Thrombin generation, Tissue Factor Microvesicles and the Endothelium in Multiple Myeloma and Pancreatic Cancer during treatment.

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Abstract

Cancer and its anti-neoplastic treatment are frequently complicated by venous thromboembolism (VTE) occurrence. Multiple myeloma (MM), a haematological malignancy and Pancreatic cancer (PC), a solid tumour are two common malignancies with similarly high VTE incidence, which worsens during treatment. Thrombin production is a key step in the pathologic evolution of VTE and may play an important role in determining VTE risk in cancer patients. The calibrated automated thrombography (CAT) assay is emerging as a reliable tool for real time estimation of thrombin generation (TG) potential, and there is a clinical need for such knowledge on the dynamic pathways underlying the thrombotic phenotype of various malignancies. Hypothetically, TG measurement may also provide a view of the haemostatic variances that exist in MM and PC as cancers with high VTE incidences. Therefore, this thesis aimed to explore the TG changes that exist in both malignancies in patients before, during and after treatment. It also explores the interaction of Tissue Factor (TF) associated with Microvesicles (MVs) or TFMVs with tumour stroma especially the endothelium, or any procoagulant changes such as thrombin production due to this interaction; and thus, aimed to study TFMVs involvement through the disruption of endothelial haemostasis. The results presented in this thesis demonstrate that solid and haematological malignant cells have significantly differing TG kinetics that may correlate with TF expression levels, and that TG parameters identified changes in MM during treatment, specifically the Lag times and Times-to-Peak parameters were progressively elevated until the third chemotherapy cycle.

In summary, procoagulant activity on the endothelium can be stimulated by TFMVs produced by cancer cells *in vitro*, and this study of thrombin as a procoagulant factor in MM and PC and its relationship to TFMVs may lead to future understanding of their important role in cancer VTE development, and thus merits further study.

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^{0}C	Degree Celcius
%	Per cent
5-FU	5-fluorouracil
aPC	Activated protein C
ANOVA	Analysis of variance
ASCO	American Society of Clinical Oncology
asTF	Alternatively spliced TF
ATCC	American Type Culture Collection
AUC	Area under the curve
BOR	Bortezomib
C of V	Coefficient of variation
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CAT	Calibrated automated thrombography
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CHH	Castle Hill Hospital
CP	Cancer procoagulant
CI	Confidence Interval
CRF	Case Report Form
CSF	Colony stimulating factor
CT	Clotting time
CTD	Cyclophosphamide, thalidomide, and dexamethasone
CTDa	Attenuated cyclophosphamide, thalidomide, and dexamethasone
CVC	Central Venous Catheter
dH20	Distilled water
DAG	Diacyl gylcerol
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DVT	Deep vein thrombosis
ECACC	European Collection of Cell Cultures
ECM	Endothelial cell matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor

ELISA	Enzyme-linked immunosorbent assay
EMP	Endothelial cell-derived MP
EPCR	Endothelial protein C receptor
ERK	Extracellular signal-regulated kinase
ESMO	European Society for Medical Oncology
ETP	Endogenous thrombin potential
GEM	Gemcitabine
fM	Final concentration of tissue factor
F	Clotting factor (suffix 'a' denotes activated factor)
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
flTF	Full-length TF
FL	Fluorescence
FSC	Forward scattered light
Gla	Gamma-carboxyglutamyl acid
hr	Hours
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEY	Hull and East Yorkshire
HRP	Horseradish Peroxidase
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
Il	Interleukin
ILV	Intraluminal vesicle
IMiD	Immunomodulatory drugs
IP3	Inositol triphosphate
IQR	Interquartile range
ISS	International Staging System
ISTH	International Society of Thrombosis and Haemostasis
kDa	Kilodaltons
LEN	Lenalidomide
LIF	Leukaemia inhibitory factor
LMWH	Low-molecular weight heparin
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
mg	Milligrams
MGUS	Monoclonal gammopathy of undetermined clinical significance
MIP2α	Macrophage inflammatory protein 2-alpha
min	Minutes
ml	Millilitres
mM	Millimolars
MM	Multiple myeloma
MMP	Matrix metalloproteinases
MP	Microparticles
MPD	Myeloproliferative disorder

mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MLC	Myosin-light chain
MUC-1	Mucin 1
MV	Microvesicles
MVB	Multivesicular body
MW	Molecular weight
MWCO	Molecular weight cut-off
Na^+	Sodium ion
$NaCl_2$	Sodium chloride
NCD	Non-communicable diseases
NCCN	National Comprehensive Cancer Network
NET	Neutrophil extracellular DNA traps
NFκB	Nuclear factor κB
NICE	National Institute for Health and Care Excellence
NO	Nitric oxide
ng	Nanograms
nm	Nanometers
NSCLC	Non-small cell lung cancer
P/S	Penicillin/streptomycin
P-selectin	Platelet selectin
PAF	P*l*atelet-activating factor
PAI	Plasminogen activator inhibitors
PAR	Protease activated receptors
PBS	Phosphate buffered saline
PC	Pancreatic cancer
PCA	Procoagulant activity
PDAC	Pancreatic ductal adenocarcinoma
PE	Phycoerythrin
PFP	Platelet-free plasma
pg	Picogram
PGI_2	Prostacyclin
Pom	Pomalidomide
PPP	Platelet-poor plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
R&D	Research and Development
RCD	Lenalidomide, cyclophoshamide, and dexamethasone
REC	Research Ethics Committee
RNA	Ribonucleic acid
ROS	Reactive oxygen species
ROTEM	Whole blood rotational thromboelastometry
SD	Standard deviation
siRNA	Small interfering ribonucleic acid
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SSC	Side scattered light
TEG	Thromboelastography

TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TG	Thrombin generation
Thal	Thalidomide
TKI	Tyrosine kinase inhibitors
TNF-α	Tumour necrosis factor-α
TNM	Tumour/Node/Metastasis
TTP	Time-to-peak
TXA	Thromboxane
tPA	Tissue plasminogen activator
UK	United Kingdom
USA	United States of America
uPA	Urokinase plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VTE	Venous thromboembolism
v/v	Volume/volume
vWF	Von Willebrand factor
WHO	World Health Organisation
µg	Micrograms
µl	Microlitres
µm	Micrometers

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Author's declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources. I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised. I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

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Chapter:1 General Introduction

1.1 Cancer

The occurrence of cancer is a global problem. Of all non-communicable diseases (NCDs) worldwide, cancer was the second highest cause of deaths in 2015 second only to cardiovascular disorders, and it accounted for 8.8 million deaths (22% of NCDs), and nearly 1 out of every 6 deaths, with worse outcomes in low-income countries (World Health Organization (WHO) Global Health Observatory Cancer Fact sheet, 2017). Latest WHO data shows that in the UK, the age-standardized incidence rate (per 100 000 population) from cancer is 272.9 in both sexes.

Historically, cancer is a disease of the elderly, with over half of the incidence in the UK in people aged 70 and above. In England, there were 299,923 new cancer registrations in 2015 equivalent to 822 per day, which represented an increase of 4,183 registrations from the same point in the previous year, with incidence in males slightly higher than in females (Office of National statistics, 2015). In this survey, breast (15.4%), prostate (13.4%), lung (12.5%) and colorectal (11.6%) cancer continue to account for more than half of the malignant cancer registrations in England for all ages combined. In the same year, the number of registrations of Pancreatic cancer (PC) cases was in 4,175 males and 4,144 females (2.8% in total) while Multiple myeloma (MM) recorded 2,686 male and 1,946 female cases (1.5%) (Office of National statistics, 2015).

1.1.1 Cancer biology and haemostasis

The haemostatic system is a complex series of biological processes that governs blood flow and coagulation, and it is often disrupted in many diseases. Various epidemiologic studies have identified several mechanisms that contribute to arterial or venous coagulopathy which includes abnormal levels of coagulation factors such as prothrombin(Poort et al., 1996), fibrinogen(van Hylckama Vlieg & Rosendaal, 2003) and other pro-/anticoagulant proteins (Griffin et al., 1981; Pabinger & Schneider, 1996; Kamphuisen et al., 2001; Danesh et al., 2005; Wolberg et al., 2005; Allen et al., 2007). Other mechanisms involve dysfunction in thrombin generation (TG)(Macfarlane & Biggs, 1953; Butenas et al., 2000; Hron et al., 2006), tissue factor (TF) clotting activity and resistance to inactivation(Bertina et al., 1994; Zoller et al., 1994), fibrinolysis inhibition(Fay et al., 1997; Meltzer et al., 2010). In cancer, there is a marked departure from normal homeostatic maintenance to prothrombotic states, which results in hypercoagulable disorders that are linked to its progress (Trousseau, 1865; Lee, 2002). There is a spectrum of clinical manifestations of haemostatic dysfunctions that can be observed in cancer patients; from venous thromboembolic (VTE) events in patients being treated with chemotherapy, to arterial embolism and disseminated intravascular coagulation (DIC), mostly in patients with haematological malignancies or metastatic cancer(Prandoni et al., 2007).

1.1.2 Cancer VTE: occurrence, risk and clinical implications

VTE is a cumulative term for pulmonary embolism (PE) and deep venous thrombosis (DVT), which are both thrombotic disorders that may occur as an early manifestation of cancer, and are important causes of cancer treatment compromise that results in high mortality rates(Khorana et al., 2007). PE develops because of small or large blood clots (thrombi) that travels as emboli to disrupt lung vessel function, while DVT occurs due to clot formation located most commonly in the deep vessels of the legs.

Virchow's triad, developed by Rudolph Virchow in 1884 has been fundamental to understanding the pathogenesis of blood clot formation in cancer patients (Virchow, 1856; Rickles & Falanga, 2009). The triad identified three specific attributes in normal homeostasis, which interplay to provide a suitable environment for thrombosis development. Similarly, cancer VTE is of multifactorial aetiology, and develops as an interplay of these three main attributes of Virchow's: blood stasis in blood vessels (due to prolonged immobilization, vascular compression), endothelium dysfunction (from direct neoplastic invasion, chemotherapy damage) and hypercoagulability (induced by cancer cell prothrombotic properties as shown in figure 1.1) that may occur in the background of genetic abnormalities(Paskauskas et al., 2008).

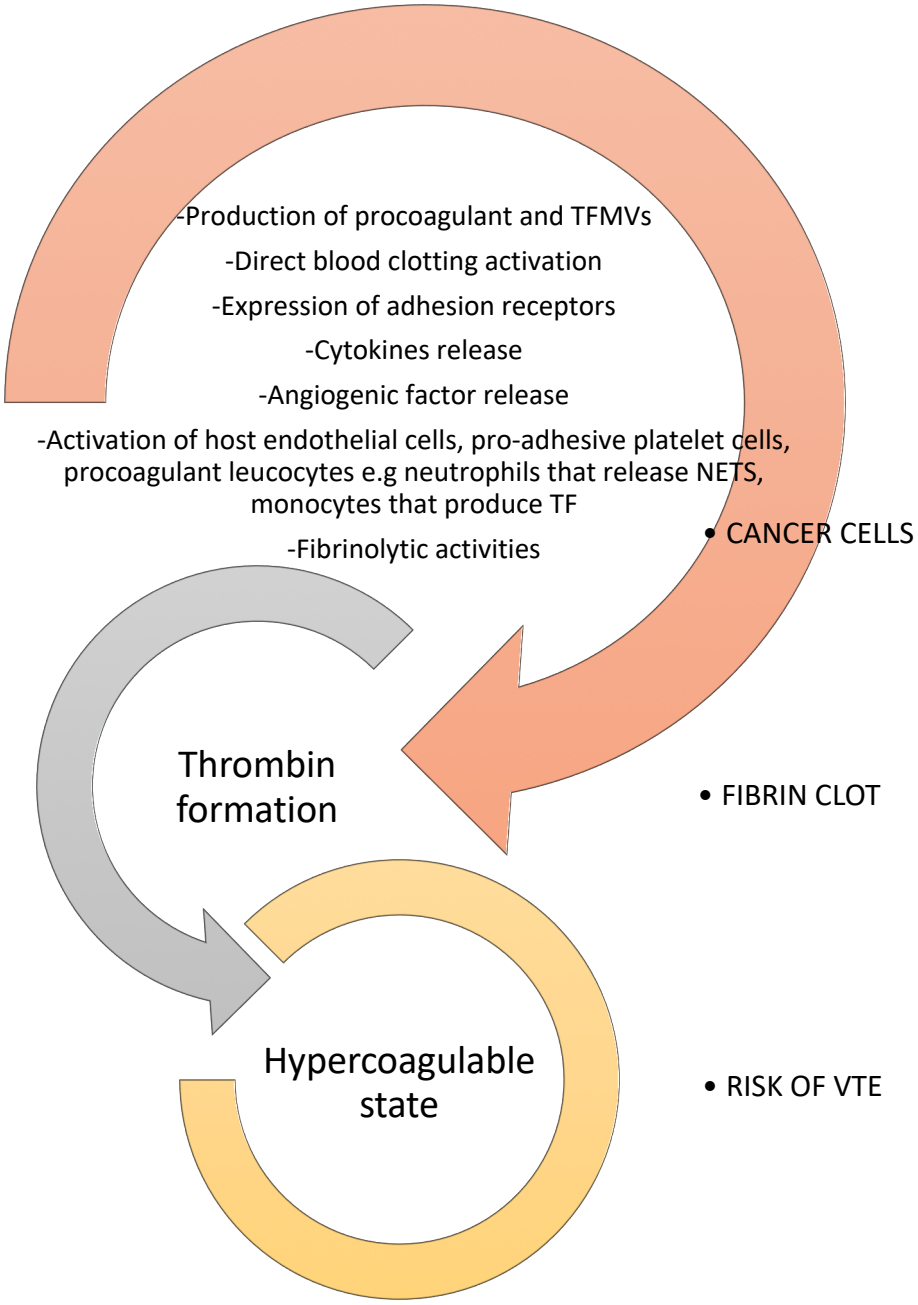


Figure 1.1 The hypercoagulable state due to prothrombotic attributes of cancer cells. Prothrombotic expressions of cancer cells such as release of inflammatory cytokines, angiogenic molecules, membrane adhesion receptors, TFMVs result in the production of thrombin that results in fibrin formation, leading to a hypercoagulable state. Abbreviations: TFMVs are tissue factor (TF) associated with microvesicles (MVs); NETS are Neutrophil extracellular traps; VTE is Venous thromboembolism. Modified from Prandoni et al(2005)(Prandoni et al., 2005)

1.1.2.1 VTE risk in Cancer

VTE was first recognised as linked to cancer by Trousseau over a hundred years ago (Trousseau, 1865; Piccioli et al., 1996). Recently, the risk of developing VTE has been identified as six to seven times increased in cancer patients when compared with non-cancer patients (Heit et al., 2000; Pabinger et al., 2013). VTE is the second most common cause of cancer deaths(Ambrus et al., 1975), and studies have consistently demonstrated VTE to be an early predictor of mortality in cancer patients (Chew et al., 2006; Paskauskas et al., 2008; Hisada & Mackman, 2017), with two-fold higher mortality in patients with PE (Naess et al., 2007). The highest risk of VTE occurrence in cancer has been determined in a large population-based cohort study to be within the first 3 months from the diagnosis of cancer(Blom et al., 2005). In addition, the occurrence of 'silent' sub-clinical VTE is possibly higher than the 15% clinically detectable VTE seen in cancer patients(Johnson et al., 1999), worsening the clinical outcome for cancer patients.

The risk of VTE occurrence is multifactorial and can be generally classified into 3 main categories; cancer-specific (qualities of the malignancy), patient-specific (consisting of patient characteristics, haemostatic system and blood counts) and therapy-specific (Figure 1.2). Cancer itself is an independent risk factor for VTE(Heit et al., 2000). Conversely, the risk of diagnosing cancer after VTE is established is also high, especially in the first 6 months to 1 year following a thrombotic incident (Sorensen et al., 1998). Although controversial, this opinion suggests that VTE could either be an indication of occult cancer many years in the making through rigorous screening, or that cancer and VTE may at the very least, share common risk factors(Prandoni & Samama, 2008). However, the efficacy of extensive screening strategies in routine practice has been called into question by a multicentre randomized controlled study due to findings of no clinical benefit in cancer patients (Carrier et al., 2015). In the study, there were no associations between the use of comprehensive or aggressive methods to screen for VTE with serious adverse events or impact on survival, although this trial was limited in its design with inherent outcome bias.

Other risk factors for VTE are patient-specific and include a wide array such as older age, obesity, dehydration, surgery, trauma, prolonged hospital stays, immobility, use of contraceptive pills or post-menopausal treatments(Franco & Reitsma, 2001). Other common risk factors include tumour mass effect, lymph node occlusion and there are also some patient conditions that have substantial VTE risk such as paroxysmal nocturnal haemoglobinuria, antiphospholipid antibody syndrome and myeloproliferative disorders(Franco & Reitsma, 2001). Myeloproliferative disorders (MPD) in particular are a collective group of haematologic disorders that involve cellular overproduction in the bone marrow and peripheral blood, in which vascular thrombotic events are one of the earliest manifestations in about 25-30% of diagnosed patients(Hachulla et al., 2000; De Stefano et al., 2008). These patient conditions may or may not be associated with genetic factors such as F5Leiden and F2 mutations, deficiencies of antithrombin III, protein C and S, which is likely to compound the risk(Anderson et al., 1992; Rosendaal, 1997; Kujovich, 2011). For example in heterozygous carriers of the F5 Leiden mutation, the regular use of contraceptive pills carries an higher multiplied VTE risk (an odds ratio (OR) = 15·6) when compared with risk carried by use of the pills alone (OR = 4) or presence of F5 Leiden mutation alone in the general populace (OR = 6)(Wu et al., 2005).

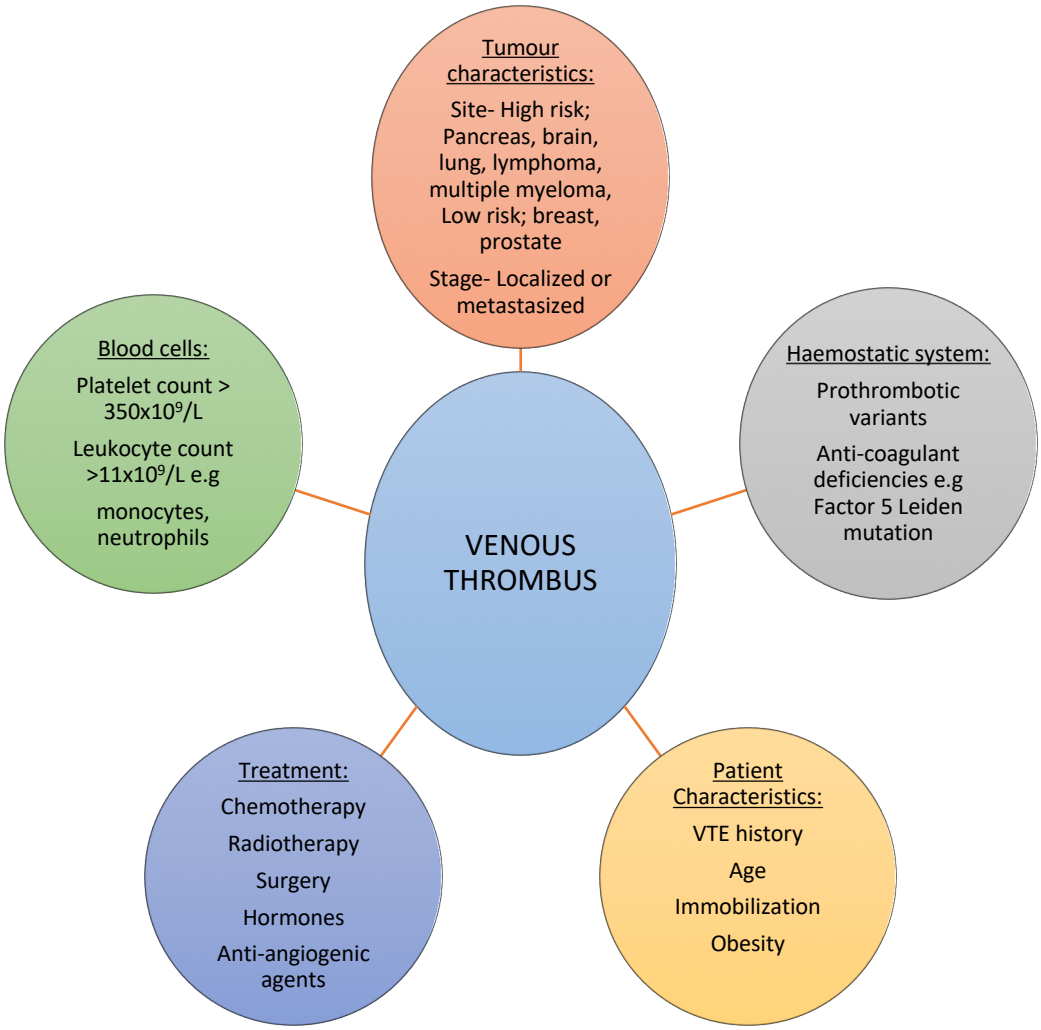


Figure 1.2 Risk factors for cancer VTE development.

Risk factors include tumour characteristics, patient characteristics, variations in the haemostatic system, type of treatment administered, concentrations of blood cells. Modified from Lyman et al, 2013(Lyman et al., 2007).

1.1.2.2 Chemotherapy and the risk of VTE

Anti-cancer therapy also poses an additional high risk of VTE. These include chemotherapy as adjuvant or neo-adjuvant, hormonal medications, antiangiogenic agents, combination treatment regimens and surgery have a pro-thrombotic effect(Falanga & Marchetti, 2012). While the actual incidence is unknown in patients receiving chemotherapy, VTE risk can be as high as 11% or more annually, making chemotherapy a major independent risk factor by itself (Otten et al., 2004). For example, VTE incidence of about 18% have been associated with Non-small cell lung cancer treated with Cisplatin-based chemotherapy in a prospective study of major vascular events (Numico et al., 2005). Furthermore, the risk of VTE can be quadrupled in cancer patients on chemotherapy as opposed to those with cancer alone(Heit et al., 2000). The incidence of chemotherapy-associated VTE may also be underestimated, due to other confounding factors that may increase the risk in cancer patients such as the use of indwelling intravenous catheters, surgery, dehydration, immobilisation and prolonged hospital stay(Otten et al., 2004). An increased VTE risk is also frequently associated with chemotherapy regimens administered in certain cancers with naturally high VTE incidences, such as pancreatic cancer and high-grade glioma, which are known for high incidence rates of up to 28%(Mandalà et al., 2007; Simanek et al., 2007) while an elevated risk from 3%(Khorana et al., 2006) to 9%(Mandala et al., 2010) due to chemotherapy has been observed in those cancers with low VTE incidence such as breast cancer.

As incidence of VTE varies by cancer type, site, dose and type of medications administered (Haddad & Greeno, 2006), chemotherapy-induced hypercoagulability in relation to specific drug combinations in treatments is a significant complication and can be a difficult study to undertake, due to the intricate pathogenic mechanisms that are likely involved. One study assessed this and reported that the use of Thalidomide as a monotherapy, carries a 5% lesser VTE risk when compared to its use in combinations for MM treatment (Singhal et al., 1999). Phase 1 clinical studies conducted by the Southern Europe New Drugs Organisation (SENDO) from 2000 to 2010 described significantly elevated VTE occurrence amongst advanced cancer patients that were administered various cytotoxic combination regimens(Mandala et

al., 2012). In another phase 1 clinical trial, cisplatin and gemcitabine combinations with anti-angiogenic agents (Semaxanib SU5416) resulted in severe thrombogenic complications in patients with solid tumours, where 9 serious thromboembolic events were evoked in 8 out of 19 patients(Kuenen et al., 2002). In addition, a meta-analysis of 15 randomized controlled clinical trials demonstrated that there was an increased VTE incidence of 12% during treatment with standard chemotherapy combinations in several advanced solid tumours, further lending support to a significant VTE association with chemotherapy(Scappaticci et al., 2007).

Although the mechanisms for this chemotherapy-related thrombotic phenomenon are poorly defined in literature, nevertheless there are various possible mechanisms that have been postulated; some involving direct endothelial damage in combination with tumour-specific products such as pro-coagulant proteinase(Falanga et al., 2013), and subsequent activation of the haemostatic cascade through monocytes or platelet induction(Walsh et al., 1992; Togna et al., 2000; Haddad & Greeno, 2006). For instance, Doxorubicin (DOX), an anthracycline agent, is used in many cancers such as MM, and it can directly induce platelet activation (Figure 1.3) and consequent thrombus formation through consistently increased cell membrane phosphatidylserine (PS) lipid exposure and PS-bearing microvesicle (MV) generation (Kim et al., 2011). Others less direct methods include stimulating the release of procoagulant MVs(Date et al., 2013), other prothrombotic proteins and inflammatory cytokines such as proangiogenic vascular endothelial growth factor (VEGF), and exerting an inhibitory effect on anti-thrombotic regulators such as protein C, S and antithrombin III as illustrated in figure 1.3. An example is the significant reduction of functional protein C levels in colorectal cancer patients on Fluorouracil (a pyrimidine analogue) infusion, which abated following treatment cessation(Feffer et al., 1989).

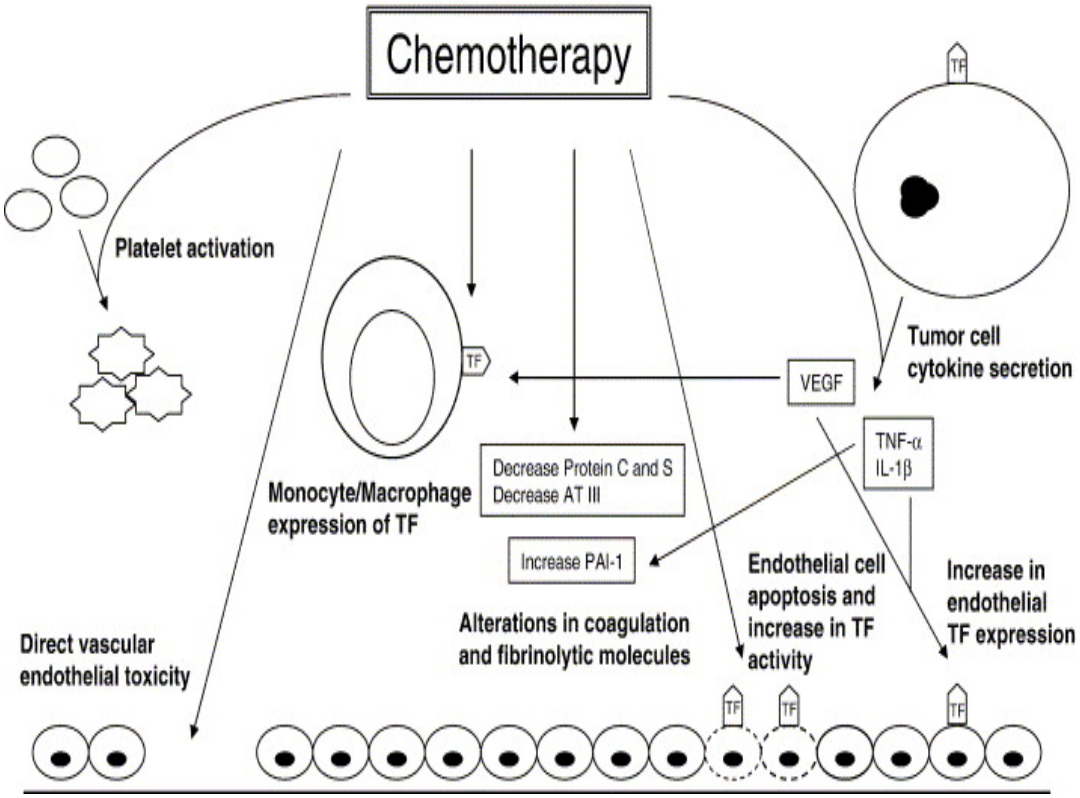


Figure 1.3 Chemotherapy and VTE.

Chemotherapy can induce varied thrombogenic effects through several mechanisms such as direct endothelial toxicity, platelet activation and aggregation, monocytic TF expression, decreased thrombin regulators such as antithrombin III (AT III), protein C and S, altered fibrinolytic activities, increase TF activity from endothelial cell apoptosis, tumour cell secretion of prothrombotic cytokines such as PAI-1, IL-1β, TNF-α, and increased vascular endothelial TF expression levels. Adapted from Haddad and Greeno, 2006(Haddad & Greeno, 2006).

Chemotherapy regimens often act at the molecular level, by involving DNA and mitochondrial damage, which induces apoptosis in cells(Caley & Jones, 2012). They interfere with cell division and stimulate widespread cell death. There are also membrane alterations caused by DNA/RNA intercalating drugs such as Cisplatin and Adriamycin, which potentiate coagulation (Ahmad et al., 1995). Increased TF activity as shown in figure 1.3 is also a marked phenomenon in cancer-related thrombosis, and a significant role has been suggested in association with MVs(Lechner & Weltermann, 2008). Other effects of chemotherapy include increased endothelial

and monocytic TF expression, stimulation of endothelial cell apoptosis resulting in more PS exposure, and alterations in coagulation and fibrinolytic molecules to support prothrombotic tendencies (Figure 1.3). However, there are still several gaps in knowledge of the specific coagulation processes that constitute prothrombotic mechanisms in cancer patients, that require filling to achieve better understanding of the VTE complications that result from chemotherapy use.

1.1.2.3 VTE prognosis and anti-thrombotic interventions

VTE occurrence in cancer patients indicates poor prognosis and early mortality(Khorana, 2010). In a population-based study of 3135 patients diagnosed with cancer, 1-year survival was only 12% in patients that experience it (Sorensen et al., 2000; Haddad & Greeno, 2006). This significant association with short-term prognosis is also reflected in a prospective observational cohort of 4458 adult cancer patients, which Kuderer et al evaluated between 2002 to 2006 in 115 U.S sites(Kuderer et al., 2008). In this study, VTE developed in 93 (2.1%) patients at a median of 38 days, while 137 died during observation at median follow-up of 75 days, and it was calculated in a multivariate analysis that VTE incidence was a significant independent predictor of early mortality all round (HR=6.98, 95%CI: 2.83–17.21; P<.0001). Due to the associated high morbidity and mortality, it is important to develop strategies that can prevent VTE and reduce the incidence in cancer patients. VTE thromboprophylaxis is safe and feasible, as has been shown by various studies that have reported considerable benefits of use of thromboprophylaxis with concurrent chemotherapy. For example, in the FRAGEM and CONKO-004 clinical trials, the incidence rates of VTE were significantly reduced in advanced pancreatic cancer with simultaneous use of Low molecular weight heparin (LMWH) and chemotherapy; from 23% in patients with no anticoagulation to 3% in patients with Dalteparin in FRAGEM and 15% in patients with no anticoagulation to 5% in patients with Enoxaparin in CONKO-004 trials(Riess et al., 2010; Maraveyas et al., 2012).

VTE is mostly preventable, and widespread antithrombotic therapies based on heparin and Vitamin K analogues are recommended in clinical use by the National

Comprehensive Cancer Network (NCCN)(Khorana, 2007), American Society of Clinical Oncology (ASCO)(Lyman et al., 2007), and European Society for Medical Oncology (ESMO)(Mandala et al., 2011) and are effective. In addition, a multinational cross-sectional study across 32 countries showed that 51.8% of hospitalized patients aged 40-72 in 358 hospitals had a VTE risk from both surgical and medical units, and a low number received appropriate prophylaxis, which further signifies the importance of hospital-wide uptake of therapeutic recommendations worldwide (Cohen et al., 2008). In England, the National Institute for Health and Care Excellence (NICE) therefore produced a set of guidelines, targeted at reducing inconsistent use and under usage of thromboprophylaxis for cancer patients in hospitals in the country(NICE, 2010). Clinical practice guidelines for the prevention of VTE recommend that hospitalized cancer patients, cancer patients undergoing surgery, as well as MM patients on chemotherapy should receive routine VTE prophylaxis(Lyman et al., 2007; Mandala et al., 2011; Farge et al., 2013). In practice, such routine anti-thrombotics recommended for hospitalized cancer patients are continued till disease control is achieved. To achieve this end, risk assessments have been regularly performed for all hospitalized patients since 2007, and the possibility of developing more sensitive risk assessment tools and biomarkers with better precision is desirable. One example is a predictive risk model developed by Khorana et al, which identifies cancer patients with high individual risk of thrombosis based on basic clinical and laboratory variables which can determine cancer patients with approximately 7% short-term risk of symptomatic thromboembolism (Khorana et al., 2008b). Thromboprophylaxis regimens with LMWH such as Dalteparin (Fragmin ™) which target pro-coagulant agents thrombin and Factor Xa(FXa), are currently indicated before and up to several weeks after surgery and in chemotherapy combinations in clinical management(Khorana, 2007). However, the clinical burden is still overwhelming in cancer patients, for instance, studies show that even while on oral anticoagulants; the incidence of recurrent thromboembolism is still twice as probable in patients with cancer (Levitan et al., 1999; Bona et al., 2000). Furthermore, these recurrent cases have even poorer survival rates, can be resistant to further oral anticoagulation and are more likely to need prolonged hospitalization(Caine et al., 2002). The emergence of new direct oral anticoagulants such as Apixaban have

provided a useful oral alternative to LMWH, due to issues that exist with non-compliance from patients averse to LMWH given by injections and prohibitive cost of long-term use in some metastatic cancers(Noble et al., 2015; Smrke & Gross, 2017). However, as oral anticoagulation efficacy is not fully established in clinical practice, careful consideration over any advantage needs to be fully assessed per patient to ensure actual clinical benefit (Carrier et al., 2014). In addition, more targeted, personalized and safer therapies need to be designed and developed to improve prognosis, particularly as these cancer patients may also have greater risks of bleeding than non-cancer patients.

1.2 Coagulation cascade

The coagulation cascade is a sequence of clotting factors in the haemostatic system, which progress in a stepwise fashion to produce coagulation in the blood. Two groups (Macfarlane in the U.K and Davie & Ratnoff in the U.S.A) first proposed this waterfall process in 1964, and described the concerted interaction of blood components that are mostly serine proteases involved in two main pathways; an intrinsic and an extrinsic-TF driven (Davie & Ratnoff, 1964; Macfarlane, 1964). More recently, a different view emerged which suggested that the development of clotting can be influenced by the cell surfaces the factors interact with, and that such clotting processes are determined by the properties of the lipid-containing cell surfaces which regulate it (Hoffman & Monroe, 2001). Consequently, the cascade theory has now largely been superseded by a proposed model where the clotting pathways is not separated distinctly into two but have overlapping and interdependent components. In this model, cells with similar phospholipid layers such as phosphatidylserine (PS) may have different roles in coagulation process which depends on the constituent of their surface receptors(Hoffman & Monroe, 2001). By highlighting the importance of specialized roles of surface receptors for coagulation proteins, this model is more adaptable and addresses some inadequacies of the preceding system. It reflects better the intricate mechanisms that may be found *in vivo*, and may aid understanding of clotting irregularities in haemostatic disorders(Hoffman & Monroe, 2001).

1.2.1 Phases of coagulation

There are 3-4 phases of coagulation in the haemostatic process, which are illustrated in table 1.1 and figure 1.4 as:

- Initiation
- Amplification
- Propagation
- Termination or Inhibition

Table 1.1 The 4 phases of cell-based model of coagulation

Modified from Hoffman and Monroe, 2001 (Hoffman & Monroe, 2001)

Initiation	**Amplification**	**Propagation**	**Termination**
Vascular endothelium and circulating blood cells are disturbed; there is interaction between TF and plasma-derived factor VII	Thrombin activation of aggregated platelets, cofactors V and VII, and factor XI on the platelet surface	Large amounts of thrombin production which leads to formation of a stable plug at the site of insult and subsequent cessation of blood loss	Restriction of the coagulation process to prevent thrombotic occlusion of vessels

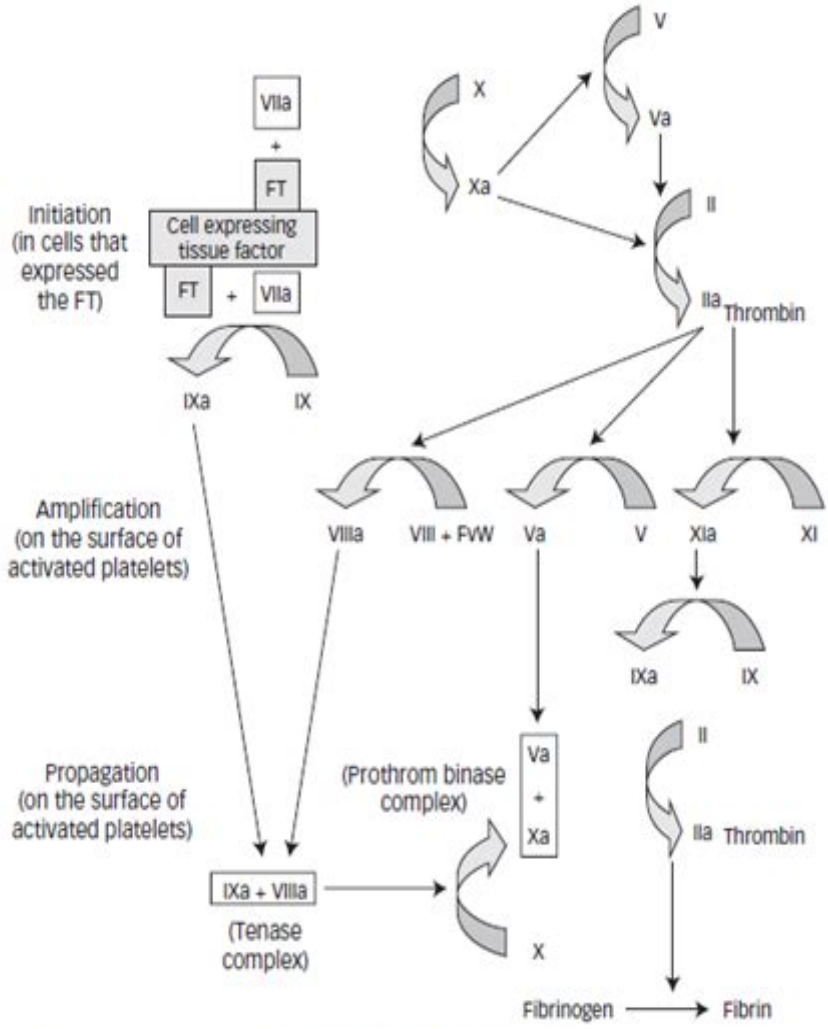


Figure 1.4 The Coagulation cascade.

Illustration of the clotting cascade based on cell surfaces comprising of the phases of coagulation -initiation, amplification and propagation from Ferreira et al, 2010 (Ferreira et al., 2010), originally adapted from Vine, 2009 (Vine, 2009). Abbreviation: FT is Tissue Factor, FvW is Von Willebrand factor.

Initiation:

There are two main methods of initiation of the clotting cascade; and TF is a key player in both(Hoffman & Monroe, 2001). There is a well-known process where TF in the cell membrane, once exposed to plasma (as explained below) is complexed with factor VII (FVII) and proteases, and this complex attracts and serves as a homing

beacon to the site of endothelial injury(Hoffman & Monroe, 2001). This localization allows platelets to aggregate and leads to further production of other cytokines that further attracts other secondary members of the clotting system, such as monocytes and neutrophils. Platelets are also stimulated to produce thrombin whose activity leads to fibrin production and clot formation. In the second more recent proposed explanation, TF-containing MVs carry circulating TF in the bloodstream (produced due to stimulating conditions *in vivo*) and these TF vehicles find and bind free platelets (that do not normally carry TF intrinsically) or platelets of an evolving thrombus plug(Conde et al., 2005). These MVs may be produced from an initial vessel wall insult or elsewhere in the body and from different types of cells, which may explain blood clot formation in a location different to the site of initial injury. This model lends itself well to cancer VTE development, and may also serve to explain how cancer MVs bearing TF may lead to indiscriminate thrombosis(Conde et al., 2005).

In the haemostatic system, TF is released from cells found in the sub-endothelium when they come into contact with the intraluminal contents within the blood. Once released, TF attaches to activated factor VII found in circulation in the plasma and forms a complex that binds to protease-activated receptors (PARs). This complex initiates the extrinsic pathway by cleaving factor X (FX) to an activated form FXa, which binds to FVa (prothrombinase) and subsequently coverts prothrombin to thrombin in the common pathway (Figure 1.4). In the intrinsic system, the TF-FVII tenase also activates Factor IX (FIX)(Davie & Ratnoff, 1964). This can be rapidly inactivated by Tissue Factor Pathway Inhibitor (TFPI) or by antithrombin III if it leaves the cell surface. If not, FIXa can combine with factor Va (FVa) in turn, which can then lead to initial thrombin production from prothrombin (Monroe et al., 1996). For the intrinsic pathway, Factor XII is converted to XIIa on contact with negatively charged surfaces (subendothelial collagen) and converts FXI to FXIa which in turn converts factor IX to IXa, which then converts factor X to Xa to enter the common pathway. These actions result in the production of thrombin in small amounts and leads to the amplification step. However, this amount of thrombin is insufficient to form a full clot, and enough quantities are only generated in subsequent steps.

Amplification:

When the endothelial breach allows sequestered TF to be exposed to intraluminal contents, the thrombin subsequently produced has three main functions. First, thrombin on TF-bearing cells in small volumes causes more platelets to adhere to the ECM matrix (Diaz-Ricart et al., 2000), by cleaving through use of its protease activated receptors (PAR), especially PAR2 located on endothelial surfaces. There is further platelet activation as well as cleaving of other factors V, VIII and XI into active components(Monroe et al., 1996). Once platelets are activated their α-granules are degranulated emptying the FV contained within in a partially activated form(Monkovic & Tracy, 1990). Secondly, this form is now fully activated by thrombin or FXa, which through its non-PAR receptors such as GPIb/IX, any thrombin that is left remains active enough to activate other coagulation factors that are found on the platelet surface. For instance, FVIII is usually produced as a complex with von Willebrand factor (vWF) bound to platelets surface, and then immediately cleaved by thrombin to release vWF (Hultin, 1985). Amplification sets the stage ahead for the assembly of a more complex structure in propagation where large-scale thrombin generation commences.

Propagation:

At this stage, thrombin necessary for clot formation is produced, once the coagulation factors are activated in the amplification stages, the tenase and prothrombinase complexes are arranged on platelet surfaces(Hoffman & Monroe, 2001). The remaining coagulation factors such as FIX(Ahmad et al., 1995), X(Cirino et al., 1997), XI(Greengard et al., 1986) in their activated forms bind to the platelet surfaces through high affinity receptors. These play different interlinked roles mediating the coagulation process. For example, FIX-Xa complexes can activate FX on platelet surfaces. FXa-Va are then formed into complexes which are necessary for extensive thrombin generation(Hoffman & Monroe, 2001). This thrombin 'burst' causes fibrinogen conversion to fibrin. Fibrin is necessary to provide a structural

scaffold for clot formation and so soluble fibrin is converted by thrombin to cross-linked fibrin which forms the haemostatic clot(Rickles et al., 2003). This clot stabilizes the platelet plug, leading to formation of a thrombus.

In tumours, overexpression of fibrin has been noted along with increased levels of fibrinogen growth factor, and this may be important in tumour invasion (Sahni et al., 2008). Fibrin production may also impair host defences by coating the tumour cell with a protective layer, to enable it to evade detection by phagocytes and avoid identification by impedance of natural killer cells (Palumbo et al., 2005). High levels of fibrinogen may also indicate poor prognosis in some malignancies such as endometrial(Seebacher et al., 2010), ovarian(Polterauer et al., 2009), renal(Tian et al., 2017) and colorectal cancer(Tang et al., 2010; Sun et al., 2014).

Termination or Inhibiton:

This is a haemostatic step to prevent blood vessels from unnecessary and indiscriminate occlusion. Here, sustained TF-induced coagulation by the TF-FVII catalytic complex is regulated in a negative feedback loop mediated by a multivalent, Kunitz-type plasma proteinase inhibitor protease called Tissue factor pathway inhibitor (TFPI) (Broze, 1995a). TFPI production is triggered when the TF-FVII complex cleaves FX to form FXa. TFPI either indirectly inhibits by forming a aggregated complex with the TF-FVII-FXa or directly by binding to FXa whereby the Xa-TFPI complex inhibits TF-FVII (Broze, 1995b). Furthermore, thrombin can bind to thrombomodulin, an anti-thrombotic protein, and the complex formed activates anti-inflammatory protein C(aPC) localized on endothelial surfaces (Stearns-Kurosawa et al., 1996). APC binds to Protein S and helps to cleave complexes of FVa-FVIIIa, preventing further indiscriminate clotting factor activations(Hoffman & Monroe, 2001). Thrombin itself can be inactivated by antithrombin III, also found on endothelium, which rapidly inhibits FIXa and FXa activities in the clotting pathways(Hoffman & Monroe, 2001).

1.3 Role of haemostatic factors in cancer thrombosis

Under normal homeostasis, the coagulation network (consisting of the intrinsic and extrinsic arms) works harmoniously through the four main steps to merge into a common pathway to generate thrombin, which in turn induces conversion of fibrinogen to fibrin and eventually construction of a blood clot. In malignant disease, cancer cells may exert a direct prothrombotic effect by stimulating the constitutive expression of TF (in association with coagulation factor VII) on tumour cell surface or endothelial stroma and thus have constant hypercoagulable ability(Contrino et al., 1996). Therefore, a few studies have suggested the possibility that in various conditions, different cancers may activate or exhibit different coagulation cascade factors and components. Baagatin et al, for instance reviewed the findings in breast cancer perioperatively, and reported changes in the coagulation factors system such as increased platelets and platelet aggregation, prolonged PT and APTT, fibrinogen, D-dimer, VWF, PAI-1, and decreased AT, PS, PC and plasminogen(Bagatin et al., 2013). A clinical study by Boersma et al examined 168 patients with malignancy and central venous catheters (CVC) and reported that those who developed thrombosis had higher FVIII (203 ±62% versus 166± 59%; p=0.023) immediately following CVC insertion(Boersma et al., 2016). This increasing trend was also supported by Kovacs et al, who identified an increasing trend of FVIII (1.71%, IQR 1.13-2.91 to 1.94%, IQR 1.33-3.06) over 2 months of administration of thalidomide-prednisolone treatment combination in myeloma patients, although there were no significant differences in those who developed VTE (1.38% 95% CI 1.27-1.49) and those who did not (1.68% 95% CI 1.52-1.85) in this study (Kovacs et al., 2015). Also, another study assessed FVII and reported a decrease in median levels (89.1%, IQR 69.7-106.0) in patients with haematological malignancy compared to healthy controls (92.7%, IQR 82.8-106.5), although this was not associated with increased VTE risk(Negaard et al., 2008). There are also additional reports of laboratory findings of other coagulation factors frequently increased in several malignancies such as FV, IX, XI which may represent independent risk factors for thrombosis (Dogan et al., 2006; Battistelli et al., 2008; Yigit et al., 2008). Although these findings of different elevated coagulation factors in various malignant conditions such as MM(Auwerda et al., 2007),

colorectal(Battistelli et al., 2008) and breast cancer(Yigit et al., 2008), further supports the role of coagulation in cancer thrombosis, the possibility that there might be distinct cancer-specific coagulation pathways for different malignancies is yet to be extensively researched. PC for instance is known to be exhibit a TF-dependent pathway of coagulation unlike MM which expresses little or no TF and may likely utilize other as yet unclear pathways in haemostasis.

The interdependency of both arms of the clotting cascade also has an impact on regulating haemostasis, for instance when TF-FVII complex from the extrinsic system can also cross-over and activate FIX and FX(Osterud & Rapaport, 1977). As a highly interconnected system, the inhibition of a part of the coagulation system is likely to only cause partial impairment of the system (Al-Horani & Desai, 2014). Moreso, various heterogenous sub-pathways or side arms might theoretically develop in certain biological conditions when specific components of arms are either more active or inactive than others. Transcriptionally, it has recently been suggested that in different tumours, there may exist alternative pathways of coagulation system regulation formed by oncogenic protein and miRNA coding genes, and that this property may be amenable to specialized biologically-based approaches to personalized anticoagulation (D'Asti & Rak, 2016). In addition, clotting systems in biological models may differ considerably in cancers of various aetiology (such as in solid and non-solid cancer types) and contribute to specific tumour qualities such as early invasion, metastasis and angiogenesis. For example, high levels of pre-operative fibrinogen (374.5 vs. 332 mg/dl, $p=0.002$) in renal cell carcinoma as compared to healthy controls have been identified as associated with significant increase in tumour size ($p=0.004$), histologic grade ($p<0.001$) and metastasis ($p<0.001$) (Erdem et al., 2014).

In order to study this theory of the role of various coagulation factors in cancer thrombosis, it would be necessary to characterize the factors that contribute in different extents to pro-thrombosis in various cancer models, and the haemostatic endpoints involved. One of such central endpoints involves thrombin, as one of the key processes in cancer metastasis usually involves TF-initiating thrombin production,

and then local fibrin deposition followed by platelet recruitment through PAR-activated signalling processes for clot formation (Camerer et al., 2004).

1.4 Thrombin

1.4.1 Thrombin structure

Thrombin, also known as factor IIa, is one of the functionally important serine proteases involved in coagulation(Davie & Kulman, 2006). It is produced as a zymogen precursor, prothrombin, from the liver and secreted into the bloodstream. The liver contains specific Vitamin K carboxylase enzymes that catalyses the Vitamin-K dependent post-ribosomal conversion of up to 12 glutamic acid residues in the N-terminus of prothrombin precursors to gamma-carboxyglutamyl acid (Gla) residues, which prevents the thrombin protease to bind to phospholipid layers of cell membranes indiscriminately(Suttie, 1980). At sites of vascular damage, prothrombin is converted by proteolytic cleavage into active thrombin by coagulation factors (FXa-FVa prothrombinase complex) downstream of the clotting cascade. These remove the Gla domain; such that thrombin can then attach to platelets in the initiation phase of coagulation, activate other factors FV, FVIII, FXI, FXIII in the amplification phase, and coverts fibrinogen to fibrin at its active catalytic site through catalytic cleavage of the Arginine-Glycine bond, during propagation as shown in figure 1.5 (Monroe et al., 1996; Hoffman & Monroe, 2001).

