem cell marker.  

The finding that the high TF expressing cell line (DLD-1) has less stem cell activity than the low TF expressing cell line (SW620) and the finding that transfecting the cells to over express TF reduces stem cell activity, whilst transfecting them to knock down TF increases the stem cell activity, provides evidence that refutes the hypothesis that in colorectal cancer cell lines TF promotes stem cell activity. The initial hypothesis was generated based on observations in squamous cell carcinoma and breast cancer. However, there is other research that concurs with the results that I have shown suggesting that in breast cancer expression of TF is a marker of a more differentiated cell type and that TF is expressed differentially to the stem cell marker EPCR. This conflicting research does cast doubt on the original hypothesis. Studies of the pathways that are activated by TF also provide a methodology by which TF may inhibit stem cell activity in colorectal cancer. My research, consistent with other research, has demonstrated that TF expression results in the phosphorylation of ERK1/2. The activation of ERK1/2 results in the differentiation of stem cell subpopulations in normal and prostate cancer cell lines with loss of stem cell marker expression and sphere formation ability. It would therefore be expected that high TF expression by colorectal cancer cells would result in increased ERK1/2 phosphorylation and therefore a more differentiated cell type and less stem cell activity. In colorectal cancer, stem cells are known to proliferate less than differentiated cells. The finding that TF over expression results in a more rapidly proliferating cell makes it less likely that it would also select for a cell with stem cell properties.

Caution does need to be used regarding over interpreting the results of in-vitro stem cell assays. Although it has been demonstrated that the colonosphere assay is able to select for cells that have a 5.5 times higher ability to form tumours in mice it is only a surrogate marker of stem cell activity. To be confident of the effect of tissue factor on stem cell activity it is necessary for in-vivo studies to be undertaken. Another possible criticism of the cancer sphere assay is the small absolute differences in CFE between conditions. It has however been shown that even small differences in sphere forming ability can reflect much larger tumour forming abilities when introduced into mouse models.
3.6.2 Tissue factor promotes anoikis resistance

Anoikis resistance is fundamental for cell survival during metastasis. TF knock down DLD-1 had reduced anoikis-resistance, however TF over expression had no effect on anoikis. Consistent with the literature the level of anoikis resistance in DLD-1 in my current study is high (greater than 50% surviving at 24 hours). This high level of anoikis resistance and also the high tissue baseline level of TF expression by DLD-1 may mean that DLD-1 is already working at its maximum ability to resist anoikis and therefore further increases in TF had no effect. Studies in BHK cells and breast cancer have shown that the TF/FVIIa complex is able to increase anoikis resistance and that this anoikis resistance is mediated via the AKT pathway and to a lesser extent the ERK1/2 pathways. My study has demonstrated that in DLD-1 the over expression of TF does not increase the phosphorylation of AKT. It may be that the phosphorylation of AKT is maximal in wild type DLD-1 or that TF does not work through AKT in DLD-1. It is conceivable that there is an inactivating mutation in AKT in DLD-1. The difference in phosphorylation of AKT with TF knock down has not been established.

The finding that reduced TF expression results in impaired anoikis resistance is consistent with clinical studies that demonstrate that TF expression by colorectal cancer is an independent predictor of the development of metastases. It is also consistent with in-vivo studies using melanoma and pancreatic cancer cell lines that demonstrated an anti-metastatic effect of TF antibodies.

The finding that reduced TF expression in DLD-1 reduces their ability to resist anoikis could be seen to be contrary to the findings that decreased TF activity increases stem cell activity. This is because anoikis resistance is a property that stem cells possess. The level of anoikis that I have demonstrated in the DLD-1 cell line is very high (approximately 50%) however the percentage of cells that produce cancer spheres is much lower (less than 1%). This suggests that many of the cells that possess anoikis resistance do not possess the other stem cell characteristics. It is conceivable that the anoikis resistance is provided by pathways that are not induced by the stem cell state. One possible pathway that this may be exerted is via the mutant K-ras gene that DLD-1 is known to possess. It has been shown that the high level of anoikis resistance seen in DLD-1 is under the control of the K-ras mutation (an effect that is also seen in other cell lines). It has also been shown that in DLD-1 the K-ras mutation does not increase the stem cell activity. This suggests that the effect that k-ras mutation has on anoikis is independent of any effects on stem cell activity. Intriguingly the k-ras mutation has also been shown in
DLD-1 to increase expression of TF\textsuperscript{220,289}. This raises the possibility that the K-ras gene is exerting its effects on anoikis via TF.

This effect of TF on anoikis has only been demonstrated in the DLD-1 cell line, therefore caution needs to be applied in extrapolation of these findings to other colorectal cell lines.

3.6.3 Levels of expression of tissue factor determine proliferation in colorectal cancer cells

Over expression of TF in both the high (DLD-1) and low (SW620) tissue factor expressing cell line resulted in increased proliferation. Paradoxically there was also an increase in proliferation with TF knock down in both cell lines. This increase in proliferation with TF knock down was less marked and occurred later than with TF over expression.

The increase in proliferation demonstrated with tissue factor overexpression is consistent with previous studies. \textit{In-vivo}, the use of a TF antibody resulted in slower growing tumours in melanoma mouse models\textsuperscript{265,266}. In lung cancer the use of a TF antibody resulted in reduced proliferation both \textit{in-vitro} and \textit{in-vivo}\textsuperscript{268}. In colorectal cancer the addition of VIIa results in increased proliferation, an effect that was blocked by the use of a tissue factor antibody and in the same study the use of a TF antibody without VIIa also resulted in an increase in proliferation\textsuperscript{270}. This apparently paradoxical effect (similar to that demonstrated in my study) may reflect the actions of feedback loops. One feedback loop that is known involves the activation of the ERK1/2 pathway. TF increases the phosphorylation of ERK1/2\textsuperscript{105} whilst ERK1/2 also suppresses the expression of TF\textsuperscript{332,333}. Depending on the balance of ERK1/2 phosphorylation and TF expression this could result in the paradoxical effects that have been demonstrated in this and previous studies.

I have demonstrated that whilst TF promotes proliferation its effects are further increased by the action of its ligand factor VIIa. The finding that TF over expression in both DLD-1 and SW620 in the absence of VIIa has pro-proliferative effects suggests that TF induced proliferation is not dependent on the presence of VIIa. It is also possible that there may be endogenous expression of VIIa by the cell lines as has been demonstrated in ovarian cancer surgical specimens\textsuperscript{334} as well as in cancer cell lines including liver, ovary, prostate, breast and thyroid\textsuperscript{335}. The finding that proliferation is increased by the presence of VIIa up to physiological levels and that with further increases it has an inhibitory effect may be the result of toxic levels being achieved or possibly due to feedback loops, an example of which has been
previously described with ERK1/2 activity being increased by the action of TF whilst the increased activity of ERK1/2 decreases TF expression.

The potential pathways that I have explored by which TF in the absence and presence of VIIa increases proliferation include:

- TF independent of VIIa activates ERK1/2
- TF-VIIa complex activation of ERK1/2
- PAR 2 activation by TF-VIIa complex that then activates ERK1/2 or AKT.

Evidence of the role of these pathways in increasing proliferation in colorectal cancer comes from various studies. In colorectal cancer the use of a ERK1/2 inhibitor in-vitro has been shown to reduce proliferation\textsuperscript{105}, an effect that is also seen in normal epithelial cells\textsuperscript{336}. The in-vitro effect of AKT phosphorylation in colorectal cancer has been demonstrated by the anti-proliferative effects of AKT antibodies\textsuperscript{337}. PAR2 increases proliferation in colorectal cancer in-vitro\textsuperscript{315} as well as in other cancers including pancreatic, gastric and prostate\textsuperscript{338-340}. This increase in proliferation is exerted via the ERK1/2 pathway.

In my study the phosphorylation of ERK1/2 in DLD-1 and SW620 in the presence or absence of VIIa reflects the increase in proliferation. This suggests that the ERK1/2 pathway may be promoting proliferation. The finding that increased TF expression alone is able to increase ERK1/2 phosphorylation suggests that the phosphorylation is not factor VII dependent. There is the possibility that factor VIIa is endogenously produced by the cell lines. There was no difference in the AKT phosphorylation or PAR2 expression with either TF over expression or VIIa concentration suggesting that the AKT pathway is not responsible for the increased proliferation. This also suggests that the ERK1/2 phosphorylation is not the result of PAR2 activation and that in these cell lines TF and VIIa do not regulated the expression of PAR2. Further studies with selective inhibition of ERK1/2, AKT and PAR2 would need to be undertaken to confirm this.

As previously discussed the TF in colorectal cancer cell lines is at least in part under the control of the K-ras mutant gene. It has also been shown by previous research that K-ras promotes proliferation in colorectal cancer cell lines via the ERK1/2 pathway\textsuperscript{341}. This again raises the possibility that the K-ras mutation is exerting its effects via TF.
The use of the Alamar blue assay is based on the level of metabolism. Its use as a proliferation marker is well established, including in the cell lines that I have studied. However, the use of the assay as a proliferation marker relies on the cells having similar levels of metabolism. This raises the possibility that the effects measured could be the result of changes in metabolism because of the action of TF and VIIa rather than proliferation. To confirm the findings an alternative proliferation marker which does not rely on metabolism could be used to determine the effect of these proteins in proliferation.

3.7 Conclusion

High TF expression by colorectal cancer is known to result in a poor prognosis and is associated with advanced stage and early metastasis. I have demonstrated two possible physiological effects of high tissue factor expression that can explain these clinical outcomes. I have demonstrated that tissue factor increases anoikis resistance, this being a requirement for metastasis. I have also demonstrated that tissue factor promotes colorectal cancer proliferation.

However, TF inhibits colorectal cancer stem cell activity. The effect of TF in inhibiting colorectal cancer stem cell activity whilst increasing proliferation is consistent with findings that in colorectal cancer the stem cell population proliferates less rapidly than the differentiated cells. It is also consistent with findings that show the pathways activated by TF are both pro-proliferative and pro-differentiation therefore moving cells away from stem-cell like behaviour.

Treatments targeted at TF may offer an anti-proliferative effect as well as an anti-metastatic effect. It is possible that targeting TF provides a method of modulating the effects of the K-ras mutation.

3.8 Limitations and future work

Although the in-vitro methods that have been used in this chapter are well described and reproducible they are merely surrogate markers for in-vivo effects of modulating protein expression and each assay has its limitations.

The cancer sphere model is well described in multiple cancer types including colorectal cancer. It is correlated with in-vivo tumour formation and therefore tumorigenicity. The finding that TF over expression suppresses stem cell activity has been demonstrated using the cancer sphere model and stem cell markers. Whilst this gives an indication regarding the effect of tissue factor on stem cells, it is necessary to determine if this effect is seen in the in-vivo setting in murine models.
with serial transplantation of cancer cells. This would allow the effect of TF inhibitors on stem cells to be determined to ensure that no therapy targeting tissue factor increases the population of cancer stem cells. To further evaluate the effect of TF on stem cells without the use of *in-vivo* models it is possible to determine the effect of TF on self-renewal of stem cells. In this assay the colonospheres are harvested, dissociated into individual cells and suspended. The number of spheres formed are then counted. If the effect of the treatment on secondary sphere formation is consistent with the effect on primary sphere formation this indicates that the cells have self-renewal, a property of stem cells.

The pathways by which tissue factor increases proliferation and also anoikis resistance need to be further evaluated by selective inhibition of downstream proteins in the AKT and ERK1/2 pathways. This can be done with selective inhibition of their phosphorylation by commercially available antibodies.

The possibility that the putative effects of the K-ras mutation may exert its effects via tissue factor raises very interesting therapeutic possibilities. Further research into reducing the effect of k-ras by targeting tissue factor is required.
4 Tumour and plasma expression of TF pathway markers in colorectal cancer

4.1 Introduction

4.1.1 Tumour expression of TF pathway proteins
Abnormal tissue expression of elements of the TF pathway have been demonstrated in various cancers including colorectal cancer\textsuperscript{221,222}. Proteins from the TF pathway that are expressed in colorectal cancer include TF\textsuperscript{221,222}, Thrombin\textsuperscript{232}, PAR 1\textsuperscript{342} and PAR2\textsuperscript{238}.

Expression of TF pathway markers is associated with a poor prognosis in a range of cancers. The limited research in colorectal cancer demonstrates that expression of TF correlates with increasing Dukes and TNM stage\textsuperscript{221} as well as an increasing risk of developing metastases\textsuperscript{222}. Expression of PAR2 is associated with high TNM stage, presence of LVI and poor differentiation\textsuperscript{238}. Thrombin and PAR1 expression has not been associated with poor prognostic factors or outcome in colorectal cancer. In prostate cancer the expression of PAR1 is associated with an advanced disease stage\textsuperscript{237}. In breast cancer expression of TF by stroma rather than epithelial cells is associated with a poor prognosis suggesting that the TF pathway may have a role in the tumour microenvironment\textsuperscript{227,228}. In colorectal cancer the site of expression (epithelial or stromal) of the TF pathway proteins has not been determined.

This chapter aims to establish the level of expression of proteins from the TF pathway in normal and malignant tissue. The site of expression will be determined and whether the expression is epithelial or stromal. The relationship between the expression levels and clinicopathological outcomes will also be determined.

4.1.2 Plasma expression of TF pathway proteins
Proteins from the TF pathway (TF, TAT, fibrinogen and d-dimer) and also markers of downstream pathway activation (P-selectin and sGPV) that are present in plasma have been proposed as biomarkers in cancer. TF is elevated in cancer compared to non-cancer controls in ovarian\textsuperscript{241}, non-small lung\textsuperscript{242}, breast\textsuperscript{185} and bladder cancers\textsuperscript{244}. In ovarian cancer increasing TF concentration correlates with reduced survival\textsuperscript{241}. Its role as a biomarker in colorectal cancer has not been investigated. TAT is elevated in many malignancies, including breast, ovarian, uterine and cervical cancers\textsuperscript{168,245,246,343}. In colorectal cancer pre-operative TAT is significantly
elevated in patients with colorectal cancer compared with non-cancer controls, and is further elevated in patients with metastases. It has also been shown that levels increase with disease recurrence.

The aims of this chapter are to determine the level of expression of proteins from the TF pathway in serum and whether there is any association with disease presence and clinicopathological factors that would suggest a role as a biomarker for colorectal cancer.

4.2 Details of the study population

A clinical cohort study was undertaken to study tumour and serum expression in patients with colorectal cancer. The study ‘CHAMPion’ is a multicentre study that had been opened at the University Hospital of South Manchester when I commenced this MD. I then opened the study in four additional sites in Manchester:

- Central Manchester NHS Foundation Trust
- East Cheshire NHS Foundation Trust
- University Hospital Salford NHS Foundation Trust
- Stockport NHS Foundation Trust.