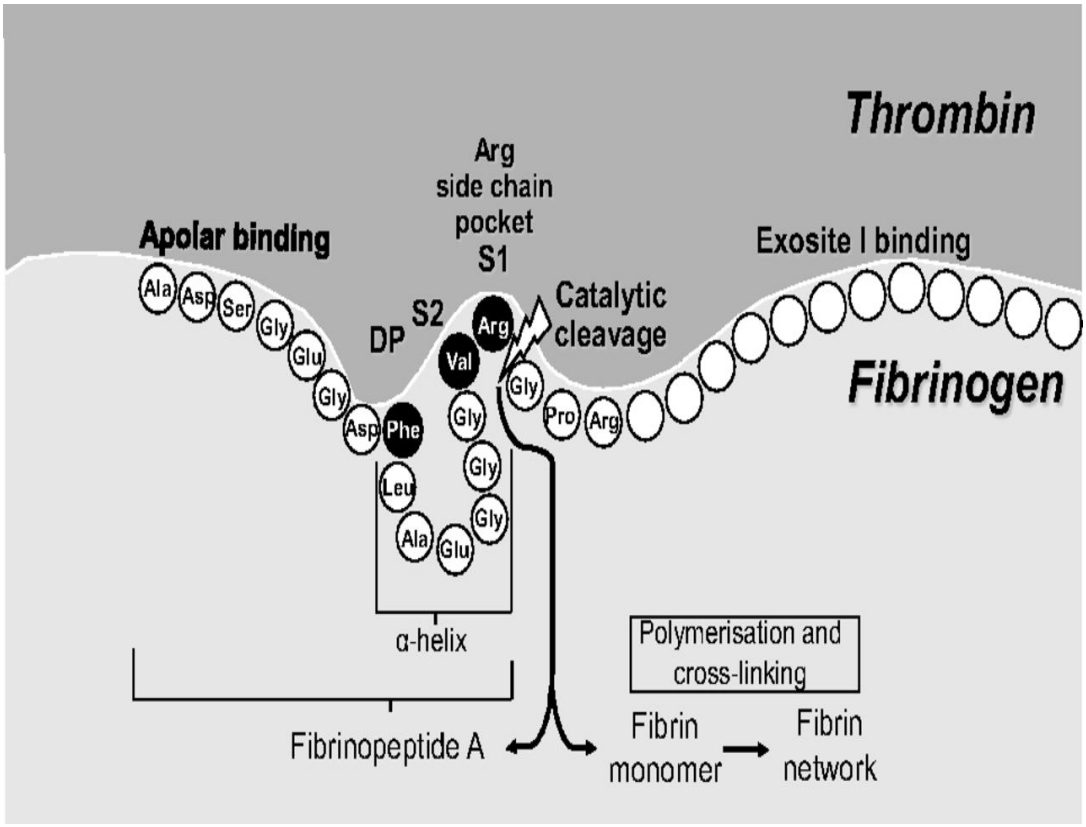


Figure 1.5 Functional role of thrombin.

Thrombin structure showing the fibrin-binding exosite 1 and the active or catalytic site. Cleavage takes place between Arginine (Arg) and the adjacent Glycine (Gly) peptide bond in the active site, leading to fibrinogen conversion to fibrin. S1 and S2 are the pockets for Arg and Val, respectively, and DP is the pocket for Phe. Abbreviations: Ala, alanine; Arg, arginine; Asp, aspartic acid; Glu, glutamic acid; Gly, glycine; Leu, leucine; Phe, phenylalanine; Pro, proline; Ser, serine; Val, valine. Adapted from Coppens et al, 2012(Coppens et al., 2012).

Thrombin is a critical link between vascular disruption, coagulation and platelet activation. Its function is mediated through a family of proteases called the PAR family(Coughlin, 1999), which are G-protein coupled signalling receptors also implicated in tumour growth and whose specific inhibition inhibits this growth(Coughlin, 2000). The family consists of PAR1, PAR3, and PAR4 which are specifically activated by thrombin, and also FXa-activated PAR1 and PAR2(Versteeg et al., 2004). PAR1 and PAR3 contain thrombin-interacting sites on their amino-terminal (N-terminal) and carboxy-terminal (C-terminal) extracellular domains

(exodomains or exosites). PAR1 is the prototype of the family, and thrombin signalling occurs when the N-terminus is cleaved by thrombin at a specific site to reveal a new N-terminus that can then bind other downstream intramembrane signalling molecules (Rasmussen et al., 1991; Vu et al., 1991). The thrombin-specific cleavage site is on a peptide chain of the PAR1 N-terminal exodomain, between residues Arginine 41 and Serine 42(Hugel et al., 1999). This site is responsible for fibrinogen conversion to fibrin by cleavage of the glycine-arginine bond (Figure 6B). The pleiotropic effects of PAR1 activation occur through coupling to various G-protein families such as $G_{12/13}$, G_q, and G_i (Figure 1.6). Likewise, PAR3 has also been derived by knockout mice gene studies and is known to mediate thrombin-triggered phosphoinositide hydrolysis in various human tissues (Ishihara et al., 1997).

Thrombin activity can be modulated in several ways, including through regulators of TG such as the platelet-expressed glycoprotein GPIba (Jain et al., 2007) or endothelial thrombomodulin(Horowitz et al., 2011). These processes determine the success of coagulation and may be linked to tumour development and spread, and potentially aid pathways of the metastatic process. For instance, Shaker et al, have identified an association between thrombin pathway activation through measurements of pre-operative D-Dimer levels in plasma with the presence of lymph node metastases in early breast cancer patients(Shaker et al., 2017), although D-dimers here may only serve as biomarkers and this study is still ongoing.

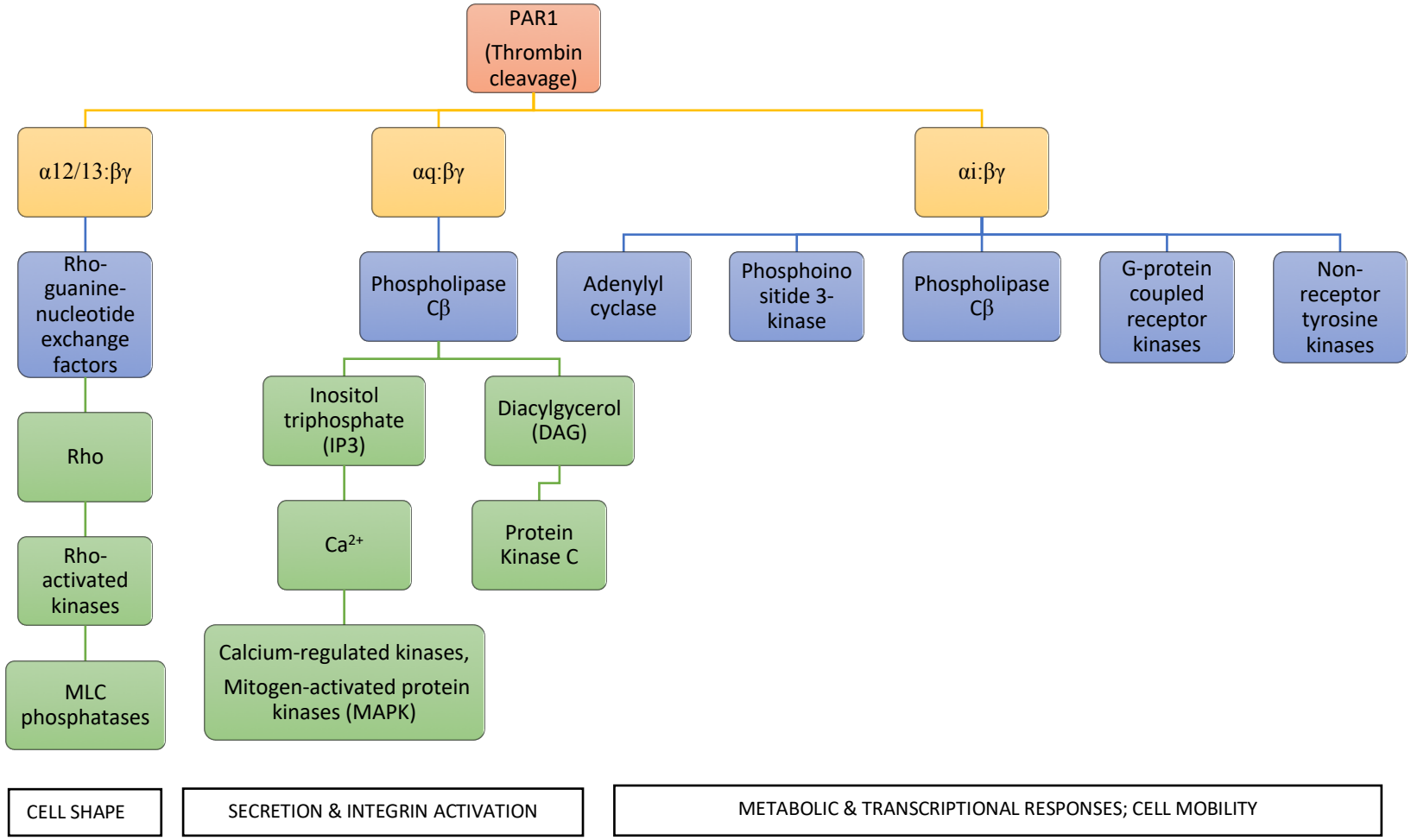


Figure 1.6 Thrombin-activated PAR1 signalling effects.

The intracellular signalling network and phenotypic effects of thrombin-activated PAR-1 signalling. Members of the G-protein families activate PAR1 signalling which leads to: Rho-mediated changes in platelet cytoskeleton, phospholipase Cβ triggering of phosphoinositide hydrolysis, calcium

mobilization, and protein kinase C activation, facilitation of platelet aggregation through calcium-regulated kinases and phosphatases, MAP kinases, and inhibition of adenylyl cyclase. Modified from Angiolillo et al (Angiolillo et al., 2010).

1.4.2 Thrombin importance in VTE

There are several reports in literature that suggests that VTE development may be associated with a change in TG. D-Dimers and fibrinogen, and to a lesser extent thrmonin-antithrmobin complex, prothrombin fragments F1 +2, protein C and S and MVs, as components of the coagulation process have been extensively studied to demonstrate some degree of efficacy in identifying patients with high risk of VTE(Samuelson Bannow & Konkle, 2018). For instance, several studies have identified elevated D-dimers markers in association with VTE events; one is a large clinical study of a subset of 111 patients with haematologic malignancies such as MM from Vienna Cancer and Thrombosis Study (CATS) where elevated D-Dimers of >1.4mg/L in the 75^{th} percentile was found positively associated with increased VTE risk of HR 1.8, IQR 1.0-3.2(Ay et al., 2009). Using clotting factor levels in the blood, TG has also shown similar promise to potentially predict risk of developing thrombosis in statistical simulation models(Brummel-Ziedins et al., 2005). Furthermore, TG measured by functional assays may also be used to predict thromboembolic events, as suggested by a cohort study of 1033 patients with various cancers where a cumulative comparison found that patients with elevated peak thrombin had a higher (11%>4%)significant likelihood of developing VTE in the first 6 months of diagnosis than in people with lower peak thrombin (Ay et al., 2011). In two prospective population-based cohorts, elevated basal peak thrombin generation was associated with subsequent risk of VTE (Lutsey et al., 2009). In a recent study, VTE has been linked to significantly higher levels of urine and plasma prothrombin fragment 1 + 2, D-dimer, lag time, time to peak, and endogenous thrombin potential(Wexels et al., 2017). However, these results although promising, are still in development and are not entirely consistent yet, probably due to the need for standardization of the methods of TG measurement.

Clinically, it may be more useful to assess the prothrombotic state in cancer patients through TG, as it is a method that comprehensively evaluates thrombin production stages such as initiation, amplification, propagation and inhibition (Figure 1.8) and

also provides a quantitative assessment of the amount of thrombin formed(van Veen et al., 2008). Furthermore, TG measurement provides an attractive method to gain insight into the complex interactions of the various factors that determine coagulation capacity and acquire composite knowledge of how it influences the hypercoagulable predisposition of distinct cancer types.

1.4.3 Thrombin measurement

Several methods exist for assessing procoagulant activity, including tests for primary and secondary haemostatic function (Table 1.2) such as prothrombin time(PT)-based clotting assays, tests for fibrin formation to degradation products and D-Dimer assays, and also newer approaches such as thromboelastography (TEG) and whole blood rotational thromboelastometry (ROTEM)(Herring & McMichael, 2012). These methods have different endpoints and varying degrees of successes, acceptance and utility over time. Of note is that the popular clotting based assays (based on calculating the prothrombin time, the partial thromboplastin time or the activated clot time) have recognized limitations. For example, clot formation occurs after generation of approximately 10nM thrombin-antithrombin, which coincides with the start of the propagation phase of the coagulation cascade, and any thrombin produced afterwards is not detected by these standard clot-based assays(Brummel-Ziedins et al., 2005). Chromogenic assays are the main methods of measuring thrombin generation kinetics (Peetz, 2016) with the calibrated automated thrombography (CAT) being a fluorogenic enhancement that may make the leap from bench-side to routine clinical practice.

Table 1.2 Tests of Coagulation function.

Types of functional tests to assess haemostasis. Modified from Heiserman, 2015 (Heiserman, 2015)

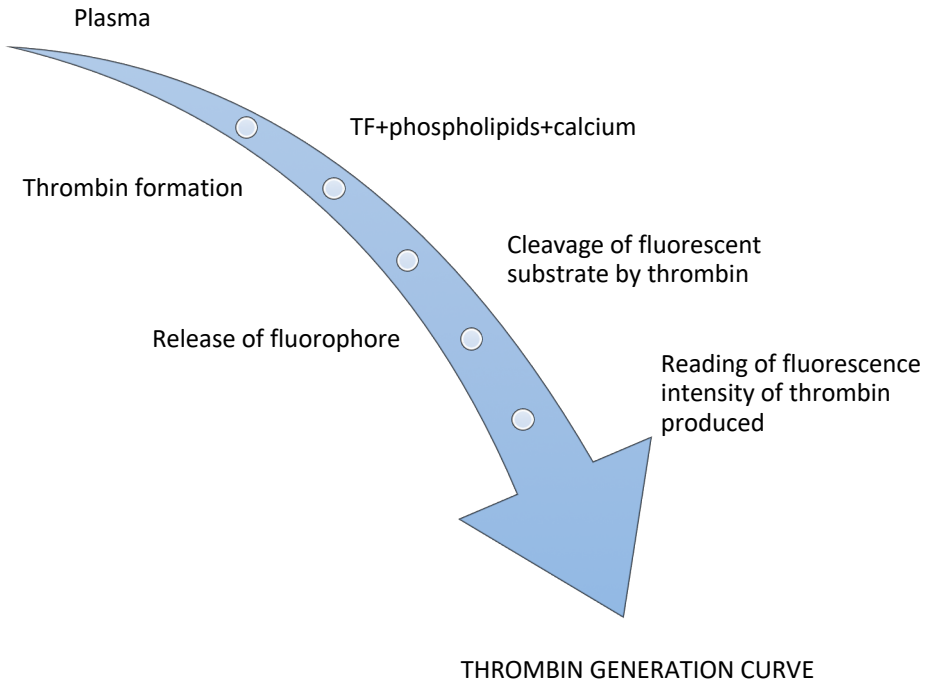
Coagulation function	Test
Vascular function	Bleeding time, tourniquet test (capillary fragility, cuff test)

Platelet function	Bleeding time, clot retraction, platelet count
Clotting ability	Prothrombin time, activated partial thromboplastin time, thrombin time
Fibrinolytic function	Clot retraction, fibrinolysin assay, D-dimer assay

The CAT assay is a global assay that can be used to evaluate and quantify the haemostatic qualities of a biological substrate(Hemker et al., 2006). By giving a dynamic picture of TG through the measurement of the amount of thrombin cleaved from a fluorogenic substrate in real time as shown in Figure 1.7A, CAT may become a useful tool in cancer related clinical scenarios. Although it has been in existence in various forms since the 1950's, it was optimized by Hemker et al (Hemker et al., 2003) into a dynamic modern version that measures five main parameters in TG as it occurs during the phases of coagulation. These parameters eventually displayed as a thrombogram curve are the Lag time, Endogenous thrombin potential (ETP), Peak thrombin produced (Peak), time-to-peak (ttPeak or TTP), and Velocity index (Vel.Index). According to Hemker et al, the Lag time in minutes refers to time thrombin formation commences, and it corresponds to the initiation and amplification stages of the coagulation cascade as illustrated in figure 1.7B (Hemker et al., 2003). The ETP is the area under the thrombogram measured in nanomolar multiplied by minutes while the Peak is the maximum nanomolar thrombin produced in the time of the assay during the propagation phase of the cascade. TTP is the duration it takes to reach maximum thrombin production in minutes. There are two other lesser parameters that can be reported; the Vel.Index which is the slope between the lag time and TTP (measured in nanomolar per minute) and the Start tail in minutes which is the time TG comes to an end (or the termination of the inhibition phase), although this value is less absolute as it can be difficult to determine when TG definitely ends(Hemker et al., 2003). With this number of endpoints, the CAT assay gives a more dynamic clotting assessment and could play a role in clinical practice to

assess clotting irregularities and abnormalities in patients with cancer related clotting disorders. However, more studies are needed to understand and address official optimization and standardization issues for the assay technique.

A)



THROMBIN GENERATION CURVE

B)

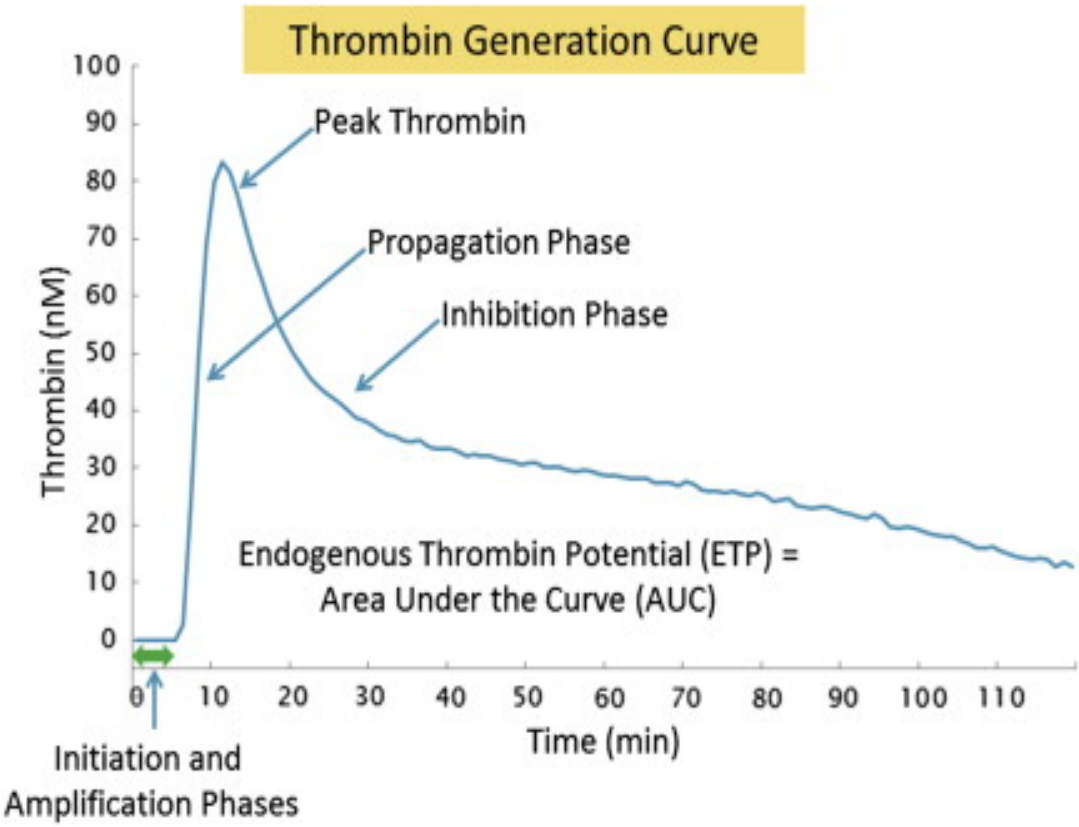


Figure 1.7 Measurement of thrombin generation in the CAT assay

A.) Basic process of the CAT assay B.) TG curve during the 4 phases of coagulation. Adapted from Centers for Medicare &Medicaid Services (CMS), Elsevier Health, (http://www.journals.elsevierhealth.com/cms/attachment/2009083251/2031129491/gr13). Accessed November 17, 2017

1.5 Tissue Factor (TF)

1.5.1 TF structure

TF (also known as factor III or formerly, thromboplastin) is a 47 kDa membrane-bound protein, that can be found in most cells of the body especially those surrounding blood vessels and is constitutively expressed in normal haemostasis(Bach, 1988; Mackman, 2009). It is a glycoprotein receptor (referred to as the cluster of differentiation CD 142) comprised of a single chain of 295 amino acids. In 1987, Morrissey et al synthesized human TF from cDNA copies as a molecular weight

precursor of 29,593 with a leader sequence of 32 amino acids, while the mature protein is a single polypeptide chain composed of 263 amino acid residues in total (Morrissey et al., 1987; Spicer et al., 1987; Morrissey et al., 1988a; Morrissey et al., 1988b). Hybridization techniques have localized TF gene to chromosome 1(Carson et al., 1985). It has 3 main domains which has specific purposes- an hydrophilic extracellular consisting of 219 residues, a C-terminal intracellular region of 21 residues and an hydrophobic transmembrane that contains 23 residues(Spicer et al., 1987). The extracellular domain contains three N-linked carbohydrate chains; one of which is an N-terminal residue that in turn consists of two fibronectin domains. These fibronectin domains have the ability to bind to FVII and VIIa (a serine protease glycoprotein)(Banner et al., 1996), and thereby exponentially increase the catalytic efficiency of TF:FVII complex(Huang et al., 2006), which triggers the coagulation cascade(Rapaport & Rao, 1995).

TF is a key initiator of the extrinsic coagulation pathways in the body and also contributes significantly to other pathways of coagulation(Steffel et al., 2006; Aird, 2012). Clotting mechanisms involving the endothelium may be heavily dependent on TF as well as phospholipids such as phosphatidylserine (PS) carried on cellular membranes (Furie & Furie, 2005; Steffel et al., 2006). TF occurs in the body in mostly three forms; the intracellular TF, the TF attached to the cell surface and the soluble form. Surface TF is normally encrypted. Decryption is known to change TF into a coagulant TF capable of inducing signalling pathways and several mechanisms have been proposed to cause this phenomenon. Some authors have suggested different mechanisms including PS external exposure to create an ideal negatively charged surface(Wolberg et al., 1999; Kirszberg et al., 2009), oxidation of the cysteine residues on TF structure(Ahamed et al., 2006), regulation by cell membrane itself through lipid rafts localization(Sevinsky et al., 1996; Awasthi et al., 2007), and monomerization as opposed to TF disulphide isomerization (dimerization) (Bach & Moldow, 1997). Notwithstanding the veracity of these postulated mechanisms, none of them have been widely agreed on as the main cause of TF decryption. However, Ettelaie et al have found that the dimerization of TF protein *in vivo*, elicited a sustained response and direct activation of various intracellular signalling pathways

such as p42/44 MAPK and JNK-SAPK, due to a possible feedback mechanism from phosphorylation of the transmembrane cytoplasmic domain of TF(Ettelaie et al., 2007).

Soluble and surface TF are also present as isoforms which may be spliced, and which have implications for tumour progression and coagulant function. The normally spliced full-length TF (fTF) and the alternatively spliced isoforms (asTF) can be found in different states in the body. Initially identified by Bogdanov in 2003, this spliced variant asTF is encoded by 5 exons unlike the fTF of 6 exons transcript(Bogdanov et al., 2003). asTF in various cancers cell lines have been linked to thrombus development as it has been identified in high quantities inside clots collected from humans, and also its ability to co-localize with platelet aggregates(Bogdanov et al., 2003). These findings have allowed suggestions that asTF may be involved in thrombus development and have specific porocagulant functions(Kocaturk & Versteeg, 2012). However further studies *in vivo* are necessary to understand its implicit clinical roles as compared to fTF in heamostasis, angiogenesis and disease pathogenesis.

1.5.2 TF-FVII complex

Like other Vitamin-K dependent factors, FVII is a single polypeptide chain - also known as stable factor or preconvertin- that consists of 406 amino acids including ten which are Gla residues(Hagen et al., 1986). Conversion to active FVIIa is accomplished by cleavage of an arginine-isoleucine bond which gives a 2 chains- a light chain containing the Gla region and a heavy chain that contains the active site(Hagen et al., 1986). Inactivation also occurs through a slower cleavage of the arginine-glycine bond(Radcliffe & Nemerson, 1976).

Normally, the endothelium performs the role of physically separating the two main initiators of coagulation apart; TF expressing cells located in the subendothelium from the circulation thereby keeping TF from activation of blood borne zymogen FVII and VIIa. However, in endothelial disruptions or conditions that promote

overexpression of TF, this balance may be thwarted resulting in excessive, indiscriminate or inappropriate coagulation. Damage to the endothelial lining causes TF-producing cells to be in contact and bind with the FVII in the blood circulation (Figure 1.9). As explained above, in the extrinsic coagulation pathway FVII is subsequently converted to FVIIa, then the TF-FVIIa complex that is formed activates FX by proteolytic cleavage (Figure 1.8). Thrombin is produced as a result, further amplified by the involvement of the intrinsic pathway, and leads to deposition of fibrin and platelet activation (Versteeg et al., 2001).

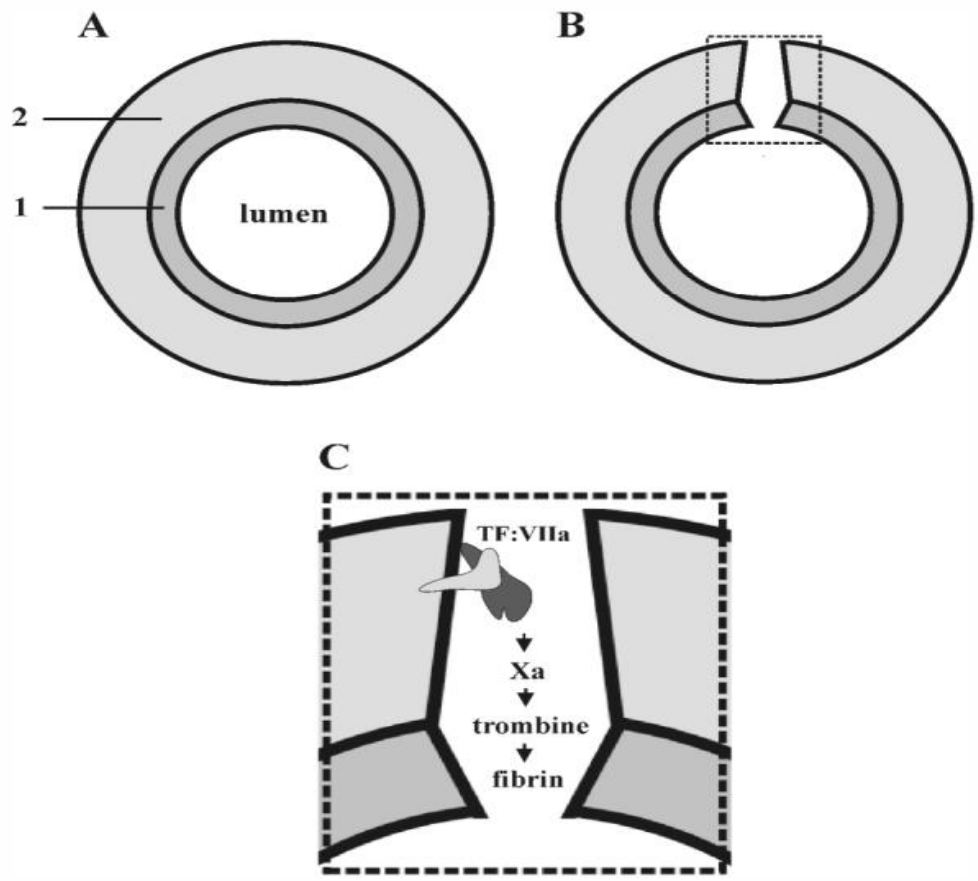


Figure 1.8 A break in the endothelial lining wall exposes extravascular TF to the bloodstream.

A) Intact vessel lumen surrounded by 1-endothelium and 2-subendothelium B) Vessel damage C) TF-VII complex formation leading to thrombin formation and ultimately fibrin deposition. Adapted from Telomerase and Cancer: Metastasis, Epigenetic mechanisms, Teratogenesis (Part 7), (https://cf.ppt-

online.org/files/slide/p/PqmJhDTBGpQsSvHnui3O70UIzjLy9Mfb4N1Rl5/slide-54). Accessed November 18, 2017

1.5.3 TF-FVII: signalling and upregulation

TF-FVII proteolytic activity seems to occur in two ways; through PAR2-dependent signalling, although this level of PAR2 activation seems to occur at higher TF concentrations and also by PAR1 and PAR2 activation mediated by FXa at lower concentrations(Riewald & Ruf, 2001). TF-FVII complex leads to transient cystolic calcium fluxes creating intracellular gradients and initiating the classical phospholipase C (PLC)/calcium pathway and phosphoinositide hydrolysis(Camerer et al., 2000). This activity highlights TF acts as a direct receptor for signalling through its cytoplasmic tail (Cunningham et al., 1999). Once TF is attached to FVII, it is coupled to a PAR receptor and this TF-FVII-PAR2 complex induce proangiogenic activities and inflammatory cytokines and growth factors (Ruf et al., 2011). It is now established that TF-FVII mediates gene modification and protein production through the activation of mitogen-activated protein kinases (MAPK) that are well known for gene transcription and translation from protein phosphorylation. There are six major genetic isoforms of MAPK including p42/p44 MAP kinase, p38 MAP kinase and stress-activated c-Jun N-terminal kinase, all with different roles in stress, inflammation and signalling (Hommes et al., 2003; Dhillon et al., 2007). Of these, malignant transformation are frequently associated with the Ras and B-raf isoforms(Dhillon et al., 2007). These isoforms induce transcription of growth factors such as vascular endothelial growth factor (VEGF), and encoding cytokines such as interleukin (IL)-1β, IL-8, macrophage inflammatory protein 2-alpha (MIP2α), and leukaemia inhibitory factor (LIF), and genes regulating cell organization and motility in metastasis (Camerer et al., 2000; Pendurthi et al., 2000). Nuclear factor-κappa B (NFkB) is another transcription molecule that is involved in downstream TF signalling(Mackman, 1995), as shown by the reduction in NFkB activity possibly through nuclear translocation that was associated with TF mRNA downregulation, in the presence of LWMH(Ettelaie et al., 2011).

TF via PAR-2 intracellular signalling also has a role outside coagulation in tumour growth, angiogenesis and embryogenesis. In cancer development, TF-FVII-PAR2 complex have been associated with recruitment of growth factors, chemokines and inflammatory cytokines to the tumour microenvironment, which are implicated in its expansion and metastasis(Ruf et al., 2011). This advancement is also known to be suppressed in the inhibition of the TF complex, which emphasizes its integral role in tumorigenesis. *In vitro*, studies of PAR-2 deficient breast cancer cells have described TF-PAR2 signalling as inducing the release of the chemokine CXCL1, which is a implicated in aggressive tumour phenotypes(Schaffner et al., 2010). In clinical cancer specimens of relapsed patients, TF and PAR2 activities have been found to be upregulated with a marked phosphorylation of TF cytoplasmic domains(Rydén et al., 2010). In glioblastoma cells, pronounced TF-PAR2 expression was observed and further antibody-inhibition experiments resulted in significantly reduced cell proliferation and spread (Gessler et al., 2010). In addition, upregulation in TF-complex activity may result in other pathologic conditions of clinical importance. For example, aberrant TF was recently identified as overexpressed in ectopic endometrial tissues in patients suffering from endometriosis(Krikun et al., 2008). This may highlight the non-haemostatic roles of TF in disease, with known associations with inflammatory cytokines such as IL-1B (Krikun et al., 2008; Krikun, 2012) and embryogenesis (Carmeliet et al., 1996).

1.5.4 TF involvement in Cancer VTE

Since 1865, cancer has been associated with thrombosis since Armand Trousseau studies described a link between them (Boccaccio & Comoglio, 2009). Most solid cancer types such as PC are known to express TF(Kasthuri et al., 2009; Welsh et al., 2012), while others including haematological cancers such as MM generally have absent or low levels as shown by Ceserman-Maus et al in a study where 55 human myeloma lines expressed no TF, unlike 90% of solid tumour lines(Cesarman-Maus et al., 2012b). Some PC may have high TF expression that could indicate poor prognosis as a result of overall tumour aggressiveness(Kakkar et al., 1995). For instance, high

TF expression which accounted for 44.2% (n=50) of a total of 113 Pancreatic ductal adenocarcinoma (PDAC) patients analysed, correlated with tumour extension (p= 0.0043), lymph node metastasis (p= 0.0043), advanced stage (p= 0.0002), and high tumor grade (p= 0.0164)(Nitori et al., 2005). In these solid malignancies such as melanoma, TF expression levels may be up to 1000-fold compared to non-metastatic cells which suggests a key role in cancer dissemination and prothrombosis(Mueller et al., 1992).

TF involvement favours a hypercoagulable state. Giesen et al, was the first to suggest that TF in the blood stream can lead to thrombus formation *ex vivo*(Giesen et al., 1999). It is now well known that tumours with highest TF expression also exhibit the highest symptomatic VTE occurrences, and this expression increases with tumour progression (White et al., 2007). Traditionally, elevated thrombotic risk is seen associated with TF in solid tumours; however, this has also been noted at similar elevated incidences in haematological cancers. In PC, for example high TF-expression has been found associated with over 25% of clinical VTE incidences by Khorana et al, 2007(Khorana et al., 2007a). A study of newly diagnosed MM before and after treatment who developed VTE have also shown increased TF expression (15.1fM) compared to those that did not (11.4fM; $p<0.001$), although this TF was associated with MVs (Auwerda et al., 2011). Cancer cells may directly activate the coagulation system themselves, notably by promoting TF expression (the primary initiator of the coagulation cascade) on the cell surface of tumour and stromal cells that can release circulating TF (Callander et al., 1992; Contrino et al., 1996; Shoji et al., 1998). Also, the potential secretion by malignant tissues of proteolytic cancer procoagulant (CP) a 68 kDa vitamin K-dependent neoplastic cysteine proteinase enzyme which activates FX (Falanga & Gordon, 1985), and stimulates platelet activation in a fashion similar to thrombin, may have further hypercoagulable effect.

TF involvement is observed widely in inflammation and angiogenesis. In angiogenic tumour-associated endothelium for instance, increased TF expression has been observed, while elevated TF antigen production levels have been correlated with VEGF expression (Nakasaki et al., 2002; Khorana et al., 2007a). Elevated TF activity

can lead to high vascular density, enhanced angiogenesis and metastatic progression in a variety of human tumours including PC, breast and colorectal cancer (Contrino et al., 1996; Nakasaki et al., 2002; Khorana et al., 2007a; Echrish et al., 2014). Metastatic potentiation from TF-dependent activation of coagulation, may occur though PAR-2 signalling (Kocaturk & Versteeg, 2012). Furthermore, as a procoagulant protein, TF elevation in association with MVs is found in several cancers, where an important role may be in inducing thrombosis through the coagulation pathway.

Notably, TFMVs levels have been elevated in treatment of leukaemia cells with the anthracyclines DOX and Daunorubicin(Boles et al., 2012) while Maraveyas et al. reported high TF antigen levels in PC patients under treatment with Gemcitabine (GEM)(Maraveyas et al., 2010). Interestingly, despite high levels of reported VTE incidences, TF expression are observed to be absent in some MM cell lines and MM patient samples from treatment groups(Cesarman-Maus et al., 2012b). VTE development in MM may therefore involve TF-independent mechanisms. These findings indicate a likely possibility that there may be different factors underlying chemotherapy-associated VTE in MM and PC, despite similarly elevated VTE risks.

1.6 Microvesicles (MVs)

1.6.1 MV Origins

MVs are small vesicular cell fragments enclosed by a phospholipid bilayer shed from activated and non-activated cells, which are involved in cellular interactions with the environment (Simak & Gelderman, 2006; Puddu et al., 2010). Also called microparticles(MPs), MVs were first reported in literature in relation to promotion of blood coagulation and were described as ‘precipitable factors’ in ultra-centrifuged plasma by Chargaff and West in 1946(Chargaff & West, 1946), and later as particulate ‘dust’ formed from activated platelets in the clotting cascade by Wolf(Wolf, 1967). The term MVs is sometimes used as a general term to include other membrane-encapsulated particles and cell products such as exosomes, apoptotic bodies,

ectosomes, phospholipid vesicles. Moreover, all these terms are used interchangeably now as composition differences that include size, morphology, protein content and buoyant density seem insufficient for a clear distinction and rigorous terminology is yet to be established (Bobrie et al., 2011).

1.6.2 MV structure and function

MVs generally vary in size from 30nm to 1µm, with the largest around 3µm (Conde-Vancells et al., 2008; Rak, 2010; van der Pol et al., 2012; van der Pol et al., 2013). They have heterogeneous compositions and may consist of anionic phospholipids, cytoplasmic inclusions, and bioactive proteins and coagulation factors. They are also known to carry genetic substance such as RNA peptides, microRNA, mRNA, and DNA between various cells without direct cell-to-cell contact(Barteneva et al., 2013) and through these transfer, have been involved in events of physiological importance such as apoptosis, inflammation, angiogenesis, and haemostasis(Morel et al., 2005; Aharon et al., 2008). Molecularly, they have specialized roles that help in intercellular signalling, cell-to-cell communication and so can be involved in disease spread, sepsis dissemination, immune surveillance and cancer metastasis.

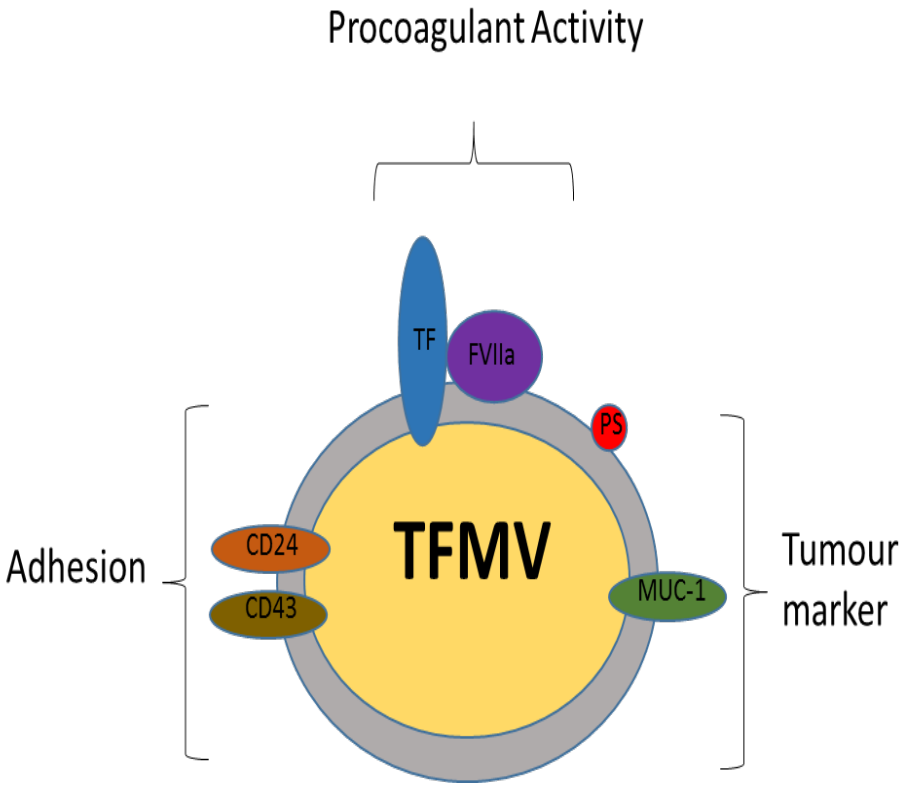


Figure 1.9 Illustration of tumour MVs and their surface proteins.

Abbreviations: TF is Tissue Factor, FVIIa is activated coagulation Factor VII, PS is Phosphatidylserine, MUC-1 is Mucin-1, CD is Cluster of Differentiation. Modified from Geddings and Mackman, 2013(Geddings & Mackman, 2013).

The structure of the MVs is a phospholipid bi-layer, anucleated and surrounding cytoplasmic constituents (Figure 1.9). The outer layer consists of negatively charged lipids including phospahatidylserine (PS) (Lechner & Weltermann, 2008) which are imbedded with distinct antigens (Figure 1.9). These antigens may reflect the source of origin of the MVs(Tilley et al., 2008) and can serve as mode of identifying and targeting the MVs, for example endothelial derived MVs are known to carry CD31 or CD146 antigen(Jimenez et al., 2003). However, it is entirely possible for cellular antigens from a specific cell type to be released and attach to MVs from a different source, or even that MVs released from a specific cell type, carrying a specific antigen, may attach to another cell type, with the aim of inducing production of MVs with 'foreign' embedded antigen. In this way, MVs from different parts of the body can mediate interactions such as metastasis and senescence (Lehmann et al., 2008). MVs

from similar or different sources might also fuse into bigger structures and share genetic material or biological parameters (Gyorgy et al., 2011a). This process may further compromise detection by obfuscating the primary antigen composition and cause difficulties in immunophenotypical identification.

The characterization of MVs has revealed specialized functions due to cell-type specific proteins from isolates of different biological samples (Conde-Vancells et al., 2008). The production of MVs is dependent on their cellular source, type of stimulus or stress, and pathologic state of parent cell (Freyssinet, 2003; Guervilly et al., 2011; Nomura & Shimizu, 2015). Previous studies have implicated MVs of different sources with the transfer and sharing of oncogenic material such as epidermal growth factor receptor factor VIII (EGRFvIII) in gliomas(Al-Nedawi et al., 2008), as vectors of genetic information (Al Dieri et al., 2002), rapid proliferation and cellular differentiation and even inducing apoptotic and procoagulant effects (Aharon et al., 2008; Wang et al., 2012). MVs shed from some tumours such as breast cancer, also contain matrix metalloproteinases and proteases that help to break down the endothelial cell matrix (ECM) and facilitate tumour invasion (Dolo et al., 1998). Moreover, in cancer coagulopathy, oncogenic pathways may be stimulated to produce MVs incorporated with surface TF antigen(Rak, 2010).

1.6.3 MV intracellular formation, release, acquisition and signalling mechanisms

The molecular pathways involved in vesicular formation seems to be poorly understood. MVs formation has been proposed to occur from 3 ways; through generation as extracellular blebbing or reverse budding and subsequent fission from cell membrane surfaces (Zwaal & Schroit, 1997), as whole exosomes extruded through intracellular endocystic development also known as multivesicular endosomal fusion with plasma membrane(Harding et al., 1984), through apoptosis as part of the breakdown products of the parent cell itself(Distler et al., 2005) figure 1.10).

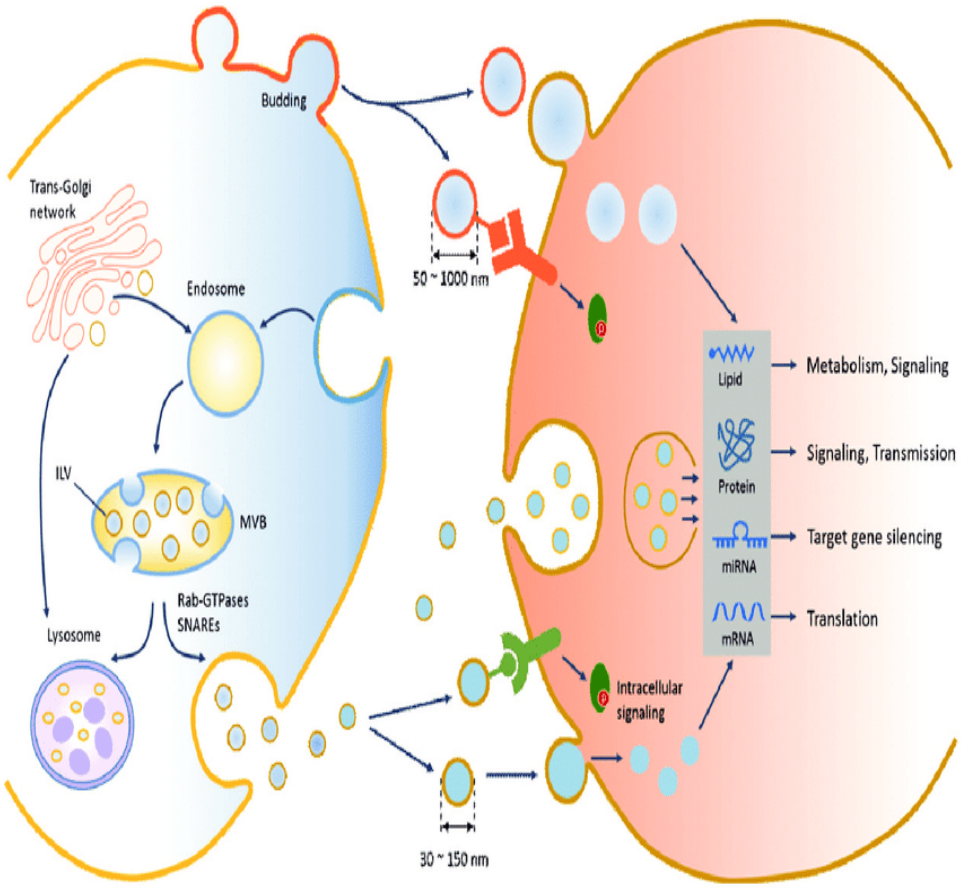


Figure 1.10 Uptake and release of small and large MVs from parent cell to neighbouring or distant cells.

Abbreviations: ILV is intraluminal vesicle; MVB is multivesicular body and SNAREs are soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) system, which are involved in the transport and fusion of MVBs with the plasma membrane (MV release). Adapted from Choi et al, 2017(Choi et al., 2017).

Because MVs are involved in different biological processes such as coagulation, various signalling pathways in the cellular microenvironment influence genetic regulation of vesiculation. In tumour development, oncogenes such as KRAS, tumour suppressor p53 are known to induce MV formation. In prostate cancer, MV formation is due to overexpression the Akt pathway activation and EGFR stimulation (Di Vizio et al., 2009; Rak, 2010). Also, Arf-6, an intracellular GTbinding protein of the ARF family, is known to promote MV shedding in various cancers such as breast cancer, gliomas and melanomas, leading to tumour cell invasion(Muralidharan-Chari et al., 2009).

This GTPase protein activates phospholipase D to stimulate recruitment of the extracellular signal-regulated kinase (ERK) to the plasma membrane(Muralidharan-Chari et al., 2009). Subsequently, ERK phosphorylates myosin light-chain kinase (MLCK) activating it, for MV cellular release. Conversely, upon inhibition of Arf-6 activation by expression of a dominant-negative Arf-6 mutant, or siRNA depletion, PKC-mediated phosphorylation of MLC occurs, which in turn blocks MV shedding(Muralidharan-Chari et al., 2009). The resultant effects of these MV-dedicated pathways in the parent cell includes outcomes such as calcium fluxes, cortical actin reorganization, altered lipid metabolism, which leads to vesicle formation(Piccin et al., 2007). Furthermore, it has been suggested that not only do oncogenes direct vesiculation, interestingly enough; they also form part of the material transferred by MVs(Al-Nedawi et al., 2008). These finding implies that MVs may function as instruments of lateral transfer between cells.

MV release:

Once vesiculation is initiated, assembly and extrusion out of the cell follows (Figure 1.10). In order to do this, lipid rafts have been postulated to be involved in MV membrane blebbing; cholesterol-rich lipid rafts are found in area of cytoplasm where MVs arise, which are also areas where factors such as TF and P-selectin glycoprotein 1 (PSGL) converge, leading to the possibility that MVs arise from such areas(Del Conde et al., 2005). Various theories have been proposed to explain how the MVs bleb from the parent membranes. One theory suggests that blebbing and release is similar to the amoeboid movement of cell motility (Paluch et al., 2006; Gadea et al., 2008), where vesicular blebs arise from zones of actomyosin contractions near the cortical cytoskeleton of the plasma membrane (El-Sibai et al., 2008; Di Vizio et al., 2009). At these front zones, swiftly forming membrane protrusions can easily arise from the hydrostatic pressure force generated by actin cross-linked proteins such as filamin and myosin which controls bleb retraction(Charras et al., 2008). Another theory implicates soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) system, in MV release through the transport and fusion of multivesicular bodies (MVBs) which are large bodies containing several intraluminal vesicles (ILV) with the plasma membrane (Figure 1.10)(Choi et al., 2017). In cancerous

cells the GTPase/ROCK signalling system, known to be regulators of cell shape, mediates bleb-associated motility (El-Sibai et al., 2008) although this process in further malignant progression is less understood.

MVs release may be physiological or pathological. In physiologic conditions it is known that the release follows a circadian rhythm (Madden et al., 2008), and levels are higher in females and affected by the menstrual cycle(Toth et al., 2007). In healthy individuals, MVs majority come from platelets and endothelium, which accounts for about 43.8% and 38.5% respectively of its sources (Berckmans et al., 2001; Shah et al., 2008), whereas this composition changes in pathologic conditions. In these conditions, MVs formed accumulate at the site of injury to contribute to thrombus formation, facilitated by attachment to the ECM through PGSL and integrin receptors found on activated platelets and endothelium, in addition to P-selectin on monocytes, leucocytes and cancer cell-derived MVs, (Falati et al., 2003; Vandendries et al., 2007; Thomas et al., 2009). These MVs may also carry TF and its accumulation may initiate thrombin generation and eventually thrombus formation.