For detailed description of the methodology of the study see section 2.2 but briefly patients undergoing curative surgery for colorectal cancer and patients undergoing similar surgery for non-cancer indications were recruited pre-operatively. They underwent, bilateral full leg duplex scans pre-operatively and at 42 days post-operation. Bloods were taken pre-operatively and on day 1, 14 and 42 post-operatively. The study is an NIHR adopted study (UKCRN portfolio no 8685) and therefore was undertaken with the aid of the research nurses at the respective hospitals.

Patients undergoing cancer surgery had tissue sampled from the cancer and distant normal tissue for immunohistochemistry (IHC).

4.2.1 Patients recruited

The study is ongoing and has continuing recruitment. The analysis undertaken in this MD is an interim analysis and is therefore underpowered. The original sample size based on the power calculation (see section 2.2.3) is for 165 cancer patients and 20 non-cancer controls.
A total of 74 patients with cancer and 11 non-cancer controls have been recruited to the study. Of the 74 cancer patients 12 have been excluded from the current analysis. The reason for the exclusions are:

- 3 underwent neo-adjuvant chemotherapy
- 3 had upper gastrointestinal malignancy
- 1 patient had pre-operative evidence of metastasis
- 1 patient had a low grade GIST.
- 2 patients had cancer in polyps and no tumour was evident on surgical histology
- 2 patients withdrew from the study.

Of the remaining 62 all had blood available for analysis and 37 had tissue available for analysis.

There were no exclusions from the control group.

4.2.2 Patient demographics

Cancer patients were older, with a higher Body Mass Index (BMI), increased prevalence of hypertension and increased use of antiplatelets than the benign controls (Table 4.1).
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Table 4.1 Demographic details of control and cancer group

<sup>a</sup>=student T test. <sup>b</sup>= Fischer’s exact test
4.2.3 Statistical analysis
For detailed description see section 2.2.12.

Data was examined to determine if it was normally distributed. The distribution was established by:

- Examining the histogram to ensure that a bell shaped distribution of the data existed
- Ensuring that the Skewness and Kurtosis are between -1 and +1
- Ensuring that the median and mean are similar
- Ensuring that the mean ± 2 SD incorporates the maximum and minimum range.

Data that was not normally distributed was log converted to normalise the distribution. Data that was log converted is presented as the geometric mean with 95% confidence intervals (CI). Statistical analysis was undertaken using parametric tests for normally distributed continuous data. Students’ T test was used to determine a difference between two populations of continuous data. If more than two factors were present the ANCOVA was used with Tukey for post hoc analysis. For correlation Pearson’s Rank correlation was used. For comparing categorical data Fischer exact test was used.

As there were multiple endpoints in the study the data was subject to repeated measures with multiple analysis for endpoints. The study is also not powered for these endpoints to be analysed and is an exploratory analysis. Due to this there is a risk of type 1 errors. The results of the analysis have an adjusted significance level, reduced to 1% (p value that is deemed to be significant is ≤0.01). Any results that are found to indicate a possible predictive role will be investigated in a future, more definitive study.

4.3 Epithelial and stromal expression of TF pathway proteins in cancer and normal tissues
To determine the expression of thrombin pathway markers on cancer and normal epithelial and stromal cells samples of tissue from the cancer and distant normal tissue were taken. The patients therefore provided their own control. From the patients recruited to CHAMPion 37 patients had tissue available for analysis. These
patients’ tissue was used for construction of the TMA. The demographics of this patient subgroup are given in Table 4.2.
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Table 4.2 Demographics of patients with IHC undertaken

Patients undergoing operations for cancer had samples of tissue from the cancer and distant normal tissue sampled (n=37). They therefore provided their own controls.
4.3.1 Details of TMA preparation, IHC and scoring.

The methods for preparation of the Tissue Micro-Array TMA and IHC are given in sections 2.2.9 and 2.2.10 but briefly representative samples of tumour and normal tissue were identified using an H+E slide. Two 1mm cores of tissue were taken from these identified areas and made into a TMA by Miss Hannah Gregson (Histopathology technician). The TMAs were stained with Haematoxylin and Eosin (H&E) to ensure that the malignant and normal areas had been captured (Figure 4.1) and underwent IHC staining for proteins from the TF pathway (TF, PAR1, PAR2 and Thrombin). An isotype control was used to ensure the specificity of the antibody. The expression of TF pathway proteins in normal and malignant tissues was determined by simultaneous scoring by myself and Dr Anna Davenport (NHS consultant Histopathologist).

A semiquantitative method of determining expression was undertaken (for detailed method see section 2.2.11) but briefly the expression of the protein was quantified into absent (score 0), low (score 1), medium (score 2) and high (score 3). The percentage of the cells that expressed each of these was also quantified (i.e. the percentage of total cells that had score 3, the total that had score 2 and the total that had score 1). An H score was calculated by multiplying the percentage of each score of the total by the score for those cells ie (3x the % of cells with score 3) + (2 x the % of score 2) + (1x the % of score 1). Examples of the staining are given; TF Figure 4.2, PAR1 Figure 4.3, PAR2 Figure 4.4 and thrombin Figure 4.5.
Figure 4.1 H&E slides of TMA for benign and malignant tissues.

TMAs prepared for both benign and malignant tissues stained with H&E to ensure that a representative section had been sampled. The example of the benign tissue demonstrates a normal cross section of colonic crypts. In the malignant example the normal morphology of the crypts is disrupted by the presence of malignant epithelial cells.
Figure 4.2 Example staining of TF in colorectal cancer tissue

TMAs were stained with TF antibodies and the expression was determined for epithelial and stromal tissues. The TF staining was both membranous and cytoplasmic in epithelial cells and membranous only in stromal cells. Examples of the intensity of staining are shown with the score allocated (red arrow identifies the area of interest). There were no cells that displayed high (grade 3) staining of the cytoplasm or in stromal cells. The isotype control is given to demonstrate specificity of the antibody. Magnification at 40x.
Figure 4.3 Example staining of PAR1 in colorectal cancer tissues

TMAs were stained with PAR1 antibodies and the expression was determined for epithelial and stromal tissues. The PAR1 staining was cytoplasmic in both epithelial and stromal cells. Examples of the intensity of staining are shown with the score allocated (red arrow identifies the area of interest). There were no cells that displayed high (grade 3) staining in the stromal cells. The isotype control is given to demonstrate specificity of the antibody. Magnification at 40x.
Figure 4.4 Example staining of PAR2 in colorectal cancer tissues

TMAs were stained with PAR2 antibodies and the expression was determined for epithelial and stromal tissues. The PAR2 staining was cytoplasmic in both epithelial and stromal cells. Examples of the intensity of staining are shown with the score allocated (red arrow identifies the area of interest). There were no cells that displayed high (grade 3) staining in the stromal cells. All the epithelial cells demonstrated some staining therefore there are none that score 0. The isotype control is given to demonstrate specificity of the antibody. Magnification at 40x.
Figure 4.5 Example staining of Thrombin in colorectal cancer tissues

TMAs were stained with Thrombin antibodies and the expression was determined for epithelial and stromal tissues. The Thrombin staining was cytoplasmic in both epithelial and stromal cells. Examples of the intensity of staining are shown with the score allocated (red arrow identifies the area of interest). There were no cells that displayed medium (grade 2) or high (grade 3) staining in the stromal cells. There were no epithelial cells that displayed high (grade 3) staining. The isotype control is given to demonstrate specificity of the antibody. Magnification at 40x.
The data for the H-scores was not normally distributed and also had zero expression as a possible result. To normalise the data it was log converted and to accommodate the results of zero a constant of 1 was added to each result (the formula for log converting was $\ln(H\text{score}) = \ln(H\text{score}+1)$). To establish the geometric mean and 95% CI the reverse was undertaken with the formula geometric mean $= \exp(\ln(H\text{score})) - 1$. All results are shown as geometric mean +/- 95% CI.

4.3.2 Localisation of expression of TF pathway proteins within epithelial and stromal cells

In epithelial cells TF expression was localised to membrane and cytoplasm (Figure 4.2). PAR1 (Figure 4.3), PAR2 (Figure 4.4) and thrombin (Figure 4.5) expression was cytoplasmic. Stromal cells TF expression was membranous and for PAR1, PAR2 and thrombin cytoplasmic.

As TF was expressed in both the cytoplasm and on the membrane of the malignant epithelium a test of correlation between the H score for expression at the two sites was performed. This was to determine if the expression of TF in the cytoplasm was reflective of the membranous expression (TF is a trans-membranous protein). There is a strong correlation between the membranous and cytoplasmic expression of TF (Pearson correlation co-efficient= 0.7 p<0.001) (Figure 4.6) implying that the expression in each site is determined by the other.

As there was such a strong correlation data is presented for the membranous staining. Cytoplasmic staining of TF was also analysed (data not shown) for each variable to ensure that there was no difference between the membranous expression and cytoplasmic expression.
Figure 4.6 Correlation of TF expression by cytoplasm and membrane in epithelial cells

Analysis undertaken with Pearson’s Rank correlation. This demonstrates a strong correlation between the expression of TF on the epithelial cell membrane and cytoplasm.
4.3.3 Epithelial expression of TF pathway markers in cancer compared to normal tissue

TF was not expressed in epithelial cells in normal tissue. It was noted that it was expressed by the wall of the vesicles of some goblet cells, however in these cells there was no other TF expression and so this was felt to be artefact and was not scored (Figure 4.7). In the epithelium TF was expressed in the membrane by 65% of the cancer specimens (n=37) and 0% of the normal epithelium (n=36). Geometric mean of the H score in the cancer specimens was 6.9 arbitrary unit (u) (95%CI 3.1 - 14.3) compared to 0u in normal epithelium (p <0.001) (Figure 4.8).

PAR1 was expressed by 84% of cancer samples (n=37) and 94% of the normal samples (n=36). There was no difference in intensity of staining between cancer and normal specimens (cancer H-score 54.5u (95% CI 29.4 – 100.6) vs benign H-score 78.3u (95%CI 59.2 – 103.3) p= 0.1) (Figure 4.8).

PAR2 was expressed by 100% of the cancer (n=37) and normal (n=36) samples. The H-scores, however, was higher in the cancer epithelial cells (cancer H score 162.0u (95% CI 143.2 – 183.3) vs normal H score 116.0u (95% CI 102.6 – 131.2) p=0.001) (Figure 4.8).

Thrombin was expressed in 100% of malignant (n=37) and normal (n=36) samples. Levels of expression were similar between cancer and normal tissue (cancer H-score of 114.8u (95% CI 96.2 – 136.9) vs normal H score 97.8u (95%CI 87.6- 108.9) p=0.2 (Figure 4.8).
Figure 4.7 TF staining of the vesicles of goblet cells in normal tissue in colorectal tissue.

It was noted that in normal tissue TF staining was evident on the wall of the vesicles of the goblet cells (identified by red arrows). This was present with no staining on the cell membrane or in the cytoplasm and was therefore felt to be artefact and was not scored.
Figure 4.8 Epithelial expression of thrombin pathway proteins in benign compared to malignant tissues.

Tissue samples taken from cancer and distant normal tissue from the same patients underwent IHC to demonstrate the expression of TF, PAR1, PAR2 and thrombin. The H-score was used to calculate the expression of the protein. Data is presented as geometric mean +/- 95%CI. Statistical analyses was undertaken using Student T test.
4.3.4 Stromal expression of TF pathway markers in cancer compared with normal tissue

The same tissues that were used to determine epithelial expression of TF, PAR1, PAR2 and thrombin were scored for expression of TF, PAR1, PAR2 and thrombin by stromal cells. Stromal fibroblast expression was specifically scored as fibroblast activity is correlated with a poor prognosis. The stromal inflammatory cells were not scored and neither was vascular endothelium as these normally express TF pathway proteins. It was noted that the stromal expression of the proteins was localised to the stroma in close proximity to (approximately 0.1mm) epithelial cells. The expression by the stroma that was within 0.1mm of epithelial cells was therefore scored.

TF was expressed by the stroma in 70% of cancer samples (n=37) and not expressed in the stoma of normal samples (n=36). The H score of TF expression was 10.1u (95% CI 4.6 – 20.7) for the cancer samples compared to 0u for the normal samples <0.001 (Figure 4.9).

PAR1 was expressed by stroma in 100% of the cancer samples (n=37) and in 53% of the normal specimens. The H score for PAR1 expression was 116.7u (95% CI 98.2 – 138.7) for the cancer associated stroma compared to 7.9u (95%CI 4.3 – 14.0) for the normal stroma (p<0.001) (Figure 4.9).

PAR2 was expressed by the stroma in 65% of the cancer samples (n=37) and 8% of the non-cancer samples (n=36). The H score for PAR2 expression was 6.8u (95% CI 3.4 – 12.9) for the cancer associated stroma compared to 0.7u (95%CI 0.2 – 1.3) for the normal stroma (p<0.001) (Figure 4.9)

Thrombin was expressed in the stroma of 70% of cancer specimens (n=37) and not expressed in the stoma of normal specimens (n=36). The H score for thrombin expression by the cancer associated stroma was 4.7u (95% CI 2.63 – 8.0) compared to 0u for the normal tissue p= <0.001 (Figure 4.9).
Figure 4.9 Stromal expression of thrombin pathway proteins in benign compared to malignant tissues.

Tissue samples taken from cancer and distant normal tissue from the same patients underwent IHC to quantify stromal expression of TF, PAR1, PAR2 and thrombin. The H-score was used to calculate the expression of the protein. Data is presented as geometric mean +/- 95%CI.

Statistical analyses was undertaken using Student T Test
4.3.5 Correlation between epithelial and stromal expression of thrombin pathway proteins

To try and investigate whether stromal expression could be an effect of epithelial expression of the thrombin pathway markers the expression of the marker in each tissue was correlated (correlation undertaken with Pearson’s co-efficient between H scores). In the benign tissue TF and thrombin staining was not present therefore this was not analysed. There was no correlation between the expression of PAR1 or PAR2 in the stroma and epithelial cells in normal samples.

There was no correlation in the malignant cells between the expression of TF and PAR1 by epithelial and stromal tissues. There was however a correlation between the stromal and epithelial expression of PAR2 (p=0.007). Thrombin approached significance with a p value of 0.04 (Table 4.3).
<table>
<thead>
<tr>
<th></th>
<th>Benign</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF</td>
<td>NA</td>
<td>R = -0.162</td>
</tr>
<tr>
<td>PAR1</td>
<td>R = 0.220</td>
<td>R = 0.187</td>
</tr>
<tr>
<td>PAR2</td>
<td>R = -0.021</td>
<td>R = 0.433</td>
</tr>
<tr>
<td>Thrombin</td>
<td>NA</td>
<td>R = 0.337</td>
</tr>
</tbody>
</table>

Table 4.3  Correlation between expression of TF pathway by epithelial and stromal cells.

The H-score of expression of TF pathway proteins by epithelial and stromal cells was correlated in benign and malignant tissues. TF and thrombin were not expressed in the normal tissue therefore analysis was not undertaken. This demonstrates that the expression of PAR1 and PAR2 by epithelial and stromal cells is independent in benign tissues. In malignant tissues TF and PAR1 expression by epithelial and stromal cells are independent but PAR2 and Thrombin expression is dependent. Correlation undertaken with Pearson’s rank correlation. R = correlation co-efficient
4.3.6 Correlation between expression of TF pathway proteins
As multiple proteins from the TF pathway are expressed by the epithelial and stromal cells in cancer it raises the possibility that the local presence of one protein is up regulating the expression of other proteins. To determine if this is the case the expression of proteins from the TF pathway by the tumour was correlated with the expression of the other TF pathway proteins. The correlation was undertaken with expression by epithelial and stromal cells. There was no correlation between any of the markers by the epithelial (Table 4.4) or stromal (Table 4.5) cells.
<table>
<thead>
<tr>
<th></th>
<th>TF</th>
<th>PAR1</th>
<th>PAR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.138</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAR2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>-0.143</td>
<td>0.305</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.4</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>37</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>-0.205</td>
<td>-0.092</td>
<td>0.108</td>
</tr>
<tr>
<td>p</td>
<td>0.2</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>N</td>
<td>37</td>
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<td>37</td>
</tr>
</tbody>
</table>

*Table 4.4* Correlation between epithelial expression of TF pathway proteins

*Correlation between the H-score of the TF pathway proteins was undertaken using Pearson's rank correlation. R= correlation coefficient, p=p-value, N= number of samples*
<table>
<thead>
<tr>
<th></th>
<th>TF</th>
<th>PAR1</th>
<th>PAR2</th>
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</thead>
<tbody>
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<td>PAR1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.312</td>
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<td></td>
</tr>
<tr>
<td>p</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAR2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.103</td>
<td>0.146</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.5</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>37</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>-0.114</td>
<td>0.002</td>
<td>0.194</td>
</tr>
<tr>
<td>p</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>N</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
</tbody>
</table>

Table 4.5 Correlation between stromal expression of TF pathway proteins

Correlation between the H-score of the TF pathway proteins was undertaken using Pearson’s rank correlation. R= correlation coefficient, p=p-value, N= number of samples
4.3.7 Expression of the TF pathway markers in colonic compared to rectal tissue

The cancers that were sampled as part of the CHAMPion study originated in both the rectum and colon. To determine if there was any difference in the expression of TF pathway markers by colonic and rectal cancers which may reflect a different biology between the two sites, the level of expression of the TF pathway proteins was explored. There was no difference in the expression of any of the markers between colonic or rectal tumours by epithelial (Figure 4.10) or stromal cells (Figure 4.11).
Figure 4.10 Epithelial expression of TF pathway proteins in colonic compared to rectal cancers.

There was no difference in the expression of TF pathway proteins between rectal and colonic cancer epithelial cells. Differences in the expression of the TF pathway proteins were determined by use of the student T Test.
Figure 4.11 Stromal expression of TF pathway proteins in rectal compared to colonic cancers.

There was no difference in the expression of TF pathway proteins between rectal and colonic cancer stromal cells. Differences in the expression of the TF pathway proteins were determined by use of the student T Test.
4.3.9 Expression of TF pathway markers in lymph node negative compared to positive tumours

Previous research has suggested that TF expression by colorectal cancer is associated with a more aggressive cancer and a cancer that is more likely to be associated with the presence of metastases\(^2^{22}3^{0}8\). Similarly PAR2 is associated with the presence of metastases and a higher TNM stage\(^2^{3}8\). The relationship between TF pathway protein expression and lymph node involvement in non-metastatic disease has not previously been explored. To determine whether any difference in expression was by stroma or epithelial cells the level of expression of the TF pathway proteins was determined in lymph node negative compared to lymph node positive cancers. In the epithelium there was no difference in expression of the TF pathway proteins between the lymph node negative and positive tumours (Figure 4.12).

In the stromal cells there was also no difference in the expression of the TF pathway proteins between lymph node negative and positive tumours. (Figure 4.13).
Figure 4.12 Epithelial expression of TF pathway proteins in lymph node positive (+ve) compared to negative (–ve) disease

There was no difference in the expression of TF pathway proteins by cancer epithelial cells in lymph node negative compared to lymph node positive disease. Differences in the expression of the TF pathway proteins were determined by use of the student T Test.
Figure 4.13 Stromal expression of TF pathway proteins in lymph node positive (+ve) compared to negative (−ve) disease

There was no difference in the expression of TF pathway proteins by cancer stromal cells in lymph node negative compared to lymph node positive disease. Differences in the expression of the TF pathway proteins were determined by use of the student T Test.
4.3.10 Relationship between expression of TF pathway proteins and T stage

To determine if the expression of TF pathway proteins was related to the T stage of a tumour the expression of the proteins by the cancer was compared to the tumour T stage. T1 tumours were excluded from the analysis as there were only two patients with IHC results with T1 tumours. ANOVA was used to determine if there was any difference between the expression of TF pathway proteins depending on the tumour T stage. Post hoc pairwise analysis was undertaken using Tukey. There was no difference between any of the variables identified for epithelial (Figure 4.14) or stromal (Figure 4.15) expression. There was also no difference in pairwise analysis.
Figure 4.14 Epithelial expression of TF pathway protein and T stage.

Analysis of expression of TF pathway proteins by epithelial cells and T stage. T1 was excluded as there were only 2 patients with IHC results and a T1 tumour. Statistical analysis was undertaken with ANOVA. There was no differences detected using ANOVA. Pairwise post-hoc analysis was undertaken with Tukey (p values shown).
Figure 4.15 Stromal expression of TF pathway protein and T stage.

Analysis of expression of TF pathway proteins by stromal cells and T stage. T1 was excluded as there were only 2 patients with IHC results and a T1 tumour. Statistical analysis was undertaken with ANOVA. There was no differences detected using ANOVA. Pairwise post-hoc analysis was undertaken with Tukey (p values shown).
4.3.11 Relationship between epithelial and stromal expression of TF pathway proteins and TNM stage

A previous study has demonstrated that colorectal TF expression is associated with a higher TNM stage\textsuperscript{221}. To determine if this was the case in this cohort of patients and to determine if any association correlated with expression by stroma or epithelial cells, the relationship between protein expression and TNM stage was explored. There was no difference in the epithelial expression of any of the TF pathway proteins between TNM stages (Figure 4.16).

In stroma there was no difference in TF, PAR2 or Thrombin expression between TNM stages. There is a suggestion of a negative association between PAR1 expression by cancer associated stroma and TNM stage, however it did not reach significance. The mean H score of TNM1 compared to TNM3 was 155.3 (95%CI 93.1 – 203.2) vs 87.9 (95%CI 53.3 – 144.6) $p=0.05$. There was no difference between TNM1 and TNM2 or TNM2 and TNM3 (Figure 4.17)
There was no difference in the epithelial expression of TF pathway proteins by the cancers between tumours of different TNM stage. Analysis was undertaken using ANCOVA. Pairwise analysis undertaken using Tukey post-hoc analysis (p-values shown).
Figure 4.17 Stromal expression of TF pathway proteins and TNM stage

There was no difference in the stromal expression of TF pathway proteins by the cancers between tumours of difference TNM stage. Analysis was undertaken using ANCOVA. Pairwise analysis using Tukey post-hoc analysis (p-values shown).
4.3.12 Relationship between TF pathway proteins and Lymphovascular invasion (LVI)

The presence of LVI is a poor prognostic indicator in colorectal cancer. Patients with LVI are at increased risk of developing metastases. The epithelial expression of TF and thrombin was lower in the LVI +ve group compared to the negative group, however neither of these reached significance (TF 1.57u (95%CI 0 – 1.57) vs 10.3u (95%CI 4.7 – 23.7) p=0.03, thrombin 84.5u (95%CI 42.9 – 165.7) vs 126.6u (95%CI 111.5 – 143.7) p=0.05. PAR1 and PAR2 did not demonstrate any suggestion of difference between the level of epithelial expression between patients with and without LVI (Figure 4.18). There was no difference in stromal expression between LVI positive and negative cancers (Figure 4.19).
Figure 4.18 Epithelial markers of TF pathway proteins in cancer with (+ve) and without (-ve) LVI

There was no difference in the epithelial expression of TF pathway proteins by the cancers between tumours with or without LVI. Analysis was undertaken with student T test, TF and PAR2 demonstrated a suggestion of a negative relationship with the presence of LVI however this did not reach significance.
Figure 4.19 Stromal makers of TF pathway proteins in cancer with (+ve) and without (-ve) LVI

There was no difference in the stromal expression of TF pathway proteins by the cancers between tumours with or without LVI. Statistical analysis undertaken with student T test. There was no difference in the expression of any of the TF pathway proteins between those tumours with LVI and those without.
4.3.13 Correlation between TF pathway protein expression and size

To determine if there is any correlation between the size of the tumour and the expression of the TF pathway protein the H-scores were correlated with the maximum diameter of the tumour using Pearson’s correlation. There was no correlation between the expression of TF pathway proteins and the size of the tumour for either epithelial or stromal expression (Table 4.6)
<table>
<thead>
<tr>
<th></th>
<th>Epithelial expression</th>
<th>Stromal expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.161</td>
<td>-0.233</td>
</tr>
<tr>
<td>$p$</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>$N$</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>PAR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>-0.252</td>
<td>-0.219</td>
</tr>
<tr>
<td>$p$</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>$N$</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>PAR2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>-0.097</td>
<td>0.062</td>
</tr>
<tr>
<td>$p$</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>$N$</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Thrombin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>-0.063</td>
<td>-0.146</td>
</tr>
<tr>
<td>$p$</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td>$N$</td>
<td>37</td>
<td>37</td>
</tr>
</tbody>
</table>

*Table 4.6 Correlation between TF pathway protein and size of the tumour*

The $H$-score for the expression of the TF pathway protein were correlated with the maximum diameter of the tumour using Pearson’s correlation. There was no correlation between the size of the tumour and either the epithelial or stromal expression of TF pathway proteins. $R^2$ = Correlation coefficient, $p$ = $p$-value, $N$ = number of samples.
4.3.14 Association between TF pathway protein and differentiation

To determine if the expression of the TF pathway protein was affected by the level of differentiation of a tumour the H scores were compared to the level of differentiation. Due to the small number of cases with poorly differentiated tumour (n=3) these were not included in the analysis. The analysis was undertaken using student T test. There was no difference in the level of expression of the TF pathway protein either by the epithelial (Figure 4.20) or stromal cells (Figure 4.21).
Poorly differentiated tumours were excluded as there were only 3 available for analysis where IHC had been undertaken. There was no difference between the epithelial expression of TF pathway proteins by well and moderately differentiated tumours. Analysis was undertaken using student t test.
Figure 4.21 Stromal expression of TF pathway proteins and level of differentiation.

Poorly differentiated tumours were excluded as there were only 3 available for analysis where IHC had been undertaken. There was no difference between the stromal expression of TF pathway proteins by well and moderately differentiated tumours. Analysis was undertaken using student t test.
4.4 Plasma markers of thrombin pathway activation

The use of TF pathway proteins and downstream markers have been proposed as possible biomarkers for cancer. This is supported by limited research suggesting TF pathway proteins are circulating at higher levels in patients with cancer and higher circulating levels are associated with a worse cancer phenotype. To determine if plasma TF pathway proteins have a role as biomarkers of the presence of colorectal cancer or as biomarkers of poor cancer phenotype, plasma levels were compared in healthy controls and cancer patients. In cancer patients, plasma levels were correlated with clinicopathological factors (lymph node involvement, LVI, tumour T stage, TNM stage). These pathological factors that are associated with a poor prognosis were used as surrogate markers of prognosis as long term follow-up is not currently available.

4.4.1 Patients recruited

Sixty two patients recruited to CHAMpion were analysed. For details of the study population and demographics see section 4.2

4.4.2 Determination of serum levels of markers of the TF pathway activation.

Analysis of the level of expression of the plasma markers of activation of the TF pathway were determined as detailed in section 2.2.6. The level of d-dimer and fibrinogen were measured by automated ELISA at the University Hospital of South Manchester (UHSM) Haematology Unit. The samples from other hospitals were centrifuged and frozen locally (for method see 2.2.5) prior to being transferred to UHSM for analysis. Previous work in our unit has demonstrated that samples that are frozen when retested after some months produce the same results as the original analysis\textsuperscript{228}. In addition the method of analysing the samples at UHSM changed during the project from miniVIDAS to TOP500. The effect of this change has also been studied in the unit and this has demonstrated that both methods produce consistent results\textsuperscript{228}.

Analysis of the other serum markers (TF, TAT, sGPV and P-selectin) were undertaken by ELISA on frozen aliquots of serum at the Institute of Cancer Sciences, Manchester (for methods see section 2.2.6.1. The sGPV and P-selectin ELISAs were validated and undertaken by myself, the TF and TAT ELISAs were validated by myself and the sample analysis undertaken by John Castle (research technician).
4.4.3 Validation of ELISA for TF, TAT, sGPV and P-selectin

Prior to analysing the serum samples the ELISA process underwent validation. This was to ensure that the kits, reagents and operator produced consistent and reliable results.

The ELISA protocol (see section 2.2.6.1) was run for each protein 6 times with Quality Control (QC) samples present. The concentration of the QC had previously been determined by the manufacturer. This ensured that the results for the assay were consistent with the stated results of the QC. The samples were run and analysed in duplicate. To ensure that each analysis passed there were a number of acceptance criteria:

- The $r^2$ for the standards was greater than 0.985
- The coefficient of variation (CV) for the standards was less than 15%
- The CV for the QC was less than 30%
- The result of the QC was within the range provided by the manufacturer.

For the analysis of the samples these were run in duplicate with QC samples at the beginning, middle and end of the plate. The plate was only accepted as having passed and the results used if the above acceptance criteria were met. In addition any individual samples that demonstrated a CV of greater than 30% were also rejected.

4.4.4 Establishment of normal distribution

None of the plasma samples displayed a normal distribution (for method of determining normality see section 2.2.12) therefore all plasma analysis required log transformation. The data is therefore presented as a geometric mean with 95% CI.

4.4.5 Association between pre-operative markers and demographic factors and co-morbidities

Levels of plasma TF pathway have previously been associated with several demographic factors.

D-dimer level has been associated with age, body mass index (BMI), hypertension, smoking and alcohol intake\textsuperscript{344,345}. Fibrinogen has been associated with age, and smoking\textsuperscript{346}. These factors were therefore assessed in this cohort. The analysis was undertaken using Pearson’s correlation for continuous data (Table 4.7) and student T test for categorical data (Table 4.8).
In this cohort the only correlations were:

- TF and age
- Fibrinogen and hypertension.