MV uptake:

Collier et al, have reported a mechanism of MV acquisition that may result in increased procoagulant potential of the endothelium, through the uptake and recycling of MVs (specifically TFMVs), and PS cell membrane exposure (Collier et al., 2013). This mechanism is an internal cellular process that occurs either by engulfment of TFMVs with subsequent intracellular degradation, TF membrane incorporation and later re-exposure by MV blebbing on the endothelial cell surface (Aharon et al., 2008; Collier et al., 2013) or by initiation of an intrinsic endothelial TF production due to stimuli from external MVs. The isolation of endothelial MVs in certain cancers such as MM have also been documented(Hall et al., 2016), and MVs produced from other sources such as monocytes have been shown to be ingested by endothelial cells to induce increased TF activity and a procoagulant nature(Aharon et al., 2008).

To aid uptake, some tumour MVs are known to express TF and more recently MuC-1 as a tumour marker (Dvorak et al., 1981; Tesselaar et al., 2007) as illustrated in figure

1.9. In the thrombus, uptake is also facilitated by the PGSL on the MVs surface, which interacts with the P-selectin on the surface of activated platelets and endothelial cells(Falati et al., 2003). In fact, elevated P-selectin expression has been identified in the endothelial vasculature of several malignancies such as lung and breast cancer, and are involved in cancer metastasis and mediating prothrombotic progression(Shamay et al., 2016). Targeting of the corresponding ligand (PGSL) on these cancer MVs may therefore confer a therapeutic advantage in order to prevent acceleration of thrombus formation(Thomas et al., 2009).

TFMVs:

A recent focus has been on cancer MVs associated with TF or TFMVs, which are known to be elevated in some cancers and may have a role in VTE and in some of the processes that promote metastasis (Table 1.3). Davilla et al for example showed that about 50% of MVs generated from breast and PC, expressed TF and generated procoagulant activity when injected into animal models, an effect removed upon addition of anti-TF antibody (Davila et al., 2008). This suggests that MVs can serve as soluble sources of TF and may mediate coagulation processes such as conversion of coagulation factor VII to VIIa which is essential for the extrinsic pathway. Furthermore, the majority of TF circulating in the blood are carried or are associated with MVs (Tilley et al., 2008), which suggests that MV detection may represent an important research tool, especially in cancer thrombosis.

Table 1.3 The clinical studies of TFMVs in cancer VTE.

Modified from Geddings and Mackman, 2013(Geddings & Mackman, 2013)

Disease	Method of measurement	Patient numbers	Conclusion	Reference
Pancreatic and Breast Cancer and VTE	TFMV activity assay	40	TFMV activity is increased in metastatic breast cancer and PC patients	Tesselaar et al, 2007(Tesselaar et al., 2007)
Pancreatic cancer and VTE	TFMV activity assay	10	Increased TFMV activity precedes VTE in PC patients	Khorana et al, 2008(Khorana et al., 2008a)
Various cancers: bone, lung, colorectal, oesophageal and VTE	TFMV activity assay	100	Cancer patients with VTE have higher levels of TFMV activity than those without VTE	Tesselaar et al, 2009(Tesselaar et al., 2009)
Various cancers: bladder, prostate, lymphoma, renal, testis	Functional chromogenic assay	66	Cancer patients with VTE have higher levels of TFMV activity than those without VTE	Manly et al, 2010(Manly et al., 2010)

Pancreatic cancer and VTE	Flow cytometry	60	Cancer patients with VTE show higher TFMV levels	Campello et al, 2011(Campello et al., 2011)
Myeloma and VTE	TFMV activity assay	122	TFMV activity is increased in MM patients and decreased after chemotherapy	Auwerda et al, 2011(Auwerda et al., 2011)
Pancreatic cancer and VTE	TFMV activity assay	348	TFMV activity is associated with mortality in PC patients	Thaler et al, 2012(Thaler et al., 2012)
Glioblastoma and VTE	Flow cytometry	61	TFMV levels are elevated in Glioblastoma multiforme before and during treatment	Sartori et al, 2013(Sartori et al., 2013)
Pancreatic cancer	TFMV activity assay	52	Higher levels of circulating TFMV or TF-related activity found correlated with increased angiogenesis	Echrish et al, 2014(Echrish et al., 2014)

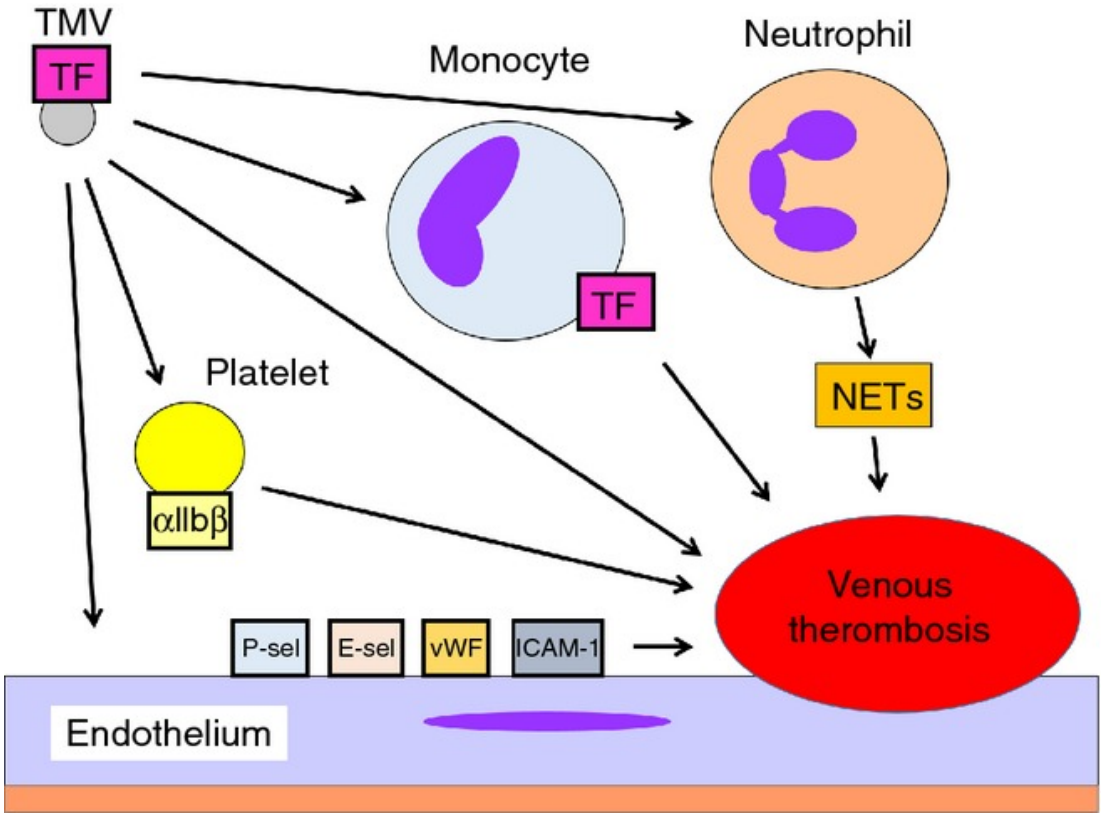


Figure 1.11 TFMVs contribute to cancer VTE.

Tumour-derived microvesicles (TMVs) may produce TFMVs for direct or indirect enhancement of VTE, through binding and activation of endothelium to activate and release adhesion molecules such as selectins, VCAM-1 (Vascular cell adhesion protein 1), binding and activation of platelets, activation of neutrophils to secrete neutrophil extracellular traps (NETs), binding of monocytes to induce more TF. Adapted from Hisada et al, 2015(Hisada et al., 2015).

Tumour cells *in vivo* and *in vitro* can release membrane TFMVs (Figure 1.11) and are known to be significantly raised in cancer compared with non-cancer patients (Davila et al., 2008; Auwerda et al., 2011; Campello et al., 2011). In some clinical studies, elevated levels of TFMVs derived from tumour have been associated with VTE in several cancers such as PC (Zwicker et al., 2009; Geddings & Mackman, 2013). They have also been associated with an aggressive metastatic process where interaction with endothelium is a key element for VTE initiation and propagation, and for the invasion of a cancer cell into stroma. TFMVs have been shown to support coagulation

in clotting and thrombin generation tests *in vitro* (Bastida et al., 1984; Davila et al., 2008) and promote the metastatic process through angiogenesis, cancer cell survival, immune suppression, and invasion (Zahra et al., 2011). For all these processes, the ability to interact with the endothelium is a key element. However, more details from future studies are needed about their interactions with the vascular endothelium, or the procoagulant changes seen because of this interaction.

Accurate TFMVs measurement is a point of challenge in MV studies(Tatsumi et al., 2014). There are two main methods to measure TFMVs; one through its functional activity in a given substrate and the other by measurement of its TF expression (Geddings & Mackman, 2013). Various tests exist to assay both endpoints; quantitative levels of TFMVs can be measured by flow cytometry and TF antigen by Enzyme-linked immunosorbent assays (ELISA) while TFMV activity can be measured by functional assays such as Zymuphen MP-activity kits, clotting-based tests, chromogenic assays, and thrombin or fibrin generation assays. Inhibitory assays of TF activity are also useful.

It is notable that even in the same patient sample; both main methods of TFMV measurement do not always correlate with themselves. In a prospective study of pancreatic patients, TF activity was assessed by a fibrin generation test that correlated with VTE development in cancer, but however not to the TFMV expression measured by flow cytometry test (van Doormaal et al., 2012). In another study, although there was a correlation of TFMV with cancer thrombosis risk, there was no correlation with a TF activity assay in the same phase II randomized controlled trial (Zwicker et al., 2013; Tatsumi et al., 2014). Furthermore, the variability in substrates used for measurement creates another level of difficulty for comparison across studies. Although plasma and serum are often used, the type of processing of this substrate may affect the results. For instance, a study found up to 80% lower levels of TFMVs in Platelet free plasma (PFP) than in Platelet-poor-plasma (PPP), probably due to the additional centrifugation steps involved in obtaining PFP, which removes more of the MVs(Lechner & Weltermann, 2008). Therefore, to improve standardization and reduce analytical variables, the Scientific Collaborative

Workshop of the International Society of Thrombosis and Haemostasis (ISTH), have recommended specific consensus protocols in isolation and measurement of MVs such as collection of blood samples in citrate containing tubes, specific centrifugation steps for sample processing, storage and flow cytometry bead detection steps(Gyorgy et al., 2011b).

1.7 Endothelium and TFMVs in Cancer VTE

The endothelium lines the lumen of blood vessels and is a vascular structure that plays a vital role in initiation of coagulation, amongst other functions in the body including angiogenesis, cell growth and adhesion, fibrinolysis, tissue remodelling. It also regulates immune responses (Figure 1.12) through anti-inflammatory mediators and vascular homeostasis (blood pressure) by controlling the smooth muscle tone of blood vessels through secretion of various vasomotor mediators. Normally, the endothelium provides a haemostatic balance between procoagulant and anticoagulant mechanisms, through active prevention of clot formation. It performs this function by production of anticoagulants Tissue Factor Pathway Inhibitor (TFPI), thrombomodulin, endothelial protein C receptor (EPCR), release of platelet inhibitors (including nitric oxide and prostacyclin), and inhibition of cellular attachments necessary for clotting(Watson, 2009; Esmon & Esmon, 2011) (Watson, 2009, Esmon and Esmon, 2011). In bleeding conditions, the endothelium performs a prothrombotic function by releasing TF into the extravascular space to initiate pathways consisting of several coagulation factors that lead to fibrin plug formation to halt bleeding. In hypercoagulable states, this regulatory balance is disrupted, and studies have indicated that the endothelium underlying vascular structures may be altered (Chirinos et al., 2005), as described previously in section 1.2.1. According to Date et al, it is possible that prothrombotic states in cancer may result from homeostatic imbalance in the endothelium with specific interactions of TFMVs or the acquisition of deficiencies of other coagulation factors(Date et al., 2017). A transformed procoagulant endothelium may not be clinically apparent at first but may eventually result in VTE manifestations such as deep venous thrombosis, which

have been established as a cause of high clinical morbidity and mortality in cancer patients (Blom et al., 2005; Blom et al., 2006).

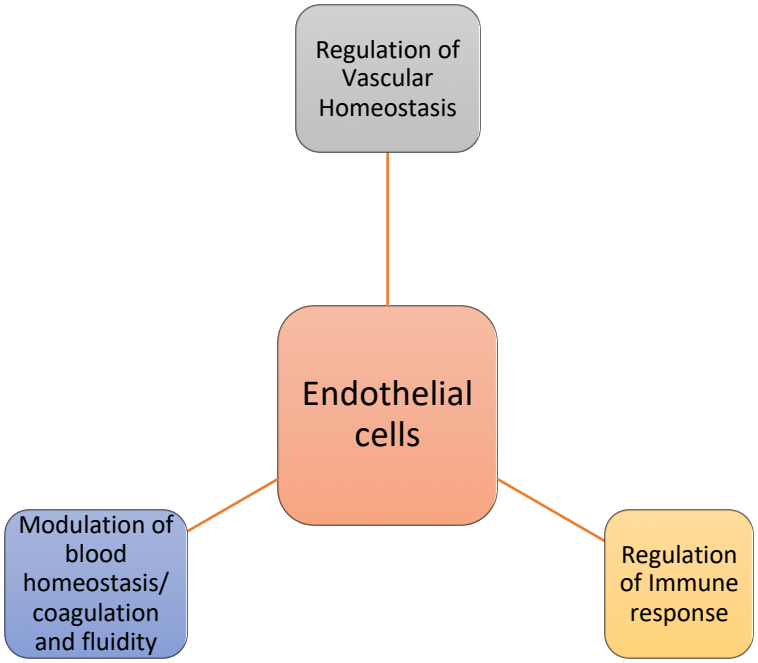


Figure 1.12 Main functions of the vascular endothelium.

The critical functions of the endothelium include regulation of vascular homeostasis, modulating blood coagulation and fluidity, and regulation of immune responses. Adapted from Speciale et al, 2014(Speciale et al., 2014).

The endothelium is important in regulating thrombin production. Specifically, it has been found that endothelial cells that over-express EPCR or are exposed to activated protein C (aPC in Figure 1.13) aid in limiting tumour invasion and metastasis. This aPC/EPCR pathway causes the reduction of the amount of endothelial produced adhesion molecules necessary for tumour growth and transmigration (Bezuhly et al., 2009). Endogenous aPC produced by endothelial cells has a protective function, through use of monoclonal antibodies against aPC such as MPC1609 and MAPC1591, which blocks its signalling and further anticoagulant activity (Van Sluis et al., 2009). On the other hand, metastasis is promoted by blocking or reduction of the production of endogenous protein C which alters the endothelial cell barrier function through sphingosine-1-phosphate receptor-1 (S(1)P(1))activation(Van Sluis et al., 2009). In all,

these anti-coagulant properties of the endothelium occur by affecting the levels of thrombin produced and have a major impact in tumour growth and metastasis success.

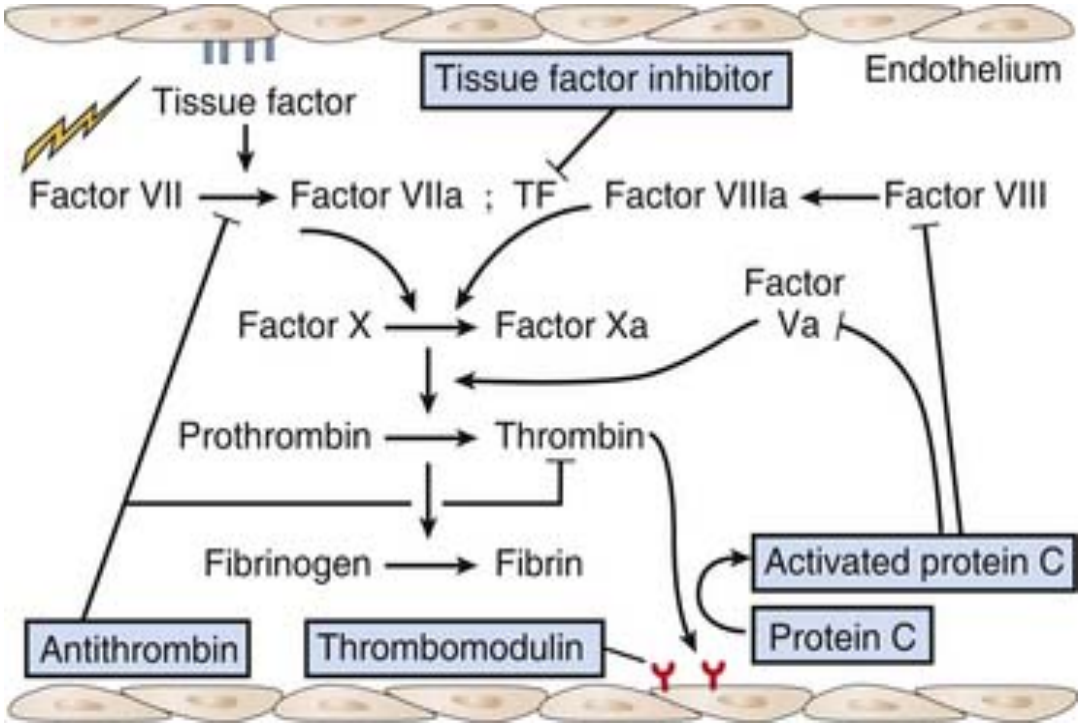


Figure 1.13 Endothelial regulation of thrombin production in the coagulation cascade.

Once coagulation is stimulated, Tissue factor (TF) interacts with factor VII which generates activated factor VII (FVIIa). The TF-FVIIa complex then conversts factor X to factor Xa whose interaction with Va results in prothrombin conversion to thrombin, and fibrinogen conversion to fibrin. There are three key regulators of this process- Protein C is activated to aPC through its interaction with cell-surface thrombomodulin, to inhibit factors Va and VIIIa activities. Antithrombin inhibits the activation of multiple factors such as factor X and thrombin. Tissue factor pathway inhibitor (TFPI) interferes directly with the TF-FVIIa complex. Adapted from Ouellette and Hazelzet, 2016(Ouellette & Hazelzet, 2016).

Numerous studies have detailed the importance of TF and TFMVs in driving coagulation pathways in the tumour endothelium over time. Such studies have demonstrated several correlations of high TF expression and activity levels with

angiogenesis, increased vascular density that may further enhance metastasis in several tumours (Contrino et al., 1996; Nakasaki et al., 2002; Khorana et al., 2007a). More recently, Echrish et al 2014, corroborated links between tumour TF, circulating TFMV and increased endothelial VEGF expression with microvessel density and invasion in pancreaticobiliary adenocarcinoma patient samples (Echrish et al., 2014). A previous study in our laboratory group has also identified endothelial MVs as being elevated in MM PPP(Hall et al., 2016). However, more work is necessary to detail TF-derived MV induction of the endothelium into procoagulant states in a cancer microenvironment, and how such mechanism differs in solid tumours and non-solid ones, some of which are now known to have similarly high rates of VTE incidence. In summary, further investigations into the contributions of the endothelium to hypercoagulability are necessary in the hope that this may shed further clarity on underlying procoagulant pathways.

1.8 Multiple Myeloma (MM)

1.8.1 Description and Pathology

Multiple myeloma is one of the well-known haematological cancers as it constitutes about 12% to 15% of hematologic malignancies (Greenlee et al., 2000). It is a malignancy that forms in a type of white blood cell called plasma cells. These plasma cells arise from post-germinal B cells of the immune system, as they are the primary component of humoral immunity and secrete immunoglobulins (Ig) composed of either kappa or lamda light chains attached to two heavy chains. MM is thus a plasma cell disorder that occurs due to monoclonal proliferation of these Igs deposited into the different parts of the body, crowding out healthy cells and leading to organ dysfunction, and ultimately death if left untreated. There are various immunoglobulins chains, which may be involved and they include IgG, IgA, IgM, IgD, and IgE, based on their composition of light chains attached to heavy chains(Cook & Macdonald, 2007). These paraprotein light chains or M-proteins are over-secreted in the bone marrow by plasma cells in the form of immunoglobulin (Ig) fragments to

form various extramedullary tissues deposits. In the kidneys these light chain deposits damage the renal tubules leading to nephropathy (Kyle et al., 2002), or are secreted into the blood leading to over-viscosity or excreted into urine as Bence Jones proteins. Various conditions exacerbate paraprotein precipitations such as infection, use of chemotherapy, radiation, dehydration (Haubitz & Peest, 2006).

1.8.2 Epidemiology

Worldwide, MM is the accounts for 0.9%-2% of all cancer deaths(Zweegman et al., 2014) and is the second most common of all haematological malignancies in the developed countries(Jemal et al., 2010). Global incidence varies substantially, although overall it is estimated that about 86,000 incident cases occur annually, accounting for about 0.8% of all new cancer diagnoses, while around 63,000 reported deaths each year(Becker, 2011). In the UK, Myeloma is the 18th most common cancer, accounting for around 2% of all new cases in 2014 (Cancer Research UK; Myeloma Incidence Statistics 2014) with a male:female ratio of around 1.3:1. Like most adult cancers, median age of presentation is over 70 years old (Palumbo et al., 2011a). Although survival is improving and has quadrupled in the past 40 years, there is still an age-discrepancy in survival trends with approximately three quarters of MM cases in England aged 15-49 survive their disease for five years or more, compared with a quarter of people 80 and above (2009-2013) (Cancer Research UK; Myeloma Incidence Statistics 2014). This may be due to higher occurrence of organ dysfunction and drug toxic effects, although the role of better hospital access and referral streams in the young may be the cause of this observation (Turesson et al., 2010; Zweegman et al., 2014).

1.8.3 Risk factors

Monoclonal gammopathy of undetermined significance (MGUS) is a benign earlier form, which is now recognized to precede most MM diagnosis (Landgren et al., 2009). While the aetiology of MM is poorly understood and remains elusive, the underlying

pathology of MM is assumed to be due to a cytogenetic malfunction from numerical or chromosomal rearrangements of transcription genes and translation to over production of paraproteins(Tricot, 2002). Primary translocation of Ig heavy chain (IgH) locus at 14q32 have been identified in over 40% of all MM(Bergsagel & Kuehl, 2003), although at least 25% have no identifiable IgH rearrangements. The main IgH translocations include 11q13 (cyclin D1), 6p21 (cyclin D3), 4p16 (FGFR3 and MMSET), and 16q23 (c-maf), with increased incidence in higher stages i.e 50% in MGUS, 60-65% in intramedullary MM, 70-80% in extramedullary MM, and >90% in MM cell lines(Bergsagel & Kuehl, 2001). There have also been reports of secondary rearrangements in later disease stages such as oncogene MYC at locus 8q24, the loss or deletion of chromosome 13, deletions and/or amplifications of chromosome 1 *et cetera* (Bergsagel & Kuehl, 2001; Sawyer, 2011).

1.8.4 Staging

Diagnosis of MM is usually made by identification of a monoclonal or phenotypically aberrant plasma cell by several methods such as immunohistochemistry or flow cytometry on bone marrow sections (Rawstron et al., 2008). The Durie-salmon classification(Durie & Salmon, 1975) was often used before 1975 but has largely been superseded by the International Staging System (ISS) developed by the International Myeloma Working Group (IMWG) which classifies patients into three risk categories (Table 1.4). The ISS has been proved to be more sensitive in predicting prognosis and guiding treatment as it is based on correlation of the patient clinical features to predict the exact tumour burden from A) extent of bone lesions B) haemoglobin level C) serum calcium level and D) M-component levels in serum and urine(Greipp et al., 2005).

Table 1.4 International Staging System (ISS) for Multiple myeloma.

Modified from Greipp et al, 2005(Greipp et al., 2005)

Stage	Criteria	Median survival in months (months)
I	Serum ß2 microglobulin <3·5 mg/l (296 nmol/l) and serum albumin ≥3·5 g/dl (35 g/l or 532 µmol/l)	62
II	Neither I or III*	45
III	Serum ß2 microglobulin ≥5·5 mg/l (465 nmol/l)	29

1.8.5 Clinical presentation and Treatment

MM often presents with bone diseases with symptoms such as frequent back pain, bone fractures and may involve focal or diffuse osteolytic lesions and spinal cord compression. The low blood cell counts in MM patients specifically of red blood cells which can lead to anaemia, and low white cell counts can predispose these patients to frequent infections from immunodeficiency while low platelets can result in easy bruising (Mehta & Singhal, 2003). In addition, signs of hypercalcemia and nervous system involvement such as muscle weakness and numbness are all commonly found in MM presentations(Coleman, 1997). Hyperviscosity due to paraproteins profusion can also lead to symptoms of stroke, dizziness, and confusion.

As complete cure is rare, MM treatment aims include disease control, quality of life enhancement, and prolong survival. Factors that generally affect treatment outcomes include advanced age, cancer burden, level of end-organ damage and the cytogenetic abnormalities present. Chemotherapy is the mainstay of treatment. Due to potential stem cell toxicities and sublethal tumour damage and risk of myelodysplasia, Mephalan-only medications have mostly been superseded by combinations with newer agents such as imunomodulatory (IMiD) drugs, which have

significant clinical improvements(Kumar et al., 2008). Autologous stem cell transplantation in combination with high dose Mephalan is also associated with high risk of AML(Mailankody et al., 2011) and toxicity and is only now used for eligible patients based on age, performance status, and comorbidities(Rajkumar, 2011).

Recently, IMiD-based regimens are commonly regarded as standards in MM management due to improved clinical outcomes(Rajkumar, 2011). First line regimens include 3 types of agents; an alkylating agent (Melphalan or Cyclophosphamide), a corticosteroid (such as prednisolone or dexamethasone (DEX), and an IMiD agent (such as Thalidomide (Thal)(Singhal et al., 1999), Lenalidomide (LEN)(Richardson et al., 2002) or Bortezomib (BOR)(Richardson et al., 2003). More recently, newer agents such as Pomolidomide (Pom) and carfilzomib are being investigated in trials for routine clinical use and all these regimens aim to drive patients into a refractory stage (Rajkumar, 2011). There are three stages of MM therapy commonly seen in most patients-induction, remission and relapse; with most patients ultimately relapsing at 5-year survival rates of 25% and 10-year survival of <10%(Blade et al., 1998). In the UK, the standard induction therapy for young people aged 60 or less who are relatively healthy is CTD (Cyclophosphamide, Thalidomide, Dexamethasone) or RCD (Lenalidomide, Cyclophosphamide, Dexamethasone) while those for patients above 60 or those with worsened states is an attenuated dose of CTDa, or MPT (Melphalan, Prednisolone, Thalidomide)(Bird et al., 2011).

1.9 Thrombosis in MM

MM is a haematological cancer well known for its thrombotic propensity, through the creation of a microenvironment with elevated levels of inflammatory and procoagulant cytokines some of which, such as IL-6 can initiate coagulation pathways and fibrinogen production (Amrani, 1990; Tricot, 2002). Other explanations proposed for this prothrombotic state include the acquired resistance to activated protein C receptor (APCR), which may allow indiscriminate prolonged clotting and whose abnormal presence has been suggested as the most frequent transitory baseline coagulation abnormality found in MM (Elice et al., 2006), and M-parapotein-

induced hyperviscosity impairment of fibrin biosynthesis and subsequent degradation (Gabriel et al., 1983; Robert et al., 1993; Carr et al., 1996).

Without treatment, the risk of VTE in MM is high. MM has been associated with increased risk for over four decades(Catovsky et al., 1970). In a large population-based study of 4 million military veterans in the US, of the 6192 patients identified with MM, 2.4% developed DVT(Kristinsson et al., 2008). These MM patients were also known to develop a 9.2-fold increase in DVT risk in comparison to other patients in the study, with the highest risk observed in the first-year post-diagnosis. In another population study in Sweden which included 18,627 MM patients and 70,991 matched controls, the risk of VTE was found to be 7.5-fold after 1 year of follow-up(Kristinsson et al., 2010). However, the use of chemotherapy is also associated with increased thrombosis, which thereby worsens the risk, with the highest level of thrombotic-related events seen in the first 6 months of treatment of newly diagnosed MM patients (Palumbo et al., 2006). Chemotherapy can cause VTE through several ways such as release of procoagulant molecules cancer cells, TF initiation, and damage to the endothelium, and different combinations carry variable VTE risks. In fact, several chemotherapy agents; single or combined have been associated with increased VTE. For instance MPT regimen in elderly patients showed an incidence of 17% versus 2% when Melphalan is used with Prednisolone only (Palumbo et al., 2006), while Thal with DEX showed 17% versus DEX only of 3%(Rajkumar et al., 2006). The newer IMiD drugs such as Lenalidomide (LEN) and Pomolidomide also carry substantial risk; for example, LEN used with DEX has an elevated risk of 11-14% before use of anti-thrombotics in refractory or relapsed MM (Weber et al., 2007). Another study reported a high rate of VTE incidence in newly diagnosed MM when DEX was used in combination with LEN versus when DEX was administered alone; 75% (9 out of 12) of the LEN/DEX arm experienced thromboembolic events such as ischaemic stroke, while no events were reported in the control arm of DEX alone(Zonder et al., 2006). This incidence reduced significantly to 15% upon administration of a prophylactic dose of 325mg Aspirin as thromboprophylaxis, which supports the effectiveness of routine thromboprophylaxis in MM patients as recommended by ASCO, ESMO, IMWG(Lyman et al., 2007; Palumbo et al., 2008; Mandala et al., 2011).

Various thromboprophylaxis agents have been used in MM clinical management, and include Aspirin, LMWH and warfarin. Several studies have concluded that these are all likely to be similarly effective in supportive management except in elderly patients where warfarin showed less efficacy when compared to LMWH(Palumbo et al., 2011b). Routine guidelines by the agencies above recommend a risk-stratified approach to thromboprophylaxis in IMiD-treated patients. For instance, in MM patients receiving Thal or LEN combination regimens, Aspirin (75 – 325mg) may be administered in low risk patients (without any risk factors)(Snowden et al., 2011). However MM Patients receiving Thal or LEN combinations that are high risk (with 2 or more MM-related risk factors such as hyperviscosity or high disease burden and 1 or more individual factors such as obesity or previous VTE) should receive high dose LMWH or dose adjusted warfarin(Snowden et al., 2011).

1.10 Pancreatic Cancer (PC)

1.10.1 Description and Pathology

The pancreas is a large endocrine and exocrine organ located at the upper part of the posterior wall of the abdomen. It is a retroperioneal organ covered in front by the stomach and the duodenum to the right side(Longnecker, 2014). It has three parts consisting of the head, body and tail, which can be further, subdivided into five regions- the head, neck, body, tail and ucinate process (Figure 1.14). Histologically, it consists of two main types of tissues, an endocrine component formed by the islets of Langerhans cells and an exocrine component of secretory acinar cells that produces digestive enzymes (Figure 1.15) such as lipase, amylase, carboxypeptidase, trypsin and chymotrypsin(Longnecker, 2014). All these hormones and enzymes mostly regulate food intake, breakdown and absorption in the body. They drain through the main pancreatic duct of Wirsung and sometimes through the minor papillae into the duodenum at the papilla of Vater(Bockman, 1993).

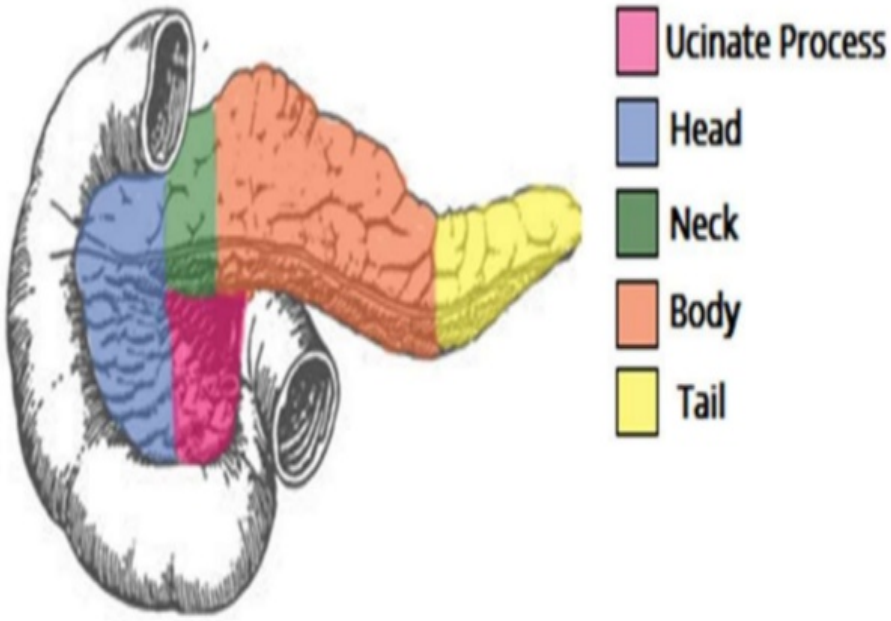


Figure 1.14 The different regions of the pancreas.

The pancreas has three main parts the head body and tail which can be divided into the five regions above. Adapted from: The pancreas, Educational healthcare resources, (http://teachmeanatomy.info/abdomen/viscera/pancreas/). Accessed August 3, 2017)

Embedded deep inside the acinar exocrine portion of pancreatic tissue, the islet of Langerhans consists of four types of cells that produce different endocrine products (Figure 1.16): alpha cells produce glucagon, beta cells comprises 65-80% of islet cells secrete insulin, delta cells produce somatostatin, and enterochromaffin cells secretes pancreatic polypeptide and digestive polypeptide hormones(Klimstra & Longnecker, 1994). These endocrine hormones; Insulin and glucagon specifically regulate glucose secretion and uptake in the cells and are vital for glucose metabolism(Aronoff et al., 2004).

Tumours of the pancreas are mostly exocrine, based on a histopathological examination over 15 years(Morohoshi et al., 1983). About 95% arise from the pancreatic duct, are called pancreatic ductal adenocarcinoma (PDAC) and can grow anywhere along the length of the pancreas though most commonly found in the head(Lillemoe, 1995). Other types are acinar cell carcinoma, solid pseudopapillary neoplasm, cystadenomas and pancreatoblastomas (Hruban et al., 2007). The endocrine section gives rise to neuroendocrine tumours that arise from the islets; a small percentage 10-30% which may be functionally producing hormones or not(Lillemoe, 1995). The functional ones include insulinomas, gastrinomas, glucagonomas, VIPomas, somastinomas.

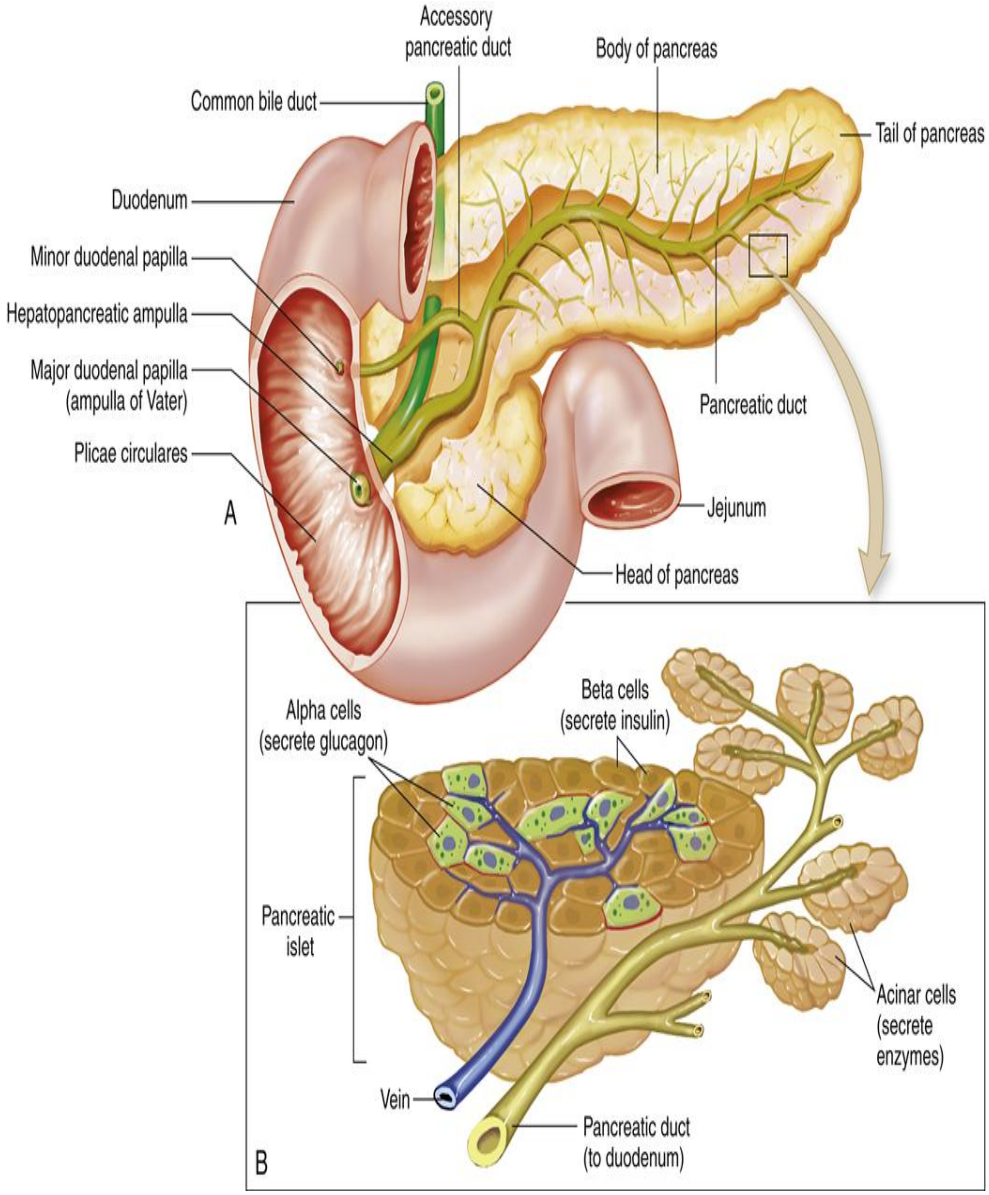


Figure 1.15 The histological tissues of the pancreas.

A) Anatomy of the pancreas in relation to surrounding structures B) Endocrine cells embedded amidst exocrine acinar cells. Adapted from Sartin, 2016(Sartin, 2016).

1.10.2 Epidemiology

Pancreatic cancer is one of the world's deadliest cancer, with most patient death occurring within 1 year of diagnosis as observed of 36,800 deaths out of 43,140 new cases diagnosed in both sexes in 2010(Jemal et al., 2003; Jemal et al., 2010). It has the lowest survival by stage of any other solid tumour (Jemal et al., 2009); a 10-year prospective study reported very low survival rates amongst 196 patients with proven

adenocarcinoma; where only 27 patients survived beyond 1 year and only 1 non-resected patient survived beyond 5 years(Gudjonsson, 1987). A comprehensive review of 37,000 hospital cases of PC has also shown overall survival of only 0.41%, with only 156 surviving patients after 5 years(Gudjonsson, 1987) and this survival rates and statistics have not improved much since then. This high mortality is mainly because of the propensity to early metastasize and spread (Pandol et al., 2009), (109 patients of the 10-year study above in fact had metastasis to the peritoneal nodes (64%) and secondly to the liver(27.4%)) with even the smallest primary lesions often show perineural and lympho-vascular invasion (Hezel et al., 2006). More recently, the 5-year survival rates have improved but differs country to country, based on management approaches such as early surgical removal, stage, absence of lymph node metastasis. Worldwide, survival is generally around 5%; in England for instance, from 2001-2006 the rate was 3%, compared with 6% in the USA for a similar study period of 1999-2005(Jemal et al., 2010).

Still regarded as largely incurable, it is the fifth most common cause of cancer death in the UK (Cancer Research UK; Pancreatic Cancer Mortality Statistics 2014) and Europe (Ferlay et al., 2007). In the U.S, PC is the fourth leading cause of cancer death (Jemal et al., 2008) and second most common gastrointestinal malignancy (American Cancer Society, Cancer Facts and Figure 2010). It is strongly related to age; in the period of 2012-2014 for example almost half (47%) of cases diagnosed on average each year were in people aged 75 and over in the UK (Cancer Research UK; Pancreatic Cancer Mortality Statistics 2014). Sex distribution is approximately equal. There were 8,817 pancreatic cancer deaths in the UK in 2014: 4,426 (50%) in males and 4,391 (50%) in females, giving a male:female ratio of around 1:1(Cancer Research UK; Pancreatic Cancer Mortality Statistics 2014).

1.10.3 Risk factors

Smoking (Blackford et al., 2009; Lynch et al., 2009), environmental tobacco exposure(Vrieling et al., 2010), alcohol intake(Genkinger et al., 2009), high consumption of fatty meals and low vegetables, fruits and Vitamin C (Lynch et al.,

2009; Thiebaut et al., 2009; Vrieling et al., 2010), obesity(Philip et al., 2013) and exposure to chemicals such as beta-naphthylamine and benzidine(Mancuso & el-Attar, 1967) and trace elements such as lead, nickel and selenium(Amaral et al., 2012) have been established as risk factors to developing PC. There has also been marked risk associated with chronic pancreatitis (Lowenfels et al., 1993; Malka et al., 2002) as well as diabetes mellitus, specifically new onset (Rosa et al., 1989; Everhart & Wright, 1995; Gupta et al., 2006). At 10% incidence, a familial risk is also considerable based on inheritance of specific familial gene clusters (Schenk et al., 2001; Petersen & Hruban, 2003; Ferlay et al., 2007; Wang et al., 2007).

The tumour arises due to accumulations of genetic mutations leading to activations of oncogenes such as the KRAS oncogene, and inactivation of various tumour-suppressor genes. For example, the first known mutations involve activating KRAS point mutations at codon 12 (from GGT to GAT or GTT, and more rarely CGT). This mutation results in glycine substitution with aspartate, valine, or arginine. Although these mutations may occur sporadically in normal pancreas tissue, they are detected in higher quantities from up to 30% of early neoplasms in to a higher frequency up to almost 100% in advanced PDAC (Klimstra & Longnecker, 1994; Rozenblum et al., 1997). Other germline mutations occur such as in INK4A and BRCA2 genes, which are associated with several variants of PC as shown in Table 1.5. Consistent with a central developmental function, growth factors also play an important part in PC growth (Li et al., 2004; Deer et al., 2010).

Table 1.5 Pancreatic tumours and common associated genetic alterations.
Adapted from Hezel et al(Hezel et al., 2006).

Pancreatic neoplasm	**Histological features**	**Common genetic alterations**
Ductal adenocarcinoma	Ductal morphology; desmoplasia	KRAS, p16INK4a, TP53, SMAD4

Variants of ductal adenocarcinoma:		
a) Medullary carcinoma	Poorly differentiated; intratumoral lymphocytes	hMLH1, hMSH2
b) Colloid (mucinous noncystic) carcinoma	Mucin pools	MUC2 overexpression
Acinar cell carcinoma	Zymogen granules	APC/_-catenin
Pancreatoblastoma	Squamoid nests, multilineage differentiation	APC/_-catenin
Solid pseudopapillary neoplasm	"Pseudo" papillae, solid and cystic areas, hyaline globules	APC/_-catenin, CD10 expression
Serous cystadenoma	Multilocular cysts; glycogen-rich epithelium	VHL
Pancreatic endocrine tumours	Hormone production	MEN1

1.10.4 Staging

PC can be comprised of three main types of tissues; papillary 39% of tumours, tubular 59% and mixed 5% (Terada et al., 1998; Tanase et al., 2006). Based on histological examination, these tumours are graded into well, moderately and poorly differentiated(Morohoshi et al., 1983) which is reflected in the standard

Tumour/Node/Metastasis (TNM) classification and staging proposed by Rindi et al for foregut tumours(Rindi et al., 2006):

T- Tumour:

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

N-Regional lymph node:

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

M-distant metastasis:

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

Staging:

Stage I = T1 N0 M0

Stage IIa=T2 N0 M0 Stage IIb =T3 N0 M0

Stage IIIa=T4 N0 M0 Stage IIIb = any T N1 M0

Stage IV = any T any N M1

1.10.5 Clinical presentation and Treatment

Usually symptomless in the early stages and are often non-specific until advanced stages. Abdominal symptoms such as recurrent discomfort, loss of appetite, nausea and vomiting, change in bowel habits such as diarrhoea, steatorrhea, and constipation may also be present(Lillemoe, 1995). Late presentation signs such as jaundice often signals head involvement and inoperability(Lillemoe, 1995). Surgery is usually advocated in early stages of PC and includes pancreaticoduodenectomy when the head of the pancreas is involved, distal pancreactectomy with splenectomy for those involving the body or tail,(Castillo et al., 1995) while Whipple's procedure removes the head, the duodenum, part of the stomach and bile duct, the gallbladder are all removed to limit extension(Whipple et al., 1935).

Chemotherapy is often used as adjunct post-surgery and as palliative in non-resectable patients(Lillemoe, 1995). Gemcitabine (GEM) combinations such as with LMWH (such as Dalteparin) has mostly replaced 5-FU based therapies (such as FOLFORINOX-oxaliplatin, irinotecan, fluorouracil, and leucovorin) due to its relatively better safety profile and efficacy, although overall outlook for survival is still poor(Heinemann et al., 2008). In some few centres, FOLFORINOX is still used as first line-treatment for advanced PC due to slightly better survival advantage, longer time before quality of life deterioration compared to GEM(Conroy et al., 2011).

The clinical experience of targeting growth factor receptors such as anti-EGFR agent, both TKIs and monoclonal antibody [mAb]) [cetuximab (Erbitux), anti-VEGF bevacizumab, and other antiangiogenic agents axitinib in PC has limited success, of little or no therapeutic merit(Goel & Sun, 2015). These therapies, for example GEM plus bevacizumab when compared to GEM with placebo may also lead to increase toxicities and no overall survival for advanced disease (Kindler et al., 2010). There remains a valid concern that from a clinical perspective, these agents also drive thrombosis lead to excess thrombotic events and therefore undermine any potential benefit obtained from use of anti-cancer therapies (Maraveyas & Johnson, 2009; Echrish et al., 2011). To date however, there is little treatment benefit in the use of growth factor targeted receptors in PC management.

1.11 Thrombosis in PC

PC has one of the highest VTE rates amongst malignancies worldwide, with incidences that reach up to 57% in patients(Khorana & Fine, 2004). In PC, an intrinsic procoagulable state exists as a complex interaction of prothrombotic and proangiogenic protein expressions. This thrombotic state further aids tumour development and expansion and accounts for poor prognosis in PC(Mandalà et al., 2007). It is surmised from several histologic studies that the tumour stroma is surrounded extensively by fibrin and the tumour is known to produce high amounts

of prothrombin, coagulation factors, angiogenic factors and fibrinogen, all leading to an excessive propensity to clot formation (Wojtukiewicz et al., 2001).

PC is widely known to have high-grade TF antigen expression (Nitori et al., 2005; Khorana et al., 2007a). This high TF expression levels have been significantly correlated with increased VTE risk as high as 26.3% (p= 0.04) (Khorana et al., 2007a), and worse treatment prognosis overall (Nitori et al., 2005). In fact, as 77% of poorly differentiated PC have a markedly elevated TF expression compared to 20% of the less-progressed well-differentiated ones, a strong linear correlation is linked between TF expression and poor histologic grade of PC(Kakkar et al., 1995), which may account for its low survival rates(Khorana & Fine, 2004). In addition, while numerous cancers can be said to express high TFMV levels, PC in particular also has one of the highest TFMV activity (Tesselaar et al., 2007; Tilley et al., 2008). Furthermore, high amounts of functional thrombin receptors such as PAR-1 can be found on PC cells, signifying an additional link in thrombin-enhanced tumour progression (Rudroff et al., 2001).

Thromboprophylaxis in PC clinical management is not used routinely as the natural evolution of VTE in aggressive advancement of PC is poorly characterized. Although VTE in PC may result in increased complications and worse survival outcomes, the actual impact of use of anti-thrombotics on survival remain uncertain. While some studies have analysed the risk of VTE associated with PC and the consequences on treatment and survival, most suggest that VTE development may signify a more aggressive cancer biologically and consequentially worse prognosis, while other studies find that there is little or no effect on overall survival. For instance, Shaib et al reviewed 201 patients with PC and reported VTE in 58 patients (28.9%) but found no survival difference between PC patients with VTE and those without VTE(Shaib et al., 2010). Few studies have also associated any clinical benefit with use of prophylactic anticoagulants; for example in a cohort of 90 patients; 72 of which had PC (49 with metastatic disease (54.4%), 24 developed VTE (26.7%)), Mitry et al retrospectively surmised that the risk of VTE was significantly reduced (HR: 0.03 [95 CI: 0.003-0.27]) with administration of thromboprophylaxis, but conversely increased in those with metastatic disease (HR: 4.4 [95CI: 1.1-17.9]) (Mitry et al., 2007).

However, in the same study there was also no difference in overall survival in patients with VTE (6.6 months) and those without (6.1 months).

1.12 Aims

Understanding the intricate mechanisms underpinning cancer thrombosis may ultimately lead to assembly of biomarkers that may predict VTE patterns in cancer patients at risk or in the general population. This is because VTE may sometimes be the first symptom of occult cancer in otherwise healthy people, and reliable markers and methods of identifying them are necessary in cancer management. Personalizable anti-coagulation may be an achievable end goal obtained by use of various measurable predictive markers of VTE risk in cancer management. Current research indicates possible clinical benefits to integrating an individual unique thrombotic phenotype produced by comprehensive methods into their VTE risk determination. A deeper understanding of the mechanisms of endothelial involvement in thrombotic events may also potentially aid in developing better therapeutic solutions in cancer management.

The overall aim of this thesis is to investigate the hypothesis that the study of thrombin generation, TF expression and TFMV activity can differentiate between the coagulation processes in two cancer types of diverse pathogenesis that are outwardly (clinically) similar in their high VTE incidences. From previous projects in our laboratory that showed that endothelial MVs are elevated in MM during chemotherapy treatment, and the finding that TFMV's levels are raised in PC patients, we have theorized that first, the endothelium may be involved in procoagulant activities during cancer development. Another theory is that as TFMV's levels are raised in PC patients, they may be correlated to the process by which thrombin is generated in such cancers. Thus, the initial aim was to examine the interaction of TFMVs that are produced from cancer cell lines with endothelial cells leading to a prothrombotic cross-talk that can be evidenced in various assays for measuring procoagulant activities including TG.

It is also known that solid cancers such as PC which is an adenocarcinoma have high levels of TF expression and thus may produce thrombin in a TF-dependent pattern, and this may differ from haematological cancers such as MM which is a plasma cell disorder that have little or no TF expression. Therefore, the hypothesis here was that the CAT assay may be used for measurement of TG in both PC and MM cell lines to allow an extensive definition of the patterns of TG which may be associated with the levels of TF produced. Furthermore, there might be differential contributions of the various factors of the coagulation cascade to these TG, from which surfaced the hypothesis that plasma deficient of one or more coagulation factors may affect the characteristics of thrombin produced in these malignancies.

In addition, it is also theorized that these patterns of TG may also be seen in patients' plasma obtained from both MM and PC patients, which may allow future use as a biomarker in identification of VTE risk in these patients.

1.12.1 Objectives

- To investigate how cancer TFMVs can interact with endothelial cells *in vitro* and confer procoagulant activity (PCA) as determined by flow cytometry and clotting assay

- To measure the thrombin generation (TG) parameters of endothelial cells that acquire PCA with a CAT fluorogenic assay

- To determine the TG characteristics of solid tumour and haematological cancer cell lines by CAT assay and highlight any significant differences in TG kinetics in various coagulation factor plasma conditions

- To evaluate the TF antigen surface expression of the cancer cell lines with flow cytometry and correlate with their TG properties

- To determine the TG properties of cancer MVs derived from these cancer cell lines through high centrifugation

- To assess the TG properties of MM and PC patient plasma samples as representatives of solid and haematological cancers, before, during and after treatment

- To measure the circulating TF antigen levels in MM and PC patient serum with quantitative ELISA.

- To correlate the TG results of these cancer patients with the circulating TF levels.

Chapter:2 Materials and Methods

2.1 Cell culture

All cells were cultured in their recommended media, grown in sterile culture flasks (Sarstedt, Leicestershire, UK), and incubated in a humidified incubator at 37°C under an atmosphere of 5% CO_2. All cell culture experiments were carried out in a Class II Biological safety hood that had sterile laminar air flow to provide sterile working conditions, which was cleaned with Trigene disinfectant (VWR International, Leicestershire, UK) and 70% v/v ethanol (Sigma-Aldrich, Poole, UK), before and after carrying out protocols. The CO_2 incubator and water bath for prewarming culture materials to 37^0C were also regularly cleaned with Virkon disinfectant (Scientific Laboratory Supplies Limited, UK) and 70% ethanol. All other equipment such as universal bottles, pipette tips boxes, were disinfected with 70% ethanol before placing in the biosafety hood in order to avoid culture contamination. All growth media were stored at 4^0C, and prewarmed in a water bath for at least 30 minutes before use. In addition, all cells were routinely tested for mycoplasma contamination (MycoProbe Assay, R&D Systems, Abingdon, UK).

2.2 Cell lines

2.2.1 HUVECs

Primary Human umbilical vein endothelial cells (HUVECs; PromoCell, Heidelberg, Germany) are isolated from umbilical cord veins (Jaffe et al., 1973) of pooled donors

(from up to four different umbilical cords). HUVECs were chosen due to their unique endothelial properties exhibited as an intermediate between those of large vessels and microvascular endothelium(Bicknell, 2004). HUVECs are readily available and have been used widely in published literature and in our laboratory especially for thrombosis-related studies. They were cultured in endothelial cell growth media (ECG; PromoCell), supplemented with FBS 0.05 v/v, Endothelial Cell Growth Supplement 0.004 v/v, Epidermal Growth Factor (recombinant human) 10 ng/ml, Heparin 90 µg/ml, Hydrocortisone 1 µg/ml (all from PromoCell). HUVECs were seeded at 1 x 10^6/ml cells in a 25cm^2 tissue culture flask and left to adhere overnight at 37^0C in a 5% CO_2 incubator. HUVECs were used at approximately 70% confluency and were utilised at passages 3-6 only.

2.2.2 Cancer cell lines

Solid tumour cells (pancreatic cancer (ASPC-1, CFPAC-1, PANC-1, MIA PaCa-2), SKOV-3 ovarian cancer, UMSCC81B Head & Neck squamous cancer, PC9 lung cancer) and malignant haematological cell lines (Multiple myeloma (MM1.S, U266B, H929), JJN3 plasma cell leukaemia, U937 histiocytic lymphoma) were cultured as described above in section 2.1.

ASPC-1, UMSCC81B, PC9 and all haematological cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI) 1640 medium (PAA; Yeovil, Somerset, UK) supplemented with 10% (v/v) FBS. CFPAC-1 and MIA PaCa-2 cells were maintained in Iscove's Modified Dulbecco's Medium (ATCC; Teddington, UK), supplemented with 10% FBS, 1% L-glutamine and for MIA PaCa-2 cells only, 2.5% (v/v) horse serum from Gibco® (Life Technologies Ltd, Paisley, UK). SKOV-3 was maintained in McCoy's 5A medium (PAA) supplemented with 1% L-glutamine (PAA) and 15% (v/v) FBS. All media were supplemented with 1% penicillin/streptomycin, which is provided as 100 units/ml penicillin, 100 µg/ml streptomycin (p/s) (Lonza, UK).

2.2.2.1 MM and other haematological cancer cells

The haematological cancer cell lines used in this study were MM.1S, U266B, H929, JJN3, U937, and U937 and were purchased from ATCC, while H929, U266 and JJN3 were a kind gift from Dr Guy Pratt (School of Cancer Sciences, University of Birmingham, UK). MM.1S was derived from peripheral blood of 42 year old female with immunoglobulin (Ig) A myeloma (Goldman-Leikin et al., 1989), U266B was derived from an IgE producing myeloma patient(Nilsson et al., 1970), H929 was derived from a patient with IgAk myeloma with malignant effusion(Gazdar et al., 1986), JJN3 is a subclone of JJN1 which is a parental cell line derived from plasma cell leukaemia in a 57 year old woman (Jackson et al., 1989), U937 was from pleural effusion of a 37 year old male with generalized histiocytic lymphoma(Sundstrom & Nilsson, 1976).

2.2.2.2 PC cells and other solid cancer cells

All solid cell lines were purchased from the American Type Culture Collection (ATCC) except PANC-1, SKOV-3, PC9 European Collection of Authenticated Cell Cultures (ECACC) and UMSCC81B (kind gift from Dr Thomas Carey, University of Michigan). ASPC-1 is a human pancreatic adenocarcinoma cell line established from ascites of a 62 year old female (Chen et al., 1982). CFPAC-1 is a metastatic ductal pancreatic adenocarinoma obtained from a 26-year-old male with cystic fibrosis and liver metastasis (Schoumacher et al., 1990). MIA PaCa-2 is derived from a 65-year-old man with pancreas adenocarcinoma of the body and tail with periaortic infiltration (Yunis et al., 1977). PANC-1 was obtained from adenocarcinoma in the head of the pancreas of a 56-year-old man with duodenal wall invasion(Lieber et al., 1975). Other solid cancer cell lines used include SKOV-3, derived from ascites of a 64 year old female with metastatic ovarian adenocarcinoma (Fogh et al., 1977). UMSCC81B is a Head and Neck Squamous Cell cancer from the University of Michigan Tonsillar squamous cancer cell line, and PC9 is a Non-small cell lung cancer line derived from well-differentiated adenocarcinoma in human lung tissue.

2.2.1 Passaging

Cells were grown to 70-90% confluency and sub cultured within this logarithmic phase, in order to maintain optimal density for continued growth. The growth medium was changed 2-3 times a week and cell viability was determined by trypan blue assay (section 2.3). Adherent cells with the exception of HUVECs were removed from culture flasks by scraping with a cell scraper (Sarstedt), while non-adherent cells were removed by pelleting with centrifugation at 320xg for 3 minutes. Once pelleted by centrifugation, for both adherent and non-adherent cells, the supernatant was discarded before resuspension of cell pellet in fresh growth media for transferral to a new flask for reseeding. HUVECs were removed by trypsinization, which entailed washing of cells in the culture flask with sterile phosphate buffered saline (PBS) twice, then addition of 3-5 ml of 0.05% trypsin ethylenediaminetetraacetic acid (Trypsin EDTA; PAA, Yeovil, UK) for 3-5 min at 37°C in the 5% CO_2 incubator. Then excess culture media of 10ml was added to the flasks and left for 3-5 minutes to neutralize trypsin action, prior to removal of the cell suspension and washing twice by centrifugation at 320xg for 3 minutes, before reseeding in fresh growth media in new culture flasks. Except for Corning® tissue culture flasks (orange top; VWR, UK) used for HUVECs culture, all other culture plastics used in this study was purchased from Sarstedt, UK.

2.2.2 Ethics statement

The use of commercially available cell lines such as those used in this study have been approved at a School level within the University of Hull.

2.2.3 Cryopreservation

To ensure high viability after recovery from storage, a cell count by trypan blue assay was performed (section 2.3) and only cells with 90% viability were cryopreserved to ensure recovery after thawing. To freeze, cell suspensions obtained as described

above were centrifuged at 320xg for 3 minutes and resuspended at a high cell concentration (3-5 x 10^6 cells/ml) in freezing media made up of 90% v/v heat inactivated fetal bovine serum (FBS; BioSera, Ringmer, UK) and 10% dimethyl sulfoxide v/v (DMSO; Sigma-Aldrich, Poole, UK). DMSO is a cryoprotective agent used to prevent formation of ice crystals during freezing, and thus reduce ionic stress on the cells. Then the cells in freezing media suspension were aliquoted into 1 ml cryovials, before these vials were placed in cryo-freezing containers ("Mr. Frosty", Nalgene®;Fisher Thermo Scientific,Roskilde, DK) filled with 100% isopropyl alcohol(Sigma-Aldrich; Poole, UK) and slowly frozen at a cooling rate of approximately 1°C per minute in a -80°C freezer. The frozen cryovials were transferred to a liquid nitrogen storage vessel after 24 – 48 hours.

To recover frozen cells, the cryovials were placed in a 37°C water bath for rapid thawing over 1-2 minutes. The surface of thawed cryovials were sprayed with 70% v/v ethanol and transferred to the class II biosafety hood. Cells were transferred from the cryovials and suspended slowly in a 50 ml sterile universal tube at 1:10 volume of pre-warmed cell medium to cell suspension. Cells were then pelleted by centrifugation at 320xg for 3 minutes to remove the DMSO-containing freezing media. Thereafter, the supernatant was discarded and cell pellets were resuspended in appropriate medium before transfer to 25 cm^2 tissue culture flasks for culturing and further assays.

2.3 Cell count and viability assay

To determine cell count, cell suspensions of adherent and non-adherent cells were removed by pipette from tissue culture flasks and pelleted by centrifugation at 320xg for 3 minutes, before resuspension in 1ml of PBS. 10µl of the cells suspended in PBS was placed into 500µl polypropylene tubes, and 10µl of 0.4% trypan blue (Sigma-Aldrich, Poole, UK) was added. This was mixed to attain homogeneity by pipetting up and down gently a few times, before 10µl was withdrawn and applied to the counting chamber on the Hawksley haemocytometer (improved Neubauer of depth 0.1mm,

1/400mm^2). The stained suspension of cells spread by capillary action into the area under the cover slip. Under a phase contrast microscope at 10x magnification, non-viable cells were identified as those stained blue by the dye because of the permeable nature of their damaged cell membranes, while viable cells were identified as those with intact membranes with no colour penetration. The cells visualized in the middle large gridded square (1 mm^2) of the haemocytometer were counted (the central blue square in figure 2.1), and the cell concentration and viability derived from the calculation formula below:

Cell concentration= Total cell count in central blue square x dilution factor of 2 x counting chamber size of 10^4/ml

% Cell viability= (cell count of viable clear cells with no colour penetration/ Total cell count in central blue square) x 100%

Final volumes for corresponding experiments were achieved through adjustment of the cell concentrations (in cell/ml) calculated from the cell count by dilution to desired volumes. Cells with ≥70% viability was used for most experiments except those involving HUVECs where >90% was used.

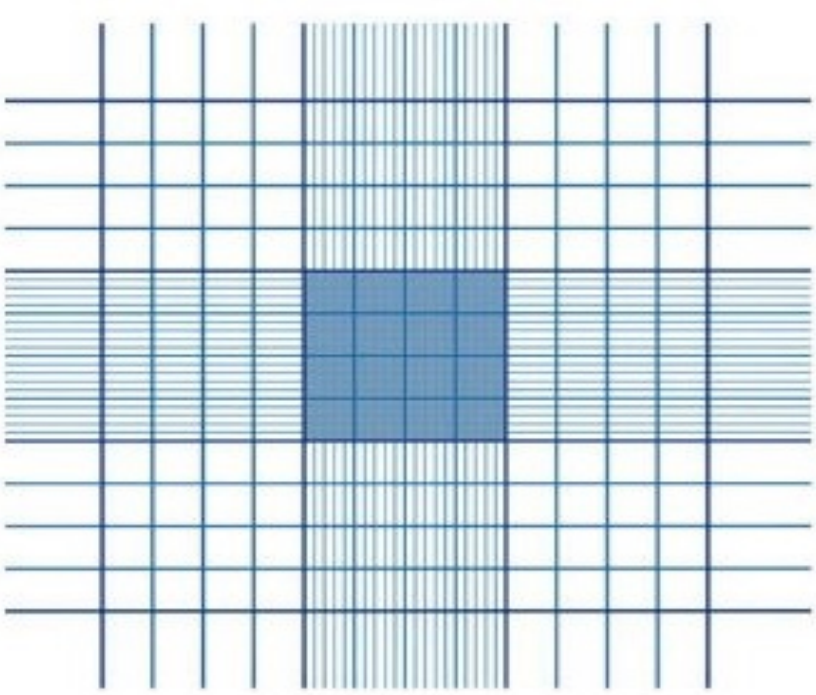


Figure 2.1 Appearance of the haemocytometer gridlines (central blue gridded square is 1 mm^2) used to count cells and estimate viability.

2.4 MV preparation

2.4.1 MV-rich media preparation:

Confluent monolayers of adherent tumour cell lines and suspensions of non-adherent cell lines were centrifuged at 320xg for 3 minutes, according to the method optimized for isolation of cell- free MV suspensions in our laboratory (Yates et al., 2011). The supernatant was collected and further centrifuged at 1000xg for another 5 minutes to remove cells and larger cellular debris, in order to obtain cell-free media containing tumour MVs, which was assessed for clotting activity with the modified prothrombin-based Clotting time (CT) assay below (section 2.5) and used for further experiments.

2.4.2 HUVECs culture in tumour-MV-rich media and platelet free plasma (PFP)

Spent growth media was removed from the HUVECs in the 25cm^2 culture flasks and discarded, then replaced with different concentration volumes (25, 50 and 100%) of the tumour MV-rich media, with the remaining volume made up with fresh ECG media. Incubation resumed for 24, 48 and 72 hours alongside control flasks of HUVECs cultured with addition of plain tumour culture media with no MVs. After incubation, the procoagulant activity (PCA) of HUVECs was then assessed with the CT assay (described in section 2.5) and the experiments were repeated four times. Based on the clotting results, HUVECs cultured in UMSCC81B MV-rich media was selected for further assessments below in Chapter 3. For all experiments, untreated controls of HUVECs cultured without media containing tumour MVs were used.

Furthermore, to assess the effect of spent tumour growth media that was added to HUVECs, the HUVECs were cultured with either spent MV-rich media or fresh MV-rich media as control. Spent MV-rich media is UMSCC81B MV-rich media removed from culture after 24 hours incubation, while fresh MV-rich has not been used in culture. Fresh MV-rich media was obtained from Vivaspin * 6 centrifugal concentrators with a Molecular weight cut-off (MWCO) of 10kDa (Sartorius Stedim Biotech AG, Göttinngen, Germany) which were used to concentrate MVs in cell-free supernatants. These are tubes that consist of high thoroughput polyethersulfone membranes used for optimal filtration of protein, antibodies, nanoparticles and MVs with >90% recovery rates. The ultrafiltration concentrator was filled with 6ml of tumour supernatant containing MVs and centrifuged twice at 4000xg for 30 minutes, then recovered with fresh tumour media. This was later used as fresh MV-rich media to replace the ECG media of HUVECs during the time course assays above, as a comparison to HUVECs cultured in spent tumour growth media. HUVECs were also incubated in the filtrate obtained.

For the CAT assay described below in section 2.8, the concentrated MVs at the bottom of the concentrating chamber was used without further recovery with fresh media.

2.4.2.1 HUVECs culture in platelet free plasma (PFP) of cancer patients

HUVECs ($1x10^6$/ml) were cultured as above and the spent media in tissue flasks replaced with 1 ml of patient platelet free plasma (PFP obtained from whole blood of cancer patients as described below in section 2.8.3). Incubation resumed for 24 hours along with control flasks of HUVECs cultured with addition of platelet-free normal control plasma (NormTrol; Helena Biosciences, Gateshead, UK). HUVECs were then harvested form the culture flasks, and the CT was assessed.

2.5 Clotting time (CT) assessment

2.5.1 Cells and cell-free supernatant CT assessment

Cells were harvested from culture flasks by centrifugation twice at 320xg for 3 minutes to remove spent media, as described above, and resuspended with 5ml PBS before cell counts as detailed in section 2.3. For neutralization experiments, cells (1 x 10^5 cells/100 µl PBS) were incubated in the presence or absence of 5 µl (5 mg/ml) of polyclonal rabbit anti-human TF (American diagnostica, Stamford, USA) in 1.5 ml polypropylene tubes, for 30 min at 37°C to block cell surface TF expression. Then CT was assessed with a Thrombotrack SOLO steel ball coagulometer (Alere, Stockport, UK). This coagulometer measures the prothrombin time (PT) of the clot formed in a sample undergoing coagulation placed in a cuvette; where a steel ball in the centre of the sample rotates along the circumference as a result of the small magnetic field generated. The liquid sample consists of either $1x10^5$ cells in 100 µl PBS suspension or cell-free media (100µl per cuvette) to which 100µl of normal plasma control (NormTrol; Helena Biosciences, Gateshead, UK) containing coagulation factors except TF, was added. The mixture was incubated for 2 minutes at 37^0C, before the start button on the coagulometer was pressed, which counts down for 3 seconds, after

which 100µl of 25mM $CaCl_2$ was added to initiate the reaction. Once the timer was initiated, the clotting reaction measures the time taken for the ball to stop rotation due to fibrin clot formation. The results are mean ± standard deviation (SD) of measurements repeated on three separate occasions n=3.

Samples, such as controls (PBS for cells and the respective media for cell-free supernatants) and NormTrol, that showed no clot in >999.9 seconds were deemed unable to support coagulation in the length of time of assay.

2.5.2 Patient Platelet-free plasma CT assessment

In both clinical studies, whole blood was collected from patients and healthy volunteer controls into sodium citrate anticoagulated tubes as described in section 2.8.3. These were processed into platelet free plasma (PFP), 100µl of which was incubated for 2 minutes each at 37^0C where clotting was initiated by 100µl of $CaCl_2$. Plasma samples that demonstrated no clot in >999.9 seconds were also deemed unable to support coagulation in this assay.

2.6 Flow cytometry

Flow cytometry is a widely used laser-based technology that is useful in multi-parameter analysis of single cells and cell subpopulations. It consists of 3 main systems; the fluidic, optical and electronic systems. It can be used to analyse fluorescent labelled antibodies that attach to specific antigens, proteins, ligands or molecules found in substrates such as cells and MVs. It can also assess the intra- and extracellular expressions specific proteins of cells or substrates and define homogenous cell populations based on their sizes and granularity. To detect these properties, cells or particles in suspension are passed through the fluidic system of the flow chamber of the cytometer. The suspension is allowed to flow haemodynamically through the core of the sheath fluid in a small nozzle, such that a laser beam that is located perpendicularly outside the sheath fluid, can focus on each

cell (Figure 2.2). Light is scattered in different directions as the cells pass through in a single file at the interrogation point. The forward, side light scatter and fluorescence emission (FSC, SSC and FL) are captured by detectors placed in the front and sides of the sheath, and cells are distinguished based on these qualities. The FSC, SSC refer to the size and granularity of the cell or particles flowing through, while florescence (FL1, FL2, FL3 or FL4) is emitted when cells or particles are stained with specific antibodies, which are coupled to fluorochromes. These fluorochromes are excited by the laser light in the cytometer and to obtain precise detection, fluorescence emitted from the 3 parameters (FSC, SSC and FL) are passed through an optical system which consists of various filters and mirrors and sensors. The emissions they detect are amplified by photomultiplier tubes (PMTs), and eventually converted into voltage pulses, which are designated events of the electrical system. The measured voltage pulse area corresponds directly to the intensity of fluorescence for that event, and this electrical signal is converted from analog into digital inputs that is detected by the external computer connected to the cytometer. Data acquired can be further used to identify cell characteristics and populations, which can be further divided into different subpopulations by using gates and various plotting options such as dot plots, scattergrams and histograms.

The flow cytometer used in this thesis was a Becton Dickinson FACS Calibur instrument (BD Biosciences, Oxford, UK), which was cleaned, serviced and calibrated on a regular basis by the BD Biosciences service team. Furthermore, data acquisition and preliminary analysis was done on an Apple Macintosh G5 computer, equipped with BD CellQuest Pro v.6.0 software (BD Biosciences).

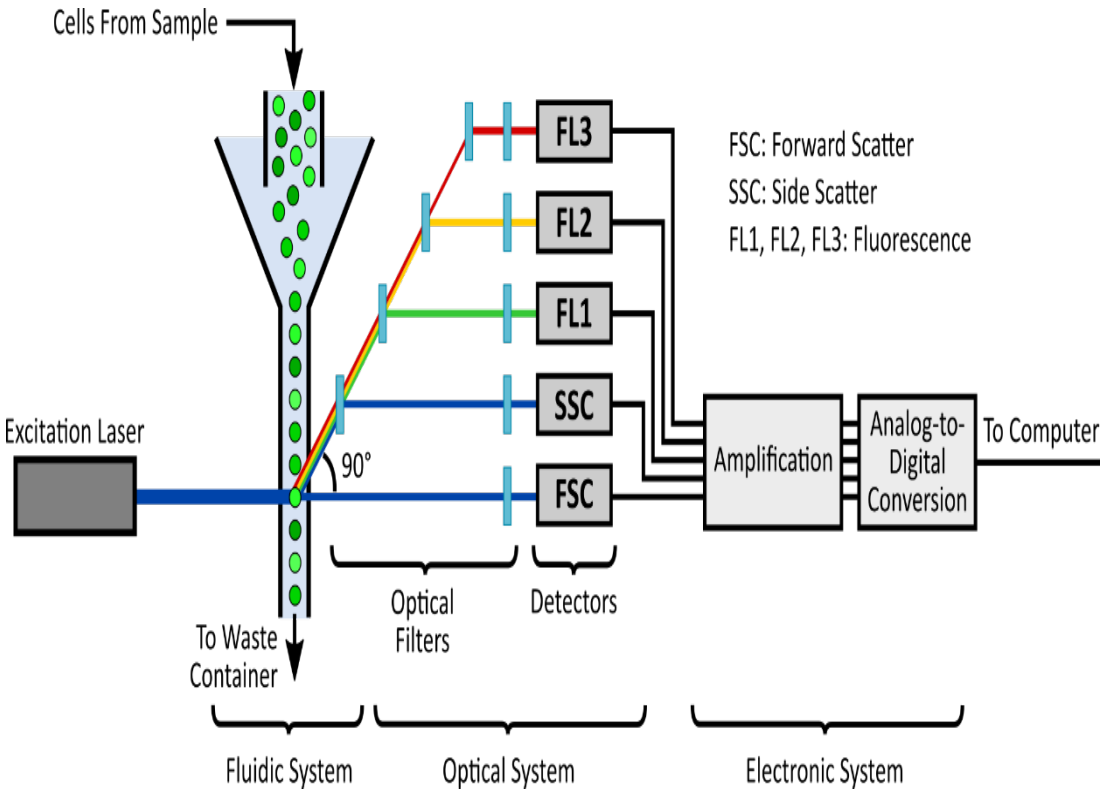


Figure 2.2 Flow cytometry illustration.

Cells or particles in suspension are passed through the cytometer in a single file hitting the laser beam at an interrogation point where light is dispersed into FSC and SSC. Fluorescence emission is also acquired from samples and all events are collected through lens and filters at similar wavelengths to corresponding detectors. The signals are further amplified and converted into an electrical current, which is detected as analysable data by the connected computer system. Adapted from: Castillo-Hair S.; FlowCal: Software for analysis and calibration of flow cytometer data, Benchling (https://benchling.com/pub/tabor-flowcal). Accessed October 1, 2017

2.6.1 Flow cytometric evaluation of cell surface TF expression in HUVECs and Cancer cells

For the detection of TF cell surface expression on HUVECs and cancer cells; 50µl of cells at $1x10^6$/ml per tube was incubated in a 5ml polypropylene tube (Sarstedt, UK) with 5µg of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD142/TF antibody (clone CLB/TF-5; Bio-Rad (formerly AbD Serotec), Kidlington, UK))

or a concentration-matched FITC-conjugated mouse anti-human IgG_1 isotype control (Bio-Rad, UK). This isotype control is used to control for changes in the level of non-specific background signal caused by primary antibodies due to non-specific binding Fc receptors present on the cell surface. The mixture was left in the dark for 30 minutes incubation at room temperature, to allow the fluorochrome-labelled antibody to bind to the target epitopes present on the specific antigen on the cell or particle surface. Then the mixture was washed twice by centrifugation at 320xg for 3 minutes with 5ml of PBS to remove any excess antigen, and then resuspended in 300µl of PBS before flow cytometry. This method of antibody staining allows the selected antigens expressed on cell surface to be detected by the flow cytometer, where the level of fluorescent signal emitted is directly proportional to the number of fluorochrome molecules conjugated to the TF antibody on each cell. This allows direct estimation of the TF antigen levels expressed in the cell population.

For each sample test tube, a minimum of 10,000 events was acquired on the cytometer. Data acquired represents the mean fluorescent intensity (MFI) of TF expression in relation to the MFI of cells stained with isotype control antibody, and the results recorded were presented as mean fluorescence ratio (CD142/TF antibody/negative control) according to the formula below:

MFIR = MFI value of the TF positive sample / MFI value of the negative isotype control sample

2.7 Confocal microscopy imaging

2.7.1 CFSE-staining protocol

For confocal microscopy, UMSCC81B cancer cells were stained with 5(6) - Carboxyfluorescein diacetate N-hydroxysuccinimidyl ester (CFSE; Abcam, Cambridge, UK ab113853) in order to obtain CFSE-stained MVs derived from UMSCC81B cells. CFSE is a cell permeable, non-fluorescent pro-dye that is cleaved by intracellular esterases in live cells to release a green fluorescent carboxyfluorescein molecule that

is membrane impermeable. UMSCC81B cells ($1x10^6$/ml) were centrifuged at 320xg for 3 minutes into a pellet before discarding the supernatant. The cell pellet was resuspended with 5ml PBS and then 10µM of reconstituted CFSE solution was added and mixed by gentle pipetting for uniform labelling, before incubation at 37^0C for 10 minutes. Staining was then quenched with equal volume of 5 ml RPMI cell culture media. The cells were washed twice with 5ml PBS at 320xg for 3 minutes to remove unincorporated CFSE before seeding in 75cm^2 culture flasks containing 15ml fresh media. After 24 hours, labelled MV-rich tumour media was harvested for addition to HUVECs in culture, by replacing the ECG media of HUVECs in 25cm^2 tissue flasks, as described above in section 2.4.2**.** Cultures of UMSCC81B without CFSE-staining were used concurrently as controls.

2.7.2 Confocal Laser scanning microscopy analysis of HUVECs incubated with tumour MVs

The $1x10^6$/ml of HUVECs cultured in CFSE-stained MV-rich media derived from UMSCC81B cells were labelled with 5µg CD142/TF: phycoerythin (PE) or isotype-matched negative control:PE antibody for 30 minutes in the dark at room temperature. Then they were washed twice with PBS by centrifugation as described above and resuspended in PBS. They were then transferred into Lumox Dishes (35mm Ø x 12mm Adherent Surface; Starstedt Ltd) at $2x10^5$ in 200 µl density, immediately after staining with PE-conjugated antibody. Confocal microscopy was performed using a Zeiss LSM710 Laser Scanning Confocal Microscope and images acquired using ZEN software version X (Zeiss Group, Oberkochen, Germany) to view HUVECs as compared to controls incubated with plain media without tumour MVs.

2.8 Thrombin generation (TG) by Calibrated Automated Thrombography (CAT) assay

2.8.1 Thrombin generation in cells

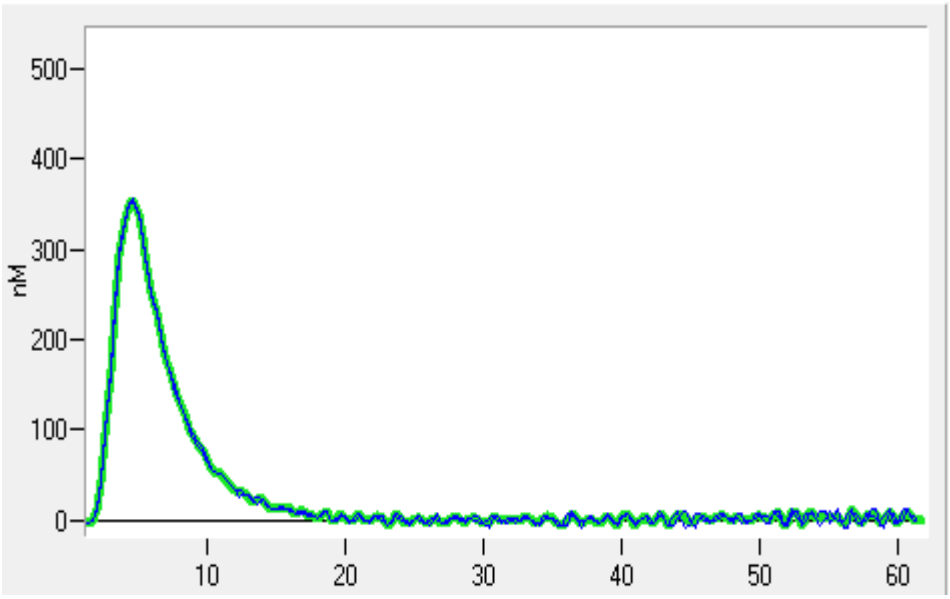
TG was assessed by the Hemker et al, 2003 method (Hemker et al., 2003) using a calibrated automated assay in real time. Here, 20µl of live cells at various cell concentrations were plated into Immunulon transparent round bottom 96-well microtiter plates (ThermoFisher Life Sciences, Loughbrough, UK) for the CAT assay. Then 20 µl of Thrombin activator/PPP-Reagent LOW (Diagnostica Stago, Theale, UK) which contained 1pM recombinant relipidated TF and 4µM phospholipids (phosphatidyl-serine, -choline, and -ethanolamine) standard preparations in HEPES-buffered saline was used as positive control, while 20µl of PBS was the negative control. Afterwards, 80µl of platelet-free normal control plasma (NormTrol) or plasma deficient in coagulation factors VII and XII were added to the microplate wells. All plasmas were purchased as freeze-dried pooled immunodepleted human plasma (Helena Biosciences, Gateshead, UK) and each reconstituted according to manufacturer's instructions in 1ml of distilled H_20.

Then the microplates were agitated and incubated to 37^0C for 10 minutes while TG was initiated by addition of 20µl of a FluCa Kit solution (Diagnostica Stago, Theale, UK) containing 0.1M $CaCl_2$ and a fluorogenic substrate (Z-Gly-Gly-Arg-amino-methyl-coumarin fluorophore). The fluorescence emission was measured by Fluoroscan Ascent® fluorometer (Thermolab systems OY, Helsinki, Finland) with excitation/emission wavelengths of 390nm/460nm. Measurement occurred up to 1 hour after automatic mixing of contents of the wells at every 20 seconds, according to the Thrombogram guide (Giesen, 2012).

Real time TG curves were calculated using Thrombinoscope™ software version 3.0.0.29 (Thrombinoscope B.V., Maastricht, The Netherlands). Each condition contained 2 wells, one test well for cell induced TG, the other a calibration well as control for thrombin activity in test well (Figure 2.3A). 20µl of Thrombin Calibrator

α_2-macroglobulin/thrombin complex (Thrombinoscope B.V., Maastricht, The Netherlands) was used in calibrator wells. Measurement in the calibration well helps correct for inner filter effects and substrate consumption(Castoldi & Rosing, 2011). Each experiment was carried out in triplicate, independently. Time to start of maximum TG or until one-sixth of the peak height is reached (Lag time), endogenous thrombin potential or area under the curve (ETP), Peak height of thrombin (Peak), time to peak (TTP or ttPeak) and velocity index (Vel.Index) of TG were amongst the parameters evaluated (Figure 1.8B). The variation that is found parallel experiments that are identical in one 96-well plate is 3–5%(Hemker et al., 2003).

A)



B)

Figure 2.3 Thrombin generation curve in a CAT assay.

A) Example of typical thrombogram readout from CAT assay experiments in this thesis. The green curve represents the fluorescence from the thrombin calibrator wells while the blue represents those from the sample test wells.

2.8.2 Thrombin generation in microvesicles

MVs were concentrated from conditioned media of cancer cells in culture by removal of 6ml of supernatant which was added to a Vivaspin * 6 centrifugal concentrator and centrifuged twice at 4000 x g for 30 minutes, then recovered with 1ml of PBS (section 2.4.2). Then, 20µl of MVs were plated into Immunulon transparent round bottom 96-well microtiter plates (ThermoFisher Life Sciences, Loughbrough, UK) for the CAT assay as described above for live cells.

2.8.3 Thrombin generation in patient platelet-free plasma

To obtain platelet-free plasma using standard venepuncture techniques, venous blood samples were taken from the antecubital vein of the patients arm into blue 3.8% tri-sodium citrated bottles for plasma. Fresh PFP was obtained by immediate centrifugation of the citrated blood collection tubes in serial steps; first at 180xg for 10 minutes at room temperature (to prevent cold-induced platelet activation) to obtain platelet rich plasma (PRP) and then at 12,000xg for an additional 10 minutes to obtain plasma free of platelets (PFP). All PFP samples were used fresh in further analysis within 4 hours of blood collection or aliquoted and stored in an -80^{0}C freezer.

For TG, frozen PFP of patients of Multiple myeloma, Pancreatic cancer and controls were defrosted at room temperature. Then they were gently pipetted up and down for <30 seconds to ensure adequate mixture. Afterwards, 20µl of PPP-Reagent LOW (Diagnostica Stago, UK) were plated into the transparent round bottom 96-well microtiter plates (ThermoFisher Life Sciences, UK) to serve as thrombin activator for the CAT assay. PFP (80µl) were then added to the plates, before warming up to 37^{0}C to commence the CAT assay as described above.

2.9 Estimation of soluble TF in patient serum by ELISA

Patient serum processed from whole blood (described in clinical methods Chapter 5) was evaluated for TF antigen concentrations using a commercially available ELISA kit

(Quantikine ELISA kit of Human coagulation Factor III/TF; R&D systems Bio-techne, Abingdon, UK). According to manufacturer's instructions, all samples were thawed in room temperature and reagents were prepared fresh by dilution to recommended working concentrations before use.

In this test, a monoclonal antibody specific for TF that is pre-coated onto a polystyrene microplate is used for a sandwich enzyme inmmunoassay. All serum samples were diluted 1:1 in Calibrator Diluent with 150µl sample + 150µl diluent. The wash buffer concentrate (25X of buffered surfactant with ProClin ® preservative) was diluted up to 500ml in distilled H_20. Once prepared, 100µl of diluted serum or standard was added to each microplate well and any TF present was bound by the antibody immobilized into the microplate wells. Then, the plate was placed on a horizontal orbit shaker (Stuart Scientific, Essex, UK) and covered up with the provided plate sealers to incubate for up to 2 hours at room temperature to ensure antigen binding to antibody. The plate was then washed twice by filling each well with 400 µl wash buffer on a microplate autowasher (Asys Atlantis; Biochrom, Cambridge, UK) to remove any unbound substances. This was followed by addition of 100 µl of Horseradish Peroxidase (HRP)-linked polyclonal TF-specific antibody per well, incubated for 2 hours at room temperature. This plate was washed again twice as above and a substrate solution at 200 µl per well (made of mixture of 100 µl colour reagent A –stabilized hydrogen peroxide and 100 µl colour reagent B- stabilized tetramethylbenzidene chromogen) was added, that turned the colour in the wells blue in relation to amount of HRP-bound to TF antigen initially attached. This colour reaction is allowed to proceed for about 30 minutes and is halted by addition of 50 µl of 1M sulfuric acid stop solution per well, to stop the action of HRP on the substrate solution and turning the wells yellow. The optical intensity of the yellow colour was immediately measured by a microplate reader (Biotek Synergy HT, Winooski, USA) at 450nm wavelength. Each sample or standard measurement was performed in duplicates and the reported intra-assay precision within the assay was CV of 2.8% while the inter-assay precision between 40 assays was 5.5 %.

2.10 Statistics

The results are expressed as mean ± standard deviation, except where indicated otherwise. Student t-tests and Analysis of variance (ANOVA with multiple comparisons) were used to determine statistically significant differences between groups. P values less than *0.05, **0.01, ***0.001 were considered significant. Pearson's correlation coefficient (*r*) was used to describe correlations and the associated student t-tests was used for statistical significance. All statistical analysis was performed with GraphPad software, version 7 (GraphPad Software, Inc., California, USA) or SPSS computer software (version 20.0 (IBM Corp.). Clinical statistics were carried out by a professional statistician (Dr Eric Gardiner) as described in chapter 5.

Chapter:3 Cancer microvesicles induce Tissue Factor-related procoagulant activity in endothelial cells *in vitro.*

Work from this chapter has been presented in the peer-reviewed publication:

Adesanya MA, Maraveyas A, Madden LA. Cancer microvesicles induce tissue factor-related procoagulant activity in endothelial cells *in vitro*. Blood Coagulation and Fibrinolysis. 2017 July;28(5):365-372.

And in the conference presentations:

Adesanya MA, Madden LA, Maraveyas A. Cancer microvesicles induces tissue factor-related procoagulant activity in endothelial cells *in vitro*. National Cancer Research Institute (NCRI) conference; Liverpool (UK), Nov 2016

Adesanya MA, Madden LA, Maraveyas A. Cancer microparticles induces tissue factor-related procoagulant activity in endothelial cells *in vitro*. 10th American Association for Cancer Research - Japanese Cancer Association (AACR-JCA) Joint Conference on Breakthroughs in Cancer Research; Maui, (Hawaii, USA), Feb 2016.

Adesanya MA, Maraveyas A, Madden LA. Procoagulant activity of tumour microparticles on the endothelium. International Society of Experimental Haematology (ISEH) 44th Annual Scientific Meeting; Kyoto (Japan), Sep 2015.

Adesanya MA, Maraveyas A, Madden LA. Procoagulant activity of tumour microparticles on the endothelium. 4th Hull York Medical School (HYMS)

Postgraduate Research Conference; Hull, (UK), May 2015. *Prize awarded for *Best Poster presentation.*

3.1 Introduction

Microvesicles (MVs) are the small particles shed from activated and non-activated cells that are involved in cellular interactions with its environment (Simak & Gelderman, 2006; Puddu et al., 2010). MVs have heterogeneous compositions and may consist of anionic phospholipids, cytoplasmic inclusions and bioactive proteins such as tissue factor (TF) (Morel et al., 2005; Aharon et al., 2008). The production of MVs is dependent on their cellular source, type of stimulus and disease state of parent cell(Freyssinet, 2003; Lacroix et al., 2010; Lacroix et al., 2013; Nomura & Shimizu, 2015) Previous studies have implicated MVs of different sources with the transfer and sharing of oncogenic material such as EGRFvIII in Gliomas(Al-Nedawi et al., 2008), as vectors of genetic information(Meziani et al., 2008) and even inducing apoptotic and procoagulant effects(Aharon et al., 2008; Wang et al., 2012); for example in cancer cells that are non-TF expressing(Shaker et al., 2016). MVs are sometimes used as a general term to include other membrane encapsulated particles secreted from a cell such as exosomes and microparticles(MP) (Ge et al., 2012). A recent focus has been on cancer MVs associated with TF, which are known to be elevated in some cancers and may have a role in VTE (Davila et al., 2008; Haubold et al., 2009) and in some of the processes that promote metastasis. However not much is known about their interactions with the vascular endothelium, although it can be hypothesized that such interactions may result in some procoagulant effects.

The role of chemotherapy is also important and is to be considered in MV generation. In a pilot study in our laboratory, the number of circulating tumour cell-derived MV in MM patients were elevated 6–8weeks after chemotherapy suggesting that the tumour cells that undergo cellular changes such as apoptosis in the presence of cytotoxic agents release MVs. Previous studies in the literature have demonstrated that MV from various cellular origins may also induce endothelial dysfunction (Freyssinet, 2003) and our finding of elevated endothelial MVs following administration of IMiD-based chemotherapy in Multiple Myeloma (MM) lends support to these studies. This pilot study in our laboratory investigated MV origins in newly diagnosed and relapsed MM patients under treatment and revealed raised

levels of endothelial MV populations(Hall et al., 2016), which therefore suggests the hypothesis that the host's response to chemotherapy treatment may also contribute to the increased thrombogenicity observed in MM patients. This is a markedly different procoagulant profile than in solid malignancies, and the involvement of known procoagulant TF and other intrinsic or extrinsic factors of the clotting cascade in various thrombotic events have yet to be determined in most cancers. Thus, this thesis study hypothesizes that TF and other coagulation factors (such as factor XII of the intrinsic and factor VII of the extrinsic clotting cascade) may influence the clotting and thrombin production profile of endothelial cells, and suggests further evaluation for the role of the endothelium and MVs during tumour interactions.

TF is a 47 kDa membrane-bound protein that is a key initiator of the extrinsic coagulation pathways in the body and also contributes significantly to other pathways of coagulation(Steffel et al., 2006; Aird, 2012). Clotting mechanisms involving the endothelium may be heavily dependent on TF and other cellular phospholipids such as phosphatidylserine (PS) carried on MV membrane(Furie & Furie, 2005). Tumour cells *in vivo* can release membrane MVs carrying TF (TFMVs), and are known to be significantly raised in cancer compared to non-cancer patients (Auwerda et al., 2011; Campello et al., 2011). In some studies, elevated levels of TFMVs derived from tumour have been associated with VTE in several cancers such as pancreatic patients (Zwicker et al., 2009; Geddings & Mackman, 2013) and in other diseases such as Irritable bowel disease (Palkovits et al., 2013). They have also been associated with the metastatic process where interaction with endothelium is a key element for VTE initiation and propagation and also for the invasion of a cancer cell into stroma, therefore facilitating the theory that their involvement is an important step for endothelial thrombogenic initiation.

Hypercoagulable states are linked to cancer (Trousseau, 1865; Lee, 2002). Thrombotic disorders are one of the early manifestations of cancer in some patients and are an important cause of cancer treatment compromise (Khorana et al., 2007c). The risk of developing VTE is 6-7 times increased in cancer patients when compared to non-cancer patients (Heit et al., 2000; Blom et al., 2005), and studies consistently

demonstrate VTE to be an early predictor of mortality(Chew et al., 2006). In prothrombotic states, studies have indicated that the endothelium lining blood vessels may be altered (Chirinos et al., 2005). Such changes have been found *in vitro* from treatment of endothelial cells with human blood products such as serum, which led to shorter coagulation time, marked intrinsic or extrinsic coagulation factor generation, and increased thrombin formation (Zhang et al., 2016). A transformed procoagulant endothelium may not be clinically apparent at first, but hypothetically may result in eventual VTE manifestations such as deep venous thrombosis (DVT), which have been established as a cause of high clinical morbidity and mortality in cancer patients (Blom et al., 2005; Blom et al., 2006). Thus, it is theorized that the assessment of the procoagulant activities of endothelial cells, after exposure to prothrombotic products of cancer cells such as TFMVs can reveal a phenotypic and functional transformation of such endothelial cells. TFMVs have been shown to support coagulation in clotting and thrombin generation tests *in vitro* (Bastida et al., 1984; Davila et al., 2008) and promote the metastatic process through angiogenesis, cancer cell survival, immune suppression and invasion (Zahra et al., 2011). For all these processes, the ability to interact with the endothelium is a key element. A deeper understanding of the mechanisms of endothelial involvement in thrombotic events may potentially aid in developing better therapeutic solutions in cancer management.

3.2 Aims

The aim of this chapter is to investigate how these cancer MVs can interact with endothelial cells *in vitro* and potentially confer procoagulant activity (PCA) through:

- Estimation of prothrombin clotting times of endothelial cells
- Evaluation of TFMV expression on endothelial cells
- Measurement of thrombin generation of endothelial cells
- Assessment of thrombin generation of endothelial cells in Factor VII and XII-deficient plasma

Detailed methodology and materials used can be found in METHODS chapter 2. Briefly, four solid cancer cell lines; Head and Neck Squamous Epithelial cell line UMSCC81B, lung cancer PC9, and pancreatic cancer CFPAC-1 and ASPC-1 which express high levels of TF (Yates et al., 2011; Welsh et al., 2012) and primary HUVECs were cultured in their respective media. After incubation in MV-rich media or platelet-free plasma (PFP obtained from cancer patients as described in chapter 5), HUVECs were assessed for clotting ability (METHODS section 2.5), TF expression on the flow cytometer (METHODS section 2.6), and MV adherence on confocal microscopy (METHODS section 2.7) and thrombin generation on the calibrated automated thrombography (CAT) assay (METHODS section 2.8). Statistical analysis was performed on GraphPad software, version 7 on the results expressed as mean ± standard deviation. Student t-tests and Analysis of variance (ANOVA) were used to compare differences in groups while relationships between CT and TF surface expression were assessed with Pearson correlation coefficient on linear regression analysis. P values less than *0.05 were considered significant.

3.3 Results

3.3.1 Optimization: Procoagulant activity of cell-free media containing microvesicles, tumour cells and HUVECs

A few experiments were carried out to optimize the methods used in this chapter. Briefly, cell-free media were obtained from tumour cell suspensions in PBS by double centrifugation; first at 320xg for 5 minutes and then at 1000xg for another 5 minutes, as described in METHODS section 2.4.2. A one-stage clotting assay (section 2.5) was used to assess the PCA of the 4 cancer cells. Cell-free media (100µl per cuvette) was mixed with 100µl of human Normal control (NormTrol) plasma for 2 min at 37°C on a Thrombotrack SOLO coagulometer. 100µl of 25 mM $CaCl_2$ was added to the mixture to initiate a clotting reaction, and then the time it takes for a clot to form (was recorded in duplicate at three independent repeats. As the reconstituted NormTrol

plasma used does not clot without the addition of exogenous TF, all cell-free media obtained from cancer cell lines with detectable cell surface TF expression were found to support coagulation in these assays. (Figure 3.1 and 3.2A). These preliminary studies also showed that subsequent serial dilutions in PBS of the cell-free media which contained tumour MVs, supported coagulation in a concentration-dependent manner (Figure 3.1).

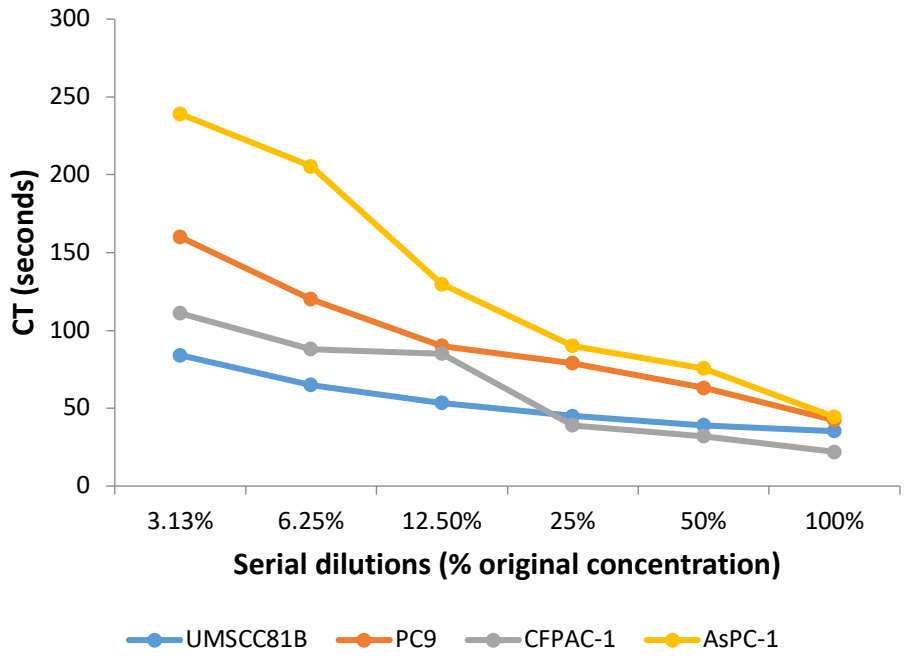


Figure 3.1 Dose-response of cell-free media containing tumour MVs.