These confounders were controlled for when undertaking statistical analysis by using ANCOVA. In addition due to the previously demonstrated strong correlation between d-dimer and age this was also corrected for, even though not identified in this current (but relatively small) patient population.
<table>
<thead>
<tr>
<th>Thrombin pathway plasma marker</th>
<th>Age Coefficient [n] (p)</th>
<th>BMI Coefficient [n] (p)</th>
<th>Alcohol Coefficient [n] (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-dimer</td>
<td>0.221 [58] (0.1)</td>
<td>-0.16 [50] (0.4)</td>
<td>-0.201 [50] (0.2)</td>
</tr>
<tr>
<td>TF pg/ml</td>
<td><strong>0.314 [59] (0.02)</strong></td>
<td>0.051 [54] (0.7)</td>
<td>-0.066 [54] (0.6)</td>
</tr>
<tr>
<td>TAT ng/ml</td>
<td>0.078 [60] (0.6)</td>
<td>0.129 [54] (0.4)</td>
<td>-0.045 [55] (0.7)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.142 [56] (0.3)</td>
<td>-0.231 [48] (0.9)</td>
<td>-0.203 [50] (0.2)</td>
</tr>
<tr>
<td>P-selectin</td>
<td>0.125 [45] (0.4)</td>
<td>0.231 [38] (0.2)</td>
<td>-0.225 [38] (0.16)</td>
</tr>
<tr>
<td>sGPV</td>
<td>-0.052 [63] (0.7)</td>
<td>-0.209 [55] (0.1)</td>
<td>-0.192 [56] 0.2</td>
</tr>
</tbody>
</table>

*Table 4.7 Correlation between plasma markers of TF pathway activation and demographics*

*Correlation between the serum marker and the continuous variable was undertaken using Pearson’s Rank Correlation. The only correlation seen was between TF and age.*
<table>
<thead>
<tr>
<th>Thrombin pathway plasma marker</th>
<th>Hypertension [n] (p)</th>
<th>Smoker [n] (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-dimer</td>
<td>[53] (0.3)</td>
<td>[61] (0.4)</td>
</tr>
<tr>
<td>TF pg/ml</td>
<td>[61] (0.3)</td>
<td>[60] (0.6)</td>
</tr>
<tr>
<td>TAT ng/ml</td>
<td>[62] (0.3)</td>
<td>[61] (0.06)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>[50] (0.007)</td>
<td>[59] (1.0)</td>
</tr>
<tr>
<td>P-selectin</td>
<td>[49] (0.9)</td>
<td>[48] (0.4)</td>
</tr>
<tr>
<td>sGPV</td>
<td>[68] (0.1)</td>
<td>[66] (0.8)</td>
</tr>
</tbody>
</table>

Table 4.8 Association between plasma markers of TF pathway activation and categorical data

Analysis of the association between serum thrombin pathway markers and presence or hypertension or being a smoker was undertaken using student t-test. Fibrinogen was found to be associated with a history of hypertension.
4.4.6 Correlation of tissue and serum markers of the TF pathway

It is suggested that the coagulation cascade is activated by the presence of prothrombotic proteins expressed by cancers. To determine if this was the case in this current cohort, the level of epithelial and stromal tissue expression of proteins from the TF pathway (as determined using the H-score) were correlated with the plasma concentration of markers of TF activation.

Epithelial expression of PAR2 correlated with plasma P-selectin ($r^2 = 0.422$ $p=0.01$). There was a near significant negative correlation ($p=0.03$) between tumour epithelial TF expression and plasma d-dimer as well as epithelial tumour PAR1 and plasma d-dimer ($p=0.02$) (Table 4.9).

Stromal expression had no significant correlations with plasma levels. The correlation between tumour stroma TF expression and plasma TF neared significance ($p=0.02$) and there was a suggestion of significance in a negative correlation between stromal TF expression and plasma fibrinogen ($p=0.03$)(Table 4.10).
<table>
<thead>
<tr>
<th>Plasma marker</th>
<th>Epithelial tissue marker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TF</td>
</tr>
<tr>
<td>TF</td>
<td>R²</td>
</tr>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>TAT</td>
<td>R²</td>
</tr>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>N</td>
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<tr>
<td>D-dimer</td>
<td>R²</td>
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<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>R²</td>
</tr>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>P-selectin</td>
<td>R²</td>
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<td>N</td>
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<td>sGPV</td>
<td>R²</td>
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<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>N</td>
</tr>
</tbody>
</table>

Table 4.9 Correlation of tissue markers (epithelial) of thrombin pathway activation and plasma markers of activation of the thrombin pathway.

Correlation using Pearson correlation. R²=correlation co-efficient, P=p value, N= number of samples
<table>
<thead>
<tr>
<th>Plasma marker</th>
<th>Stromal tissue marker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TF</td>
</tr>
<tr>
<td>TF</td>
<td>R²</td>
</tr>
<tr>
<td></td>
<td>P</td>
</tr>
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<td></td>
<td>N</td>
</tr>
<tr>
<td>TAT</td>
<td>R²</td>
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<td></td>
<td>P</td>
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<td></td>
<td>N</td>
</tr>
<tr>
<td>D-dimer</td>
<td>R²</td>
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<td></td>
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<td></td>
<td>N</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>R²</td>
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<td></td>
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<td></td>
<td>N</td>
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<tr>
<td>P-selectin</td>
<td>R²</td>
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<td></td>
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<td></td>
<td>N</td>
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<tr>
<td>sGPV</td>
<td>R²</td>
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<td></td>
<td>P</td>
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<td></td>
<td>N</td>
</tr>
</tbody>
</table>

Table 4.10 Correlation of tissue markers (stromal) of thrombin pathway activation and plasma markers of thrombin pathway activation.

Correlation using Pearson’s correlation. $R^2$=correlation co-efficient, $P$=p value, $N$= number of samples
4.4.7 Pre-operative markers TF pathway activation in cancer compared to control

Pre-operative markers of TF pathway activation are elevated in a range of cancers. In colorectal cancer, plasma d-dimer, P-selectin and TAT are elevated in patients with colorectal cancer compared to benign controls. To determine if any of the markers were able to discriminate between patients with cancer and benign controls in the CHAMPion cohort plasma levels were compared. Only P-selectin demonstrated a tendency towards significance (geometric mean 34.3 ng/mL (95%CI 29.8 – 39.5) for benign disease and 40.2 ng/mL (95%CI 37.5 – 44.3) for cancer (p=0.05)). There was no association between the level of any other pre-operative marker of TF activation and the presence of cancer (Figure 4.22).
Figure 4.22 Pre-operative plasma markers of thrombin pathway activation in cancer and control patients.

There was a suggestion of significance in the level of P-selectin with a higher level in the patients with malignant disease compared to benign disease. There was no difference with the other potential biomarkers. Analysis undertaken using ANCOVA for d-dimer and TF controlling for age, ANCOVA for thrombin controlling for HT. Others analysed using student T-test. Presented as geometric mean +/- 95% CI.
4.4.8 **Plasma markers in rectal compared to colonic cancer**

All patients recruited had tumours located in either the rectum or colon. To determine if the level of the plasma marker was affected by the position of the tumour plasma levels were compared in colonic and rectal cancers. TAT demonstrated a difference being significantly higher in colon compared to rectal cancer (5.7ng/mL (95%CI 4.5 – 7.4) vs 4.0ng/mL (3.6 – 4.5) p=0.01). sGPV demonstrated a possible trend for being higher in colonic compared to rectal cancers (52.5ng/mL (95%CI 48.0 – 57.5) vs 44.4ng/mL (95%CI 39.7 – 39.7) p=0.03). Conversely TF demonstrated a possible trend for rectal being higher than colonic (45.2pg/mL (95%CI 40.7 – 50.3) vs 41.9pg/mL (95%CI 39.1 – 44.9) p=0.04). d-dimer, TAT and P-selectin did not demonstrate any difference in their plasma levels of between colonic and rectal cancers (Figure 4.23)
Figure 4.23 Plasma markers of thrombin pathway activation in rectal and colonic tumours

Analysis undertaken using ANCOVA for d-dimer and TF controlling for age, ANCOVA for thrombin controlling for HT. Others analysed using student T-test. Presented as geometric mean +/- 95% CI.
4.4.9 Plasma TF pathway markers in lymph node and lymphovascular involved cancer

D-dimer is elevated in patients with lymph node involvement in gastro-oesophageal cancers and breast cancer\textsuperscript{228,256,347}. Specifically to colorectal cancer d-dimer and fibrinogen have been associated with lymphatic involvement\textsuperscript{250,260,348}. In this current cohort of patients, I determined the plasma levels of the TF pathway proteins in the presence or absence of lymph node involvement or lymphovascular involvement. No markers were able to discriminate between the presence and absence of lymph node involvement (Figure 4.24), or lymphovascular involvement (Figure 4.25).
Figure 4.24 Plasma markers of thrombin pathway activation in lymph node positive (+ve) and negative (-ve) disease

ANCOVA used for d-dimer with correction for age, and for thrombin with correction for hypertension. Paired T test used for TAT, sGPV and P-selectin. Data presented as geometric mean +/- 95% CI
Figure 4.25 Plasma markers of the thrombin pathway activation and the presence (+ve) and absence (-ve) of lymphovascular invasion (LVI)

ANCOVA used for d-dimer with correction for age, and for thrombin with correction for hypertension. Paired T test used for TAT, sGPV and P-selectin. Data presented as geometric mean +/- 95% CI
4.4.10 Plasma makers of TF pathway activation correlated to T stage of tumour

In colorectal cancer an elevated d-dimer level has been associated with serosal penetration by the cancer (T4 tumour)\textsuperscript{250}. No other protein from the TF pathway has been shown to be associated with T stage in previous works. In this analysis for TF the T1 tumours were not included in the analysis as there were only two patients with T1 disease and TF levels quantified. There was no difference in the level of any of the plasma proteins in relation to the T stage of the tumour. The level of d-dimer was higher in T4 compared to T3 tumours (951.6pg/mL (95%CI 630.1 – 1434) vs 563.4pg/mL (95%CI 432.5 – 795.0) and this neared statistical significance (p=0.05), however there was no difference between T4 tumours and T1 tumours (p=0.5)(Figure 4.26).
Figure 4.26 Plasma markers of thrombin pathway activation with T stage.

T1 was not analysed for TF as only 2 patients with T1 disease had TF levels available. ANOVA was used for TAT, sGPV and P-selectin with post hoc analysis using Tukey. Ancova used for d-dimer and TF (with correction for age) and fibrinogen (with correction for HT).
4.4.11 Plasma markers of TF pathway activation and TNM stage

D-dimer has in a previous study been shown to be elevated in higher TNM stage colorectal cancers\(^{250}\). In this cohort there was no difference in d-dimer, TF, TAT, sGPV or P-selectin between different TNM stages. Fibrinogen demonstrated a significant increase in expression between TNM1 and TNM2 (3.5mg/dL (95%CI 2.8 – 4.3) vs 4.0mg/dL (95%CI 3.7 – 4.4) \(p=0.006\)) however there was a significant lower expression in TMM2 compared to TNM3 (4.0mg/dL (95%CI 3.7 – 4.4 vs 3.3mg/dL (95%CI 2.8 – 3.8) \(p=0.01\)). There was no significant difference between TNM1 and TNM3 \((p=0.7)\) (Figure 4.27).
ANOVA used for TAT, sGPV and P-selectin with post hoc analysis using Tukey. Ancova used for d-dimer (with correction for age) and fibrinogen (with correction for HT).
4.4.12 Plasma markers of TF pathway activation and differentiation

Expression of the plasma markers of the TF pathway proteins were correlated with the level of tumour differentiation. There was no difference in the plasma level of TF pathway proteins between the levels of differentiation (Figure 4.28)
Figure 4.28 Plasma markers of thrombin pathway activation and cancer differentiation.

ANOVA used for TAT, sGPV and P-selectin with post hoc analysis using Tukey. Ancova used for d-dimer and TF (with correction for age) and fibrinogen (with correction for HT).
4.4.13 Correlation of plasma markers of TF pathway activation with tumour size

There was no correlation between tumour size and TF, TAT, P-selectin or sGPV. However, fibrinogen correlated with tumour size (correlation coefficient 0.481, p<0.001). There was a possible correlation between tumour size and plasma d-dimer (correlation coefficient 0.262, p=0.05) (Figure 4.29).
Figure 4.29 Correlation between plasma markers of activation of TF pathway and size of tumour.

Natural log of TF pathway marker plotted against size of tumour. Pearson's correlation undertaken. CC = correlation coefficient.
4.5 Discussion

4.5.1 Epithelial Expression of TF pathway proteins in colorectal cancer

There is limited previous research regarding the level of expression of TF pathway proteins in colorectal cancer. This study has demonstrated no expression of TF by normal colorectal epithelial cells. TF staining was evident in the wall of secretory vesicles of the goblet cells but was not present in either the cell membrane or cytoplasm. As there was no expression by either the cell membrane or cytoplasm this goblet cell expression was felt to be an artefact by Dr Anna Davenport (consultant histopathologist). A previous study using frozen sections demonstrated expression in the epithelium of normal colorectal specimens but the authors of the study have not provided pictures of the expression of TF in normal colorectal tissue or specified where the expression was localised. It is possible that the expression that they have seen is in the vesicle of the goblet cell. The majority (70%) of the cancer specimens expressed TF in epithelial cells. This finding is consistent with previous studies that have also demonstrated expression of TF by colorectal cancers. These previous studies have demonstrated that this expression is associated with advanced TNM and Duke's stage and an enhanced ability to form metastases. This relationship has been demonstrated in metastatic cancers that were excluded from my study. In my study of localised cancers I have not demonstrated any association with either lymph node involvement or advanced TNM stage. It may be that TF has a role in the development of distant metastases rather than in local and lymph node disease. It is also worth noting that the numbers of my current study are small and that the study may be underpowered to detect a difference.

In my current study of colorectal cancer I demonstrated expression of PAR1 in normal mucosa. One paper has been published that explores the expression of PAR1 in normal and malignant tissues. They demonstrated no PAR1 expression in non-cancer control patients, however only three patients were included in this group. In the cancer group (12 patients) 11 expressed PAR1 in the tumour. Interestingly in the patients with cancer PAR1 was expressed by normal mucosa distant to the tumour. This expression of PAR1 in normal tissue in patients with colorectal cancer has not been explained but could reflect a fold change within the colon. The control group that I used consisted of patients acting as their own controls with a normal sample remote from the cancer and I found no difference in the expression of PAR1 by the epithelial cells in the cancer compared to the
patient’s own control tissue. This raises the possibility that PAR1 expression occurs in normal colon tissue of cancer patients but not in normal colon tissue of non-cancer patients. As the previous study only demonstrated the lack of PAR1 expression in three non-cancer patients it is possible this is an error and that it is expressed in normal colorectal tissue. To determine if this is the case it would be useful to determine the expression of PAR1 by epithelial cells in more patients without a colonic cancer. There was no association between the expression of PAR1 and poor prognostic factors (lymph node involvement, high T stage or high TNM stage). There are no previous studies that have analysed this.

PAR2 was expressed by normal epithelial cells but its expression was significantly stronger (with a higher H-score) in the epithelium of cancers. This is consistent with a previous study that has analysed its expression in malignant and normal colorectal epithelium. PAR2 expression has also previously been demonstrated in other normal epithelial cells of the GI tract including the oesophagus and small intestine. The increase in expression in cancer tissues was seen in both the epithelial cells and in the cancer associated stroma, the stromal expression has previously not been studied in GI tissues. I have not demonstrated any relationship between the epithelial expression of PAR2 and clinicopathological factors unlike the previous study that involved samples from 152 patients. It is possible that once recruitment is complete and all samples analysed the finding may be replicated in this current study.

Very limited previous research has been undertaken regarding the expression of thrombin by colorectal tissues. One previous study has demonstrated the presence of thrombin in colorectal tumours with no specification as to whether the expression was epithelial or stromal. In prostate tissue it is expressed by normal stroma but not epithelial cells whilst in cancer it is expressed by both stroma and epithelium. I have demonstrated that it is expressed in normal epithelial colorectal cells, and does not demonstrate any up regulation in cancer compared to normal epithelium. There is no association with any clinicopathological factors identified.

4.5.2 Stromal Expression of TF pathway proteins in colorectal cancer

The importance of stroma in colorectal cancer has been demonstrated by previous research with colorectal tumours containing a higher proportion of stromal cells being associated with a worse prognosis in colorectal cancer. Not only is the presence of increasing amount of stromal cells associated with a poor prognosis but also the activation of the stromal cells is associated with a poor prognosis. In
colorectal cancer the presence of activated stromal cells is demonstrated by the
expression of fibroblast activation protein (FAP) and stromal derived factor (SDF).
The expression of both FAP and SDF are associated with reduced survival and
advanced disease\textsuperscript{63 352 353}.

This significance of TF expression by cancer associated stroma has previously been
demonstrated in breast cancer where stromal TF expression is associated with a
poor prognosis\textsuperscript{227 228}. The stromal expression of TF in breast cancer is co-localised
with stromal expression of smooth muscle actin (SMA) which is an indicator of
fibroblast activation\textsuperscript{237}. SMA expression in breast cancer is associated with a poor
prognosis\textsuperscript{229}. This suggests that TF is activating the stoma and that, due to
interactions with the malignant epithelial cells, it may be influencing the behaviour of
the malignant cells.

The differentiation between epithelial and stromal expression of TF has not
previously been explored in colorectal cancer. This study has demonstrated that TF
expression, as in breast cancer, is up regulated in cancer associated stroma. It was
also noted that the expression was only in the stroma closely associated with
epithelial cancer cells with a penetration of only 0.1mm into the cancer stroma. This
raises the possibility that the stromal TF expression is as a result of interaction
between the stromal cells and the epithelial cells. To determine if the expression in
the stroma is a bystander effect of expression by the epithelial cells the level of
expression in epithelial cells and stromal cells was correlated. The lack of correlation
found suggests that stromal expression of TF is independent of epithelial TF
expression. Unlike in the breast cancer studies\textsuperscript{227 228} the stromal TF expression did
not correlate with surrogate markers of a poor prognosis such as lymph node
involvement, T stage, TNM stage or lymphovascular involvement. It was not
possible to determine if the expression of TF is associated with a long term
prognosis due to the length of follow-up. This will be analysed once adequate
follow-up to allow for the development of recurrence or death has been undertaken.
The lack of correlation between the TF pathway markers and prognostic indicators
may be a result of the small numbers of patients who have been analysed to date
and there may be a positive correlation evident once recruitment has been
completed.

This study has, for the first time, demonstrated that PAR1 expression is up regulated
in the stroma of colorectal cancer. In breast cancer stromal expression of PAR1 is
up regulated in cancer compared to benign controls, and is associated with reduced
overall and disease free survival\textsuperscript{228}. In prostate cancer stromal expression of PAR1 is associated with elevated PSA and advanced disease state\textsuperscript{237}. In my study there is a suggestion of an inverse relationship between PAR1 expression by stroma and lymph node involvement although this did not reach significance (p=0.05). No other associations have been seen with other clinicopathological factors.

The expression of PAR2 was also up-regulated in the stroma of colorectal cancer compared to the stroma of benign colorectal tissue. This has not previously been investigated in colorectal cancer but has been seen in breast cancer\textsuperscript{228}. In breast cancer it was not associated with any difference in long term prognosis but was associated with poor pathological predictors including HER2 positive and highly proliferating cancers. In this current study no relationship between PAR2 expression and clinicopathological indicators of poor prognosis has been demonstrated.

Thrombin was not expressed in normal stroma but was expressed in cancer associated stroma. In prostate cancer it has been demonstrated in the stroma of normal tissue whilst in cerebral metastasis from clear cell renal cell carcinoma its expression by the stroma has been increased, a finding also noted in breast cancer\textsuperscript{228,230,231}. In this study, as in the other studies there is no association between the expression of thrombin by the stroma and indicators of poor prognosis.

\textbf{4.5.3 Summary of expression of TF pathway proteins by colorectal cancer and future work}

The finding that proteins from the TF pathway are up-regulated in colorectal cancer is consistent with other tumours. The findings in previous studies showed that the expression of these proteins is associated with a poor prognosis suggesting that they may have a role in promoting the cancer. This has not been demonstrated in the current cohort and may reflect the fact that only small numbers of patients have been included in this analysis. It may be that once recruitment is complete a correlation becomes evident. This study has for the first time also demonstrated that the proteins from the TF pathway are expressed by the cancer associated stroma. This expression is only in the peritumoral stroma and this suggests that there is interaction between the stroma and epithelium that involves the TF pathway proteins. It may be that this interaction between the epithelial cancer cells and the cancer associated stroma may be a potential target for treatment. The lack of correlation between the expression of the TF pathway proteins in the epithelial cancer cells and the stroma suggests that the stromal expression is not just a
reflection of the epithelial expression of the proteins. It is also interesting that there is not a correlation between the expressions of different proteins of the TF pathway. This raises the possibility that these proteins are being induced by other pathways or as the result of different mutations in the cancers rather than been induced as part of the TF pathway. Further work needs to be undertaken regarding examining the interaction between the stroma and epithelial cells and also larger numbers of patients with full follow-up need to be examined to determine if the expression of the TF pathway proteins has a prognostic effect. Using the samples that are available these could be co-stained with TF pathway proteins and FAP or SDF to determine if in colorectal cancer the presence of the TF pathway proteins is activating the stromal cells.

The in-vitro work that I have carried out has demonstrated that the presence of TF increases the proliferation of malignant epithelial cells. The finding that TF is over expressed in the cancer associated stroma raises the possibility that this increases proliferation. To determine if this is the case correlation between TF expression in the stroma and proliferation markers (for example Ki67) could be undertaken to determine if this is having a stimulatory effect. In the in-vitro setting the use of a co-culture system with fibroblast cell lines that have been transfected to over express TF and colorectal epithelial cells would allow the interaction to be explored further and act as a model for any treatments targeting the interaction between the stroma and the epithelial cells.

**4.5.4 Plasma proteins from the TF pathway as biomarkers in colorectal cancer.**

This study sought to determine if plasma proteins from the TF pathway have a role as a biomarker in colorectal cancer. To determine if there was any potential role of the proteins as biomarkers their level in serum was compared to clinicopathological factors. It was necessary to use these surrogate markers of prognosis as there is insufficient follow up to allow detection of recurrence, development of metastases or death.

The proteins that were selected to be studied as markers of the activation of the TF pathway were; TF which is an instigator or the pathway, TAT as a marker of thrombin generation, d-dimer as a marker of fibrinolysis and therefore the presence of established clot, P-selectin and sGPV as a marker of the action of thrombin on platelets.
There has been limited research into the use of the TF pathway proteins as biomarkers in cancer and in respect of TF and sGPV this is the first research into their use in colorectal cancer. To be useful as a biomarker the level of the protein need to be able to satisfy a number of criteria. These are that they can:

- Differentiate patients with a cancer from those without
- Differentiate early from advanced disease
- Demonstrate a change with recurrence of disease.

This study is not able to offer any data regarding change of level with the development of recurrence of disease as the levels had only been monitored in the perioperative period.

4.5.4.1 Ability to differentiate patients with a cancer from those without

None of the potential biomarkers were able to definitively differentiate between cancer and benign controls. There was a suggestion that P-selectin may be raised in patients with cancer compared to control patients (with a p value of 0.05) which is consistent with a previous study\textsuperscript{261} but there were no other markers in the CHAMPion study that demonstrated such a difference in expression in colorectal cancer compared to benign control.

Previous studies have demonstrated a difference in the level of d-dimer and TAT between colorectal cancers and benign controls. In a study of 96 cancers and 40 controls d-dimer was significantly raised in colorectal cancer compared to non-cancer controls\textsuperscript{250}. This study incorporated more patients than were included in the interim analysis of the data from the CHAMPion study. The study that demonstrated that the level of TAT is raised in patients with colorectal cancer compared to healthy controls was again a larger study than this current analysis\textsuperscript{188}. Once the CHAMPion study has completed recruitment it will be a larger study than either of these earlier studies. If no difference is seen between the level of these proteins in the presence and absence of cancer, provide evidence that these proteins are not useful in differentiating patients with and without cancer.

4.5.4.2 Differentiate early from advanced disease

The study was limited to patients with local disease as patients with metastatic disease were excluded. The clinicopathological factors in patients with local disease that are clinically relevant and have a bearing on the management of patients with colorectal cancer are:

- Presence of lymph node involvement
- Stage of the tumour (T stage)
- Presence of lymphovascular invasion
- TNM stage (which amalgamates tumour T stage and nodal involvement).

None of the potential biomarkers displayed any significant relationship to the presence of lymph node involvement, T stage or lymphovascular involvement. Fibrinogen levels were significantly higher in TNM2 compared to TNM1 but there was no difference between TNM1 and TNM3.

There is limited published evidence regarding the association between serum level of TF pathway proteins and clinicopathological outcomes. The level of TF in colorectal cancer has not been previously been studied. The finding that TAT is not affected by clinicopathological factors is consistent with previous research. In a cohort study of 93 patients with colorectal cancer plasma d-dimer was elevated in patients with higher TNM stage, T4 disease and lymphatic invasion. In this previous study they noted that d-dimer was positively correlated with the age of the patient, however they made no correction for this in the analysis. It is possible that this is a confounding factor in the previous study. The inability for fibrinogen to accurately differentiate between clinicopathological factors is consistent with a previous study. This large study of 341 patients did not demonstrate any difference in fibrinogen levels with lymph node involvement, T stage or TNM stage. They did, however demonstrate that patients with metastatic disease have a higher pre-operative fibrinogen. P-selectin has also previously been shown to not be correlated with clinicopathological factors but is increased in metastatic disease.

The tumour size significantly correlated with plasma level of fibrinogen, there is also a possible correlation with d-dimer. This suggests the presence of tumour mass is related to the expression of these markers. However, if fibrinogen and d-dimer are not able to accurately discriminate between benign and malignant disease then this is of limited use clinically.

4.5.5 Summary of the use of TF pathway proteins as a biomarker in colorectal cancer and future work

This study using surrogate markers of prognosis has suggested that none of the proteins that have been measured in the plasma of patients with colorectal cancer have the potential to be a reliable biomarker in colorectal cancer. The use of the TF pathway proteins as biomarkers will need to be re-evaluated once full clinical follow-up has been completed. The plasma proteins in the TF pathway lack the ability to
differentiate between patients with and without cancer, and also to differentiate between clinically important clinicopathological factors. The study is ongoing and further analysis will be undertaken once this is complete but given the overlap in values between the benign and malignant group it is unlikely that any of the proteins will be of clinical use in the setting of localised cancer. Previous studies have suggested that they may have a role in detecting the presence of metastatic disease. It is not possible to analyse this in this study as patients with metastatic disease were not included. The biomarker with the most potential would appear to be P-selectin. A future study with measurement of P-selectin at diagnosis and in follow-up to detect any change on recurrence of disease may be warranted. Further analysis once actual patient outcomes are available will further clarify if the proteins have a role as a biomarker.
5 Thrombotic response to surgery in colorectal cancer

5.1 Introduction

Colorectal cancer is associated with an increased risk of VTE. The evidence regarding the incidence of VTE in colorectal cancer patients has come from retrospective cohort studies\textsuperscript{154,158-160}. Patients with poor prognosis cancers are more likely to develop a VTE post-operatively than good prognosis cancers\textsuperscript{354}.

A study by Iverson et al\textsuperscript{168} in the 1990s demonstrated that patients with colorectal cancer had an increased thrombotic response (as demonstrated by increased plasma expression of TAT and fibrin degradation products) compared to non-cancer control patients and that colorectal cancer patients who developed a DVT postoperatively had evidence of activation of the clotting system pre-operatively. In a study of 176 patients with colorectal cancer undergoing surgery a raised pre-operative d-dimer was associated with an increased risk of postoperative DVT\textsuperscript{305}. Pre-operative activation of the clotting system increasing the risk of developing a post-operative DVT has also been demonstrated in other studies incorporating a range of cancers\textsuperscript{207-209,211}.

The increased risk of developing a postoperative VTE in colorectal cancer patients has led to recommendations that patients undergoing surgery for colorectal cancer should receive extended course venous thromboprophylaxis\textsuperscript{191,192}. In extended course venous thromboprophylaxis the use of LMWH is continued for 28 days from surgery rather than the conventional in-hospital treatment. This has significant health and cost implications. The ability to identify patients who are at risk of developing a VTE would allow targeted thromboprophylaxis, therefore reducing the cost and health burden of this prophylaxis. The finding that pre-operative activation of the clotting system may be able to predict post-operative development of a VTE leads onto the suggestion that this could be a potential biomarker for predicting the development of a VTE and therefore targeting thromboprophylaxis to those most at risk. Plasma markers are able to identify the activation of the clotting system. The markers used in this thesis are TF (the instigator of the intrinsic clotting pathways), Thrombin-antithrombin (TAT) the complex formed between thrombin and its inactivating protein antithrombin, d-dimer a breakdown product of fibrinogen, fibrinogen, P-selectin and sGPV which are released when platelets come into contract with thrombin (which is formed as part of the common pathway).
The aims of this chapter are to

- Establish the current incidence of DVT in colorectal cancer patients undergoing curative surgery
- Identify patient subgroups at increased risk of VTE, that would benefit from extended course venous thromboprophylaxis
- Determine if serum markers of activation of the TF pathway are predictive biomarkers of the development of a post-operative VTE.

To achieve these aims, patients recruited to the CHAMPion clinical study underwent pre- and post-operative duplex scans and monitoring of markers of activation of the TF pathway in the peri-operative period to allow the development of a DVT, the presence of a thrombotic state and the role of potential biomarkers to be established.