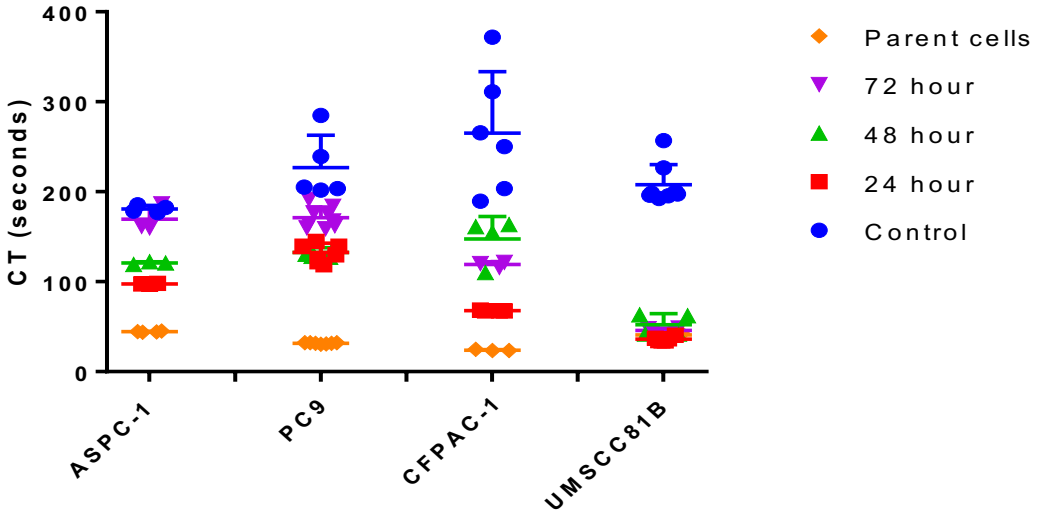
The dose-response of CT with serial dilutions of cell-free media from four solid cancer cell lines: Head and Neck Squamous Epithelial cell line UMSCC81B, lung cancer PC9, and pancreatic cancer CFPAC-1 and ASPC-1 showed a concentration-dependent curve for each of the cell lines. CT was measured with a one-stage clotting assay.

Further initial studies show that clotting ability was lost from filtrate obtained from concentration of tumour media containing MVs in Vivaspin concentrator of MWCO 10 kDa, as described in METHODS section 2.4.2. HUVECs cultured in the filtrate also showed no clotting in the times measured suggesting that the majority of the clotting activity was in fact associated with MVs as the main proportion of MVs were presumably removed by the filtration step. There was also no clotting seen in HUVECs supernatant before and after incubation with tumour MV-rich media. Also, CFSE-

staining as described in section 2.7.1 did not alter the PCA of MV-rich media produced from stained UMSCC81B (CT= 25.57+/- 2.8 seconds; p=0.67) compared to MV-rich media from UMSCC81B without stain (CT=27.43+/3.81 seconds).

3.3.2 Procoagulant activity of HUVECs cultured in cell-free media containing UMSCC81B tumour microvesicles (MV-rich media)

HUVECs incubated in UMSCC81B media containing MVs, showed the shortest PCA times measured on the CT assay (section 2.5) amongst the 4 cancer cell lines used to obtain MV-rich media (Figure 3.2A), and so was selected for further assays and analysis. HUVECs showed no significant difference in CTs cultured with either spent MV-rich tumour media or fresh MV-rich tumour media (p=0.97). Relative to controls (unexposed HUVECs), exposed HUVECs also exhibited PCA in a tumour media concentration-dependent but not time-dependent manner, with the shortest CT (48.59 +/-5.72 seconds) seen with 100% concentration of UMSCC81B media containing MVs (Figure 3.2B). There is a statistically significant overall difference (*p=0.0113) of the mean CTs (88.4+/-34.5 seconds) of HUVECs exposed to media derived from UMSCC81B, from the control HUVECs (234.2 +/- 53 seconds) that were cultured without UMSCC81B MV-rich media influence (Table 3.1, Figure 3.3). However, there was no significant difference overall between HUVECs CT in NormTrol and HUVECs CT in factor XII deficient plasma (p=0.0842).



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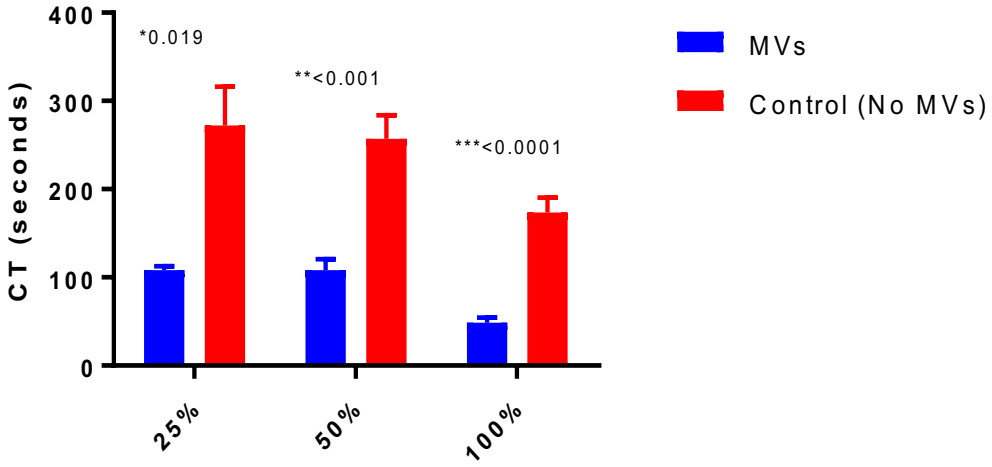


Figure 3.2 Clotting times of HUVECs cultured in MVs-rich media of solid cancer cell lines (parent cells).

A.) HUVECs cultured in UMSCC81B MV-rich media shows the shortest CTs of <50 seconds amongst the 4 solid cancer cell lines media assessed over 24-72 hours and was selected for further assays. HUVECs control was cultured in tumour media without MVs. Data represents mean +/- S.D of n=4 or more B.) HUVECs harvested from 25%, 50% and 100% concentration of tumour media containing MVs and the corresponding controls of 25%, 50% and 100% media concentration without MVs. This shows the shortest CT at 100% MV media concentration at 48.59 +/- 5.72

seconds compared with control at 174 +/- 16.8 seconds (***p<0.001; ANOVA). Each value represents mean of 3 +/- S.D. Data significant at $p < 0.05$.

Table 3.1 Clotting times (CT) of HUVECs cultured in UMSCC81B MV-rich media over time.

% Concentration of MV-rich tumour media used over 24-72 hour	CT of HUVECs with NormTrol Plasma	CT of Control HUVECs with NormTrol Plasma	t-tests comparing HUVECs and Control HUVECs in NormTrol Plasma
0	-	-	-
24 hour			
25%	113.2±1.4	293.6±47.1	*<0.05
50%	110.8±19.1	328.6±51.8	**<0.01
100%	54.9±12.5	282.1±13.2	***<0.001
48 hour			
25%	117.8±3.5	216.3±37.6	0.06
50%	94.7±14.8	284±50.4	**<0.01
100%	45.3±4.7	156.9±9.1	***<0.001
72 hour			

25%	98.7±2.8	440.3±18.2	***<0.001
50%	124.5±39	182.9±25.3	0.2
100%	41.0±0.3	171.2±1.9	***<0.001

HUVECs were cultured in 0, 25%, 50% and 100% concentrations of cell-free supernatants of UMSCC81B cells containing MVs for 24, 48, 72 hours and compared to untreated controls. Data represents mean +/- S.D of n≥3, significance at *p< 0.05, **p<0.01, ***p<0.001, Multiple t tests for comparison of results for HUVECs CT with Normal plasma (NormTrol).

A)

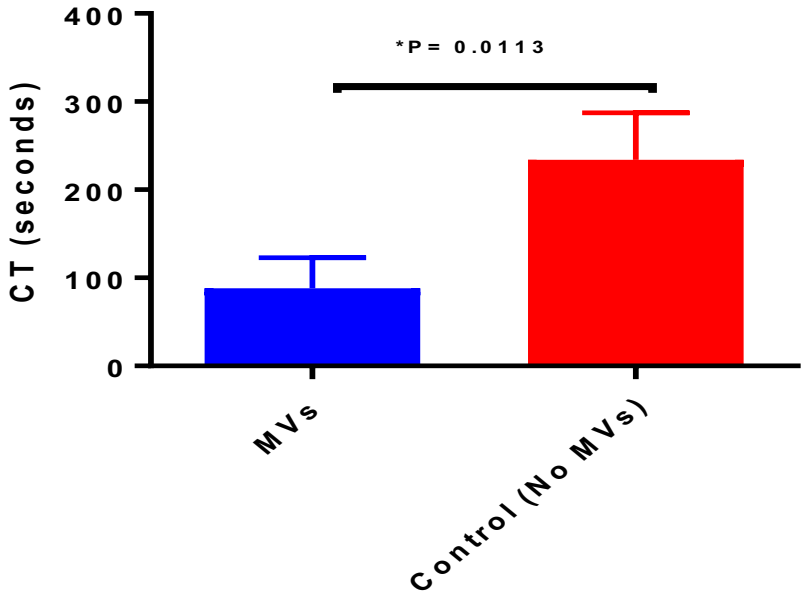


Figure 3.3 Effect of tumour MVs on the CTs of HUVECs incubated in UMSCC81B MV-rich media and control media without UMSCC81B MVs.

Over 24-72 hours, the overall mean clotting time in HUVECs cultured in tumour media of 88.4+/-34.5 seconds and 234.2 +/- 53 seconds in controls, showed a significant difference (*p= 0.0113; t-test) between both groups with 62.3% increase in clotting ability upon addition of MVs to HUVECs media. Data represents mean +/- S.D of n=4

3.3.3 Tissue Factor surface expression on HUVECs cultured in MV-rich tumour media

Flow cytometry analysis showed a distinct subpopulation of HUVECs that had acquired TF expression (Figure 3.4). The percentage of this subpopulation was dependent upon the concentration of tumour media added (containing UMSCC81B MVs) to the HUVECs culture and not the duration of incubation. The range of TF-positive HUVECs was determined to be 0, 4.2(+/-1.4), 12.5(+/-3.7) and 45.9(+/-18.7)

% for MV-positive media concentration of 0, 25, 50 and 100% (n=5) (**M1** in Figure 3.4). The percentage of TF-positive subpopulation (**M1**) increased by 4.2(+/-1.4)%, 12.5(+/-3.7) %and 45.9(+/-18.7)% as the concentration of tumour media containing MVs used in HUVECs incubation increased from 25% to 100%. There was no difference seen by increasing the duration from 24 to 72 hours.

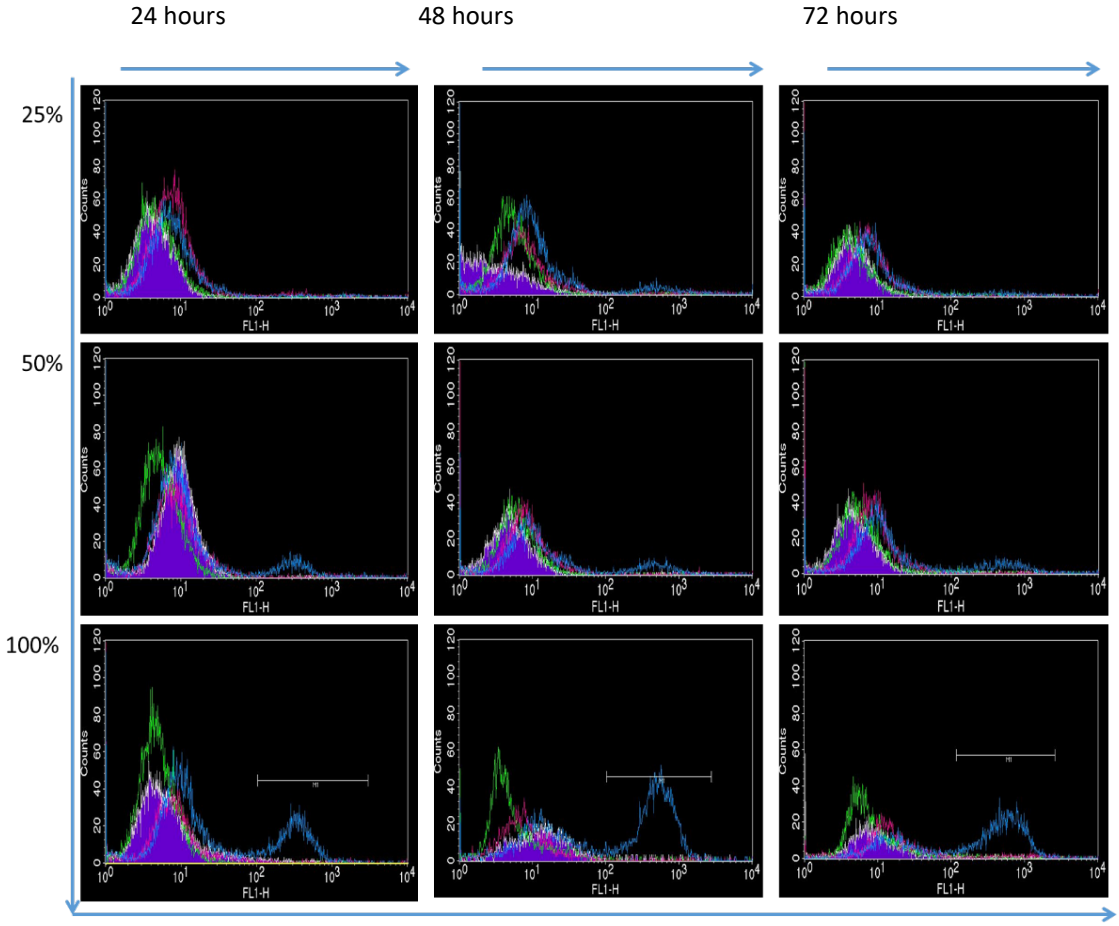
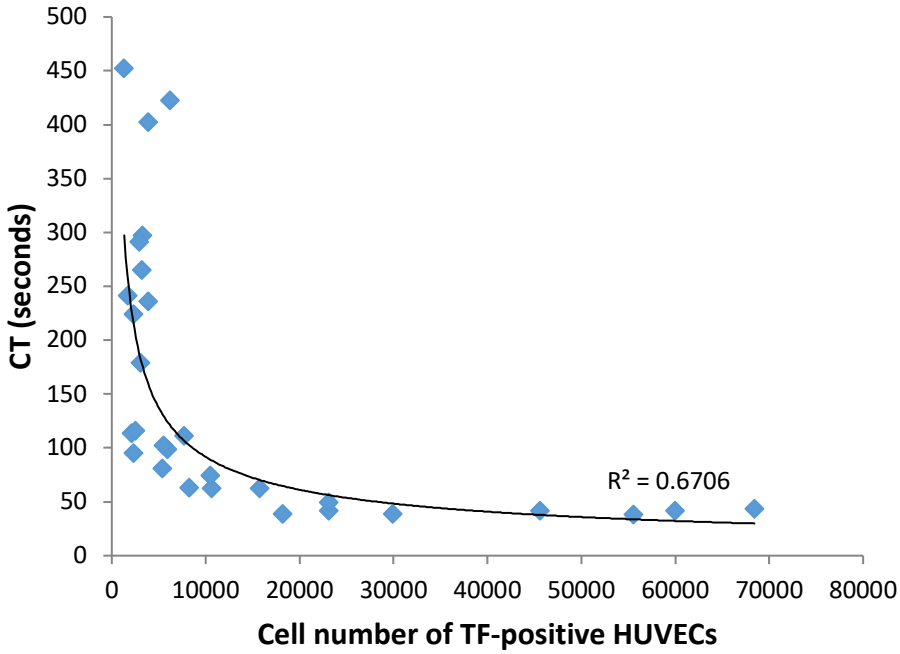


Figure 3.4 Time course assay showing TF surface attachment to endothelial cells. HUVECs were incubated with cell-free media with or without tumour MVs at various concentrations from 25% to 100% at 24, 48, 72 hours. Cells were then washed and labelled with 5µg of CD142:FITC monoclonal antibody with mouse anti-human IgG_1 as isotype control. Mean Fluorescence intensity (MFI) for each time point was measured on a flow cytometer with the HUVECs incubated without tumour MVs as negative controls. ■ IgG_1 isotype in HUVECs control, ■ IgG_1 isotype in test HUVECs, ■ TF expression in HUVECs control, ■ TF expression in test HUVECs

Correlating the actual number of TF-positive HUVECs per CT assay (derived from the % expressing TF, at 100,000 cells per assay) to coagulation times obtained showed a logarithmic relationship (Figure 3.5A); a finding that exists for tumour cells that has been previously reported in our laboratory (Welsh et al., 2012). Here, a near linear relationship existed between CT and TF expression on a Log_{10}/Log_{10} plot such that high TF expression tends to result in short CT times, $r^2 = 0.7$ (Figure 3.5B).

A)



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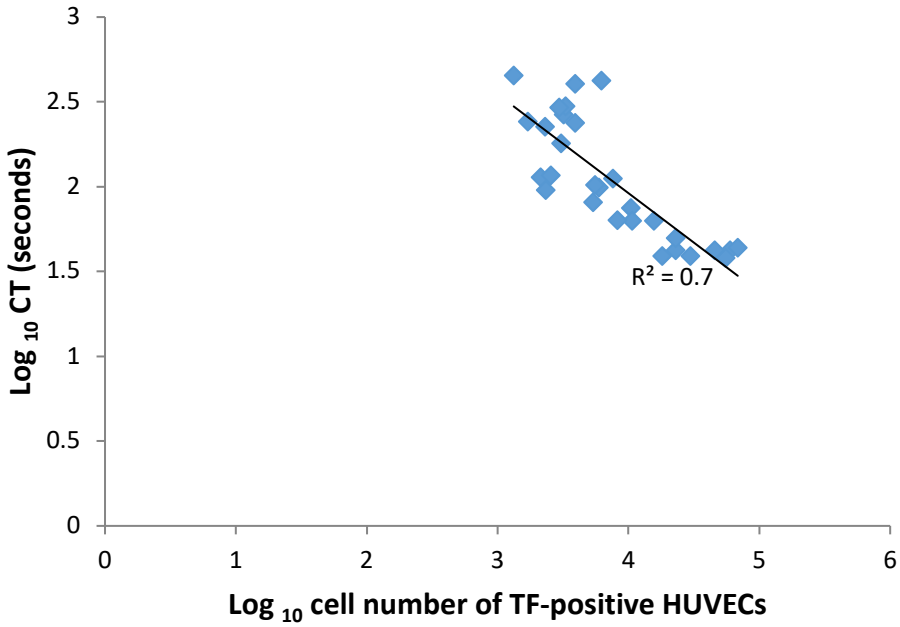


Figure 3.5 The cell surface TF expression of HUVECs cultured in media containing MVs of UMSCC81B cells.

Following 24-72 hour cell culture, HUVECs were assessed by flow cytometry and plotted against their average CT from a one-stage clotting assay. A) Inverse correlation of TF expression with CT such that as TF expression increases, CT decreases. B.) Near linear relationship of CT with TF expressed per 10^5 HUVECs number on Log_{10}/Log_{10} transformed data using regression, r^2=0.7.

3.3.4 The effect of TF-blocking on HUVECs cultured in MV-rich tumour media

To confirm the previous findings above that demonstrate a relationship between the TF and the CT of HUVECs cultured in MV-rich tumour media, the contribution of TF in the PCA of HUVECs was explored by the addition of anti-TF antibody to neutralize TF surface expression on HUVECs. Briefly, HUVECs cell suspensions (1 x 10^5 cells/100 µl PBS) were pre-treated with an anti-TF antibody (5 µg/ml) for 30 min at 37°C as detailed in METHODs section 2.5.1, and the CT time assessed in triplicate. This anti-

TF blocking resulted in a decrease of the procoagulant effect significantly by 56% from 214+/-5.2 to 424.23 +/-11.8 seconds, although it did not abolish CT completely in the times measured from the HUVECs cultured in 100% tumor media concentration (complete replacement of HUVECs ECG media with tumour MV-rich media) (Figure 3.6).

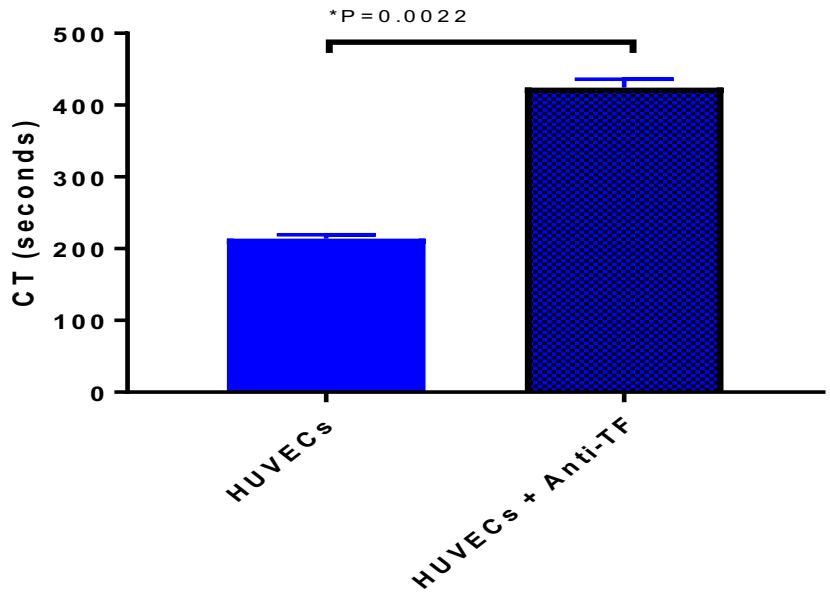


Figure 3.6 Effect of TF-inhibition on CT of HUVECs cultured in MV-rich tumour media over 24 hours.

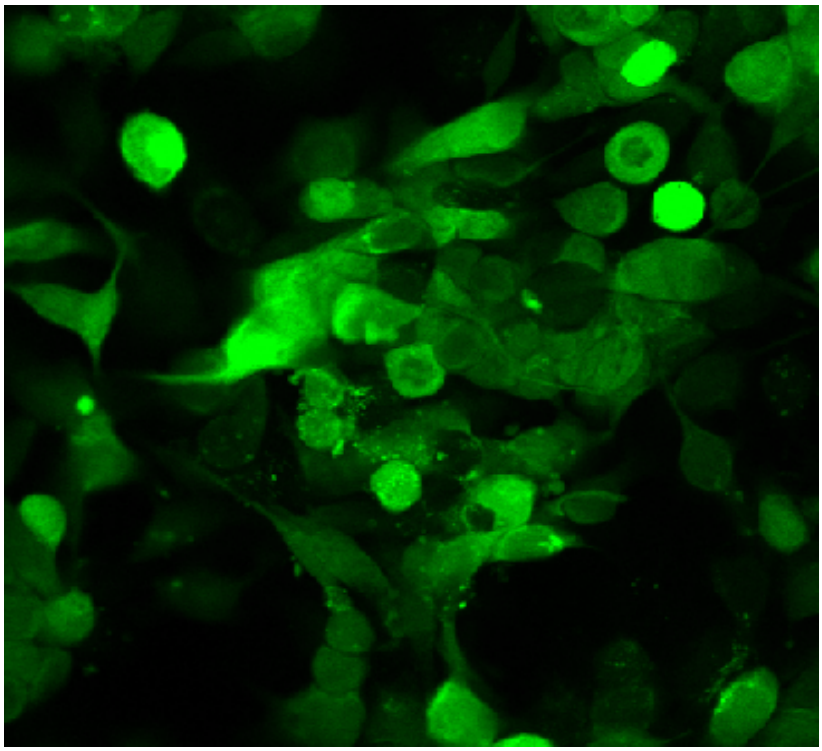
Inhibition of coagulation by incubation of TF-positive HUVECs with anti-TF competitive blocking agent reduced the procoagulant effect significantly by 56 %(*p=0.0022; t-test). Each value represents mean of 3 +/- S.D. Data significant at $p < 0.05$.

3.3.5 Localization of TFMVs on HUVECs endothelial cell surface after 24 hour incubation in MV-rich tumour media

CFSE- intracellular staining of UMSCC81B cells during cell culture was used to fluorescently label released MVs prior to incubation with HUVECs (as described in section 2.7.1). After incubation HUVECs acquired fluorescence corresponding to uptake of CFSE-labelled MV, which was confirmed by confocal microscopy. In figure

3.7, confocal microscopy showed UMSCC81B cells stained with CFSE, HUVECs expressing CFSE-labelled MVs derived from UMSCC81B media and control HUVECs.

A)



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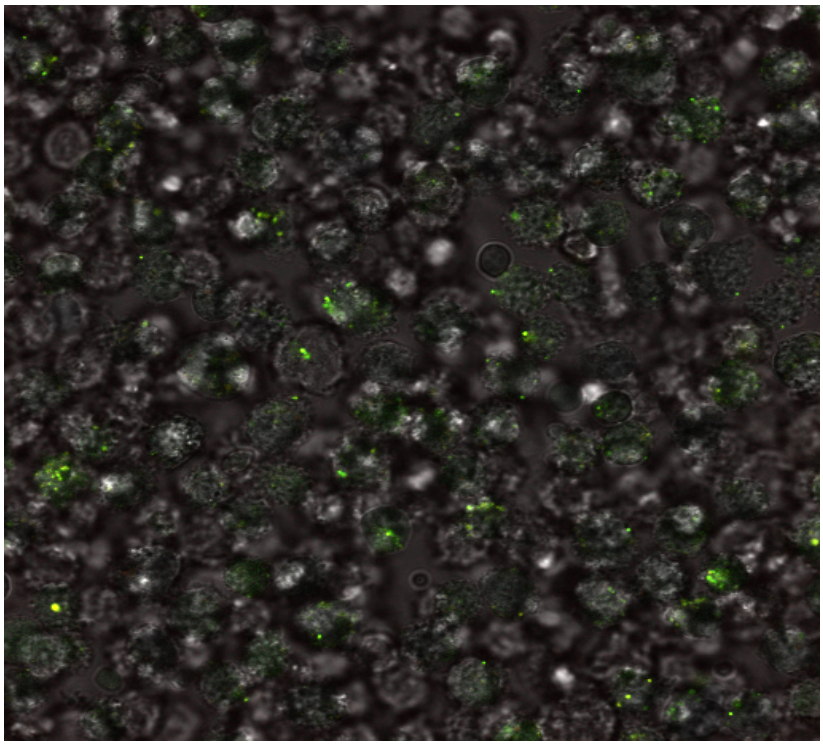

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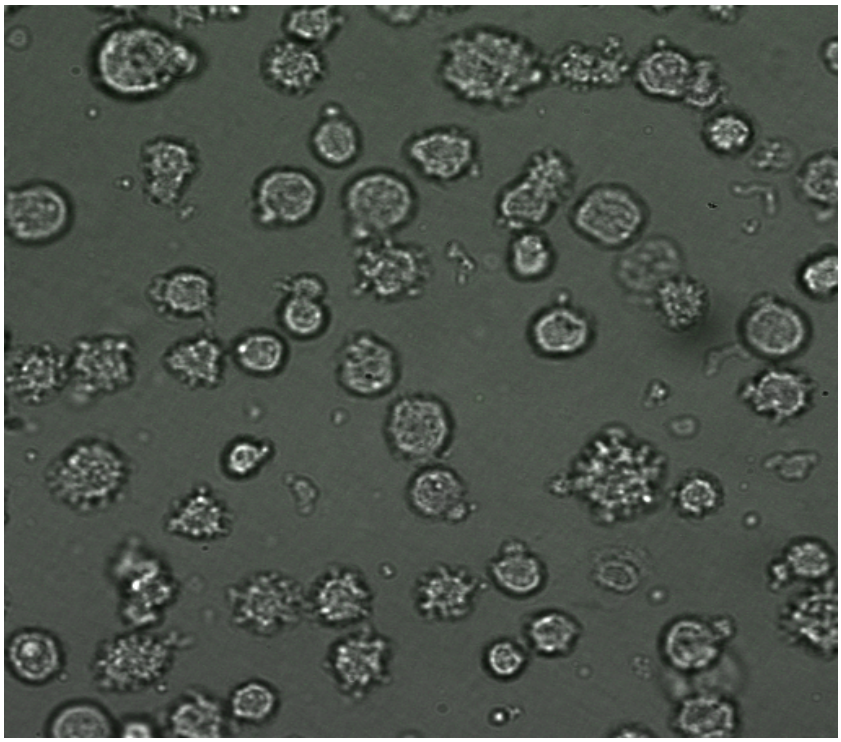


Figure 3.7 Localization of TFMVs on HUVECs cell surface after 24 hour incubation in MV-positive media from cancer cells.

A) UMSCC81B's producing CFSE-labelled tumour MVs B.) HUVECs acquiring CFSE-stained MVs C.) Control HUVECs cultured without MV presence.

3.3.6 Cell-induced Thrombin generation (TG) in HUVECs cultured in MV-rich tumour media

HUVECs harvested from MV-rich media (HUVECs TF) and fresh tumour media (HUVECs control) was assessed for thrombin generation (TG) in a Calibrated automated thrombography (CAT) assay (as described in METHODs section 2.8.1), at 1 x 10^6/ml cell concentration per microplate well. As negative controls, HUVECs cultured in ECG media without any treatment (HUVECs plain) and platelet-free plasma (plasma only) was also assessed for TG. The TG in 3 plasma conditions was measured as described for the cancer cell lines in section 2.8.1 using NormTrol or plasma deficient in coagulation factors VII (FVII) and XII(FXII), to assess if procoagulant HUVECs are of the TF-FVII dependent coagulation type. Overall, ANOVA

tests with multiple comparisons showed significant differences between the means ±S.D of the TG values obtained (Table 3.2, Figure 3.8).

Table 3.2 Thrombin generation in HUVECs cultured in MV-rich tumour media.

	Platelet-free plasma		Lag times (minutes) /p value vs NormTrol	ETP (nM/minute) /p value vs NormTrol	Peak thrombin (nM) /p value vs NormTrol	TTP (minutes) /p value vs NormTrol
HUVECs plain	NormTrol		9.073±0.74	1568±11	137±1	14.56 ±0.6
	Factor	XII-deficient	8.63±4.16	1320±41	65±16	23.14±3.41
	Plasma		/0.96	/0.34	/*0.04	/*0.011
	Factor	VII-deficient	18.79±0.62	-	12±1	60.7±4.11
	Plasma		/***0.0001		/**0.0034	/***0.0001
HUVECs Control	NormTrol		6.47±1.13	1521±144	133±34	13.27±3.69
	Factor	XII-deficient	8.33±1	1502±154	85±35	20.52±2.96
	Plasma		/0.45	/0.96	/*0.013	/***0.0001
	Factor	VII-deficient	15.31±2.93	1299±11	69±22	25.52±1.32
	Plasma		/***0.0001	/0.59	/*0.013	/***0.0001
HUVECs TF	NormTrol		2.58±0.73	1547±139	177±16	6.15±0.56
	Factor	XII-deficient	3.67±1.27	1663±132	147±23	7.028±1.11
	Plasma		/0.63	/0.15	/0.058	/0.8
	Factor	VII-deficient	9.42±4.68	1562±53	104±3	22.05±1.54
	Plasma		/***0.0002	/1.1	/**0.0028	/***0.0001

plasma	NormTrol		21.14±2.35	1524±3	154±1	67.67±1.45
only	Factor	XII-deficient	55.66±4.72	-	26/**0.0029	60.67±1
	Plasma		/***0.0001			/0.097
	Factor	VII-deficient	62.67±1.16	-	12/**0.0011	68.68±0.4
	Plasma		/***0.0001			/0.94

HUVECs plain= HUVECs cultured with ECG media, HUVECs control=HUVECs cultured in fresh Roswell Park Media Institute (RPMI) tumour media, plasma only= plasma (NormTrol, Factor VII or Factor XII-deficient) were all used as controls for HUVECs TF (HUVECs harvested from culture in MV-rich media of TF-positive UMSCC81B). Values represent mean ± S.D of n=3.

3.3.6.1 Lag times

In NormTrol plasma, as shown in table 3.2, Lag times was shortest significantly (p=0.0001) in HUVECs TF at 2.58±0.73 minutes when compared to the controls (HUVECs plain and HUVECs control). Lag times of HUVECs plain (9.07±0.74 minutes) was 2.6 minutes longer than that of HUVECs control (6.47±1.13 minutes), and also over 60% longer than lag times of HUVECs TF of 2.58±0.73 minutes.

However, the overall Lag times was prolonged in FVII-deficient plasma compared to NormTrol plasma (Table 3.2). For instance, Lag times of HUVECs TF in NormTrol of 2.58±0.73 minutes increased significantly (p=0.0002) by 72.6% to 9.42±4.68 minutes in FVII-deficient plasma. Also, Lag times of HUVECs control increased by over 136% from 6.47±1.13minutes in NormTrol to 15.31±2.93 minutes (p=0.0001) in FVII-deficient plasma. Results obtained from HUVECs in factor XII-deficient plasma were similar to those in NormTrol plasma (Figure 3.8A).

3.3.6.2 Endogenous Thrombin Potential (ETP)

In NormTrol plasma, no significant differences (p=0.9305) were observed in the ETP of the 3 types of HUVECs assessed (Table 3.2); HUVECs TF had ETP of 1547±139 nM/minute while HUVECs control had 1521±144 and HUVECs plain had ETP of 1568±11 nM/minute.

Although there was no significance, HUVECs control in FVII-deficient plasma overall had slightly less thrombin generation potential (1299±11 nM/minute) than in NormTrol plasma (14.5%, 1521±144 nM/minute) and FXII-deficient plasma (13.5%, 1502±154 nM/minute). In addition, HUVECs TF ETP slightly increased from 1547±139 nM/minute in NormTrol plasma to 1663±132 in FXII-deficient plasma but also showed no statistical significance on further analysis. Both HUVECs plain in FVII-deficient plasma, as well as plasma deficient in both FVII and FXII (plasma only) were incapable of attaining any thrombin potential that was recordable on the assay (Figure 3.8B).

3.3.6.3 Peak thrombin

In NormTrol plasma, peak thrombin of HUVECs TF (177±16nM) was significantly higher (p=0.03) than HUVECs plain of 137±1nM and HUVECs control of 133±34 (Table 3.2).

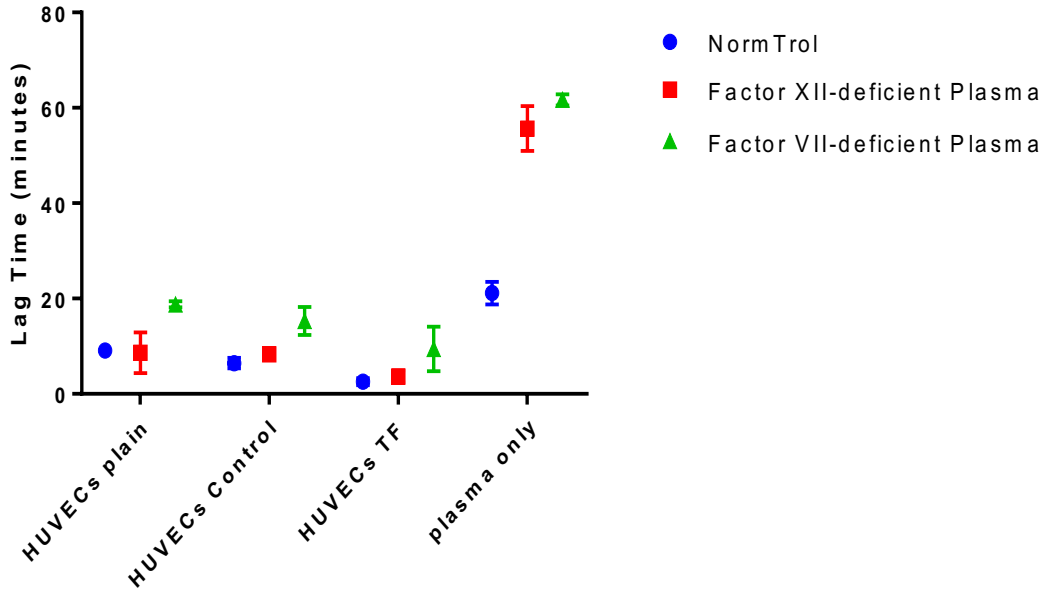
Peak thrombin produced by HUVECs TF and controls in FVII-deficient plasma was overall significantly less than in FXII-deficient and NormTrol plasma (Table 3.2, Figure 3.8C). For instance, HUVECs TF produced average peak thrombin of 177±16nM in FXII-deficient plasma and 147±23nM in NormTrol, which was 41.6% and 29.3% respectively higher than the 104±3nM of thrombin produced in plasma devoid of FVII. HUVECs control also produced less thrombin (69±22 nM) in FVII-deficient plasma compared to the 85±35 and 133±34 nM generated respectively in FXII-deficient and NormTrol plasma. These values were significant (p=0.0028) by 2-way ANOVA testing.

3.3.6.4 Times-to-peak (TTP)

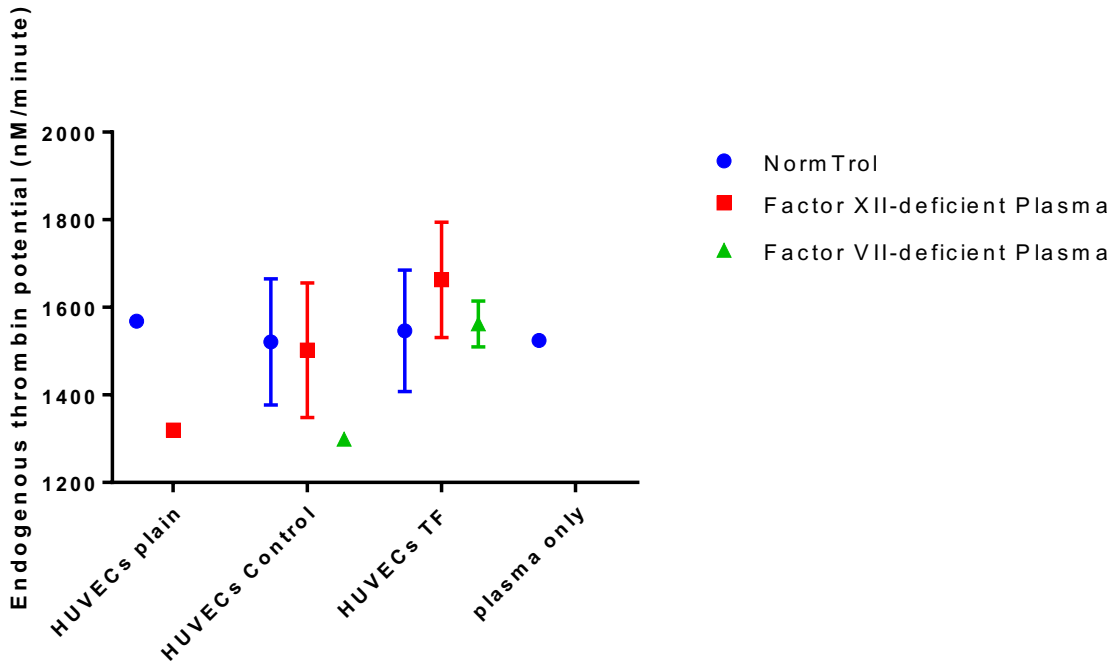
In NormTrol plasma, the TTP of HUVECs TF was the shortest significantly (p=0.0058) at 6.15±0.56 minutes when compared to the controls (Table 3.2). The TTP of HUVECs plain (14.56±0.6 minutes) was 1.29 minutes longer than that of HUVECs control (13.27±3.69 minutes), which was also 57.7% longer than TTP of HUVECs TF at 6.15±0.56minutes

These TTP values were similar to values obtained in Lag times, with those from HUVECs in FXII-deficient plasma closely similar to values generated in NormTrol and higher that those in FVII-deficient plasma. For instance, the TTP of HUVECs TF in FVII-deficient plasma of 22.05±1.54 minutes was 72% significantly higher than that obtained in NormTrol at 6.15±0.56minutes (p=0.0001). Also, TTP of HUVECs plain was significantly and markedly prolonged to 60.7±4.11 minutes (p=0.0001) in FVII-deficient plasma compared to NormTrol plasma (Table 3.2, Figure 3.8D).

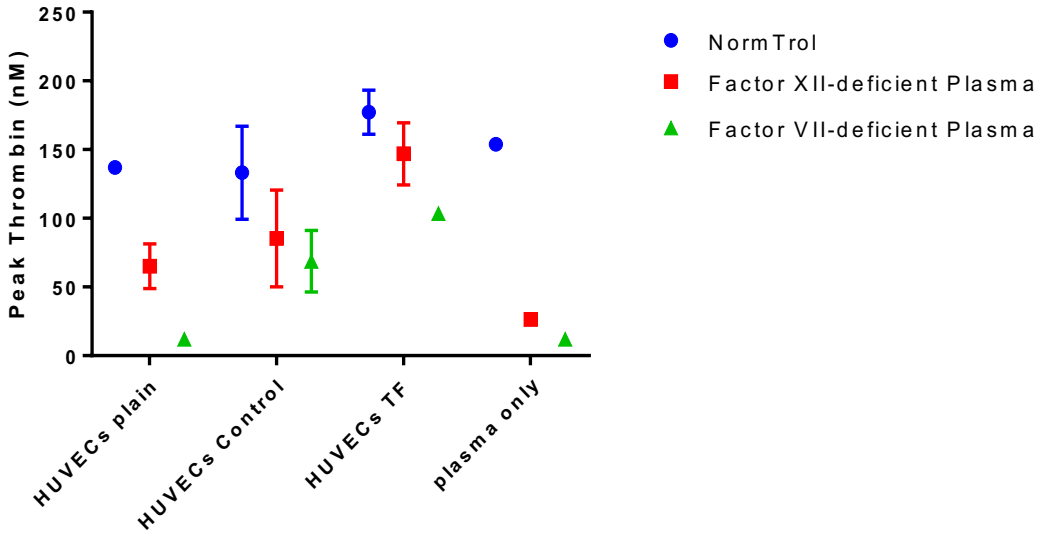
A)


Lag Time (minutes)
80
60
40
20
0
HUVECs plain
HUVECs Control
HUVECs TF
plasma only
NormTrol
Factor XII-deficient Plasma
Factor VII-deficient Plasma

B)


Endogenous thrombin potential (nM/minute)
2000
1800
1600
1400
1200
HUVECs plain
HUVECs Control
HUVECs TF
plasma only
NormTrol
Factor XII-deficient Plasma
Factor VII-deficient Plasma

C)



D)

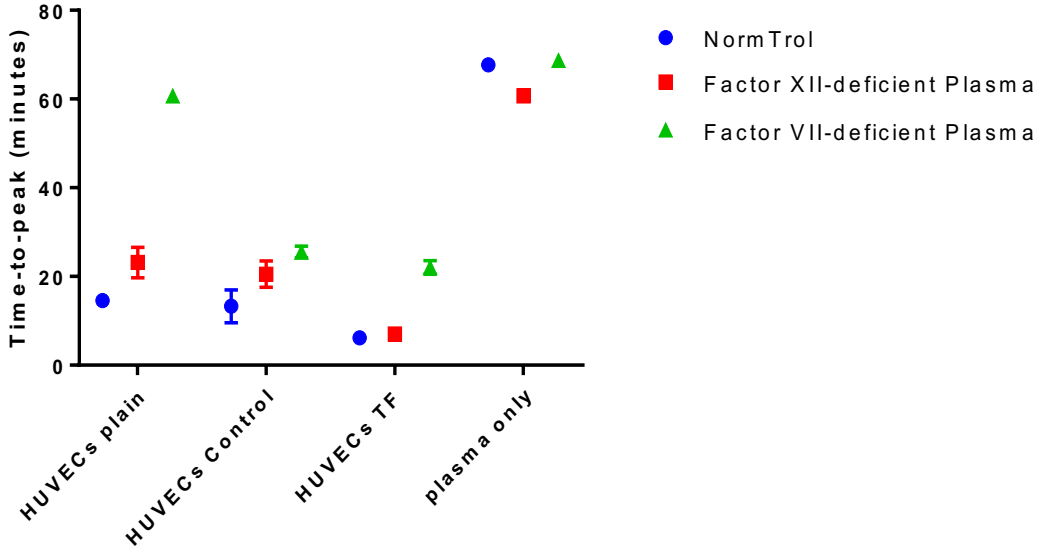


Figure 3.8 Thrombin generation in HUVECs cultured in MV-rich tumour media.

A) Lag times B) ETP C) Peak thrombin D) Time-to-peak**.** HUVECs plain was HUVECs cultured with Endothelial cell growth (ECG) media, HUVECs control was HUVECs cultured in Roswell Park Memorial Institute (RPMI) media, plasma only was NormTrol or coagulation factor-deficient plasma which were all used as controls for HUVECs TF (HUVECs harvested from culture in MV-rich media of TF-positive UMSCC81B). Values represent mean +/- S.D of n=3.

3.3.7 Procoagulant Activity of HUVECs after 24 hour incubation in MV-positive patient plasma

HUVECs were cultured for 24 hours in 1ml of defrosted platelet-free plasma (PFP) (METHODS section 2.4.2) derived from whole blood of Multiple myeloma (MM) and Pancreatic cancer (PC) patients before treatment (described in section 2.8.3). Prior to this, the mean CT of PFP of both MM and PC patients were determined (section 2.5.2) as 438 seconds and 265 seconds respectively, before the CT and TF expression of HUVECs on flow cytometry were assessed. PFP from PC and MM patients before treatment had previously been determined to contain MVs in published data from our group (Echrish et al., 2014; Hall et al., 2016). HUVECs viability reduced from >95% to <74% after 24 hours of culture in PFP. Although there was a 14.4% slight reduction of CT between control HUVECs and test HUVECs cultured in PC PFP (from 320 seconds to 274 seconds), this difference was insignificant ($p=0.091$) and there was no quantifiable TF expression on flow cytometry, hence only n=2 repeats were performed. HUVECs cultured in MM PFP did not show any clot formation in 2 repeats, and so this experiment was discontinued.

3.4 Discussion

Although TFMVs have been associated with pathogenesis of thrombosis in studies (Tesselaar et al., 2007), there are controversies regarding the production, initiation and expression of TF in pathologic amounts on endothelium during the growth and expansion of a tumour, due to its complex physiology. A few studies have explored the role of TFMV induced coagulation activity of the endothelium under cancer progression (Maiolo et al., 2002; Collier et al., 2013). In this study, the changes in PCA and TF surface expression in HUVECs under the direct influence of tumour microvesicles, may mark the transfer of hypercoagulability and the acquisition of an endothelial phenotype which can initiate and sustain clotting. The distinct population of HUVECs that acquired TF via tumour MV were shown to then promote coagulation in a cell number dependent manner with a near logarithmic relationship

demonstrated (Figure. 3.5) as has been previously shown for tumour cell-mediated coagulation in our laboratory(Welsh et al., 2012), that was also dependent upon cell surface TF expression(Tesselaar et al., 2007). The TF expression detected on HUVECs, after incubation with tumour media was consistent within the 2nd and 3rd log decade of fluorescence (Figure 3.4) which may suggest that the tumour MVs released during culture are also consistent in TF expression.

As removal of MVs in tumour media resulted in no PCA or surface TF expression, the results of this study suggest that such hypercoagulability transfer from cancer cells to endothelial cells may likely involve tumour MVs. A previous study reported the induction of procoagulant effect by monocytic-derived MVs in endothelial cells(Aharon et al., 2008). Co-cultures of HUVECs with HeLa and HL60 cells in a 4 hour incubation, have also previously been reported to support the presence of a clotting-defined, two-way interaction between tumour cells and endothelial cells in a TF-related manner(Maiolo et al., 2002). This interaction may also vary depending on other features. For example, although TF is involved significantly in the PCA of HUVECs exposed to tumour MVs, the activity is not completely dependent on TF as anti-TF blocking did not completely abolish clotting (Figure 3.6). Therefore, other factors such as the presence of phospholipids (for example phosphatidylserine and phosphatidylcholine) and their receptors, may contribute to a lesser degree in HUVECs PCA. Moreover, there was no significant overall variation between HUVECs TG in NormTrol and Factor XII deficient plasma ($p=0.0842$), denoting that this clotting interaction may not be dependent on factor XII presence in intrinsic coagulation pathways.

There may be two mechanisms behind the acquisition of TF by endothelial cells. The first is extracellular and is possible through the release of TF from MVs and subsequent attachment to the endothelial cell surface. The other is an internal process either by engulfment of TFMVs with subsequent intracellular degradation, TF membrane incorporation and later re-exposure by blebbing on the endothelial cell surface (Aharon et al., 2008; Collier et al., 2013) or by initiation of an intrinsic endothelial TF production due to stimuli from external MVs. An alternative is that

both might occur at the same time, or specific stimulus and conditions might dictate the prevalence of one mechanism rather than the other. HUVECs have been shown to internalize MVs *in vitro* through a process of phospholipid carrier via integrin αvβ3 and lactadherin (Terrisse et al., 2010). The engulfment and recycling of MVs through the Rab family of golgi-endosomal transport network has also been shown with two different TF presence levels under a short 4 hour duration(Collier et al., 2013). Further downstream, the implication of TF-VIIa-protease-activated receptor-2 or PAR-2 signalling systems in thrombin generation (and activation of other trans-membrane G-protein-coupled receptors) leads to transcription of more prothombotic genes, amplification of signal transduction cascades in the host cells and facilitates tumour establishment (Rickles et al., 2003; Ruf et al., 2011). Of note, is the possibility that PAR-1 can also trans-activate PAR2(O'Brien et al., 2000), which promotes additional thrombin generation response in the endothelium and tumour environment(Shi et al., 2004). Also, TF has various variants and isoforms such as the systemically active flTF, and levels of encryption that may be produced under different oncogenic influences which may ultimately affect the PCA(Bogdanov et al., 2003; Bach, 2006; Wang et al., 2012).

Endothelial cells can differentiate into different cell classes or types depending on the kind of stimulation received from their microenvironment (Lacorre et al., 2004). *In vivo* this plasticity is usually seen only with high doses of activation agents such as interleukin-6, lipopolysaccharides, tumour necrosis factor alpha (Sprague & Khalil, 2009; Adams et al., 2013). Furthermore, endothelium from different body areas reflect specific tissue demands and are heterogenic (Aird, 2012), adding another level of complexity to the stimulation processes of the endothelium. This may explain the non-uniform acquisition of TF observed in distinct HUVEC populations as the source is from pooled donors; however, the data was consistent across differing batches of HUVECs. In addition, although the data was inconclusive regarding differences seen in HUVECs cultured in actual patient plasma, this does not reflect *in vivo* situations, and may be due to temperature differences, or insufficient quantities of plasma used that may be unable to induce a significant procoagulant change in the endothelial cells.

Although activated cells may acquire procoagulant activity similar to their parent cells, not much is known about their actual ability to generate thrombin. Thrombin is an important constituent of the clotting cascade, with the ability to initiate clot formation. In this chapter, it is shown that HUVECs associated with tumour MVs can produce thrombin, in sufficient quantities greater than the controls (Table 3.2, Figure 3.8). This thrombin generation also varies depending on the amount of coagulation factors, specifically factor VII present in the plasma. This result can be understood in view of the relationship of TF to factor VII, in the formation of a complex that initiates coagulation pathways. This relationship may also have important consequences in the method of recruitment of endothelial cells in prothrombotic conditions, such as cancer development. Lag times and TTP are also shown to be significant parameters amongst the TG parameters assessed, suggesting a further role in standardization of the TG assay.

In conclusion, HUVECs acquired a procoagulant phenotype in the presence of increasing concentrations of tumour MVs expressing TF. This acquired procoagulant activity was shown to be dependent on the number of HUVECs that subsequently express TF. It was also demonstrated that the procoagulant state once initiated may have TF-independent drivers, can produce thrombin that is important in clot formation and dependent on factors of the coagulation system, and for the first time suggest that this may not be a 'systemic' effect, but subpopulations of endothelial cells have different susceptibilities that may contribute to the HUVEC-MVs interaction.

Chapter:4 Thrombin generation in live haematological and solid cancer cells and microvesicles determined by calibrated automated thrombography.

Work from this chapter has been presented in the peer-reviewed publication:

Adesanya MA, Maraveyas A, Madden LA. Differing mechanisms of thrombin generation in live haematological and solid cancer cells determined by calibrated automated thrombography. Blood Coagulation and Fibrinolysis. 2017 December;28(8): 602–611.

And in the conference presentations:

Adesanya MA, Maraveyas A, Madden LA. Characterization of the thrombin generation potential of haematological and solid cancer microvesicles by calibrated automated thrombography. Haematology Society of Australia and New Zealand, the Australian & New Zealand Society of Blood Transfusion and the Australasian Society of Thrombosis and Haemostasis (HAA) Annual Scientific Meeting; Melbourne (Australia), November 2016.

Adesanya MA, Madden LA, Maraveyas A. The influence of coagulation factors on thrombin generation in solid tumour and haematological cancer cells by calibrated automated thrombography. European Haematology Association (EHA) Scientific Conference on Bleeding Disorders; Barcelona (Spain), September 2016- *Oral

presentation of Published abstract in official supplement of *Haematologica*; August 16, 2016 101(s2): 1-11.

Adesanya MA, Maraveyas A, Madden LA. Characterization of the thrombin generation potential of Pancreatic cancer and Multiple myeloma by calibrated automated thrombography (CAT). 8th International Conference on Thrombosis and Hemostasis Issues in Cancer (ICTHIC); Bergamo-Milan (Italy), April, 2016-*Published abstract in *Thrombosis Research*: 140 (S1);S186.

4.1 Introduction

Thrombin is a key enzyme in haemostasis; it is a serine protease that forms the end product of the common pathway. The coagulation pathway is composed of intrinsic factors (such as factor XII), and extrinsic factors such as tissue factor (TF) and factor VII complex that ultimately lead to thrombin generation (TG). Multiple myeloma (MM) and pancreatic cancer (PC) manifest a dysfunction of the haemostatic system that gives rise to a high incidence of thrombotic complications, particularly during systemic cancer treatment phase(Khorana et al., 2007b; Dumitrascu et al., 2010; Carrier et al., 2011). However, it is hypothesized that thrombosis may signify different haemostatic processes in both types of cancer. For example, it has been speculated that thrombosis in MM does not influence cancer behaviour and is mostly as a result of treatment, whereas in solid cancers such as PC, it is seen as a sign of aggressive tumour behaviour and poor prognosis indicator (Zangari et al., 2010; Cesarman-Maus et al., 2012a). Furthermore, molecular mechanisms involved in PC thrombosis development, such as TF expression, cyclo-oxygenase-2 (COX-2) and plasminogen activator inhibitor 1 (PAI-1) up-regulation, may ultimately not be important in MM-associated thrombosis due to its intra-vascular nature (Boccaccio & Comoglio, 2009; Cesarman-Maus et al., 2012a). To date few studies have been reported on TG variances using cancer cells themselves. There is limited knowledge on the thrombin generation kinetics in cancer (such as the measurement of thrombin output in various substrates, characterizing and profiling of thrombin generation, the influence of external pathways) and clinical and preclinical (*in vitro*) data in this setting are currently sparse. Therefore, the work in this chapter was undertaken to define TG in solid and haematologic cancers such as PC and MM.

Although several methods exist for assessing procoagulant activity, chromogenic assays are the cornerstone of measuring TG kinetics with the calibrated automated thrombography (CAT) developed as a fluorogenic optimization that may become a useful tool in cancer related clinical settings (Peetz, 2016). Technical optimization is ongoing to make it a point of care test. The assay gives a dynamic picture of TG by measurement of the amount of thrombin cleaved from a fluorogenic substrate in real

time. Hemker et al (Hemker et al., 2003) developed the modern version of the CAT assay that measures 5 main parameters in TG: Lag time, Endogenous thrombin potential (ETP), Peak thrombin produced (Peak), time to peak (ttPeak or TTP), and Velocity index (Vel.Index). With this number of endpoints, the CAT assay gives a more comprehensive clotting assessment and could play a role in clinical practice to assess haemostatic discrepancies in patients with cancer-related clotting disorders. However, there still exists official standardisation issues for the assay results, and more studies are needed to address them in detail.

The traditional substrates for the CAT assay, as developed by Hemker et al, have been platelet poor and platelet-rich plasma. Few studies have documented the use of other substrates including whole blood(Ninivaggi et al., 2012; Tappenden et al., 2016), tumour cells(Takamiya & Sakata, 2008), lysed cells(Marchetti et al., 2012). Although the components of the coagulation cascade are well characterised in haemostasis, there are limited experimental data directed at understanding their relative importance in the coagulopathy of solid versus non-solid malignancies. Various factors of the cascade have been implicated in tumour cell sensitivity to cell-associated procoagulants, including factor XII (Hageman factor) which was found to be necessary for initiation of intrinsic coagulation in malignant haematopoeitic cells via procoagulant phosphspholipids (phosphatidylserine or PS) on charged surfaces(Kunzelmann et al., 2004). In addition, Marchetti et al investigated the residual capacity of cancer cells to generate thrombin, after TF-FVII inhibition, which revealed a possible TF-FVII independent pathway that may occur in these cells(Marchetti et al., 2012). Thus, it is hypothesized that factors VII and XII may have distinct roles in thrombus formation (Kuijpers et al., 2014) as members of the extrinsic and intrinsic pathways respectively, and understanding their contribution to the process of cancer-associated thrombosis could inform clinical approaches to treatment and prophylaxis.

4.1 Aims

The aims of this chapter were to:

- compare the characteristics of the thrombin curves generated by human solid malignant cell lines (with an emphasis on Pancreatic cancer) versus the curves generated by haematological malignant cell lines (with an emphasis on Multiple myeloma cell lines)
- explore the role of the intrinsic and extrinsic pathways in both cell lines by investigating TG in an environment of factor VII and XII deficient human plasma
- assess Tissue factor (TF) expression in these cell lines, in order to gain an insight into its relationship with TG.

Detailed methods for the experiments in this chapter are described in METHODS chapter 2. Briefly, seven solid tumour cells (pancreatic cancer ASPC-1, CFPAC-1, PANC-1, MIA PaCa-2, ovarian cancer SKOV-3, Head & Neck squamous cancer UMSCC81B and lung cancer PC9) and 5 malignant haematological cell lines (Multiple myeloma MM.1S, U266B, H929, plasma cell leukaemia JJN3 and histiocytic lymphoma U937) were cultured as described in METHODS section 2.1. The cells and their MVs released into cell-free media were assessed for TG on a calibrated automated assay in real time. Time to start of TG (Lag time), endogenous thrombin potential (ETP), Peak height of thrombin (Peak), time to peak (TTP or ttPeak) and velocity index (Vel.Index) of TG were evaluated. TF cell surface expressions were also measured with 5µg of anti-human CD142: FITC antibody by flow cytometry (METHODS section 2.6) and the mean fluorescence ratio or MFR (CD142/negative control) for each sample was evaluated and repeated three times on different days. Furthermore, at decreasing cell concentrations ($5x10^6$ - $4x10^4$ cells/ml), UMSCC81B suspended in PBS (100µl per cuvette) was assessed for prothrombin times as previously described in the clotting assay (METHODS section 2.5) (Welsh et al., 2012). Relationships between TG parameters (Lag time, ETP, Peak, TTP, Vel.Index) and TF cell surface expression was assessed with Pearson correlation coefficient on linear regression analysis.

4.2 Results

4.2.1 Thrombin generation in solid tumour and haematological malignancy cell lines in platelet free plasma

Initially, 12 different cell concentrations were used to identify the optimal concentration at which TG signal is detectable in both solid and haematological cancers by the CAT assay. TG was assessed as described in section 2.8 in decreasing cell concentrations from ($5x10^6$ - $4x10^4$ cells/ml) and was observed to be concentration dependent in both main cancer types (Table 4.1). Although ETP (Figure 4.1B) in both solid and haematological cells remained similar, there were linear decreases in Lag times (Figure 4.1A) and TTP (Figure 4.1D) such that as concentration decreases both increased in duration. The inverse was seen for peak thrombin (Figure 4.1C) and Vel. Indices (Figure 4.1E). Lag times and TTP also correlated very strongly (r=0.98, Figure 4.2) amongst all the 5 TG parameters measured. Furthermore, haematological cells had overall smaller TG curves compared to the solid cancer cells. UMSCC81B, CFPAC-1 and AsPC-1 cell lines initiated TG quickest within 1.5±0.2 minutes in NormTrol, followed by SKOV-3 1.6±0.2, PC9 at 1.8±0.1, PANC-1 4.9±1.1 while MIA PaCa-2s are least at 7.6±1.4 minutes. Amongst the haematological cells, U937 was the quickest and required 9.8±1.6 minutes to initiate TG while MM.1S was the slowest, taking 16.2±2.8 minutes (Table 4.1). The intra assay coefficient of variation (C of V) of Lag times, ETP and the rest of the TG parameters were 4.78%, 3.53% and < 5% respectively, while the inter assay C of V of all the TG parameters were < 10%. All cell lines did not hydrolyse the fluorogenic substrate when plasma was absent, and as a result, no reaction occurred in the assay in this condition and no TG was recorded.

Table 4.1 Thrombin generation parameters of 12 cancer cell lines and low 1pM TF standard preparation.

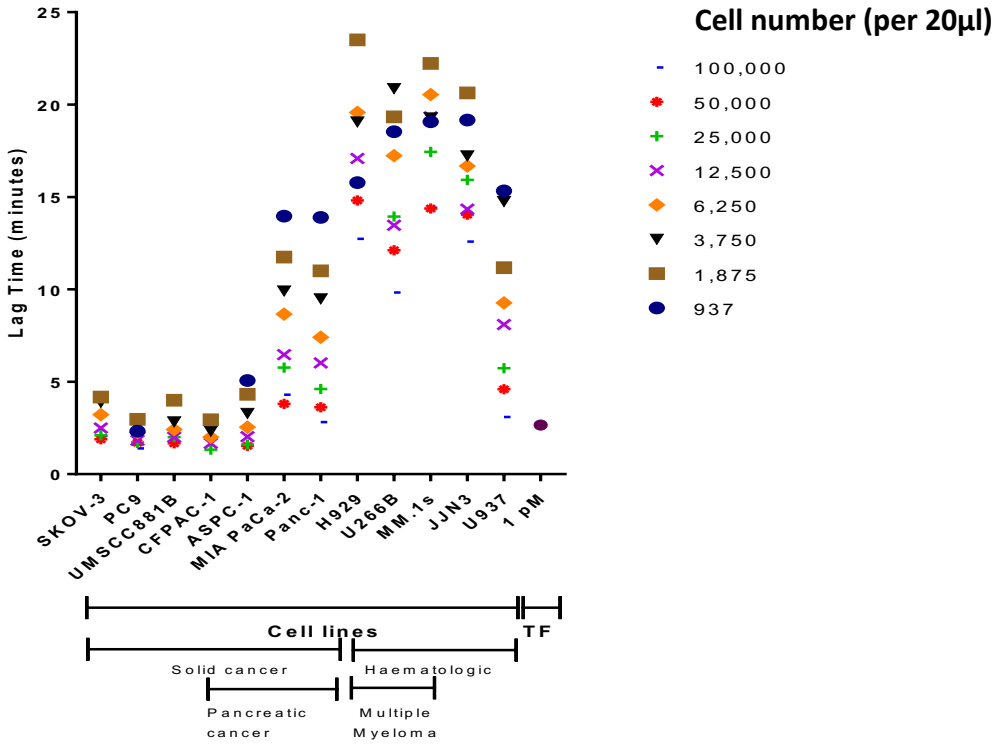
	Platelet free plasma	Lag time (minute)	ETP (nM/minute)	Peak thrombin (nM)	TTP (minute)	Vel. Index (nM/minute)
SKOV-3	NormTrol	1.6±0.2	1299±63	182±14	4.6±0.2	61.6±6.6
	F XII-deficient	1.9±0.3	1730±79*	173±24	5.3±0.1	50.8±3.4
	FVII-deficient	11.8±5.4**	992±172*	65±23**	20.3±6.8***	8.1±4.5***
PC9	NormTrol	1.8±0.1	1265±137	174±10	4.8±0.1	58±3.4
	F XII-deficient	1.5±0.1	1825±119*	233±62*	4.7±0.5	74.7±28.9
	FVII-deficient	9.2±0.1*	1610±132*	121±20*	15.2±4.2**	18.9±9.7***
UMSCC81B	NormTrol	1.5±0.2	1331±203	207±23	4.4±1.1	81.5±26.9
	F XII-deficient	1.7±0.1	1473±142	220±10	4±0.1	94.4±11
	FVII-deficient	10±4.4**	1510±120	104±64***	16.8±8**	18.9±15.8***
CFPAC-1	NormTrol	1.5±0.2	1294±47	217±12	3.7±0.2	95±16.4
	F XII-deficient	1.6±0.2	1722±107*	244±19**	4.2±0.3	93.7±16.6
	FVII-deficient	10.8±4.3***	1332±154	105±38***	17.6±6.1**	19±13.6***
ASPC-1	NormTrol	1.5±0.2	1264±79	162±12	4.8±0.8	50.8±13.4

	F XII-deficient	1.5±0.2	1751±124*	181±19**	6.7±0.8	41.3±12.9
	FVII-deficient	16.8±6.5**	1066±495	60±19***	26±6.8***	6.7±2.5***
MIA PaCa-2	NormTrol	7.6±1.4	1080±107	71.±14	15.1±1.4	9.7±2.7
	F XII-deficient	5.1±2.6	1306±312	99±9	15.9±0.8	23.6±41.9
	FVII-deficient	19.7±6.3***	1261±304	84±32	28.1±6.8***	13.5±11.3
PANC-1	NormTrol	4.9±1.1	1166±96	92±27	12.6±2.2	14.4±6.2
	F XII-deficient	3.8±0.6	1474±331*	95 ±41*	13.5±2.7	11.5±7.4
	FVII-deficient	21.8±3.6***	1104±96	87±27	30.2±3.2**	10.6±3.6
H929	NormTrol	13.2±2.1	1122±71	90±23	19.7±2.8	14.6±5.9
	F XII-deficient	16.5±2.9	1001±305.	49±16	28.5±4.2*	4.2±1.8
	FVII-deficient	21.3±4.7**	1319±385	77+36	30.3±6.4**	9.6±6.4
JJN3	NormTrol	15.4±1.6	1084±108	67±8	23.1±1.7	8.7±1.1
	F XII-deficient	15.3±3.4	825±151	38±5	30.3±6.4*	2.5±0.4
	FVII-deficient	23.6±0.9**	797±155	44±13	34.0±0.8**	4.3±1.3
U266B	NormTrol	15.2±1.3	1086±108	71±12	22.7±1.6	9.6±2
	F XII-deficient	19.3±3.4	682±39**	35±9	34.0±4.0***	2.4±0.4
	FVII-deficient	23.7±3.1**	861±20	47±12	34.8±4.7***	4.4±1.6
U937	NormTrol	9.8±1.6	999±115	60±20	17.8±1.1	7.6±1.7

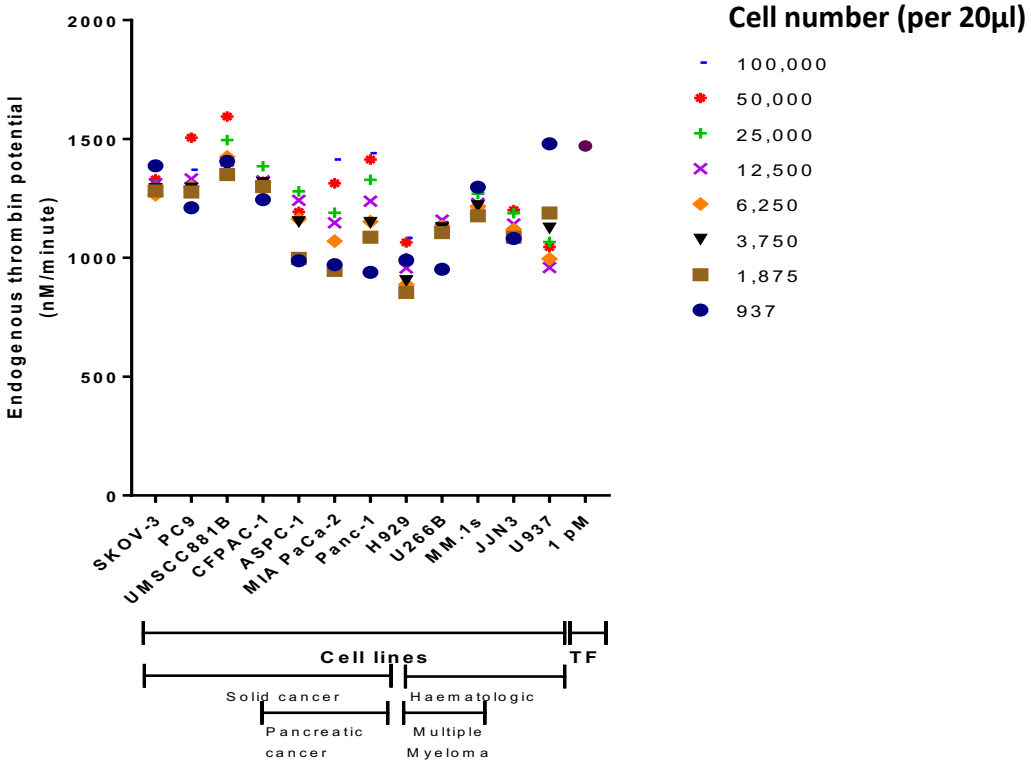
	F XII-deficient	7.6±1.4	670±12	32±3	26.6±5.2*	1.8±0.3
	FVII-deficient	28.0±7***	968±244	46±5	39.2±6.4***	4.2±0.5
MM.1s	NormTrol	16.2±2.8	986±102	65±12	21.7±4.4	9.5±2.8
	F XII-deficient	20.4±9.0	665±95	34±7	35.8±9***	2.3±0.8
	FVII-deficient	29.4±2.4***	702±10	37±6	40.7±2.7***	3.2±0.5
TF standard	NormTrol	2.6±0.1	1399±175	254±6	5.4±0.1	93.5±3.7
	F XII-deficient	2.1±0.1	2011±164***	328±8**	5.0±0	111.6±0.6
	FVII-deficient	14.3±1.8***	1722±70*	219±24	18.5±2***	53.6±11.3***

Cells were collected and washed in PBS at concentration of 0.63 x 10^6/ml to induce TG in 3 different platelet-free plasma (NormTrol, Factor XII-deficient and Factor VII-deficient). TG parameters include Lag time, ETP, Peak, TTP and Velocity Index. Results are mean of n=4 ± S.D performed in duplicates. P values from two-way ANOVA multiple comparisons with NormTrol as baseline compared with Factor VII-deficient plasma and Factor XII-deficient plasma. Significance is at *p< 0.05, **p<0.01, ***p<0.001.

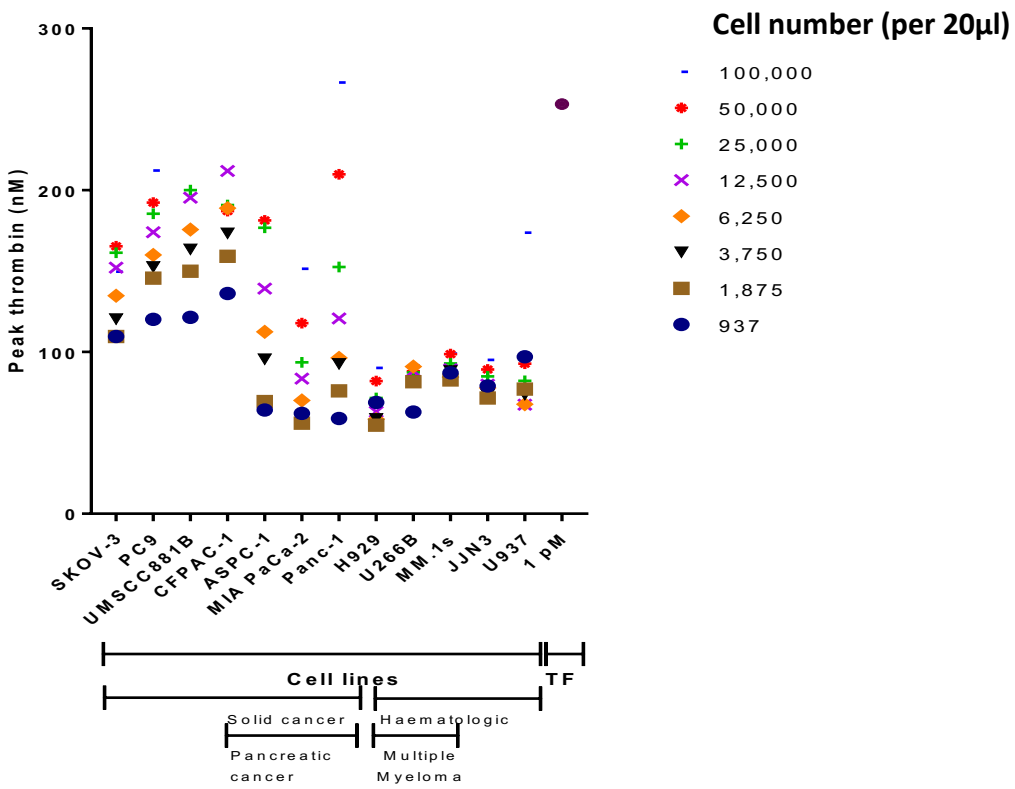
A)


Lag Time (minutes)
25
20
15
10
5
0
SKOV-3
PC9
UMSCC881B
CFPAC-1
ASPC-1
MIA PaCa-2
Panc-1
H929
U266B
MM.1s
JJN3
U937
1 pM
Cell lines
TF
Solid cancer
Haematologic
Pancreatic cancer
Multiple Myeloma
Cell number (per 20µl)
100,000
50,000
25,000
12,500
6,250
3,750
1,875
937

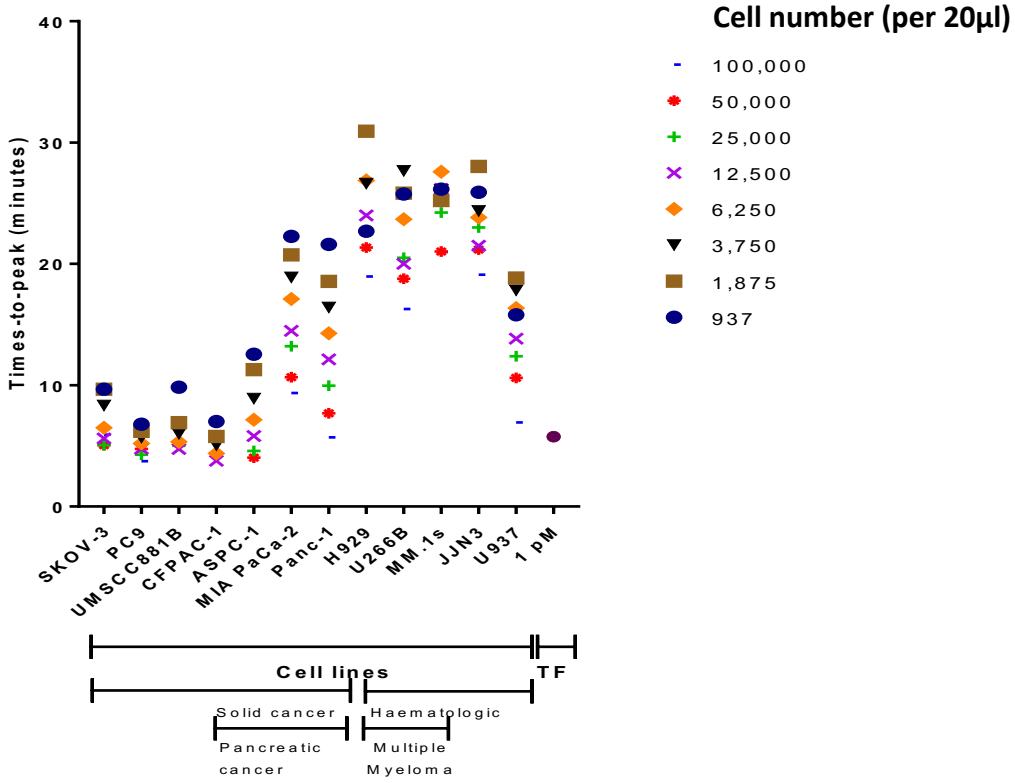
B)


Endogenous thrombin potential (nM/minute)
2000
1500
1000
500
0
SKOV-3
PC9
UMSCC881B
CFPAC-1
ASPC-1
MIA PaCa-2
Panc-1
H929
U266B
MM.1s
JJN3
U937
1 pM
Cell lines
TF
Solid cancer
Haematologic
Pancreatic cancer
Multiple Myeloma
Cell number (per 20µl)
100,000
50,000
25,000
12,500
6,250
3,750
1,875
937

C)


Peak thrombin (nM)
300
200
100
0
SKOV-3
PC9
UMSCC881B
CFPAC-1
ASPC-1
MIA PaCa-2
Panc-1
H929
U266B
MM.1s
JJN3
U937
1 pM
Cell lines
TF
Solid cancer
Haematologic
Pancreatic cancer
Multiple Myeloma
Cell number (per 20µl)
100,000
50,000
25,000
12,500
6,250
3,750
1,875
937

D)


Times-to-peak (minutes)
40
30
20
10
0
SKOV-3
PC9
UMSCC881B
CFPAC-1
ASPC-1
MIA PaCa-2
Panc-1
H929
U266B
MM.1s
JJN3
U937
1 pM
Cell lines
TF
Solid cancer
Haematologic
Pancreatic cancer
Multiple Myeloma
Cell number (per 20µl)
100,000
50,000
25,000
12,500
6,250
3,750
1,875
937

E)

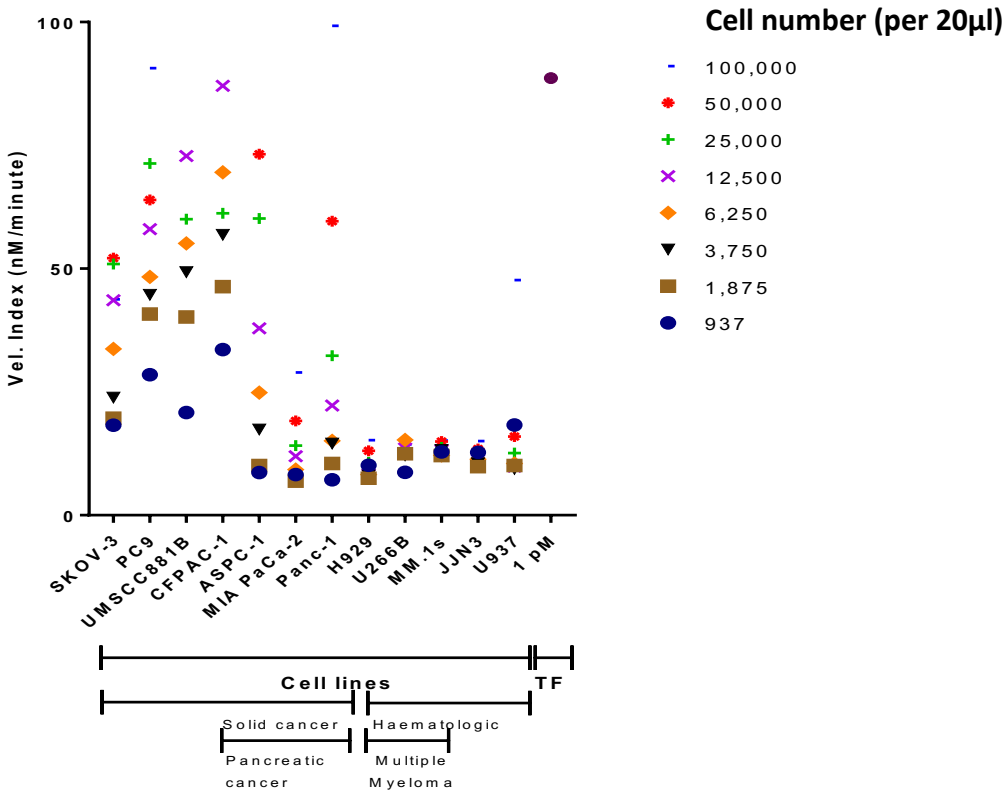


Figure 4.1 Thrombin generation with increasing cell numbers.

A) Lag times B) ETP C) Peak thrombin D) Times-to-peak E) Velocity Index. TG was induced by 8 different concentrations of 12 cancer cell lines (from 100,000 to 937 cells/20µl) in platelet-free plasma with low 1 pM TF standard preparations as controls. Results are mean of n=3 performed in triplicates (intra assay C of V of Lag times, ETP and the rest of the TG parameters were 4.78%, 3.53% and < 5% respectively, inter assay C of V of the TG parameters were < 10%). The 5 TG parameters measured by calibrated automated thrombography (CAT) assay are Lag times, Endogenous thrombin potential (ETP), Peak thrombin (Peak), Times-to-peak (TTP or ttPeak), Velocity index (Vel.Index).

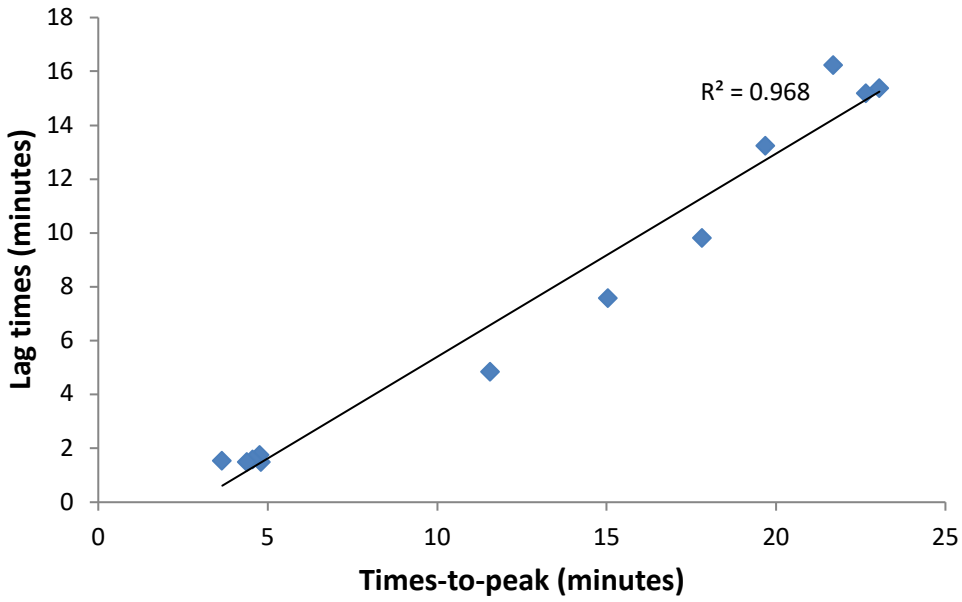
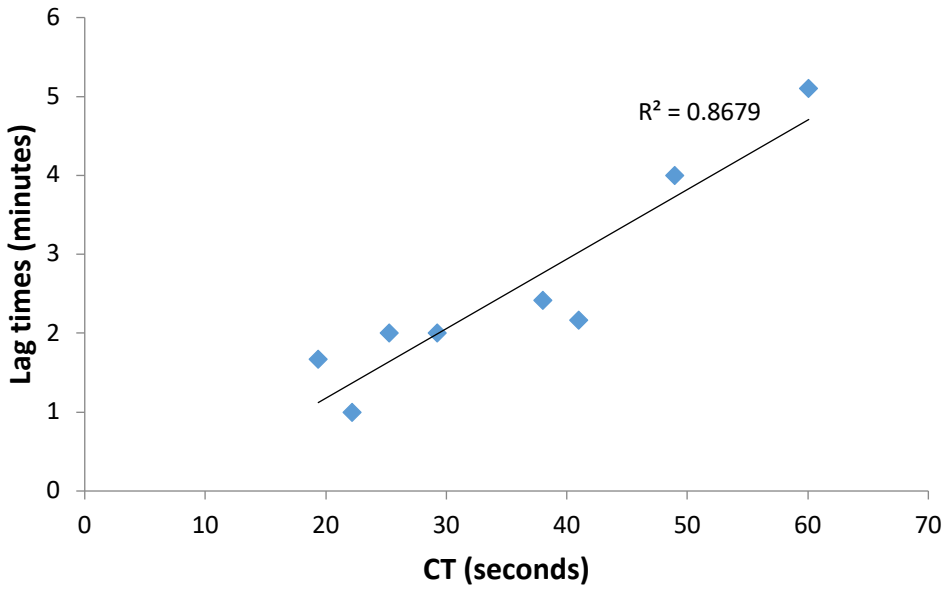


Figure 4.2. Correlation of Lag times and TTP from solid tumour and haematological malignancy cell lines in platelet free plasma

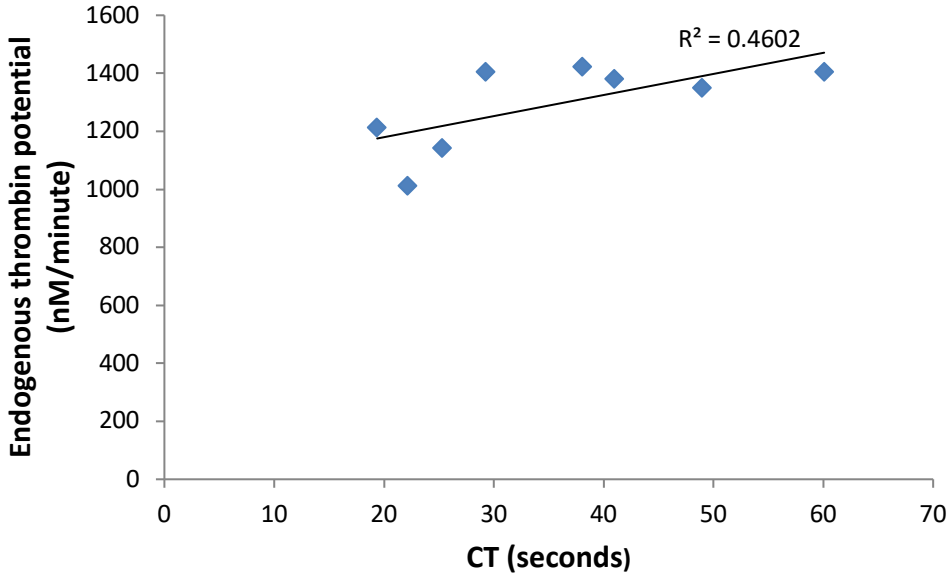
4.2.2 Clotting times and Thrombin generation

To compare the TG parameters with another test of procoagulant activity, UMSCC81B cell lines at decreasing concentrations were selected for a prothrombin assay performed with a coagulometer as described in section 2.5. When compared with TG parameters, CT for UMSCC81Bs had the highest correlation with the thrombin Peak (r=-0.99, p= 0.0007) figure 4.3C, followed by times-to-peak in figure 4.3D (r=0.92, p=0.0006) and lag times in figure 4.3A (r=0.9, p=0.0001). It also correlated less strongly with ETP in figure 4.3B (r=0.68, p=0.003).

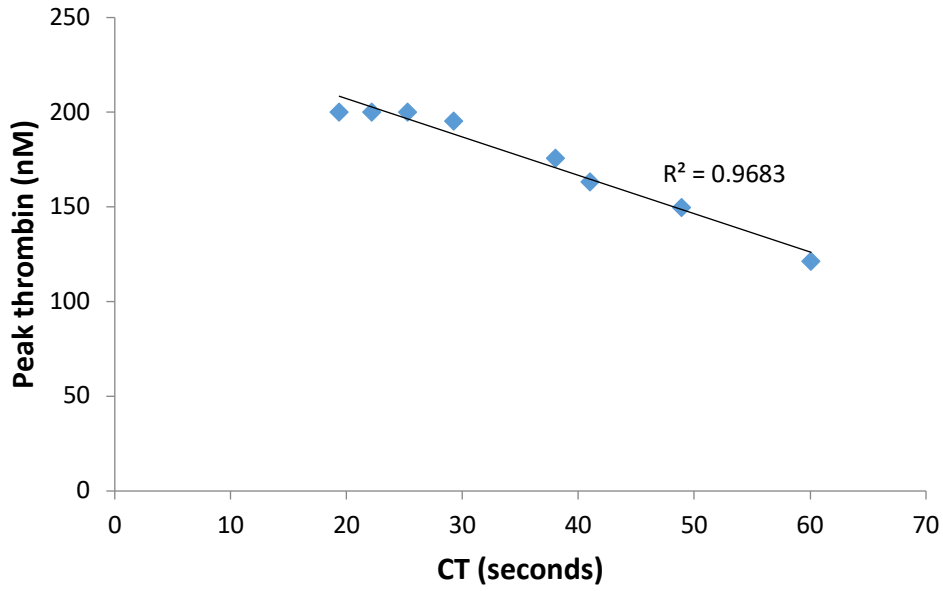
A)


Lag times (minutes)
CT (seconds)
R² = 0.8679
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70

B)


Endogenous thrombin potential (nM/minute)
CT (seconds)
R² = 0.4602
0
200
400
600
800
1000
1200
1400
1600
0
10
20
30
40
50
60
70

C)



D)

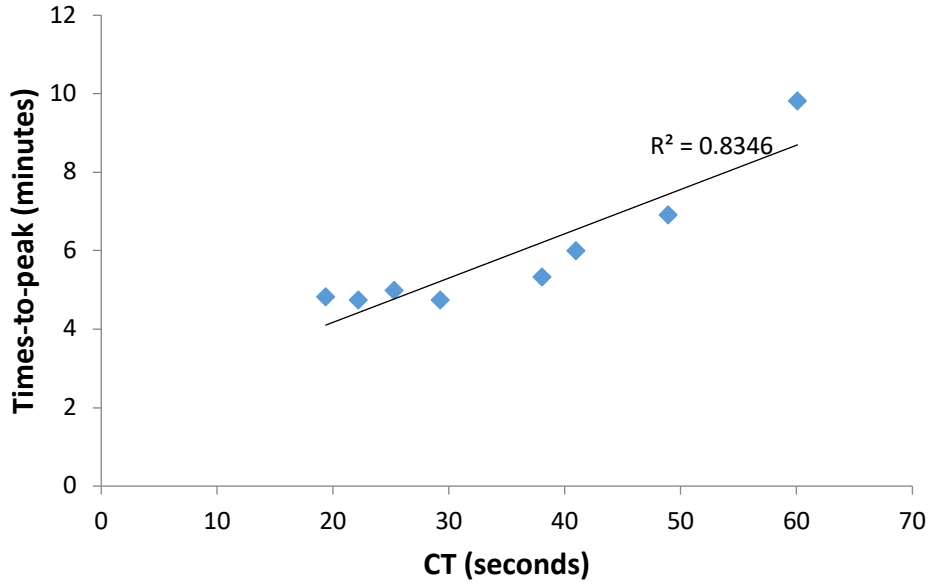


Figure 4.3 Clotting times (CT) versus Thrombin generation (TG).

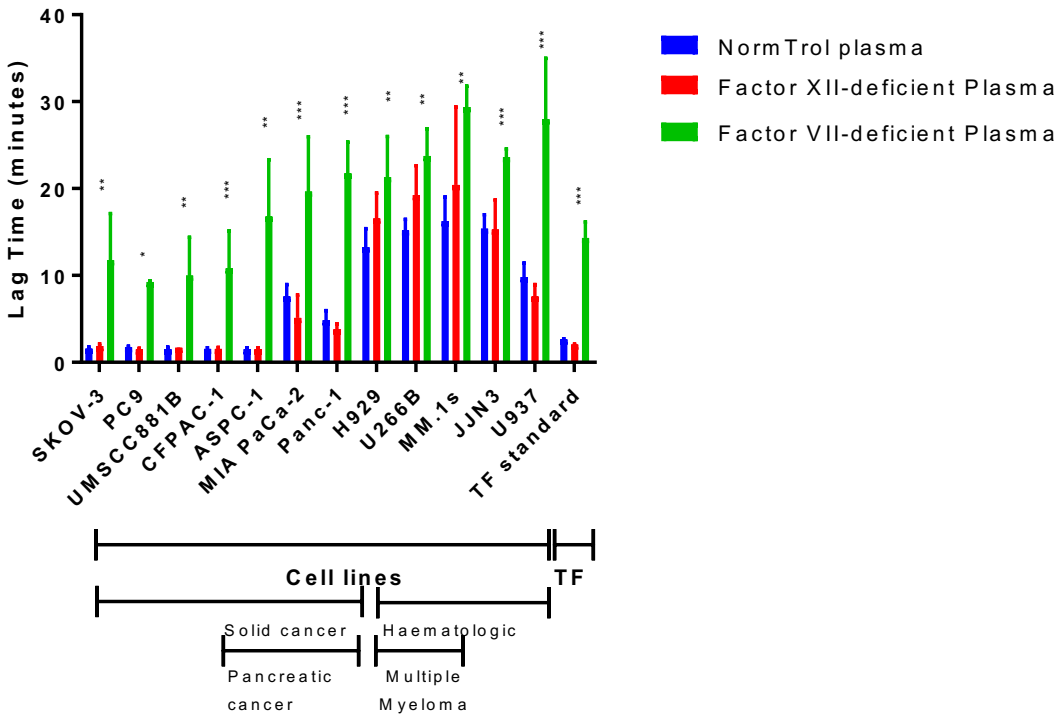
A) Lag times:CT B) ETP:CT C) Peak:CT D) Times-to-peak:CT. TG parameters from the CAT assay were compared with clotting times from the one-step Thrombotrak Solo coagulometer. UMSCC81B cancer cell lines were used at decreasing concentrations (5, 2.5, 1.25, 0.63, 0.3, 0.15, 0.08, 0.04 x 10*6/ml) for both assays. CT for UMSCC81Bs had the highest correlation with the thrombin Peak (Pearson coefficient r=-0.99, p= 0.0007), followed by times-to-peak, lag times and ETP.

4.2.3 Influence of coagulation factors on thrombin generation in solid tumour and haematological malignancy cell lines

TG parameters measured in NormTrol and coagulation factor VII-deficient plasma indicated significant differences (Table 4.1, Figure 4.4), but TG parameters in NormTrol and factor XII-deficient plasma were not significant, especially in solid tumour cell lines. For instance, ASPC-1 had a 1.5±0.2 minute Lag time in NormTrol and under factor XII-deficiency, the duration of which was prolonged significantly by 91% (p=0.0001) to 16.8±6.5 minutes with factor VII absence in plasma. Interestingly, there was a smaller change (less prolongation) in haematological cells, such that absence of factor VII in platelet-free plasma prolonged Lag times but to a much lesser extent than was observed in solid tumour cells (Figure 4.4A). For example, Myeloma U266B showed a lag time of 15.2±1.3 minutes in NormTrol plasma which increased significantly or lengthened (p=0.0014) by 36% to 23.7±3.1 minutes in factor VII-deficient plasma, but only increased to 19.3±3.4 minutes (p=0.158) in factor XII-deficient plasma.

In all cell lines, Lag times (Figure 4.4A) and TTP (Figure 4.4B) were the TG parameters that showed the highest number of significant changes under the conditions described, followed by Peak thrombin produced, Vel. index, and ETP (Table 4.1). TTP, for example (Figure 4.4B) showed that haematological cells have a different profile to solid tumour cells as there were significant differences observed in TG in both factor VII and XII-deficient plasma when compared to NormTrol plasma, unlike in solid tumour cells where most differences were only seen in factor VII-deficient plasma compared to NormTrol. U937 for example had a TTP of 17.8±1.1 minutes in NormTrol plasma which became significantly prolonged by 33 %(p=0.019) to 26.6±5.2 minutes in factor VII-deficient plasma, and by a much larger extent of 55% (p=0.0001) to 39.2±6.4 minutes in factor-XII deficient plasma

A)



B)

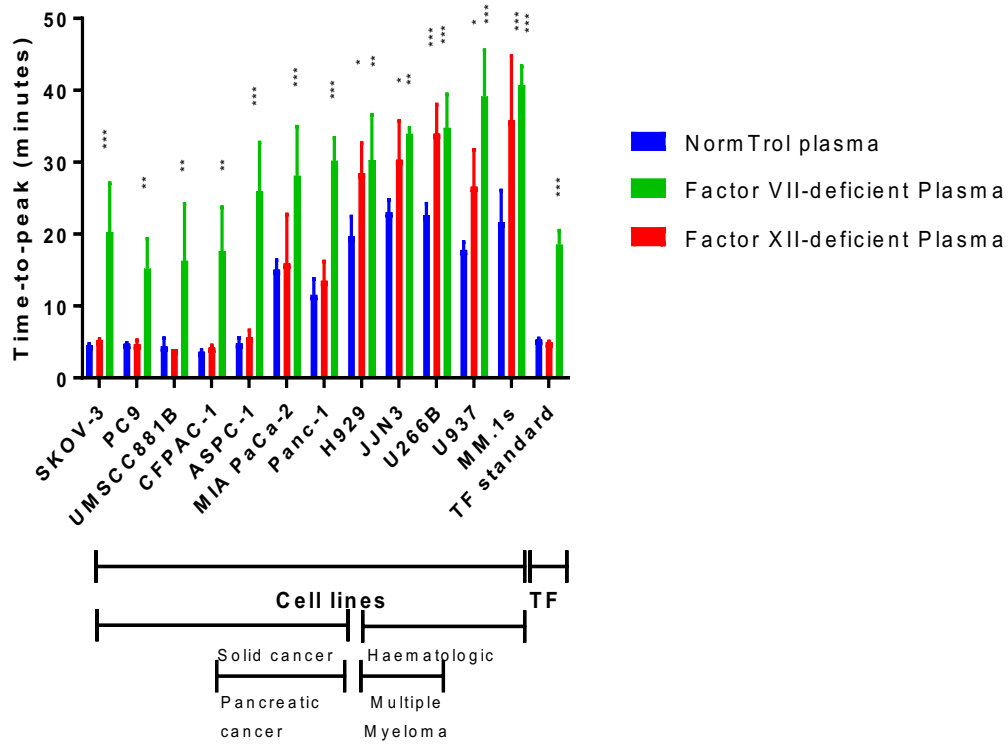
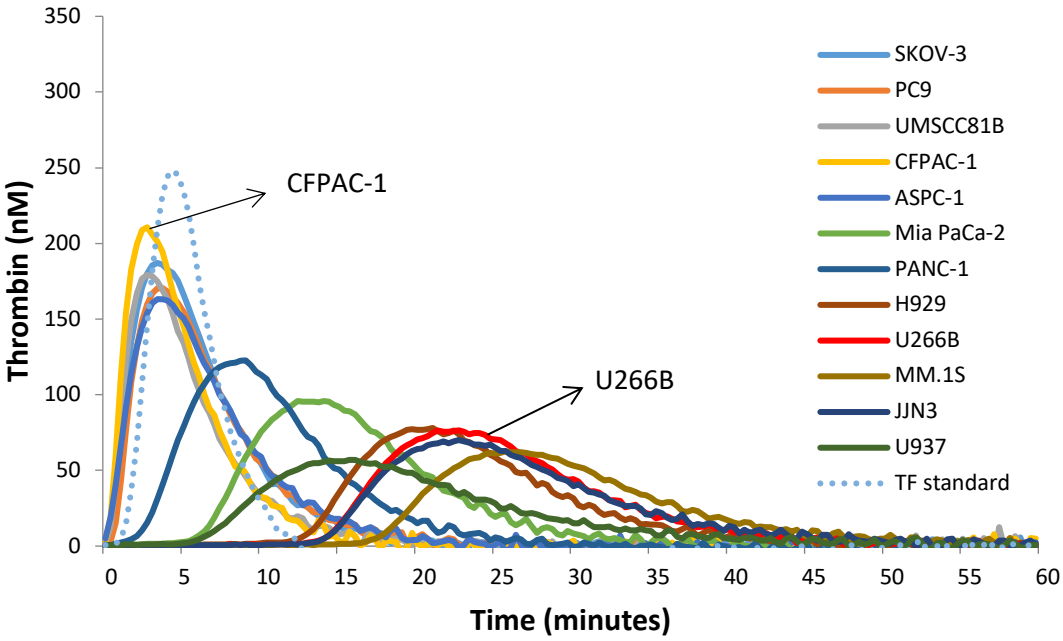


Figure 4.4 Influence of coagulation factors on thrombin generation in cell lines.