5.2 Study population

5.2.1 Patients

For details of patients recruited to the CHAMPion study and their demographics see section 4.2. For details of the statistical analysis undertaken see section 4.2.3. As in chapter 4, due to the nature of multiple testing the level of significance has been reduced to 1% (therefore for a test to be significant the p value is <0.01).

5.3 Incidence of VTE in patients undergoing curative surgery for colorectal cancer and benign disease

5.3.1 Methodology

Patients undergoing surgery for colorectal cancer and control patients undergoing similar surgery for benign disease were prospectively recruited. Patients were excluded if they had a history of DVTs, significant (non-cancer) risk factors for VTE and if they were on anticoagulation (not including thromboprophylaxis). For more details of inclusion and exclusion criteria see section 2.2.2. Patients recruited underwent duplex Ultrasound imaging pre-operatively and 6 weeks post-operatively. The patients were also subjected to blood analysis at the pre-operative stage, day 1, day 14 and 6 weeks post-operatively.

5.3.2 Incidence of DVT

Of the 54 patients with colorectal cancer who underwent pre-operative duplex scans four (7%) were found to have incidental pre-operative DVTs. These were all below
knee DVTs. None of the 11 non-cancer controls had pre-operative DVTs, suggesting an increased risk of developing DVTs in the cancer group, although this was not statistically significant (p=0.5).

Forty-eight colorectal cancer patients (all of whom had had negative pre-operative scans) underwent duplex scans at 42 days after surgery, of these three (6%) were diagnosed with a DVT. Two patients with negative pre-operative scans did not undergo the scan at day 42. There were no symptomatic VTEs or incidental VTEs diagnosed on alternative imaging. The total incidence in the cancer group was 13%. In the non-cancer control group there were no postoperative DVTs detected in the 8 patients who underwent duplex scans and no symptomatic or incidental VTEs, again suggesting increased risk for the cancer patients to develop a DVT but this failed to reach statistical significance (p=0.6).

All patients received venous thromboprophylaxis in the peri-operative period (with LMWH and compression stockings on a risk-adjusted basis according to preoperative risk assessment and scoring according to NICE guidance). Of the three colorectal cancer patients who developed post-operative DVTs, 2 (66%) had received extended course (28 days from the day of surgery) venous thromboprophylaxis with LMWH.

5.3.3 Demographics of colorectal cancer patients who developed DVTs
To determine if there were any demographic characteristics that increase the risk of a patient with colorectal cancer developing a DVT the characteristics of the patients who had DVTs pre-operatively (Table 5.1), post-operatively (Table 5.2) and at any time (Table 5.3) were compared. There were no differences in the demographics of the patients who developed DVTs at any time point compared to those remaining DVT free.
<table>
<thead>
<tr>
<th></th>
<th>No pre-operative DVT</th>
<th>Pre-operative DVT</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>70 (43 –90)</td>
<td>70 (62 – 79)</td>
<td>0.9(^a)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td>30:24</td>
<td>3:1</td>
<td>0.6(^b)</td>
</tr>
<tr>
<td><strong>WHO Status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>47</td>
<td>3</td>
<td>0.3(^b)</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>28</td>
<td>28</td>
<td>0.9(^a)</td>
</tr>
<tr>
<td><strong>Alcohol weekly consumption</strong></td>
<td>5 (0 – 30)</td>
<td>6 (0 – 16)</td>
<td>1.0(^a)</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>34</td>
<td>2</td>
<td>0.6(^b)</td>
</tr>
<tr>
<td>No</td>
<td>20</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10</td>
<td>1</td>
<td>0.6(^b)</td>
</tr>
<tr>
<td>No</td>
<td>44</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>Antiplatelet use</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>17</td>
<td>1</td>
<td>1.0(^b)</td>
</tr>
<tr>
<td>No</td>
<td>37</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 Demographics of patients diagnosed with a pre-operative DVTs compared to DVT free.

\(^a\)= student t test, \(^b\)= Fischer exact test.
<table>
<thead>
<tr>
<th></th>
<th>No post-operative DVT</th>
<th>Post-operative DVT</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>69 (43 – 87)</td>
<td>72 (61 – 90)</td>
<td>0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>26:18</td>
<td>2:1</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>WHO Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>36</td>
<td>3</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>28</td>
<td>28</td>
<td>0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alcohol weekly consumption (mean)</td>
<td>5 (0 – 30)</td>
<td>2 (1 – 2)</td>
<td>0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>28</td>
<td>2</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>16</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
<td>0</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>36</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Antiplatelet use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13</td>
<td>1</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>33</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2 Demographics of patients who developed post-operative DVTs compared to those remaining VTE free

<sup>a</sup>= student t test, <sup>b</sup>= Fischer exact test.
<table>
<thead>
<tr>
<th></th>
<th>No DVT</th>
<th>DVT</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>69 (43 –87)</td>
<td>71 (61 – 90)</td>
<td>0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>26:18</td>
<td>5:2</td>
<td>0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>WHO Status 0</td>
<td>36</td>
<td>6</td>
<td>0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>WHO Status 1</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BMI Mean (range)</td>
<td>28 (21 – 42)</td>
<td>27 (21 – 35)</td>
<td>0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alcohol weekly consumption (mean)</td>
<td>5 (0 – 30)</td>
<td>4 (0 – 16)</td>
<td>0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hypertension Yes</td>
<td>28</td>
<td>4</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hypertension No</td>
<td>16</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Diabetes Yes</td>
<td>8</td>
<td>1</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetes No</td>
<td>36</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Antiplatelet use Yes</td>
<td>13</td>
<td>2</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Antiplatelet use No</td>
<td>31</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3 Demographics of patients who develop DVTs at any time compared to those that remained VTE free.

<sup>a</sup> = student t test, <sup>b</sup> = Fischer exact test.
5.3.4 Histological characteristics of patients who develop DVTs

Histological variables of the patients who developed DVTs in the pre-operative stage (Table 5.4), post-operative stage (Table 5.5) and at any time (Table 5.6) were compared with those that did not. The only factors that neared significance in determining those that developed DVTs post-operatively was the presence of node positive disease (p=0.02) and increasing TNM stage (p=0.03), although this was not seen for the development of pre-operative DVTs.
<table>
<thead>
<tr>
<th>T stage</th>
<th>No pre-operative DVT</th>
<th>Pre-operative DVT</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>2</td>
<td>1</td>
<td>0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T2</td>
<td>14</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>23</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>14</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Node +ve</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>15</td>
<td>1</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>39</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>LVI present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>15</td>
<td>1</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>33</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Size of tumour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (mm)</td>
<td>44</td>
<td>29</td>
<td>0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>range</td>
<td>(15 – 80)</td>
<td>(5 – 50)</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>3</td>
<td>0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.4 Pathological results for patients who were diagnosed with DVTs pre-operatively compared to those that were VTE free.

<sup>a</sup> = student t-test, <sup>b</sup> = Fischers exact test.
<table>
<thead>
<tr>
<th></th>
<th>No post-operative DVT</th>
<th>Post-operative DVT</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>3</td>
<td>0</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T2</td>
<td>10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>19</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>12</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Node +ve</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>3</td>
<td>0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>34</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>LVI present</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>2</td>
<td>0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>30</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Size of tumour</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (mm)</td>
<td>45</td>
<td>36</td>
<td>0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>range</td>
<td>(15 – 80)</td>
<td>(25 – 45)</td>
<td></td>
</tr>
<tr>
<td><strong>TNM stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>0</td>
<td>0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.5 Pathological results for patients who were diagnosed with DVTs post-operatively with no preoperative DVT compared to those that remained VTE free.

<sup>a</sup> = student t-test, <sup>b</sup> = Fischers exact test.
<table>
<thead>
<tr>
<th></th>
<th>No DVT</th>
<th>DVT</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>3</td>
<td>1</td>
<td>0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T2</td>
<td>10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>19</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>12</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Node +ve</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>4</td>
<td>0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>34</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>LVI present</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>3</td>
<td>0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>30</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>Size of tumour</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (mm)</td>
<td>45</td>
<td>32</td>
<td>0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>range</td>
<td>(15 – 80)</td>
<td>(5 – 50)</td>
<td></td>
</tr>
<tr>
<td><strong>TNM stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>3</td>
<td>0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.6 Pathological results for patients who were diagnosed with DVT at any time compared to those that remained VTE free.

<sup>a</sup> = student t-test, <sup>b</sup> = Fischers exact test.
5.3.5 **Tissue markers in patients who develop DVTs**

To determine if the tissue expression of proteins from the TF pathways were related to the development of DVTs, the H scores of the cancer specimens in the patients who developed DVTs were compared to those that did not (for method of calculating H scores see section 2.2.11). The proteins from the TF pathway that were analysed were TF, PAR1, PAR2 and thrombin. Thirty-seven patients had tissue available for analysis. Of these one patient did not undergo a pre-operative duplex and two patients did not complete the duplex scan at day 42. These three patients were therefore excluded from analysis. Of the remaining 34 patients available for analysis five were diagnosed with a DVT during the study. There were no other non-DVT VTEs diagnosed. Patients with DVTs had significantly lower epithelial expression of TF compared to the patients who did not develop DVTs 0.7u (95%CI 0 – 3.2) vs 7.7u (95%CI 3.0 – 18.0) p=0.004 (Figure 5.1). There was no difference in the stromal expression of any of the proteins between those patients who did, and did not develop DVTs (Figure 5.2).
Figure 5.1 Epithelial markers in patients who develop DVT at any time compared to those that were DVT free

Analysis of epithelial expression of TF pathway proteins in patients who developed DVTs compared to those that did not. There were no non-DVT VTEs diagnosed. Expression was evaluated by use of the H score. Analysis was undertaken using student T test.
Figure 5.2 Stromal markers in patients who develop DVTs at any time compared to those that were DVT free.

Analysis of stromal expression of TF pathway proteins in patients who developed DVTs compared to those that did not. There were no non DVT VTEs diagnosed. Expression was evaluated by use of the H score. Analysis was undertaken using student T test. Y axis split for clarity in TF, PAR2 and thrombin.
5.4 Thrombotic response to surgery

Previous studies have compared the thrombotic response to surgery undertaking bowel resections for benign and malignant indications. It has been shown that there is an increased thrombotic response to surgery in patients with cancer compared to those undergoing similar surgery for benign indications\(^{168}\). There have been no studies that examine the effect of clinicopathological factors of malignancy on the thrombotic response to surgery. To undertake this investigation, the effect of surgery on thrombotic markers was examined in the peri-operative period. Plasma thrombotic response was determined by measuring TF, TAT, d-dimer, fibrinogen and P-selectin and sGPV, preoperatively and at day 1, 14 and 42 post operatively in colorectal cancer patients and patients undergoing surgery for benign indications. This allowed comparison between surgery for benign and malignant conditions. It also allowed comparison between clinicopathological subgroups within patients with cancer. Differences between the levels of the markers of thrombotic response were identified using ANCOVA to allow control for confounders (age for TF and d-dimer, hypertension for fibrinogen, see section 4.4.5 for analysis of confounders), T test was used for TAT, sGPV and P-selectin if two outcomes were present, ANOVA was used if three or more outcomes were present. To detect effect on the rate of change of markers of thrombosis the Generalised Estimating Equation (GEE) was used. This allows the pre-operative values and any known confounding factors to be controlled for individual patients.

5.4.1 Correlation between markers of the TF pathway

If an individual marker of the TF pathway activation is raised and indicates the presence of a thrombotic state then it would be expected that other markers of the activation would be present. To investigate this, plasma levels of TF pathway markers were correlated using Pearson’s Rank Correlation.

There was a possible correlation between d-dimer and TAT at all-time points but this only reached significance at 14 days post-surgery\( (p=0.003, \text{ Table 5.9}) \). The significance level for the correlation between d-dimer and TAT at the other time points were: pre-operation 0.03 (Table 5.7), day 1 post- operation 0.04 (Table 5.8) and day 42 post- operation 0.02 (Table 5.10). Other possible correlations were pre-
operative d-dimer and fibrinogen (p=0.02) and TAT and sGPV at day 1 post surgery (p=0.03).
Table 5.7 Correlation of plasma biomarkers of activation of the TF pathway pre-operatively.

Analysis undertaken with Pearson’s rank correlation. $R^2$ = correlation co-efficient, $P$ = p value, $N$ = number of patients.
<table>
<thead>
<tr>
<th></th>
<th>TF</th>
<th>TAT</th>
<th>D-dimer</th>
<th>Fibrinogen</th>
<th>P-selectin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TAT</strong></td>
<td>$R^2$</td>
<td>-0.184</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P$</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$N$</td>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D-dimer</strong></td>
<td>$R^2$</td>
<td>-0.208</td>
<td>0.324</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P$</td>
<td>0.2</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$N$</td>
<td>38</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fibrinogen</strong></td>
<td>$R^2$</td>
<td>0.153</td>
<td>-0.06</td>
<td>0.058</td>
<td></td>
</tr>
<tr>
<td>$P$</td>
<td>0.4</td>
<td>0.713</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$N$</td>
<td>39</td>
<td>40</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P-selectin</strong></td>
<td>$R^2$</td>
<td>0.216</td>
<td>-0.347</td>
<td>0.064</td>
<td>0.048</td>
</tr>
<tr>
<td>$P$</td>
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<td>0.06</td>
<td>0.7</td>
<td>0.792</td>
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</tr>
<tr>
<td>$N$</td>
<td>30</td>
<td>30</td>
<td>40</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td><strong>sGPV</strong></td>
<td>$R^2$</td>
<td>-0.109</td>
<td>0.434</td>
<td>0.354</td>
<td>-0.148</td>
</tr>
<tr>
<td>$P$</td>
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<td>0.08</td>
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</tr>
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<td>$N$</td>
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<td>24</td>
<td>25</td>
<td>26</td>
<td>29</td>
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</tbody>
</table>

Table 5.8 Correlation of plasma biomarkers of activation of the TF pathway at day 1 post-surgery

Analysis undertaken with Pearson’s rank correlation. $R^2$ = correlation co-efficient, $P$ = p value, $N$ = number of patients.
<table>
<thead>
<tr>
<th></th>
<th>TF</th>
<th>TAT</th>
<th>D-dimer</th>
<th>Fibrinogen</th>
<th>P-selectin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TAT</strong></td>
<td>R²</td>
<td>0.188</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D-dimer</strong></td>
<td>R²</td>
<td>-0.177</td>
<td><strong>0.495</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
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<td>N</td>
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<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fibrinogen</strong></td>
<td>R²</td>
<td>-0.120</td>
<td>0.092</td>
<td>-0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.5</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>34</td>
<td>33</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td><strong>P-selectin</strong></td>
<td>R²</td>
<td>0.219</td>
<td>-0.256</td>
<td>0.173</td>
<td>-0.166</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.3</td>
<td>0.2</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>26</td>
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<td>27</td>
<td>27</td>
</tr>
<tr>
<td><strong>sGPV</strong></td>
<td>R²</td>
<td>-0.027</td>
<td>-0.302</td>
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<td>-0.029</td>
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<tr>
<td></td>
<td>P</td>
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<td>0.2</td>
<td>0.9</td>
<td>0.9</td>
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<tr>
<td></td>
<td>N</td>
<td>21</td>
<td>19</td>
<td>21</td>
<td>21</td>
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</tbody>
</table>