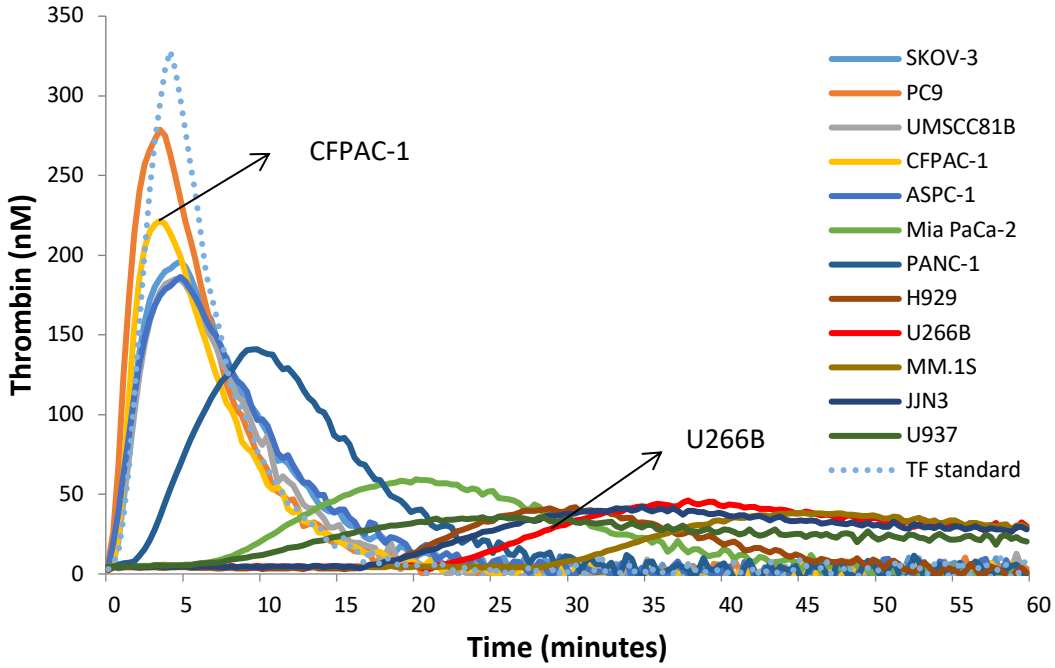
A) Lag times B) Time-to-peak TG in solid cancer cell lines such as PC was compared with haematological (such as MM) cell lines *in vivo* on the CAT assay, each at constant cell number of 0.63 x 10^6/ml. Results are mean of n=4 ± S.D performed in duplicates. *P values are from two-way ANOVA multiple comparisons with NormTrol as baseline compared with Factor VII-deficient plasma and Factor XII-deficient plasma. Significance is at *p< 0.05, **p<0.01, ***p<0.001

Thrombin generation curves were produced by the CAT assay for the 12 cancer cell lines in 3 types of platelet-free plasma; NormTrol (Figure 4.5A), Factor XII-deficient plasma (Figure 4.5B) and Factor VII-deficient (Figure 4.5C). Under coagulation factor VII-deficient plasma conditions, TG in the majority of cell lines and control was affected, with significant delay in initiation, volume and times to reach peak, and TG was not completed in all the MM cells in 60 minutes. For instance, CFPAC-1 thrombin production reduced from 220 to 120 nM in factor VII-deficiency, a 45% reduction, while TG remained near 225nM in factor XII-deficiency. Conversely, for haematological cells only U937 retained the ability to complete its TG curve in 60 minutes when factor XII was absent in plasma, at a reduced volume and velocity (from 17.8±1.1 to 39.2±6.4 nM/min) while the others such as U266B did not complete TG in the duration of experiment.

A)


Thrombin (nM)
Time (minutes)
CFPAC-1
U266B
SKOV-3
PC9
UMSCC81B
CFPAC-1
ASPC-1
Mia PaCa-2
PANC-1
H929
U266B
MM.1S
JJN3
U937
TF standard

B)


Thrombin (nM)
Time (minutes)
CFPAC-1
U266B
SKOV-3
PC9
UMSCC81B
CFPAC-1
ASPC-1
Mia PaCa-2
PANC-1
H929
U266B
MM.1S
JJN3
U937
TF standard

C)

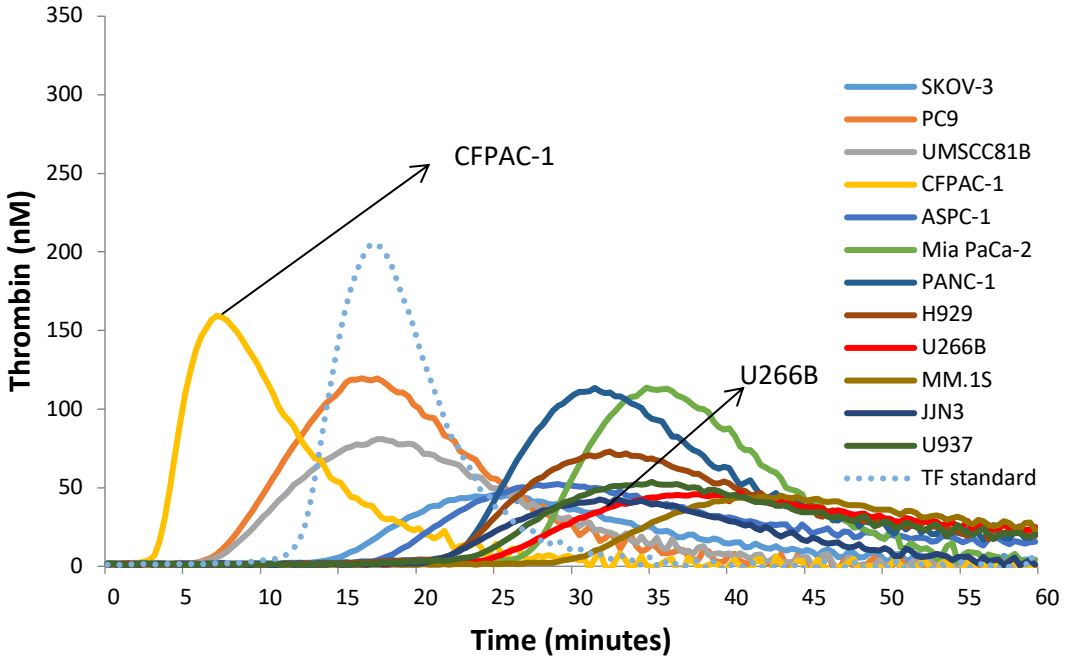


Figure 4.5 Thrombin generation curves of solid and haematological malignant cell lines.

The graphs show the differences in TG in 3 types of platelet-free plasma: A) NormTrol B) Factor XII-deficient and C) Factor VII-deficient; induced by 12 cancer cell lines with low 1pM TF as control. Pancreatic CFPAC-1 and Multiple myeloma U266B are highlighted here as examples to compare the differences in TG.

4.2.4 Influence of coagulation factors on thrombin generation in microvesicles released by cells from solid and haematological malignant lines

In NormTrol and coagulation factor VII-deficient plasma, TG in MVs produced by solid cancer and haematological cell lines (detailed in section 2.8.2) followed similar patterns as their parent sources (Table 4.2; Figure 4.6). Overall Lag times were shorter in MVs of solid tumours in NormTrol plasma compared to haematological ones. Furthermore, apart from U937 MVs, none of the MVs from the hematologic lines was capable of generating enough thrombin in the 3 types of platelet-free plasma used (NormTrol, Factor VII-deficient and Factor XII-deficient) in the time allotted per assay

of 60 minutes. Similarly to the parent cell lines, TG in FXII-deficient plasma was alike to TG in NormTrol, while the TG parameters measured in FVII-deficient plasma were significantly reduced.

Generally, Lag times were not affected by the absence of FXII except in MIA PaCa-2 MVs where it was reduced by approximately 50% from 8.77± 3.28 to 4.52± 2.06 minutes, although this change was not of any significance ultimately ($p=0.12$; two-way Anova). UMSCC81B MVs Lag times occurred too fast to be recorded by the assay, which can only measure activity every 10-20 seconds (discussed in METHODS section 2.8).

Table 4.2 Thrombin generation parameters in Microvesicles from 5 cancer cell lines and low 1pM TF standard preparation.

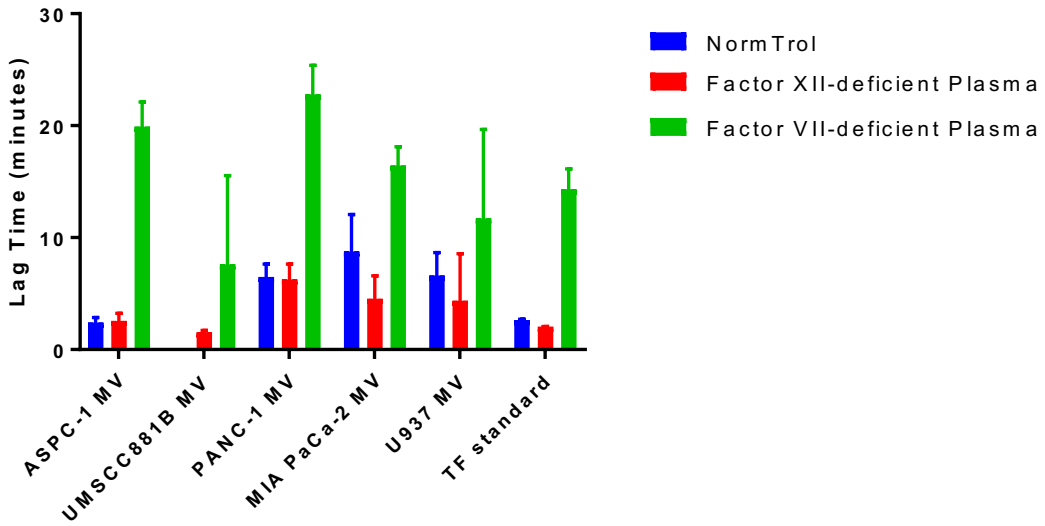
	Platelet free plasma	Lag time (minute) /P value	ETP (nM/minute) /P value	Peak thrombin (nM) /P value	TTP (minute) /P value	Vel. Index (nM/minute) /P value
UMSCC81B MV	NormTrol	-	-	-	-	-
	F XII-deficient	1.54±0.17 /0.72	1535±227 /***0.0001	220±89 /***0.0001	4.24±0.45 /0.46	90.21±57.6 /***0.0005
	FVII-deficient	7.62±7.91 /**0.0031	1031±235 /***0.0002	99±62 /*0.024	13.89±10.9 /***0.0007	21.32±18.7 /0.49
ASPC-1 MV	NormTrol	2.42±0.43	880±149	105±46	6.73±1.68	28.46±20.12
	F XII-deficient	2.56±0.67 /0.99	1105.69±221.64 /0.18	112±48 /0.92	7.404±2 /0.96	26.25±19.68 /0.98
	FVII-deficient	19.92±2.21 /***0.0001	126.2±37.05 /***0.0003	17±16 /**0.0068	32.97±6.81 /***0.0001	1.46±1.58 /0.16
PANC-1 MV	NormTrol	6.48±1.15	840.58±54.93	74±19	12.7±2	12.88±4.93

	F XII-deficient	6.28±1.38 /0.99	1018.56±133.28 /0.41	83±24 /0.88	13.7±2.76 /0.93	12.03±5.54 /0.99
	FVII-deficient	22.78±2.6 /***0.0001	769.94±344.02 /0.87	27±6 /0.093	34.22±4.8 /***0.0001	2.505±0.89 /0.63
MIA PaCa-2 MV	NormTrol	8.77±3.28	558.61±53.53	39±8.61	15.86±2.95	5.5±1.34
	F XII-deficient	4.53±2.06 /0.12	651.59±9.1/ 0.89	35.3±11.71 /0.99	21.62±7.78 /0.23	2.25±1.47 /0.97
	FVII-deficient	16.44±1.68 /***0.0029	541.75±239.13 /0.99	24.47±9.95 /0.75	29.43±2.14 /***0.0002	2.14±0.93 /0.96
U937 MV	NormTrol	6.62±2.051	859.46±139.14	95.49±4.4	13.38±4.01	12.87±10.93
	F XII-deficient	4.36±4.2 /0.63	1059.77±428.82 /0.51	41.43±0.81 /0.31	10.57±9.56 /0.74	25.05±1.35 /0.082
	FVII-deficient	11.71±7.96 /0.13	967.91±244 /0.81	70.43±31 /0.76	18.53±9.62 /0.39	11.29±7.19 /0.99
TF Standard	NormTrol	2.64±0.064	1399±174.82	254±6.14	5.37±0.11	93.54±3.65
	FXII-deficient	2.05±0.017 /0.95	2011±164 /*0.0014	328±8 /*0.016	4.98±0.08 /0.99	111.64±0.6 /0.37

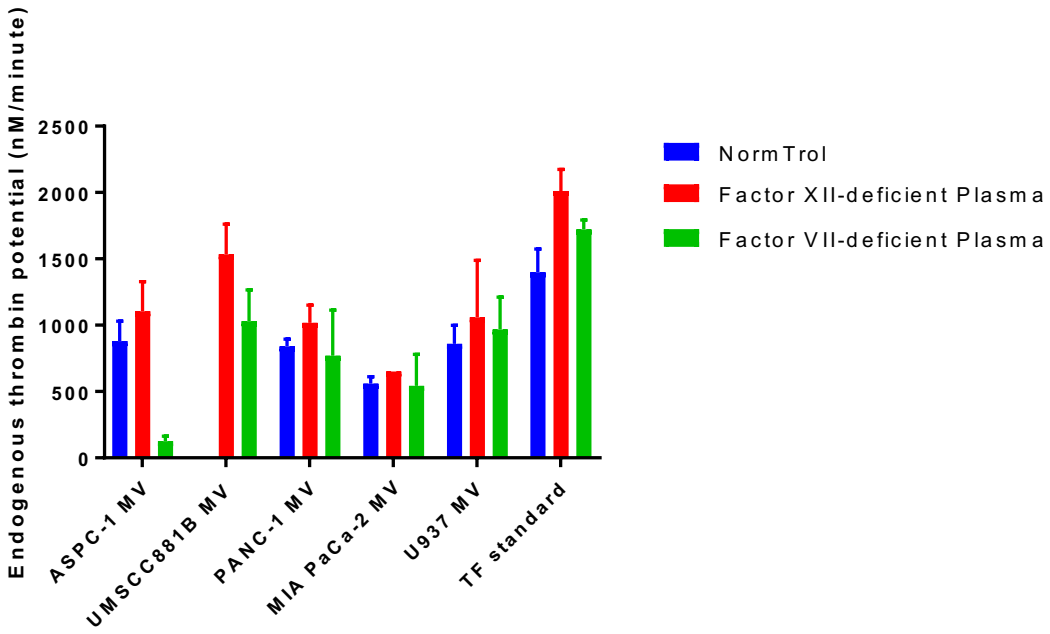
	FVII-deficient	14.32±1.83 /***0.0001	1722±70 /0.102	219±24 /0.31	18.52±1.96 /**0.0013	53.633±11.25 /*0.019

MVs from tumour cells at concentration of 0.6 x 10*6/ml were concentrated in PBS to induce TG in 3 different platelet-free plasma (NormTrol, Factor XII-deficient and Factor VII-deficient). TG parameters include Lag time, ETP, Peak, TTP and Velocity Index. Results are mean of n=4 ± S.D performed in duplicates. P values from two-way ANOVA multiple comparisons with NormTrol as baseline compared with Factor VII-deficient plasma and Factor XII-deficient plasma. Significance is at *p< 0.05, **p<0.01, ***p<0.001.

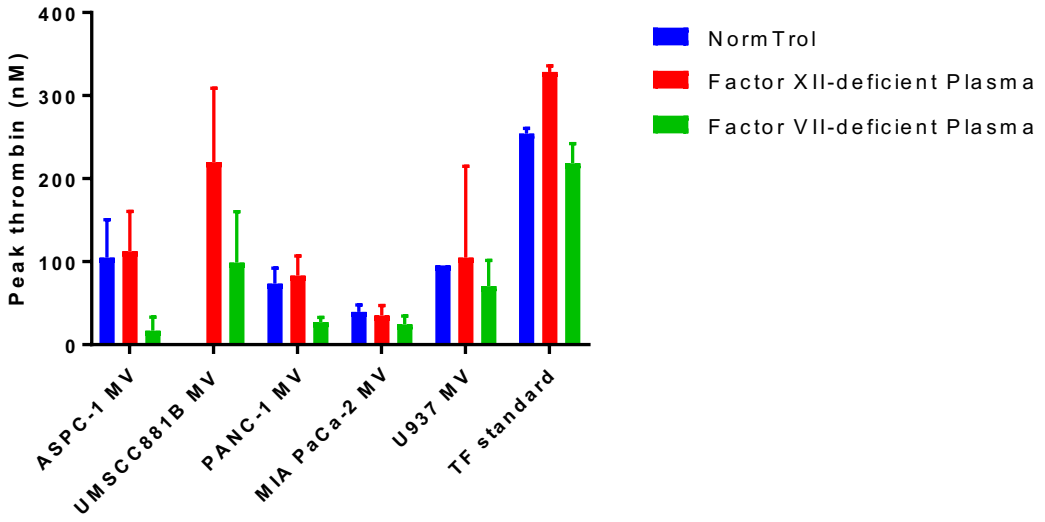
A)


Lag Time (minutes)
30
20
10
0
ASPC-1 MV
UMSCC881B MV
PANC-1 MV
MIA PaCa-2 MV
U937 MV
TF standard
NormTrol
Factor XII-deficient Plasma
Factor VII-deficient Plasma

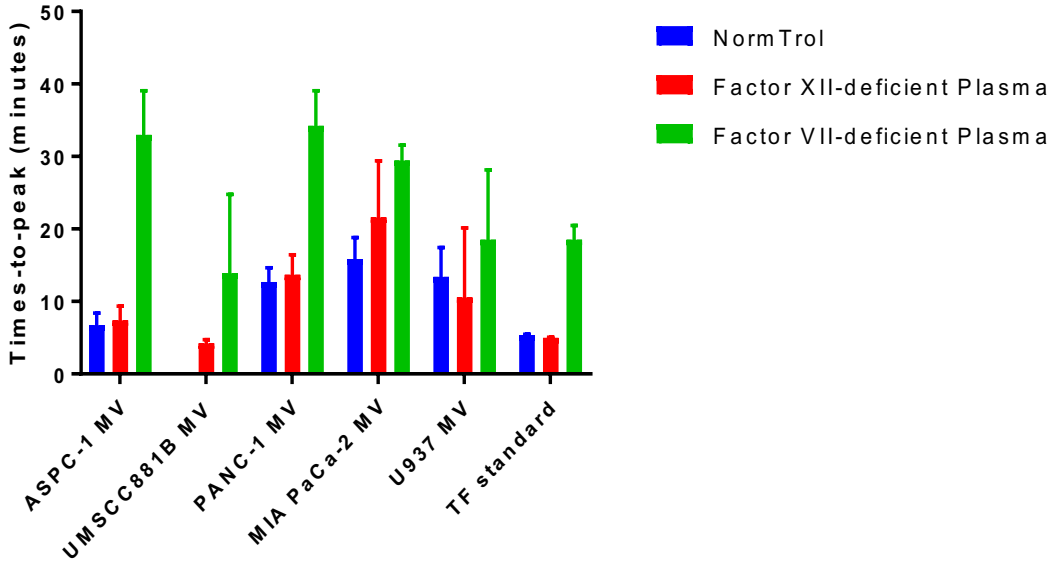
B)


Endogenous thrombin potential (nM/minute)
2500
2000
1500
1000
500
0
ASPC-1 MV
UMSCC881B MV
PANC-1 MV
MIA PaCa-2 MV
U937 MV
TF standard
NormTrol
Factor XII-deficient Plasma
Factor VII-deficient Plasma

C)


Peak thrombin (nM)
400
300
200
100
0
ASPC-1 MV
UMSCC881B MV
PANC-1 MV
MIA PaCa-2 MV
U937 MV
TF standard
NormTrol
Factor XII-deficient Plasma
Factor VII-deficient Plasma

D)


Times-to-peak (minutes)
50
40
30
20
10
0
ASPC-1 MV
UMSCC881B MV
PANC-1 MV
MIA PaCa-2 MV
U937 MV
TF standard
NormTrol
Factor XII-deficient Plasma
Factor VII-deficient Plasma

E)

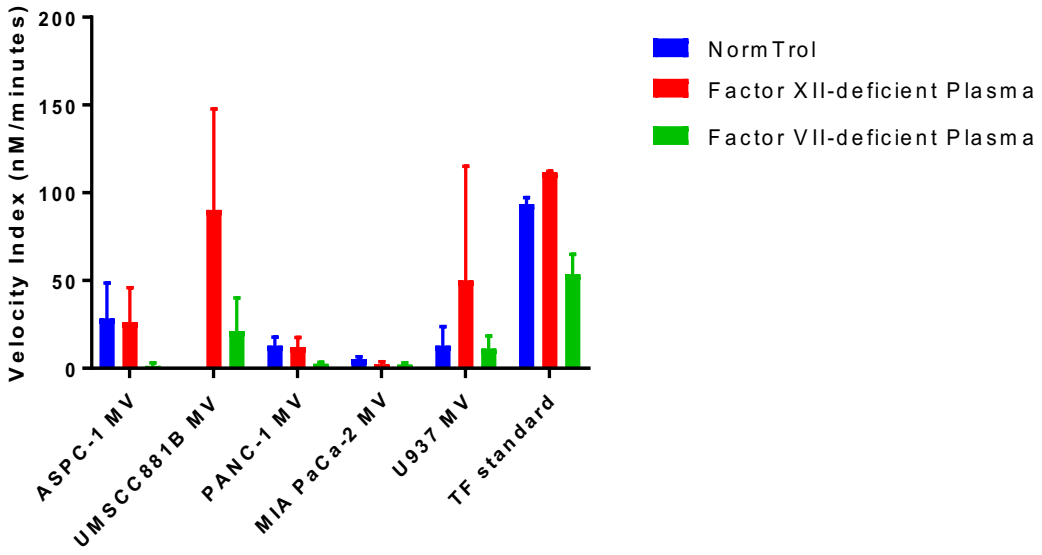


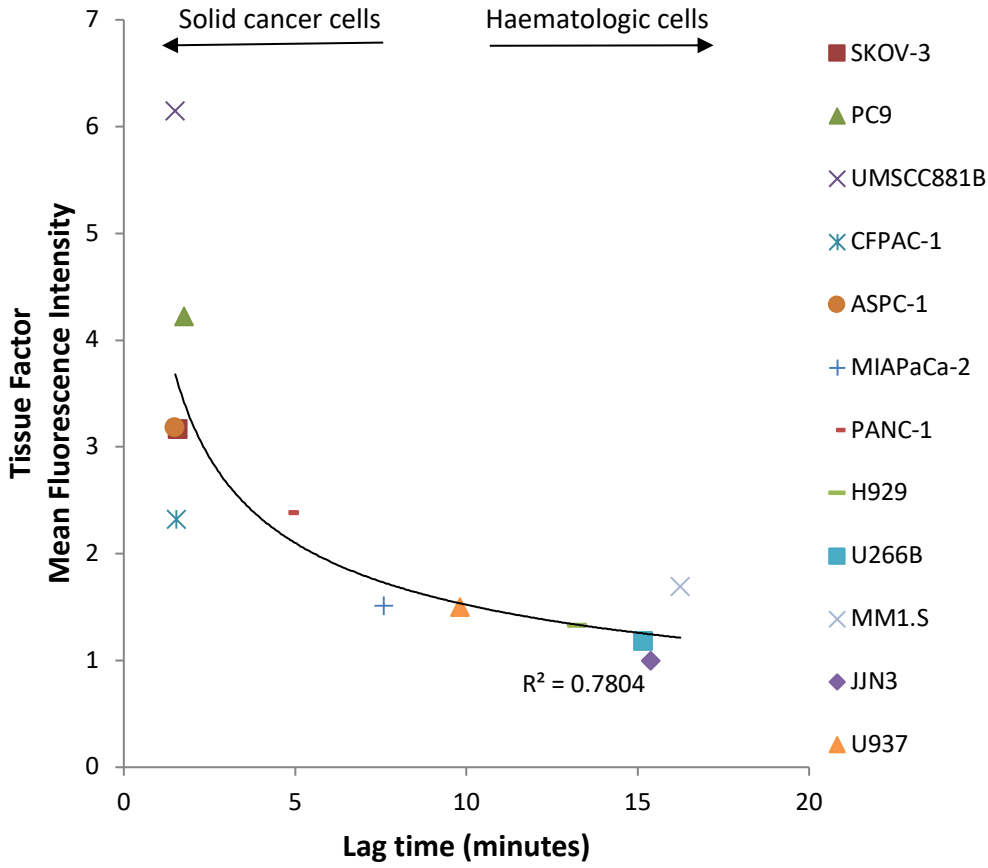
Figure 4.6 Influence of coagulation factors on thrombin generation in cancer MVs. A) Lag times B) ETP C) Peak thrombin D) Times-to-peak. E) Velocity Index. TG in MVs (per 20µl of PBS) were obtained from cancer cell lines of PC (ASPC-1, PANC-1, MIA PaCa-2), UMSCC81B and haematologic U937 and was measured on the CAT assay. Results are mean of n=3 ± S.D performed in duplicates.

4.2.5 Influence of tissue factor on cell-induced thrombin generation from solid tumours and haematological malignancy cell lines in platelet-free plasma

As a measure of the influence of cell TF expression on TG of cell lines, mean TF cell surface expression was assessed by flow cytometry and plotted against average TG parameters using data generated at 0.63 x 10^6 cells/ml. There was variability in TF expression as expected across the cell lines however; the solid cancer cell lines expressed higher TF values than haematological cells (Figure 4.7). While UMSCC81B expressed the highest TF amongst solid cancer cells overall, CFPAC-1 expressed the highest TF amongst PC cells and JJN3 the lowest of all cell lines. An inverse correlation was seen upon correlation of TF expression with TG parameters Lag time (r^2=-0.78, p=0.036) (Figure 4.7A) and TTP (r^2=0.78, p=0.002) (Figure 4.7B). Upon Log_{10}/Log_{10} transformation of data with regression, a near linear relationship of these 2 TG

parameters in NormTrol with TF expressed per 10^5 cells was displayed. Correlation with TG parameters obtained from factor VII-deficient conditions showed weak correlations of low significance ($r^2 < 0.5$) and no relationship with factor XII-deficient conditions. Also, the TG parameters ETP, Vel.Index and peak height correlated weakly with TF cell surface expression ($r^2=0.65$, $p=0.000000009$, $r^2=0.61$, $p=0.004$ and $r^2=0.69$, $p=0.0000079$ respectively).

A)



B)

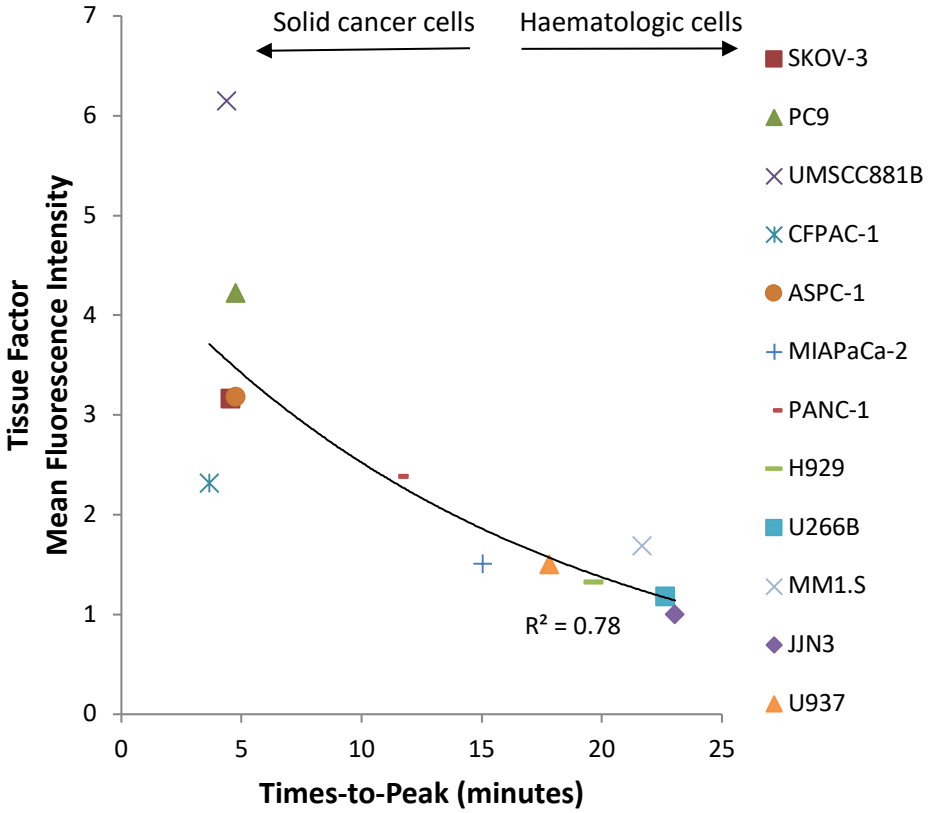


Figure 4.7 Influence of Tissue Factor (TF) on Thrombin generation in cell lines.

A) TF:Lag times B) TF:Times-to-peak. Following 24 hour cell culture, flow cytometry was used to assess mean TF surface expression of cell lines at 0.63 x 10*6/ml cell concentration. There was an inverse correlation of TF expression with averaged TG parameters such that as TF expression increases, Lag times and times-to-peak decreases. There existed a near linear relationship of TG parameter (Lag times, Times-to-peak) in NormTrol with TF expressed per 10^5 cells on Log_{10}/Log_{10} transformed data using regression where R^2 values are 0.78 and 0.75 respectively. Results are mean of n=3 performed in triplicates.

4.3 Discussion

Cancer cell lines exhibit TG in a cell concentration dependent manner. Of the five TG parameters, lag time and time-to-peak highlighted the differences between solid and haematological tumours and were dependent on TF expression. Coagulation driven by solid tumour was also more TF-FVII dependent than for haematological cells, which was both factors VII and XII dependent, which suggests that there is a role for factor XII in the absence of factor VII in non-solid malignancies.

An ETP of 30% or higher has been linked to clinical situations where bleeding tendencies were absent or low, and therefore to the severity of observed bleeding(Al Dieri et al., 2002). In plasma, several studies suggest that the peak thrombin height and ETP are considered most important (Duchemin et al., 2008), but none has been conclusively determined for other substrates. Lag times reported here were defined as the time in which 20nM of thrombin is formed (Hemker et al., 2003) and the importance of this initiation phase has been previously described in coagulation studies with limited factor V component (Butenas et al., 1997). Peak thrombin may reflect the ability of the cell lines to generate thrombin; and it also correlated with a one-step clotting assay (Figure 4.3). This could mean that although the height of thrombin produced may reflect clotting time, it may not necessarily indicate the absolute capacity of the cell lines to generate thrombin.

Pancreatic cancer displays a TF-driven coagulation process when compared to MM that is not as dependent on TF-related coagulation (Yates et al., 2011; Welsh et al., 2012). As to be expected, PC and other solid cancer cell lines exhibited a stronger overall TG profile in this study with larger volumes of thrombin produced than MM cells (Table 4.1). TG parameter differences characterize cancer cell lines, which may reflect how thrombin is formed *in vivo* and potential response to therapy. In this study, MM cells generally have slower lag times and may continuously generate thrombin for a longer period (Figure 4.5). A further distinction could also be made between the PC cell lines, where the fastest lag times and times-to-peak of those such as ASPC-1 and CFPAC-1 may be explained by increased cell surface mucin, unlike for

PANC-1 and MIA PaCa-2 (Lieber et al., 1975; Chen et al., 1982; Deer et al., 2010) have less Mucin and which are slower to initiate TG. Although UMSCC81B has the highest TF cell surface expression this did not necessarily translate into the cell line with the highest total amount of thrombin produced, as only a little TF is needed to produce the initial thrombin necessary for amplification to the propagation phase of the clotting haemostatic cascade (Monroe & Hoffman, 2006).

Compared to low TF control, factor VII exerted more influence on TG than factor XII in both PC and MM. However, factor XII had a greater influence on TG in the MM cells and almost none in PC (Figure 4.4). Factor VII and factor XII are members of the extrinsic and intrinsic contact-activated coagulation pathways involved in thrombus formation(Kuijpers et al., 2014). They may also be involved in other functions such as inflammation and angiogenesis *in vivo* (Ruf et al., 2011; Woodruff et al., 2011). Lack of both factors caused changes in TG parameters, which was consistent across cell lines. Solid tumour cells, such as PC express more TF, compared to haematological cell lines.TF-factor VII complex has been suggested to drive coagulation from a damaged vessel wall while factor XII may act at more distant locations (Nickel et al., 2015) and may modulate stability of thrombus independent of thrombin generation(Konings et al., 2011). TF-dependent pathways are known to utilise factor VII which activates the PAR-2 pathway and leads to a TF-FVII complex that activates the extrinsic clotting pathway (Rickles et al., 2003; Ruf et al., 2011). Although, TF-factor VII removal from the assay caused the most profound change in the TG kinetics, it is interesting to note that there was no complete abolishment in both cell groups, highlighting the possibility of an alternative pathway being utilized in these situations. Marchetti et al also reported a similar observation upon blocking of TF activity with anti-TF antibody during TG (Marchetti et al., 2012). Moreover, our study implies that although TF cell expression correlates with some particular TG parameters, it does not singularly determine the level of thrombin produced.

Haematological cells under factor XII-deficient conditions behaved differently from solid cancer cells by generating less thrombin in a longer time with slower initiation. It could be suspected from this result that some level of inhibition of the contact

pathway is lifted, such that though lower levels of thrombin height is reached, there is continuous thrombin production beyond 60 minutes. Although factor XII-deficiency is not associated with excessive bleeding its involvement in stabilising thrombus clot may contribute to increased risk of emboli formation (Kuijpers et al., 2014). It has also been suggested that some reduction in the levels of factor XII may result in higher cardiovascular risk and thrombotic complications (Woodruff et al., 2011; Renné et al., 2012). Also, though it can be bypassed by other coagulation cascade members, factor XII may still be essential and its importance comes to prominence in myeloma-related thrombosis in the absence of other constituents(Woodruff et al., 2011), as seen here when TG persisted in smaller quantities after factor VII absence. Moreover, continuing TG may be responsible for persistent initiation of the clotting cascade leading to continuous clotting formation in clinical scenarios as suggested by Duchmein et al (Duchemin et al., 2008). This study has indicated a possible large intrinsic pathway reserve for haematological cells and to a lesser extent solid cancer cells, and highlights differential contributions to TG by both the intrinsic and extrinsic coagulation pathways. This is supported by a previous study that has highlighted the possibility of a large intrinsic pathway reserve in human plasma samples of different origins (Rice et al., 2016).

The absence of factor XII in plasma may allow coagulation through an alternative TG pathway. For example, a recent study found that in some solid cancers such as prostate and pancreatic cancer, TG might be triggered by polyphosphate exposure on their plasma membranes in a factor XII-dependent manner (Nickel et al., 2015). Therefore, the importance of the role of the intrinsic pathway may not be restricted to non-solid cancers alone. Furthermore, a switch from one factor-dependency to another might be possible in other cell lines and substrates.

To conclude, this chapter has shown that cells from solid tumours exhibit greater thrombin generation potential and are faster to initiate coagulation than haematological cells. The use of factor VII deficient plasma had a greater influence in thrombin generation in the solid tumour cells suggesting a greater reliance on the

extrinsic, TF-driven pathway in these cells, which may persist when evaluated in cancer patients *in vivo*.

Chapter:5 Thrombin generation (TG) and Tissue factor (TF) involvement in Multiple Myeloma and Pancreatic Cancer

PART A

5.1 Introduction of Clinical Studies

In order to study the thrombin generation (TG) patterns in Multiple myeloma (MM) and Pancreatic cancer (PC), two main clinical studies were conducted, and the work demonstrated in this thesis is a sub study of these pre-existing studies. The main studies were both prospective cohort trials; one involved MM patients and was titled 'Study of apoptosis related changes and endothelial responses of multiple myeloma patients treated with chemotherapy' and the other involved PC patients entitled 'A Study of the effect of resection of localized Pancreatic Cancer on tissue factor (TF)-promoted pathways of Thrombosis and Angiogenesis markers'. These studies were commenced from previous PhD projects in our laboratory at different time points over the past 9 years; Dr Hussein Echrish was the initial Principal investigator (PI) of PC study from 2008, which was taken over by Dr Jessica Hall in 2011 who also commenced the MM study. This thesis project started during 2014, and during this period, the ongoing PC study was amended substantially from a surgical-only treatment to include a separate arm for PC patients on chemotherapy treatment, and compare this with chemotherapy effects on TG in the MM study. Further details on both studies are described below.

5.2 Design and Patient groups

The recruitment process included identification of eligible patients by the Chief Investigator -Professor Anthony Maraveyas or by the co-investigator Dr Hazem Sayala at Multidisciplinary team meetings (MDT) held at the Queen's centre for Oncology and Haematology, Castle Hill Hospital (CHH) at Hull and East Yorkshire (HEY) Trust in the National Health Service (NHS). Good clinical practice (GCP) guidance was followed in the consent process; briefly, the research nurse or doctor approached these patients for detailed explanation of the study objectives. If interested, they were provided with pertinent information (Patient information sheets and consent forms; (see appendix A and B for MM and localized PC respectively) to recruit them to the study. Patients could reject participation or opt out during the study. Once written and informed consent is given, the data managers registered their clinical and demographic data onto the trial database, and scheduled hospital visits were recorded.

While the surgical arm of the PC study has been completed, the other studies are all ongoing with a target to recruit 50 MM patients, 50 PC in the chemotherapy arm, as well as 20 Myeloproliferative disorders (MPD), 20 Cholecystitis as inflammatory controls for MM and localized PC respectively. During the tenure of this studentship as from 2014 10 MM (6 newly diagnosed and 4 relapsed), 7 PC surgery, 9 PC on chemotherapy patients were recruited and used for data collection, along with 6 MPD and 12 Cholecystitis controls. For overall data collection, samples obtained by the previous investigators mentioned above that remained in long term storage were also used (Figure 5.1), as the actual number of samples received after 2014 was less than expected due to several logistic issues. TG from CAT assay was prioritized over others for these analyses, when sample volumes were low. In addition, all patients recruited during this thesis project were link-coded and anonymized according to study protocols so as to maintain 'blinding' of the laboratory experiments to avoid bias and 'unblinding' occurred only prior to data analysis.

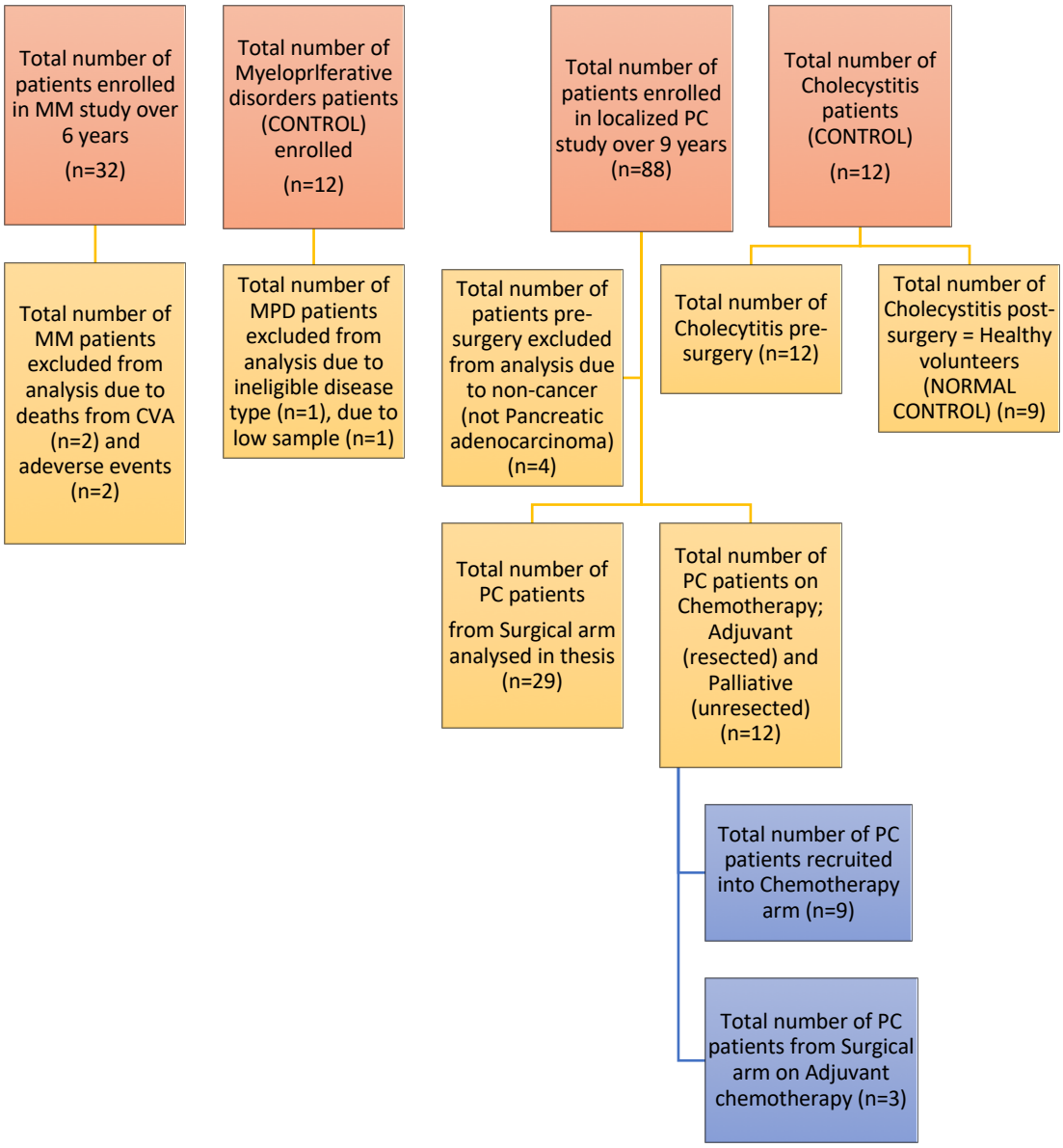


Figure 5.1 FLOW DIAGRAM of patients in both MM and PC clinical studies.

The 4 main patient groups assessed were MM, Myeloproliferative disorder (MPD) control, PC and Cholecystitis control

5.3 Clinical methods: Blood sample collection and processing

At the scheduled visits, pre-treatment baseline and non-baseline samples of all patients in the study (MM, MPD, PC and Cholecystitis) were collected as per the schedules of events (on each visit before any treatment or surgery was administered, listed in Table 5.1 and Table 5.2) for assays, which included haematological tests

where the haemoglobin level, white blood cells and platelet counts that were checked by the CHH laboratory. Paraprotein levels were also assessed for MM patients before, during and after the chemotherapy courses, and the MM disease immunophenotype categorized in bone marrow aspirates at the Haematological Malignancy Diagnostic Service (HMDS) in Leeds, UK. Using standard venepuncture techniques, venous blood samples were taken from the antecubital vein of the patients arm into sample BD Vacutainer tubes (BD biosciences, UK); a yellow serum-separator bottle, and two blue 3.8% tri-sodium citrated bottles for plasma. All samples were treated and processed the same way into serum and platelet-free plasma (PFP). As described in section 2.8.3, fresh PFP was obtained by immediate centrifugation of the citrated blood collection tubes in serial steps; first at 180xg for 10 minutes at room temperature (to prevent cold-induced platelet activation) to obtain platelet rich plasma (PRP) and then at 12,000xg for an additional 10 minutes to obtain plasma free of platelets (PFP). All PFP samples were used fresh in further analysis within 4 hours of blood collection or aliquoted and stored in an -80^{0}C freezer. Serum samples were obtained by leaving the serum-separator bottle to stand upright for 30 minutes to allow clot formation. Serum was also processed within 4 hours of collection stored and stored same as PFP. Frozen PFP was used for CAT assays (section 2.7), while fresh PFP was used for PCA assessment with a prothrombin time clotting assay as described in METHODS section 2.5, although the fresh PFP results were not evaluated further due to low numbers. Serum was used for TF antigen level estimation by ELISA (section 2.8) as illustrated in figure 5.2.

Table 5.1 Blood sampling schedule for MM patients and MPD controls enrolled in the study

Timepoints	Newly diagnosed MM patients	Relapsed MM patients	Myeloproliferative group (control)
Baseline (T1)	X	X	X
day 8, cycle 1	-	X	-
day 1, cycle 2 (T2)	X	X	-
day 1, cycle 3 (T3)	X	X	-

8 weeks post chemotherapy(T4)	X	X	-

Table 5.2 Blood sampling schedule for localized PC patients (surgical and chemotherapy arms) and Cholecystitis controls enrolled in the study.

Timepoint	Resected	Unresected	Laproscopic - Cholycystectomy	Cholecystitis (Control)
Baseline	X	X	X	X
Post-surgery/ Pre-chemotherapy	X	X	X	X
8-12 week Chemotherapy	X	X	-	-
24 week Chemotherapy	X	X	-	-
8 weeks post chemotherapy	X	X	-	-

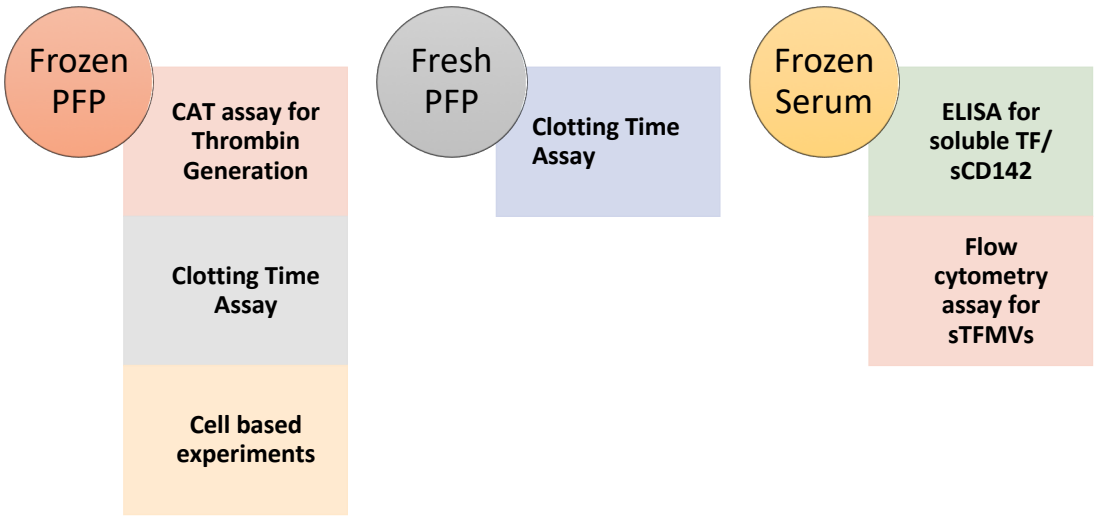


Figure 5.2 Serological experiments performed in both MM and PC clinical studies.

Abbreviations: PFP is platelet free plasma. CAT is Calibrated automated thrombogram assay. ELISA is Enzyme-linked immunosorbent assay. sCD142 is serum Tissue Factor/CD 142 antigen. TFMVs is Tissue Factor Microvesicles (Appendix C)

5.4 Clinical studies description

5.4.1 Clinical History of MM patients

The current study was initially authorized as a clinical trial of an investigational medicinal product (CTIMP) by the Medicines and Healthcare products Regulatory Agency in 2012, and granted approval by the Research Ethics Committee/REC (ref 12/YH/0328) and the Hull and East Yorkshire (HEY) Research and Development (R&D) department. It was approved for reopening in 2014. The study initially studied the effects of novel chemotherapy treatment of MM on the clotting pathways, platelets and the endothelium. These patients received an induction chemotherapy of either CTD (Cyclophosphamide, Thal, DEX) given as a 28 day or an intensified 21 cycle regimen, or a LCD (LEN, Cyclophosphamide, DEX) or LD (LEN+DEX) therapy.

Eligibility criteria for the study included patient's age ≥ 18 years, ability to give informed written consent and a confirmed diagnosis of symptomatic MM through the presence of a paraprotein in serum and/or urine, organ damage (such as renal insufficiency, osseous appearances) or symptoms considered by the clinician to be MM-related. Patients that required treatment for their myeloma either at presentation or at the time of relapse were included. Those excluded included patients with other conditions known to elevate MV levels such as active infection, uncontrolled hypertension, diabetes mellitus with HBA1C indicative of poor diabetic control, recent myocardial infarction <3 months, rheumatoid arthritis or other inflammatory process in active phase (e.g. psoriasis). Also excluded were patients treated with long-term anti-coagulants (e.g. warfarin for DVT or AF) and those with recent thrombosis still on secondary prophylaxis. A control group for this study was included as patients with intrinsic thrombotic potential in myeloproliferative

disorders (MPD) such as chronic myeloid leukaemia, myelofibrosis, polycythaemia rubra vera.

Blood samples were collected at baseline pre-chemotherapy T1, after first cycle T2, after second cycle T3, and at end of chemotherapy T4 according to table 5.1 schedule. In addition, samples were not collected at all the time points for several reasons such as patient withdrawal from the study, severe illnesses and other adverse events. Overall, total number of newly diagnosed and relapsed patients recruited was 32; the samples collected for various assays and analysed at T1 was 28 (10 after 2014), while there were 20 in T2 (8 after 2014), 18 in T3 (7 after 2014) and 12 after end of treatment T4 (3 after 2014). One patient sample at baseline T1 haemolysed and was the sample excluded from analysis. Only one patient entered a 3rd cycle of chemotherapy and this, as well as the 4 from end of week 1 timepoint collected from relapsed patients, was excluded from analysis due to low numbers. In total, 12 patients were recruited for the MPD control and only the baseline bloods were processed (METHODs section 5.3) and analysed.