Table 5.9 Correlation of plasma markers of activation of the TF pathway at 14 days post-surgery

Analysis undertaken with Pearson’s rank correlation. $R^2$ = correlation co-efficient, $P = p$ value, $N = number$ of patients.
<table>
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<th>TF</th>
<th>TAT</th>
<th>D-dimer</th>
<th>Fibrinogen</th>
<th>P-selectin</th>
</tr>
</thead>
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<tr>
<td><strong>TAT</strong></td>
<td>R²</td>
<td>-0.162</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
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<tr>
<td></td>
<td>N</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D-dimer</strong></td>
<td>R²</td>
<td>0.190</td>
<td>0.411</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>P</td>
<td>0.3</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>32</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fibrinogen</strong></td>
<td>R²</td>
<td>-0.13</td>
<td>0.34</td>
<td>0.335</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.5</td>
<td>0.06</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>32</td>
<td>31</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td><strong>P-selectin</strong></td>
<td>R²</td>
<td>0.367</td>
<td>-0.002</td>
<td>0.112</td>
<td>-0.015</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.07</td>
<td>0.9</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>26</td>
<td>23</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td><strong>sGPV</strong></td>
<td>R²</td>
<td>-0.074</td>
<td>-0.301</td>
<td>-0.047</td>
<td>0.167</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.7</td>
<td>0.2</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>21</td>
<td>18</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 5.10 Correlation of plasma markers of activation of the TF pathway at 48 days post-surgery

Analysis undertaken with Pearson’s rank correlation. $R^2$ = correlation co-efficient, $P = p$ value, $N = number$ of patients.
5.4.2 Thrombotic response to surgery for cancer compared to benign conditions

The thrombotic response to surgery in patients undergoing operations for cancer and benign indications was compared. There was no difference at any of the time points between plasma level of TF, TAT, d-dimer, fibrinogen, P-selectin or sGPV in the patients undergoing surgery for cancer or benign indications. There was also no difference in the rate of change of the expression of the TF pathway proteins between patients with benign and malignant indications for their surgery as determined by GEE (Figure 5.3).
Figure 5.3 Thrombotic response to surgery in colorectal cancer vs benign surgery.

Analysis at each time point undertaken with ANCOVA for TF and d-dimer (with control for age) and Fibrinogen (with control for hypertension). TAT, P-selectin and sGPV analysed with student T test. GEE analysis for rate of change analysis between benign and malignant indications. GEE analysis undertaken with control for the pre-operative level of expression of the marker and the confounders corrected for in ANCOVA. b= benign, c= cancer
5.4.4 Thrombotic response in laparoscopic compared to open surgery

The thrombotic response comparing open and laparoscopic surgery has been examined in cholecystectomy, with no difference seen between the two groups\textsuperscript{355, 356}. The difference between open and laparoscopic colorectal surgery has not been examined. The surgical approach was split into open (including laparoscopic converted and laparoscopic assisted) and laparoscopic surgery. D-dimer at day 14 demonstrated a possible significant elevation in the open compared to the laparoscopic group (p=0.03). The other markers of activation of the haemostatic pathway also demonstrated an increase in response in the open surgery group although this was not statistically significant. Using GEE (controlling for pre-operative levels of expression of the biomarker and any confounders), open surgery has an increased thrombotic response compared to laparoscopic surgery as demonstrated by increased plasma d-dimer and TAT (Figure 5.4).
Figure 5.4 Thrombotic response in laparoscopic compared to open surgery

Analysis at each time point undertaken with ANCOVA for TF and d-dimer (with control for age) and Fibrinogen (with control for hypertension). TAT, P-selectin and sGPV analysed with student T test. GEE analysis undertaken for rate of change analysis between the open and laparoscopic approach. GEE analysis undertaken with control for the pre-operative level of expression of the marker and the confounders corrected for in ANCOVA. o = open, l = laparoscopic.
5.4.5 **Thrombotic response to surgery in lymph node positive compared to negative disease.**

Patients who underwent surgery for colorectal cancer but did not have a complete excision of the cancer have an increased thrombotic response following surgery compared to those whose cancer was removed\(^\text{168}\). I therefore sought to determine if an increased tumour burden as determined by the presence of lymph node involvement had an increased thrombotic response compared to those without lymph node involvement. There was no difference in the plasma level of the markers of activation of the clotting pathway (TF, TAT, d-dimer, fibrinogen, P-selectin and sGPV) at any point between patients with or without lymph node involvement and there was no difference in the rate of change of the proteins as determined by use of GEE (Figure 5.5).
Figure 5.5 Thrombotic response to surgery in patients with lymph node positive compared to lymph node negative cancers

Analysis at each time point undertaken with ANCOVA for TF and d-dimer (with control for age) and Fibrinogen (with control for hypertension). TAT, P-selectin and sGPV analysed with student T test. GEE analysis undertaken for rate of change analysis between the lymph node positive and lymph node negative disease. GEE analysis undertaken with control for the pre-operative level of expression of the marker and the confounders corrected for in ANCOVA. (+ve = lymph node positive, -ve = lymph node negative).
5.4.6 **Response to surgery by T stage**

The thrombotic response to surgery in cancers with different T stage tumours was analysed. The analysis was undertaken at individual time points using ANCOVA for TF and d-dimer (with control for age) and fibrinogen (with control for hypertension). ANOVA was used for TAT, sGPV and P-selectin. There was no difference in the expression of TF pathway proteins at any time point depending on the tumour T stage. There was also no difference in the rate of change of expression of any of the proteins as determined by GEE (Figure 5.6).
Figure 5.6 Thrombotic response to surgery by T stage

Analysis at each time point undertaken with ANCOVA for TF and d-dimer (with control for age) and Fibrinogen (with control for hypertension). TAT, P-selectin and sGPV analysed with ANOVA. GEE analysis undertaken for rate of change analysis between the different T stage disease. GEE analysis undertaken with control for the pre-operative level of expression of the marker and the confounders corrected for in ANCOVA. T1 disease not analysed as only 2 patients had T1 disease.
5.4.7 Response to surgery by TNM stage

The thrombotic response to surgery in relation to tumour TNM stage was analysed to determine if the TNM stage of the tumour influenced the thrombotic response. Patients with TNM stage 4 disease were not included as patients with metastatic disease were excluded from the study. There was no difference in the thrombotic response to surgery as a result of the TNM stage, with no significant difference in values at any of the time points for any of the TF pathway proteins. There was also no difference in the rate of change of the expression of the proteins as demonstrated by GEE analysis (Figure 5.7).
Figure 5.7 Thrombotic response by TNM stage

Analysis at each time point undertaken with ANCOVA for TF and d-dimer (with control for age) and Fibrinogen (with control for hypertension). TAT, P-selectin and sGPV analysed with ANOVA. GEE analysis undertaken for rate of change analysis between the different TNM stage disease. GEE analysis undertaken with control for the pre-operative level of expression of the marker and the confounders corrected for in ANCOVA. TNM1 = TNM stage 1, TNM2 = TNM stage 2, TNM3 = TNM stage 3.
5.5 Predictive Biomarkers for development of VTE

The ability to predict patients who are at increased risk of developing a post-operative VTE would be beneficial in the management of an individual patient’s peri-operative thromboprophylaxis. Previous studies have suggested that pre-operative markers of activation of the TF pathway are able to predict the development of post-operative DVTs:

- Plasma TF in pancreatic cancer correlates with the development of DVT\(^\text{207}\). No similar study has been undertaken in colorectal cancer.
- Plasma TAT levels were increased in patients subsequently developing DVT in a study of all abdominal cancers (with no subset analysis of colorectal cancer)\(^\text{209}\), however a study of pre-op TAT in colorectal cancer did not demonstrated any association\(^\text{168}\).
- D-dimer had a predictive role in a study that recruited patients with different types of cancers (with no subgroup analysis of colorectal cancer)\(^\text{208}\), and also in two colorectal specific studies\(^\text{168 305}\).
- In a study incorporating any cancer P-selectin was increased in patients who subsequently developed post-operative DVT\(^\text{211}\). No colorectal cancer specific analysis or study has been undertaken.
- sGPV has not had its role in predicting the development of VTE analysed.

To determine the predictive value of the TF pathway proteins and markers of platelet activation, the plasma level were compared in:

- Patients who developed a DVT at any time compared to those that did not.
- Patients who had a pre-operative DVT compared to those that did not.
- Patients who developed a DVT in the peri-operative period (diagnosed 6 weeks post-surgery).

5.5.1 Plasma markers in patients who developed DVTs

There were no differences in the pre-operative plasma level of the markers of activation of the TF pathway in patients who developed a DVT at any time compared those that did not (Figure 5.8).

When patients who were diagnosed with a pre-operative DVT were analysed separately (compared to those with a negative Duplex scan) there was a suggestion of significance in the level of TAT with a reduced level in patients diagnosed with a DVT \((5.6 \text{ng/mL (95\%CI 4.6 – 6.8)} \text{ vs 2.6 ng/mL (95\%CI 0.9 – 7.2)} \text{ p}=0.03)\). There
were no differences between the other potential biomarkers in patients with and without VTE (Figure 5.9).

When patients who developed a DVT in the post-operative period were compared to those remaining VTE-free there was no difference in the pre-operative levels between those who developed a DVT and those that did not. The level of d-dimer had a trend to be higher in the patients who did develop a post-operative DVT compared to those who did not (DVT developed post operatively 1264.5ng/mL (95%CI 853.7 – 1873.0); no DVT 688.1ng/mL (95%CI 563.6 – 840.2), p=0.1). P-selectin was not analysed since only two of the patients included subsequently developed post-operative DVTs. Preoperative TF, TAT, Fibrinogen, P-selectin and sGPV did not display any difference between patients who subsequently developed VTE compared to those remaining VTE free (Figure 5.10).
Figure 5.8 Plasma markers in patients who developed DVTs compared to those who did not.

Analysis undertaken with ANCOVA for TF and d-dimer (with control for age) and Fibrinogen (with control for hypertension). TAT, P-selectin and sGPV analysed with student T test.
Figure 5.9 Serum markers in patients with pre-operative DVT compared to those with no pre-operative DVT

Analysis undertaken with ANCOVA for TF and d-dimer (with control for age) and Fibrinogen (with control for hypertension). TAT, P-selectin and sGPV analysed with student T test.
Figure 5.10 Pre-operative serum markers in those who develop a post-operative DVT compared to those that remained DVT free.

Analysis undertaken with ANCOVA for TF and d-dimer (with control for age) and Fibrinogen (with control for hypertension). TAT, sGPV analysed with student T test. P-selectin not analysed as only 2 patients with results developed a post-operative DVT.
5.6 Discussion

5.6.1 Development of VTE in colorectal cancer patients

This study is the first to screen patients for DVTs pre and post-operatively who are undergoing similar operations for benign and malignant colorectal indications. In addition to screening for DVTs by the use of venous duplex the presence of non-DVT VTEs was sought by any clinical presentation or incidental diagnosis. Stender et al\textsuperscript{305} screened patients undergoing operations for colorectal cancer pre-operatively and post-operatively and the study by Iverson et al\textsuperscript{168} screened patients undergoing operations for colorectal cancer postoperatively. By screening patients before and after surgery for both malignant and benign indications this study is uniquely able to identify any difference in the incidence of DVTs in the peri-operative period in patients undergoing surgery for malignant or benign disease. Although there is no statistical difference yet in this study between the incidence of DVTs in the cancer group compared to the benign controls (p=0.5), the fact that none of the benign patients developed DVTs suggests that cancer patients are at increased risk of VTE. Once recruitment is complete it may be that the difference between the incidence of VTE in patients undergoing surgery for malignant compared to benign indication will become statistically significant.

This research demonstrates that a sizable proportion (7%) of patients have incidental DVTs present prior to surgery for colorectal cancer, this figure being only of patients with asymptomatic VTE as patients who were diagnosed with a VTE prior to being included in the study were excluded. This is consistent with Stender et al\textsuperscript{305} who diagnosed 8% of patients pre-operatively with an asymptomatic DVT. It is important that these patients can be diagnosed early as patients with cancer are at risk of propagating their clot and therefore developing larger DVTs and also PEs with all the inherent risks\textsuperscript{200,201}. These patients with established DVTs need to receive full therapeutic measures to prevent propagation of their VTE disease. The use of pneumatic calf compression devices is now routine in the peri-operative prevention of DVTs but there is a suggestion (but no direct evidence) that the use of these devices in patients with DVTs may increase the risk of the clot migrating\textsuperscript{357}. The presence of an established DVT is, according to the manufacturers, a contraindication for their use. The high level of incidence of DVTs pre-operatively and the implications that their presence has in terms of patient management raises the possibility that all patients should be screened pre-operatively for a DVT to guide appropriate peri-operative management.
In this study the incidence of DVT in patients undergoing cancer surgery is 13%. This level will be an underestimation as patients who had a symptomatic DVTs prior to surgery, or a VTE that was diagnosed as part of the pre-operative staging, were excluded from this study. It is also worth considering that this study excluded patients with advanced disease who are known to have a higher incidence of DVTs. The total level of development of VTE is lower than that detected by Iverson et al (15% vs 22%). This reduction may reflect the changes in practice (including the use of extended course thromboprophylaxis) that have developed over the 20 years since the previous study was undertaken.

Of the three patients who developed DVTs in the peri-operative period, two had received extended course venous thromboprophylaxis. This suggests that the level of anticoagulation achieved by routine thromboprophylaxis was not sufficient to prevent the development of a thrombosis in these patients.

The incidence of DVT in the peri-operative period in this study was 6%. It is difficult to make a comparison with the study by Stender et al regarding the incidence of DVT as they report the total incidence of DVTs over a year at 11%. There is therefore no direct comparison with our data that can be made.

In an effort to identify patients who are at increased risk of developing a DVT the demographic and histological factors for those who developed DVTs and those who remained VTE free were compared. Patients with lymph node positive disease were possibly at increased risk of developing a DVT than those without lymph node involvement, however no other demographic or histopathological risk factors were identified.

It has been suggested that the expression of TF by the tumour may increase the risk of developing a VTE. Evidence to support this has come from clinical studies in pancreatic and ovarian cancers. This study has demonstrated that the patients who developed a DVT had a lower epithelial expression of TF than patients remaining VTE free. There is no other association between the expression of proteins from the clotting pathway by the tumour and the development of a VTE in colorectal cancer in this current study. However the number of patients analysed is small and this analysis will be re-examined once recruitment is complete.

5.6.2 Thrombotic response to surgery

It has previously been demonstrated that patients undergoing surgery for cancer demonstrate an increased thrombotic response when compared to similar surgery.
for benign indications. This effect has been seen in surgery for lung cancers\textsuperscript{358} and in colorectal cancer\textsuperscript{359}. The difference in thrombotic response has not been demonstrated in this current study. This may be the result of the small number of patients that have been analysed.

In addition to the lack of difference in the thrombotic response between surgeries undertaken for benign and malignant indications, there was also no difference in the thrombotic response in relation to clinicopathological factors including lymph node involvement, T stage and TNM stage. The lack of difference between those patients with and without lymph node involvement is contrary to the finding that patients with lymph node positive disease appear to be more at risk of developing a VTE. This raises the possibility that the thrombotic response, as demonstrated by an increased expression of the proteins from the TF pathway, does not translate into an increased risk of developing a DVT.

It is noteworthy that patients who undergo open (or laparoscopic converted to open) procedures have an increased thrombotic response to surgery when compared to those that had a completely laparoscopic procedure. The physiology behind this is unclear as the difference in surgical trauma between a laparoscopic and open approach to resection of colorectal cancer is small. It may reflect other factors, for example laparoscopic surgery reduces exposure of the visceral organs to the atmosphere with resultant reduction in the desiccation of tissue. A previous meta-analysis based on post-hoc analysis of randomised controlled studies into laparoscopic surgery for colorectal cancer has not demonstrated a difference in the incidence of DVTs between the open and laparoscopic approach\textsuperscript{360}. This lack of correlation between the thrombotic response and risk of developing a DVT again suggests that there may be no link between the development of a DVT and the thrombotic response as measured by the serum proteins of the TF pathway. It is possible that the relationship is present but due to the numbers currently available for analysis in this study it has not been identified.

5.6.3 Biomarkers for prediction of DVTs postoperatively

Previous research has suggested that plasma proteins from the TF pathway may have a role as a predictor of the development of a post-operative VTE. This would be clinically valuable as it would allow selected extended prophylaxis for those patients that are at higher risk. This study has demonstrated that the plasma biomarkers analysed fail to identify patients with DVTs diagnosed both pre-operatively and post-operatively. Pre-operative d-dimer is higher in patients who
subsequently developed a post-operative DVT however this was not significant. Previous studies have suggested that a raised d-dimer is able to predict the development of a VTE post-operatively\textsuperscript{168,208,305}. It is possible that when all patients have been recruited to the CHAMPion study and the data has been analysed this difference will be statistically significant. There was no trend of differences in the levels of the other markers between patients who did and did not develop post-operative DVTs. Two studies have previously looked at whether TAT is able to predict the development of a post-operative VTE. One study (that incorporated a large range of cancers) demonstrated that it was able to predict the development of a post-operative VTE\textsuperscript{209}, another study (only incorporating colorectal cancer) demonstrated the converse and that TAT was not able to predict a VTE\textsuperscript{168}. The current study supports this tumour-specific study that in colorectal cancer TAT is not able to predict the development of a post-operative DVT. P-selectin has been shown in a study including multiple different cancers to be able to identify patients at risk of developing a VTE\textsuperscript{211}. There was no specific colorectal analysis. This study suggests that in colorectal cancer P-selectin does not have this ability.

5.6.4 Further work and limitations
The current analysis is limited by the number of patients who have been recruited. The study has ongoing recruitment and a final analysis will be undertaken once the recruitment is complete.

The finding that patients develop DVTs despite undergoing extended course thromboprophylaxis raises the possibility that patients are not adequately anticoagulated with the current regime. There have been no studies examining the dosing requirement in colorectal cancer for anticoagulation. There has been a study examining the anticoagulation in the critical care setting with LMWH (by using anti-factor Xa assay) which has demonstrated that current treatment (40mg enoxaparin) does not achieve adequate anticoagulation\textsuperscript{361}. A study in colorectal cancer to determine the optimum dosing regime would perhaps allow a further reduction in the incidence of post-operative DVTs.
6 Discussion

There is a two way link between thrombosis and colorectal cancer. The presence of colorectal cancer is a major risk factor for the development of a VTE\(^ {153, 154, 158-160}\), which is further increased by treatment of the cancer\(^ {161-163}\), and the presence of a VTE appears to be associated with a poor cancer prognosis\(^ {215-217}\).

The causative link between cancer and thrombosis is subject to a number of explanations and may be multifactorial. The link may be the abnormal prothrombotic proteins (which form part of the TF pathway) that are expressed by the tumours. These proteins may have a role in activating the coagulation system resulting in a hypercoagulable state\(^ {170, 172}\). These same prothrombotic proteins may also have an effect on the cancer biology in promoting the development of the cancer at a cellular level\(^ {107, 221, 228, 265-267, 271, 272, 274, 278, 285, 312}\).

The ability to use the proteins from the TF pathway, either expressed on the cancer specimens or in the plasma as a biomarker, for cancer presence, aggressive disease or development of a VTE would have valuable roles in cancer diagnosis and follow-up, the rationalisation of cancer treatment and also individualisation of venous thromboprophylaxis. Suggestions that this may be possible have come from studies in a range of cancers\(^ {168, 221, 222, 241-244, 247-250, 260}\).

The presence of a prothrombotic cancer niche has also been suggested as having a role in breast cancer, with the TF pathway proteins being expressed by cancer associated fibroblasts\(^ {227, 228}\). If the presence of the prothrombotic cancer niche could be demonstrated in colorectal cancer this would allow a novel research avenue to target this interaction.

The objective of this research was to further clarify the role that TF has in the development of cancer at a cellular level and the role that proteins from the TF pathway may have as a biomarker in colorectal cancer and of thrombosis.

In chapter 3 I set out to determine the possible cellular effects of TF on cancer cells \textit{in-vitro}.

The first objective was to determine if TF was able to promote the action of colorectal cancer stem cells. The cancer stem cell theory has come to prominence as a model of treatment resistance in cancers over recent years. There has been some evidence that in breast cancer and vulval squamous cell carcinoma TF may have a role in promoting cancer stem cells\(^ {228, 287}\). This role, if demonstrated in
colorectal cancer, would provide an explanation for the link between the presence of TF expression by the cancer and a poor cancer prognosis. The work that I undertook investigated the effect of TF on colorectal cancer stem cells. This made use of two established techniques of determining stem cell activity. These were the use of cancer stem cell markers (in particular ALDH) and the cancer sphere assay. These techniques both demonstrated that TF was actually inhibitory to the action of colorectal cancer stem cells, appearing to promote the cancer cells to a more differentiated state. This inhibition was seen in both wild type cell lines with differential expression of TF and also in cell lines transfected to over and under express TF. This effect, although contradicting the work done in breast and vulval squamous cell carcinoma is consistent with other work done in breast cancer\textsuperscript{288}. The downstream activation of ERK1/2 by TF and factor VIIa that I have demonstrated would also be consistent with TF promoting stem cells to a more differentiated state as this action of ERK1/2 has previously been demonstrated\textsuperscript{292-294,325}.

The second objective was to determine the effect of TF on anoikis resistance. It has been shown in breast cancer and in baby hamster kidney cells that TF and its ligand factor VIIa have a role in promoting anoikis resistance\textsuperscript{107,286,285}. To determine if this same effect was seen in colorectal cancer an anoikis assay was undertaken to determine the effect of TF expression on colorectal cancer cell line anoikis. This work determined that reduced levels of TF expression impaired DLD-1’s ability to resist anoikis. It has previously been demonstrated that the high level of anoikis resistance in DLD-1 is under the control of the k-ras mutation\textsuperscript{219}. It has also been previously demonstrated that in colorectal cancer the expression of TF is under the control of k-ras\textsuperscript{330}. This leads to the suggestion that the putative effects of k-ras (the pathway by which they are exerted is to date unknown) may operate at least partially through TF. The effect of TF increasing anoikis resistance is also consistent with clinical studies that have demonstrated that TF expressing colorectal cancer have a higher metastatic potential\textsuperscript{221,226}.

The third objective was to determine the effect that TF has on proliferation in colorectal cancer cell lines. It has previously been shown that in colorectal cancer the action of TFs ligand factor VIIa has an effect of increasing proliferation and that this was negated by the use of a TF inhibitor\textsuperscript{270}. In lung cancer, melanoma and colorectal cancer TF inhibition has been shown to reduce proliferation\textsuperscript{268,269}. In the colorectal cancer cell lines studied the over expression of TF increased proliferation. This increase in proliferation was further increased by the addition of the TF ligand
factor VIIa. This is consistent with clinical studies that have demonstrated more aggressive tumour phenotypes with TF expressing tumours\textsuperscript{221}. One possible pathway by which the effect of TF and VIIa may exert its pro-proliferative effects is by the increased phosphorylation of ERK1/2. This work has demonstrated that TF alone is able to increase the phosphorylation of ERK1/2, an effect that is further increased by the action of factor VIIa. It has previously been shown that the action of ERK1/2 results in increased proliferation\textsuperscript{105}.

The unexpected finding that knock down of TF in the colorectal cancer cell lines also had an effect of increasing proliferation (although to a lesser extent than TF over expression) has actually been previously demonstrated\textsuperscript{270}. One possible pathway by which this could happen is again by the action of ERK1/2. It has been shown (by this study and others) that TF promotes the phosphorylation of ERK1/2\textsuperscript{105}. It has also been shown previously that ERK1/2 has a role in the suppression of TF expression\textsuperscript{316}\textsuperscript{317}. The interference of this, or other as yet undefined pathways, may explain this paradoxical effect. The finding that reduced TF expression can increase proliferation does however demonstrate the need for careful research regarding the effect of TF on proliferation before consideration of using the effect that TF has on proliferation as a therapeutic target.

In chapter 4 I set out to determine the expression of TF pathway proteins in colorectal cancer tumours and also in the plasma of patients with colorectal cancer. It has previously been demonstrated that there is aberrant expression of TF pathway proteins by colorectal cancer\textsuperscript{222}\textsuperscript{232}\textsuperscript{308}\textsuperscript{342}. This study confirms this is the case in all the TF pathway proteins studied including TF, PAR1, PAR2 and thrombin. This study has also for the first time demonstrated that the expression of the TF pathway proteins is not just epithelial but also stromal. Additionally it has demonstrated that the stromal expression is only when it is in close (0.1mm) contact with the cancer epithelial cells. This suggests that there is an interaction using proteins from the TF pathway between the epithelial and stromal cells in a tumour. This study has not demonstrated any link between the expression of the TF pathway proteins and prognostic indicators although this link has been demonstrated by other studies\textsuperscript{306}\textsuperscript{292}. In breast cancer the stromal expression of proteins from the TF pathway are associated with a poor cancer prognosis\textsuperscript{211}\textsuperscript{212}. Whilst this has not been demonstrated in this cohort of colorectal cancer patients it might become evident once long term follow-up has been achieved.
The use of plasma markers of activation of the TF pathway has been suggested as a possible biomarker for aggressive disease. In this study none of the markers of TF pathway activation were able to differentiate between patients with or without colorectal cancer or between poor clinicopathological markers. P-selectin neared significance in the difference between the presence and absence of colorectal cancer and this warrants further evaluation. The findings to date do suggest that with the possible exception of P-selectin the use of plasma markers of TF pathway activation do not have a role as biomarkers in colorectal cancer.

In chapter 5 I set out to determine the incidence of VTE in patients undergoing surgery for colorectal cancer and matched benign indications. This demonstrated that there was a 7% incidence of incidental VTEs present in the patients undergoing surgery for colorectal cancer before they had commenced their treatment. It also demonstrated that a further 6% of patients undergoing surgery for colorectal cancer developed DVTs in the perioperative period. This was despite the use in hospital of venous thromboprophylaxis in all patients and of extended course venous thromboprophylaxis in two thirds of patients who developed a peri-operative DVT. This incidence is consistent with previous studies. The continued development of DVTs despite the use of anticoagulation does suggest that this may be delivered at an insufficient dose in these patients, as has been suggested by a study of the actual level of anticoagulation achieved by venous thromboprophylaxis. The large pre-treatment incidence of DVTs raises the possibility that patients should be screened pre-operatively for the presence of a DVT as this will be at risk of propagating and migrating during the patient’s treatment. It has been suggested by the limited analysis undertaken that patients with lymph node positive disease are at increased risk of developing a perioperative VTE and that these patients may benefit from more aggressive anticoagulation. None of the patients undergoing surgery for benign indications were diagnosed with a pre-operative or perioperative VTE. Although increased incidence of VTE in the group of patients compared to the benign controls did not reach significance it does suggest that the presence of malignancy is promoting the hypercoagulable state in these patients.

To determine if the proteins from the TF pathway have a role in the prediction of development of a VTE the levels of the markers were correlated with the development of a VTE. Only d-dimer demonstrated any suggestion of being at a higher level pre-operatively in patients who developed a peri-operative DVT. This was not statistically significant but that may reflect the underpowered nature of this study. Previous studies have suggested that d-dimer may have a role in the pre-
operative prediction of the development of a perioperative DVT\textsuperscript{168, 208, 305}. Once recruitment is complete this study may confirm these previous findings in which case this would be a valuable tool to use in the risk stratification of patients with regard to VTE risk when undergoing surgery.
7 Conclusion

This thesis has demonstrated that proteins from the TF pathway are abnormally expressed in colorectal cancer by both epithelial and stromal cells. The expression of these proteins has not, in this study, been shown to have a relationship with prognostic indicators. Long term follow-up results are awaited to determine if the expression of these proteins correlates with early recurrence, metastasis or death.

In addition to demonstrating the abnormal expression of the TF proteins in colorectal cancers this thesis has also demonstrated that the action of TF has effects on the cancer at a cellular level. The action of TF increases the rate of proliferation of colorectal cancer cells and also appears to have a role in anoikis resistance. This raises the possibility that inhibition of TF may have a role as an anti-proliferative and anti-metastatic treatment in colorectal cancer. The finding that TF promotes colorectal cancer stem cells to a more differentiated cell type raises the possibility that using TF antibodies as a cancer treatment may result in increased treatment resistance. This needs further careful evaluation by appropriate in-vivo studies.

The development of VTE in patients with colorectal cancer is common and presents a serious risk to the patients. This study has demonstrated that a significant number of patients with colorectal cancer have a pre-operative asymptomatic DVT. These patients are at risk of propagating this clot during their treatment. The use of routine diagnostic tools to establish the presence of the DVT may affect patient outcome if appropriate treatment measures are undertaken. The finding that patients are continuing to develop VTEs despite venous thromboprophylaxis following current recommendations suggests that these recommendations need to be re-visited, possibly following studies where actual levels of anticoagulation offered by the prophylactic treatment have been established.

Proteins from the TF pathway have been proposed as biomarkers of disease prognosis and of risk of developing a VTE. This study has suggested that there is not a use for these proteins as a biomarker of colorectal cancer. There is a suggestion that d-dimer may have a role as a biomarker for the development of a post-operative VTE and this requires further evaluation. If this was found to be the case this would have a valuable clinical role in risk stratifying patients with regards to prophylactic strategies to prevent the development of a VTE.
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