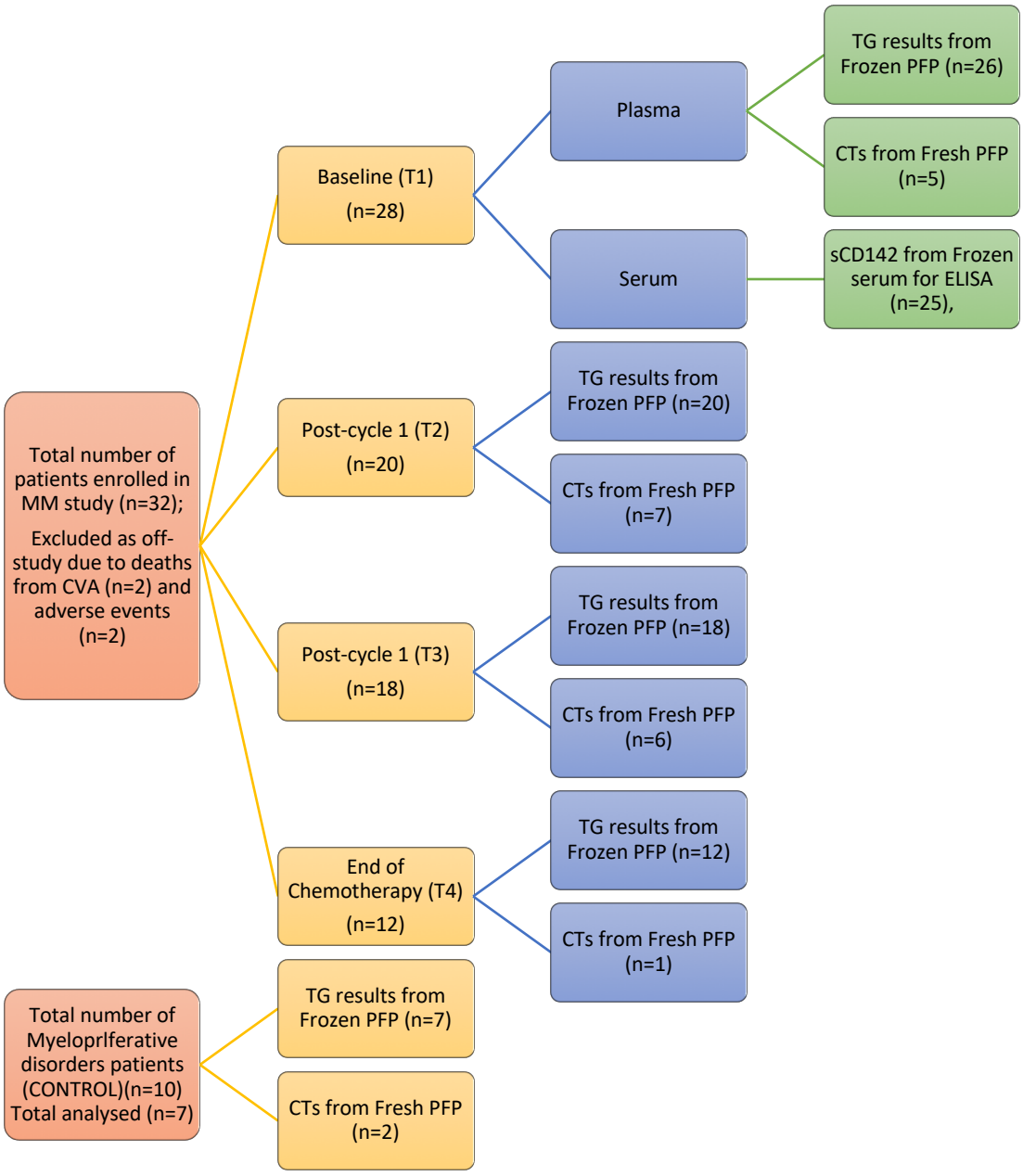


Figure 5.3 FLOW DIAGRAM of patients in MM study

5.4.2 Clinical History of PC patients

The study was initially approved by the REC (ref: 08/H1305/59) and by the NHS Trust research and development (R&D) / NHS Trust organization (number RO721) in 2008. This study initially titled 'Pancreatectomy and Tissue Factor' was started to study the effects of tumour removal from PC patients on TF-related thrombotic pathways. To

facilitate one of the secondary objectives of this thesis the ongoing PC study was amended substantially to include a separate arm to study the effects of chemotherapy on localized PC patients undergoing non-surgical treatment, and compare this with chemotherapy effects in the MM study (Version 3.0, August 2016). This amendment of the PC study was granted a favourable opinion by the REC in September 2016 and approved by Health Research Authority (IRAS 3284) at end of October 2016. Recruitment commenced shortly afterwards and included PC patients on palliative treatment, as well as those on adjuvant and neo-adjuvant chemotherapy.

5.4.2.1 Surgical Arm of PC study:

For the surgical arm, eligibility criteria included all patients fit for surgical resection and overall 88 patients were recruited into the study over a period of 9 years as these all had attempted surgery for malignant conditions and non-malignant ones, which were detected and excluded after histology was performed on the resections. 3 patients proceeded after surgery to week 8 of adjuvant chemotherapy and these were included in the chemotherapy arm analysis below. In this study, 29 patients were available overall but due to incomplete and missing samples, TG assays were performed on 21 patients (including those obtained by previous PI's) with confirmed pancreatic adenocarcinoma (PDAC), pre-cancerous malignant pancreatic conditions and cholangiocarcinoma (shown in Figure 5.4) while non-malignant conditions (n=4) were excluded. Most patients received either a Whipple's total or partial pancreatic resection (Resected group in Table 5.2) while others judged to be unresectable (Unresected group in Table 5.2) had either a bypass or palliative chemotherapy to relieve symptoms. A cholecystitis group was recruited as age and sex-matched controls and their pre and post-operative blood samples processed as described in section 5.3.

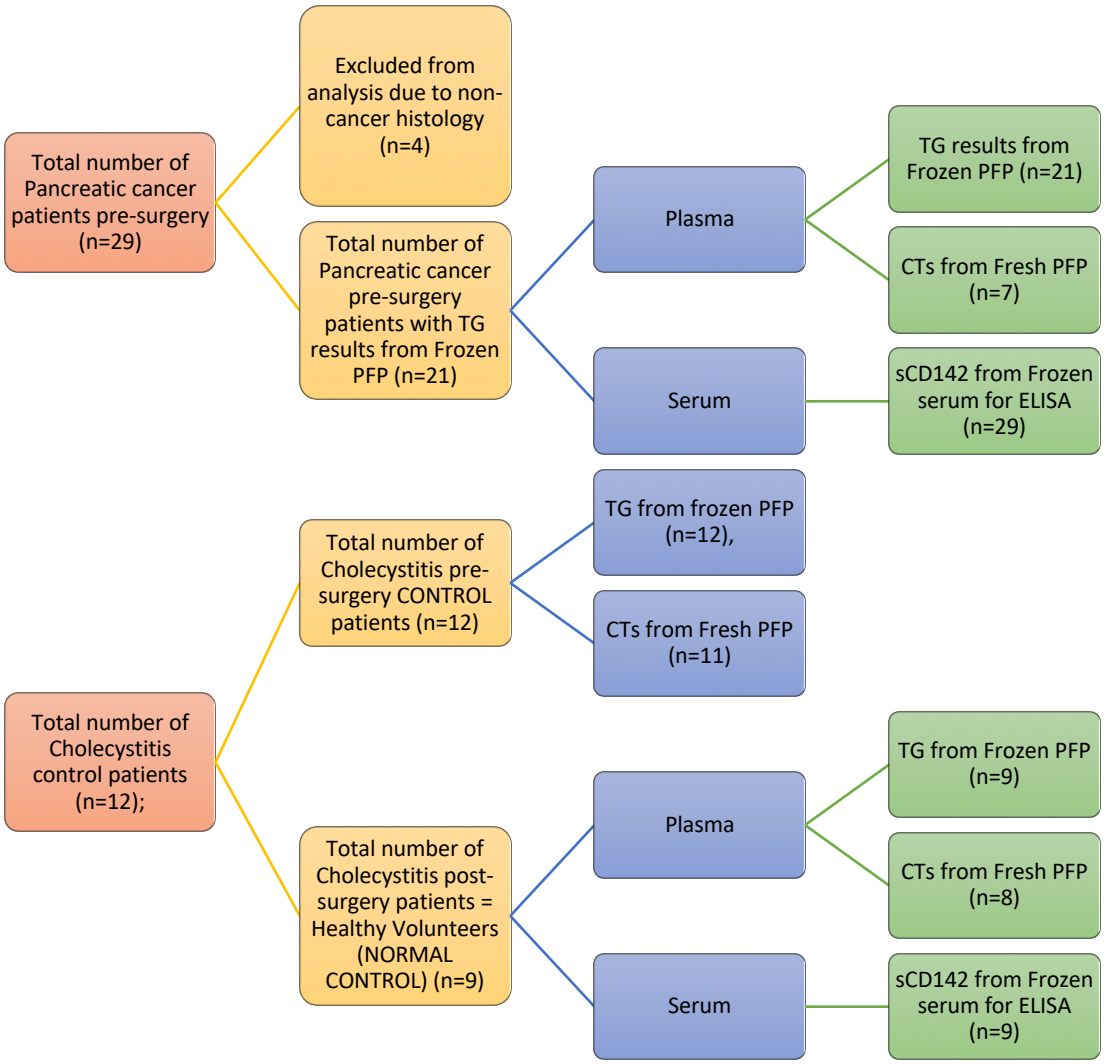


Figure 5.4 FLOW DIAGRAM of patients in Surgery arm of PC study

5.4.2.2 Chemotherapy Arm of PC study:

For the PC chemotherapy arm, the sampling schedule in the study protocol (see Table 5.3) involved obtaining pre-chemotherapy baseline bloods at the initial patient visits, then twice during chemotherapy at weeks 8 or 12 and weeks 24, and finally at 8 weeks after the end of the chemotherapy regimen. 12 patients were placed on chemotherapy before the end of this thesis study, however due to inadequate, haemolysed or missed samples, only 9 of these had enough samples at the different timepoints to be analysed. Also included for in this group were the 3 patients from

the surgical arm that had adjuvant or palliative chemotherapy, as shown in figure 5.5. The cholecystitis group recruited above remained the controls for this group. Most of these PC chemotherapy patients were placed on Gemcitabine (GEM)-based chemotherapy regimen (monotherapy or combined with Paclitaxel) or Modefied de Gramont 5-Fluorouracil (5-FU)-based therapy. Due to low recruitment numbers of study population, data analysis was performed on the TG results from overall 9 patients on chemotherapy (resected and non-resected) only at baseline and 6 at week 8-12. There were less than 3 patients in week 24 and at end of chemotherapy during this study; therefore, these data were not included in the final analysis.

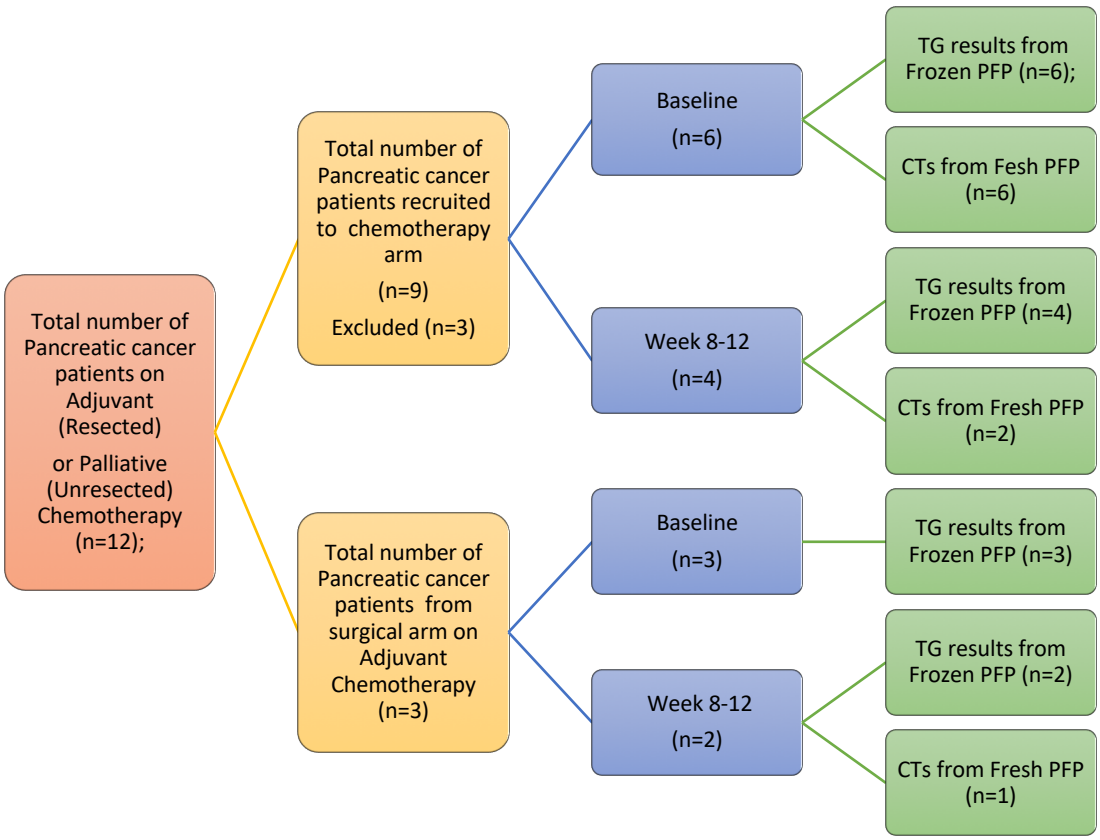


Figure 5.5 FLOW DIAGRAM of patients in Chemotherapy arm of PC study

5.5 Clinico-pathologic details

The clinico-pathologic details of the 4 independent patient groups (MM, PC, MPD and Cholecystitis) in both studies are summarized in Table 5.3 below. The data obtained from both clinical studies in this chapter was analysed on GraphPad software, version 7 (GraphPad Software, Inc., California, USA) where the median (Interquartile range/IQR) haematological parameters in both MM and PC patient groups and the respective controls were calculated. Student t-tests or one-way analyses of variance (ANOVA) were used to compare values within independent patient groups. P values of <0.05 were assumed as statistically significant.

Table 5.3 Summary of clinico-pathological details at baseline pre-treatment for patients recruited into PC and MM studies

Patient characteristics	MM (n=28)	MPD Control (n=10)	PC (Surgery) (n=29)	PC (Chemotherapy) (n=12)	Cholecystitis Control (n=12)	P value at baseline (one-way ANOVA)
Male/female	15/13	7/3	16/13	5/7	5/7	0.47
Age	71.14 ± 1.65	71.91 ±- 2.43	64.86 ± 1.87	69.64 ± 2.16	71.25 ± 2.31	0.039
Paraprotein concentration (mean ± SD; g/l)	27.10 ±15.40	-	-	-	-	-
Ig Myeloma type:						
IgG	12	-	-	-	-	-
IgA	5	-	-	-	-	-
Others	10	-	-	-	-	-

Haematological parameter (mean ± SD)						
Haemoglobin (g/dl)	11.29±0.30	14.01 ±1.36	12.35±0.37	12.21 ± 0.59	13.92 ± 0.30	<0.0001
Platelet count ($x10^9$/l)	201.10 ±14.42	291.30 ±45.53	284.80 ± 22.88	287± 36.54	243.40 ± 12.81	0.0155
WCC count ($x10^9$/l)	6.41±1.99	7.27±3.06	9.02 ± 0.75	8.96 ± 0.70	6.92 ± 0.39	0.0022

N.B. P values between the 4 patient groups (MM, PC, MPD and Cholecystitis) was obtained by one-way ANOVA tests and values of <0.05 were assumed as statistically significant.

5.5.1 MM Patient characteristics

5.5.1.1 Age and Sex of MM patients

The average age of the MM and MPD control patients analysed are similar (p=0.803) with no difference on analysis 71.14 +/- 1.65 years for MM while those for MPD patients is 71.91 ± 2.43, n=10. The male to female ratio for MM patients was 1.15:1 (15 males to 13 females) while those for MPD was 2.3:1 (3 out of 10 were females), as shown in table 5.3.

5.5.1.2 Demographics

Out of the 26 MM patients analysed for Thrombin generation (TG), 4 patients were hypertensive. All patients received anti-thrombotic treatment of Dalteparin (Fragmin®) from prophylactic doses of 5,000 units and above, and there were no symptomatic DVT or PE recorded during treatment. There were no serious adverse events (SAEs) observed during the collection of samples for this thesis project, although it should be noted that prior to 2014, 2 patients suffered from cerebrovascular accidents (CVAs) and both were newly diagnosed patients receiving CTD within the T1 cycle (associated with progressive disease). Another 2 patients were withdrawn from the study due to adverse events of suspected respiratory tract infection or pulmonary oedema and placed on alternative chemotherapy regimens. Furthermore, as determined by the treating physician, patients also received antibiotics and antiemetic prophylaxis during treatment according to local hospital protocols.

5.5.1.3 Haematological parameters in MM patients

Table 5.4 Summary of haematological parameters in MM patients over the duration of treatment

Mean +/- S.D	T1 (n=28)	T2 (n=20)	T3 (n=18)	T4 (n=12)	P value vs. T4* (Student t-test)
Haemoglobin level (g/dl)	11.29 ±0.30	10.79 ±0.34	10.92 ±1.18	12.05 ±1.94	0.992 0.272 0.367 -
Platelet count (x10^9/l)	201.1 ±14.42	247.1 ±81.54	251.9 ±83.9	259.5 ±95.09	0.3147 0.9571 0.9893 -
WCC (x10^9/l)	6.41 ±1.99	5.96 ±2.16	6.1 ±1.75	6.04 ±1.65	0.9059 0.9993 0.9999 -
Paraprotein concentration (g/l)	27.10 ±15.40	17.98 ±12.96	14.0 ±10.16	12.51 ±6.35	0.0050 0.6283 0.9854 -

It is known that a pre-chemotherapy Haemoglobin (Hb) level of <10.0 g/dl, platelet count of ≥ 350 x10^9/l and White cell counts (WCC) > x10^9/l have all been individually correlated with an increased cancer VTE risk(Khorana et al., 2008b). In this thesis, although VTE endpoints were not studied, all the Hb values obtained at different time points except at T4, were below the normal reference range (men=13.5 to 17.5 g/dl and women 12.0 to 16.0 g/dl) as provided by the Castle Hill Hospital laboratory that performed the tests. Platelet counts and WCC counts obtained were all however within normal reference ranges. In table 5.4, the average Hb level of MM patients

pre-treatment at T1 was 11.29+/-0.3g/dl while after the first, second, and end of chemotherapy it was 10.79+/- 0.34, 10.92+/-1.18, and 12.05+/-1.94g/dl respectively. There was a slight decrease from T1 of 11.29g/dl after the first cycle was administered to T2 10.79 g/dl (mean difference 0.5 +/- 0.53, p=0.99) and this reduction remained until a return to pre-treatment levels at the end of chemotherapy. Overall, when compared to T4 values, Hb values at different timepoints were not significantly different (p=0.253; one-way ANOVA). However, the Hb values at baseline were significantly lower than the MPD control group at 14.01 +/- 1.36g/dl (p=0.0001), which was within normal reference range (Table 5.3).

The platelet counts show no significant variation across time during chemotherapy (p= 0.0753). As seen in table 5.3, platelet counts of MM patients at baseline 201.10+/-14.42 $x10^9$/l (n=28) were 31% lower compared to MPD control 291.30± 45.53, n=10), and this difference was of significance (p= 0.0166). This result was similar to that observed for WCC, with significant variances between MPD control and baseline MM (p=0.0022), but none observed during the duration of chemotherapy (p=0.123).

The mean paraprotein levels for MM patients were 27.17 +/-15.4g/l at T1, which declined significantly (p= 0.0023; one-way ANOVA) in a downward trend with administration of chemotherapy in figure 5.6, indicating reduction in paraprotein plasma viscosity as a treatment response. This mean paraprotein level at T1 was higher than at T4 (12.51+/-6.35g/l), and this difference highly statistically significant (p=0.005). In addition, IgG was found to be the most frequent subtype (12 out of 20; 60%) and kappa the major light chain (10 out of 20; 50%) out of the paraproteins.

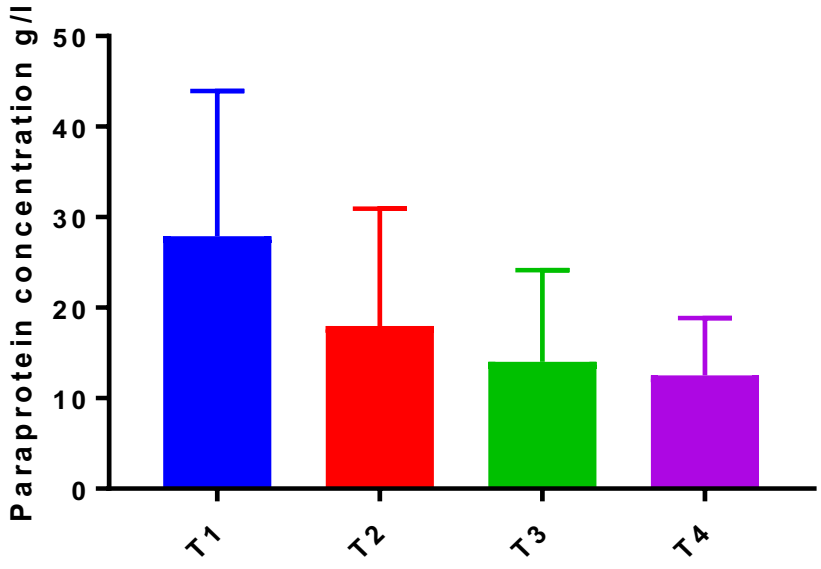


Figure 5.6 Total Paraprotein (g/l) in patients with MM before, during, and after chemotherapy.

MM patients prior to chemotherapy administration (T1, n= 28), after the first cycle of chemotherapy but before start of the second (T2, n=20), after second cycle but before start of the third (T3, n=18), and 6-8 weeks after the end of chemotherapy treatment at minimum 3 cycles (T4, n=12).

5.5.2 PC Clinical characteristics

5.5.2.1 Age and Sex of PC patients

The mean +/- SD age of the 29 localized PC patients included in this thesis project was 64.86 ± 1.87 years, while that of the Cholecystitis control group of 12 patients is 71.25 ± 2.31 years, and there was no statistical difference in these groups at p=0.0582. Furthermore, out of the PC patients, 16 were male and 13 females with a male:female ratio of 1.23:1. The cholecystitis group had 5 males out of 12 cases with a 0.71:1 male:female ratio.

5.5.2.2 Demographics

Out of the 29 PC cases analysed, 9 patients were hypertensive, while 11 drank alcohol in frequent amounts (2 units per week and above). In the study, 9 patients were diabetic, and while 2 were current cigarette smokers, another 2 were ex-smokers having quit over 20 years ago. Also 5 of these patients received anti-thrombotic Dalteparin at 5,000 units subcutaneously prophylactically, and one of these patients already had a thrombotic event (DVT).

5.5.2.3 Haematological parameters of PC patients

The mean Hb concentration for PC patients at baseline was 12.35 ± 0.37 g/dl, and this is statistically significantly less than the control group of 12 Cholecystitis patients 13.92 ± 0.30 g/dl taken at pre-surgery time points (p= 0.0107; student t-test).

The platelet count in blood samples of PC patients at baseline was 284.8 ± 22.88 per 10^9/l which was 14.4% higher than the counts for Cholecystitis control cases at 243.40± 12.81, n=12 (p=0.24; t-test) table 5.3. However, this difference did not reach any significance.

The WCC of PC and Cholecystitis patient groups were significantly different; with 23.28% higher counts in PC 9.02 ± 0.75 x10^9/l, relative to control 6.92 ± 0.39 x10^9/l at a statistical difference of (p= 0.017; t-test).

5.5.3 PC on Chemotherapy Clinical characteristics

5.5.3.1 Age and Sex of PC chemotherapy patients

The patients recruited and ultimately analysed for the chemotherapy arm (including those on adjuvant therapy from the surgical arm) had a mean age group of 69.64 ± 2.16 years; n=12 (Table 5.3). This value was similar (p= 0.6162, t-test) to the mean

age of the cholecystitis control cases, which was 71.25 ± 2.31; n=12. Out of these PC chemotherapy patients, 5 were male and 9 were female (male:female ratio is 0.55:1).

5.5.3.2 Demographics

Of the overall PC patients (n=12) placed on chemotherapy (including those on adjuvant therapy from the surgical arm of n=3), 3 were hypertensive, and one drank alcohol only socially. In addition, 5 of these patients had Diabetes and 1 smoked 11-20 packs of cigarette per day while the other was an ex-smoker.

5.5.3.3 Haematological parameters of PC chemotherapy patients

On the average, the mean +/- SD Hb concentration of patients analysed in the chemotherapy arm of PC study was 12.21 ± 0.59g/dl (n=12), while that of the control cholecystitis group before treatment was 13.92 ± 0.30g/l (n=12). This lower concentration of Hb in PC on chemotherapy patients was significant at p=0.0104; t-test.

The mean platelet counts in PC on chemotherapy was 287.0 ± 36.54 x 10^9/l and was higher compared to the values for cholecystitis control patients at 243.40 ± 12.81 x 10^9/l, although this difference held no significance (p=0.193; t-test) as seen in table 5.3.

The number of white cells present in PC chemotherapy patients baseline blood was 8.96 ± 0.70 x10^9/l, and was significantly higher (p=0.0127; t-test) than those found in Cholecystitis control 6.92 ± 0.39 x10^9/l.

PART B

5.6 The Assessment of Thrombin generation (TG) and Tissue Factor (TF) involvement using the Calibrated Automated Thrombogram (CAT) assay in Cancer patients

Patients with Multiple myeloma (MM) and Pancreatic cancer (PC) are known to be affected by severe antineoplastic treatment-related thrombophilia that manifests as a high incidence of clinical VTE events, with up to a third of these patients experiencing VTE in the absence of thromboprophylaxis(Khorana et al., 2007b). An important factor that has been associated with increased VTE risk is the use of chemotherapy, especially immunomodulatory (IMiD)-based therapies in MM (Zangari et al., 2010)and mostly Gemcitabine (GEM) in PC(Khorana & Fine, 2004). In both cancers, the development of thrombosis before and during treatment involves the generation of thrombin as part of the coagulation process. There is little knowledge of the dynamics of thrombin activation in the context of cancer coagulopathies driven by various malignancies and their treatments and whether markers of this state as measured by *in vitro* tests vary between different cancers. In chapter 4, it was shown how thrombin generation (TG) differs between solid cancers cell lines and haematological ones *in vitro*, with specific emphasis on the various kinetic parameters that correlated positively with tumour-derived Tissue Factor (TF) in PC, which distinguished it from MM cell lines. The hypothesis therefore generated by these findings was that the CAT assay, as a real time measurement of thrombin as it is formed in substrates, may be able to identify similar differences in the dynamics of thrombin production between these two malignancies (MM and localized PC) in the clinical setting. In this chapter, this hypothesis is tested by exploring baseline TG differences between the two malignant states before, during and after treatment for the effect of treatment on the production of thrombin, and to explore the relationship of TF to thrombin development in both malignancies.

5.7 Aims

The preceding subchapter part A describes the two main clinical trials from which MM and PC patients and their respective control groups of Myeloproliferative disorders (MPD) and Cholecystitis patients were recruited. In this part B, platelet-free plasma (PFP) samples obtained from the processing of whole blood of both cancer patients and controls were assessed on the CAT assay; detailed in METHOD section 2.8.3. Here, the aims were to:

- assess characteristics of TG through 4 main parameters (Lag times, ETP, Peak thrombin, TTP) from the TG curves generated from fluorescence emitted from thrombin-cleaved conjugates formed per test well in the assay (METHODS section 2.8)
- evaluate serum samples for soluble TF antigen with quantitative ELISA kits (METHODS 2.9).

5.8 Statistics

The data obtained from both clinical studies was analysed on GraphPad software, version 7 (GraphPad Software, Inc., California, USA) where the median (Interquartile range/IQR) haematological parameters in both MM and PC patient groups and the respective controls were calculated. Then further statistical analysis on acquired data from CAT and ELISA assays was performed by Dr Eric Gardiner on SPSS computer software (version 20.0 (IBM Corp.) using a SPSS MIXED procedure (with no random effects) that accommodated for missing data points across time. Two correlation structures for the variable measurement within a patient were used; the first assumed covariances per patient with an unstructured correlation matrix with no predetermined pattern, while the second is a heterogenous first order autoregressive covariance structure that assumes that the variances of the measurements at each time point can differ. For both structures, the marginal means (+/- standard error (S.E)) were used to compare variations over time points and cancer type with paired

student's T-tests. One-way ANOVA tests such as the non-parametric Kruskal-Wallis (KS) tests based on medians was used for comparing the pre-treatment baseline results of the 4 independent patient groups (MM, PC, MPD and Cholecystitis). P values of <0.05 were assumed as statistically significant.

5.9 Results

5.9.1 Longitudinal study of the TG parameters in MM patients before, during, and after chemotherapy

Whole blood samples were collected and processed into frozen PFP (methods section 2.8.3 and 5.3) for assessment on the CAT assay from patients at different timepoints of treatment (Table 5.5); baseline pre-chemotherapy T1, after the first cycle of chemotherapy T2, after the second cycle T3, and at 8 weeks after the end of chemotherapy T4. Only 2 MM patients entered a 3rd cycle of chemotherapy and only 1 patient into a fourth cycle, and both were not included in analysis. A mixed marginal model (as described in section 5.8 above), which accommodated missing time points per patient, was used to estimate mean values. Tests of significance of differences between T1, T2, T3 with T4 as a reference time point, were performed using t-tests on these mean TG values. T4 was chosen as a reference time point to demonstrate differences between patients with disease in-situ (T1, T2 and T3) and those in which the disease is cured or in remission (which it represents), and to align with previously published results recorded in our laboratory(Hall et al., 2016). P values of <0.05* were deemed significant statistically. The main significant results were observed between Lag times and TTP at T3 and T4, as explained in detail below. Velocity index was not evaluated in this chapter, as in previous chapter 3 and 4 it gave similar results to Peak thrombin and added no new information.

Table 5.5 Summary of CAT parameters for MM PFP before, during and after chemotherapy.

Time-points during chemotherapy	Patient numbers	Lag times- *P value vs. T4	ETP- *P value vs. T4	Peak- *P value vs. T4	TTP- *P value vs. T4
T1	26	0.40	0.18	0.79	0.70
T2	20	0.11	0.81	0.59	0.09
T3	18	*0.04	0.89	0.65	*0.03
T4	12	-	-	-	-

Each parameter obtained at various time points (T1, T2 and T3) were compared to T4 at end of chemotherapy treatment. Significant *P values was at < 0.05.

5.9.1.1 Lag times

The lag time (the time to start of maximum TG), of each time point was measured in MM PFP samples. In table 5.6 and figure 5.7, the median (IQR) lag times in MM PFP before chemotherapy was 3.12 (1.67-9.83) minutes. This duration was faster than lag times at T2 of 3.60 (1.67-9.5), and at T3 of 3.67(2-17), before returning to baseline levels once chemotherapy ceased at T4 of 3.17 (1.67-4.33) minutes.

Statistical analysis using a marginal model (an autoregressive covariance structure) for the 26 MM patients showed that the mean lag times at T3 are 1.85 minutes (S.E = 0.824 minutes; (*p=0.037)) significantly higher than T4. This difference in mean lag time between T3 and T4 was also of borderline significance under an unstructured covariance model (p=0.05). Although lag times at T2 seemed higher than at T4 as well, however no other differences were statistically relevant.

Table 5.6 Lag times of PFP of MM patients before, during, and after chemotherapy showing the statistical difference in the 4 timepoints on a covariance model.

Time-points during chemotherapy	Patient numbers	Median (IQR) Lag times	*P value vs. T4
T1	26	3.12(1.67-9.83)	0.40

T2	20	3.60(1.67-9.5)	0.11
T3	18	3.67(2-17)	*0.04
T4	12	3.17(1.67-4.33)	-

N.B. Prior to statistical analysis, a marginal covariance model that estimated missing data patterns was fitted to all values obtained for each patient. Under a specified assumption, this model facilitated the estimation of the marginal means for each time point, which was analysed by Student's t tests to determine significant variations. P values of <0.05 were assumed as statistically significant.

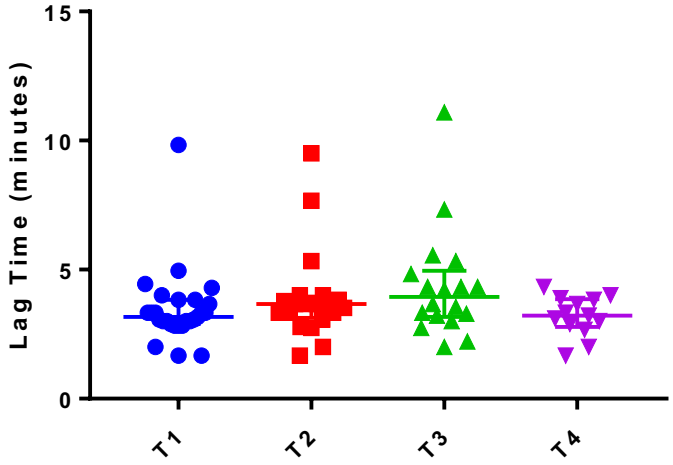


Figure 5.7 Lag times of PFP of MM patients before, during, and after chemotherapy.

Lag times of PFP in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1, n= 26), after first cycle of chemotherapy and before start of the second (T2, n=20), after second cycle and before start of the third (T3, n=18) and 6-8 weeks after the end of chemotherapy treatment at minimum 3 courses (T4, n=12). The horizontal bar represents the median levels and the error bars represent the IQR.

5.9.1.2 Endogenous thrombin potential (ETP)

The endogenous thrombin potential or area under the curve, corresponding to the total amount of thrombin formed (ETP in nM/min) was measured for all analysed MM PFP. Over the duration of treatment, the median (IQR) was T1 1429 (676-2843)

nM/minute and was similar to that of T2 1442 (27-2526) and T3 of 1459 (25-2272) nM/minute. This trend changed at T4 when the ETP was reduced to 1234 (803-2124) units, as shown in figure 5.8. However, as seen in table 5.7, when inserted into a mixed marginal model, there were no statistically significant differences between time points for either covariance structure model (autoregressive or unstructured) used.

Table 5.7 ETP of PFP of MM patients before, during, and after chemotherapy showing no statistical difference in the 4 timepoints on a covariance model.

Time-points during chemotherapy	Patient numbers	Median (IQR) ETP	*P value vs. T4
T1	26	1429(676-2843)	0.18
T2	20	1442 (27-2526)	0.81
T3	18	1459 (25-2272)	0.89
T4	12	1234(803 -2124)	-

N.B**.** Prior to statistical analysis, a marginal covariance model that estimated missing data patterns was inserted to all values obtained for each patient. Under a specified assumption, this model facilitated the estimation of the marginal means for each time point, which was analysed by Student's t-tests to determine significant variations. P values of <0.05 were assumed as statistically significant.

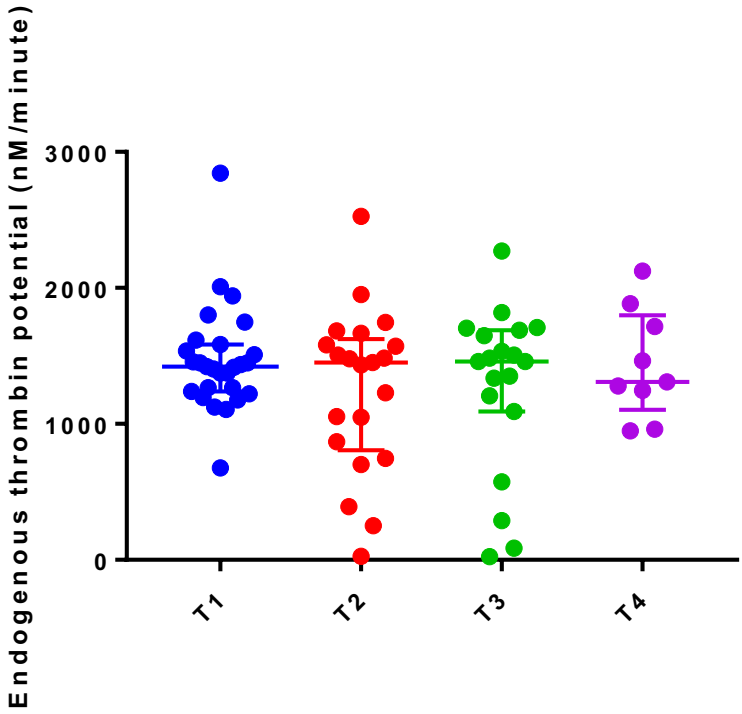


Figure 5.8 ETP of PFP of MM patients before, during, and after chemotherapy.

ETP of PFP in newly diagnosed and relapsed MM patients before chemotherapy administration (T1, n= 26), after first cycle of chemotherapy and before start of the second (T2, n=20), after second cycle and before start of the third (T3, n=18) and 6-8 weeks after the end of chemotherapy treatment at minimum 3 courses (T4, n=12). The horizontal bar represents the median levels and the error bars represent the IQR.

5.9.1.3 Peak thrombin

The peak height or maximum amount of thrombin formed (in nM thrombin) was measured for MM patient samples. As shown in figure 5.9, the median (IQR) values for peak thrombin at T1 of 243 (112-448) nM was similar to that of T2 at 263 (1-375) and T3 and T4 at 241 (4-388) and 264 (90-404) nM respectively. However, there were no statistically significant trend was observed between time points using a mixed modelling covariance structure to analyse the mean (Table 5.8).

Table 5.8 Peak of PFP of MM patients before, during, and after chemotherapy showing no statistical difference in the 4 timepoints on a covariance model.

Time-points during chemotherapy	Patient numbers	Median (IQR) Peak	*P value vs. T4
T1	26	243(112-448)	0.79
T2	20	263(1-375)	0.59
T3	18	241 (4-388)	0.65
T4	12	264(90-404)	-

N.B. Prior to statistical analysis, a marginal covariance model that estimated missing data patterns was fitted to all values obtained for each patient. Under a specified assumption, this model facilitated the estimation of the marginal means for each time point, which was analysed by Student's t-tests to determine significant variations. P values of <0.05 were assumed as statistically significant.

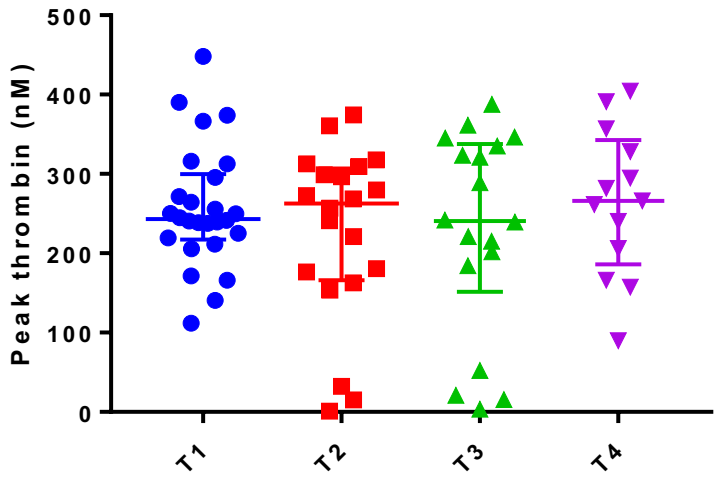


Figure 5.9 Peak thrombin of PFP of MM patients before, during, and after chemotherapy.

Thrombin Peak measured from PFP in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1, n= 26), after the first cycle of chemotherapy but before start of the second (T2, n=20), after second cycle but before start of the third (T3, n=18), and 6-8 weeks after the end of chemotherapy treatment at minimum 3 cycles (T4, n=12). The horizontal bar represents the median levels and the error bars represent the IQR.

5.9.1.4 Time-to-peak (TTP)

The time until the peak thrombin height is reached (TTP) in minutes was measured in MM samples. Median (IQR) TTP at baseline T1 was 6.06 (3.56-13.83) minutes which increased over time to T2 of 6.66 (3.44-19.67) and peaked at T3 of 6.85 (4-32.33) minutes (Figure 5.10). This value decreased to 5.84 (3.67-9.44) minutes after the last course of chemotherapy T4.

Similar to Lag times, statistical analysis was performed using a mixed marginal model (an autoregressive covariance structure) in order to accommodate for missing time points for the 26 MM patients. This covariance marginal model confirmed that the mean TTP was 3.18 (S.E = 1.33) minutes longer at T3 relative to T4 at end of chemotherapy. This difference between T3 and T4 was of significance under the autoregressive covariance structure model (p=0.026), shown in table 5.9. Other differences observed were not found as significant. These two variables of the CAT assay (Lag times and TTP) also showed a strong correlation > 0.9 after analysis with Pearson tests.

Table 5.9 TTP of PFP of MM patients before, during, and after chemotherapy showing the statistical difference in the 4 timepoints on a covariance model.

Time-points during chemotherapy	Patient numbers	Median (IQR) TTP	*P value vs. T4
T1	26	6.06(3.56-13.83)	0.704
T2	20	6.66(3.44-19.67)	0.096
T3	18	6.85(4-32.33)	*0.026
T4	12	5.84(3.67-9.44)	-

N.B. Prior to statistical analysis, a marginal covariance model that estimated missing data patterns was fitted to all values obtained for each patient. Under a specified assumption, this model facilitated the estimation of the marginal means for each time point, which was analysed by Student's t-tests to determine significant variations. P values of <0.05 were assumed as statistically significant.

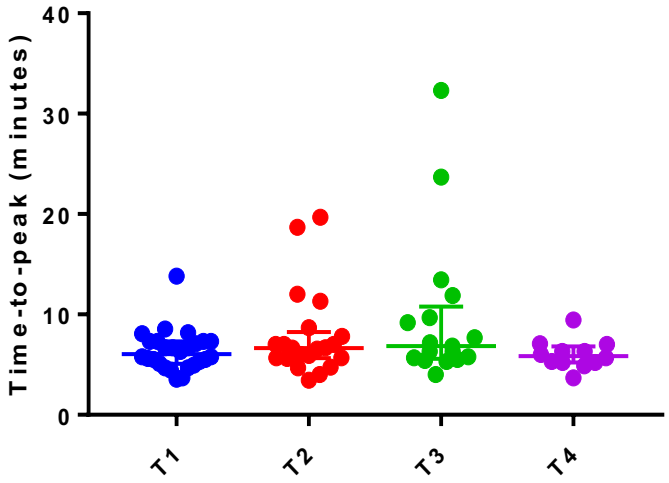


Figure 5.10 TTP of PFP of MM patients before, during, and after chemotherapy.

TTP of PFP in newly diagnosed and relapsed MM patients before chemotherapy administration (T1, n= 26), after first cycle of chemotherapy and before start of the second (T2, n=20), after second cycle and before commencement of the third (T3, n=18) and 6-8 weeks after the end of chemotherapy treatment at minimum 3 courses (T4, n=12). The horizontal bar represents the median levels and the error bars represent the IQR.

5.9.2 Baseline studies of TG parameters in Multiple Myeloma (MM) patients and Myeloproliferative disorders (MPD) control with localized Pancreatic Cancer (PC) patients and Cholecystitis control, before treatment administration.

The median values for all TG parameters obtained by CAT assay for the MM and the MPD control at baseline pre-treatment, with localized PC and Cholecystitis control before surgical treatment are shown in table 5.10. For all four TG variables, data from 66 patients were used: n=21, n=12, n=26 and n=7 from the PC pre-surgery, cholecystitis pre-surgery control, MM pre-treatment baseline T1 and MPD baseline control groups respectively. Overall, differences observed between the four groups

were not statistically significant except for variances of the ETP parameter (Kruskal-Wallis statistic = 10.28, df=3, p=0.016) and this is described further below.

Table 5.10 Summary of median (IQR) TG parameters in 4 independent patient groups; MM and the MPD control, PC and Cholecystitis control before treatment.

	Group 1: MM (n=26) median (IQR)	**Group 2: MPD (n=7) median (IQR)**	**Group 3: PC (n=21) median (IQR)**	**Group 4: Cholecystitis pre-surgery (n=12) median (IQR)**	**Overall P value between groups (ANOVA)**
Lag times (minutes)	3.12(1.67-9.83)	2.67(2.11-4.17)	3.33(2.40-10.89)	3(1.67-4.00)	0.083
ETP (nM/minute)	1429(676-2843)	1162 (645-1356)	1214(745-1782)	1276(673-1795)	0.016
Peak(nM)	243(112-448)	177(80-292)	223(73-346)	210(82-290)	0.078
TTP (minutes)	6.06(3.56-13.83)	6(4.56-10.17)	6(4.11-16.33)	6.33(4.33-8.67)	0.899

TG parameters between groups were compared with one-way ANOVA (Kruskal-Wallis tests). P values of <0.05 were assumed as statistically significant.

5.9.2.1 Lag times

The median (IQR) Lag times in MM patients before treatment commenced T1 seemed slightly higher at 3.12 (1.67-9.83) minutes than MPD controls at 2.67 (2.11-4.17), shown in figure 5.11. This value also seemed lower than that of PC, which had the longest lag times before surgery at 3.33 (2.4-10.89) minutes, and was 33 seconds longer than the lag times for Cholecystitis pre-surgery control at 3.00 (1.67-4) minutes. None of these differences however reached statistical significance when Kruskal-Wallis (KS) tests (p=0.083) were used to assess differences in estimated medians between these four independent groups.

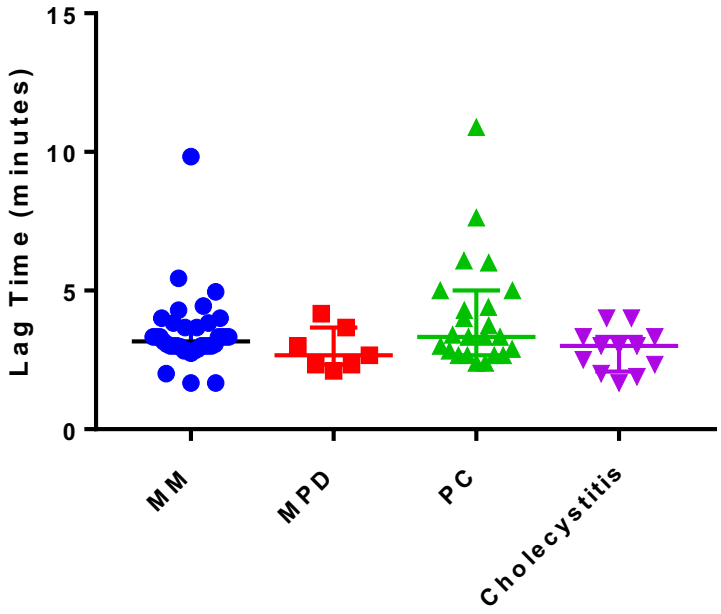


Figure 5.11 Lag times comparison between PFP of patient groups.

Lag times of MM patients (n=26) and MPD control (n=7) prior to treatment; Lag times of PC patients (n=21) and Cholecystitis control (n=12) before surgery, measured by the CAT assay. The horizontal bar represents the median levels and the error bars represent the IQR.

5.9.2.2 Endogenous Thrombin Potential (ETP)

There was an overall significant trend found amongst groups for the ETP (p=0.016). The median (IQR) for MM before treatment at T1 of 1429 (676-2843) nM/minute was higher than that of the MPD control 1162 (645-1356), shown in figure 5.12. Pairwise comparisons adjusting for multiple testing by a Bonferroni correction showed that this difference was significant (p=0.025). This median value for MM at T1 was also higher than PC before surgery of 1214 (745-1782) nM/minute, which was slightly lower than Cholecystitis pre-surgery control group with 1276(673-1795) nM/minute, however no statistical difference was found in these 3 groups.

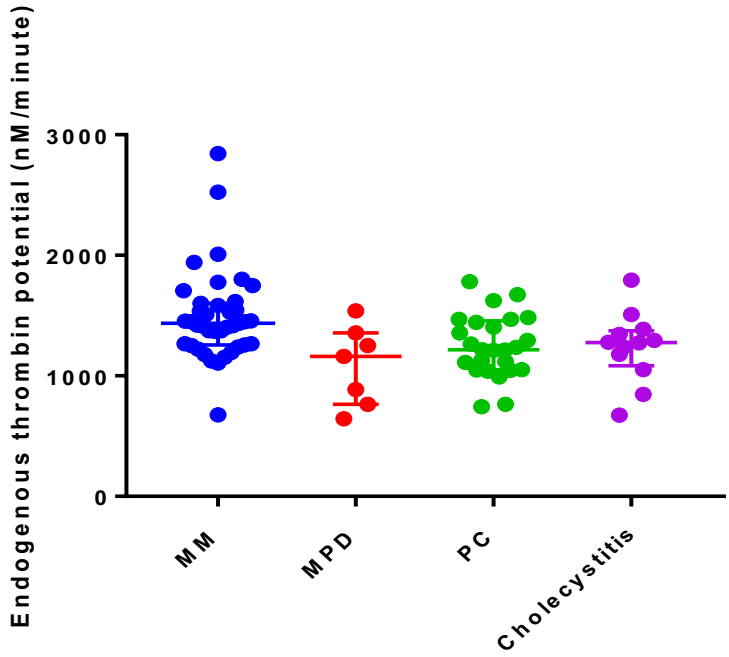


Figure 5.12 ETP comparison between PFP of patient groups

ETP of MM (n=26) and MPD control (n=7) prior to treatment; ETP of PC patients (n=21) and Cholecystitis control (n=12) before surgery, measured by the CAT assay. The horizontal bar represents the median levels and the error bars represent the IQR.

5.9.2.3 Peak thrombin

In the samples studied, the median peak thrombin reached for MM at T1 243(112-448) nM was higher in quantity than the 7 MPD controls of 177.30 (80-292) nM, shown in figure 5.13. This MM median was similar to that of PC before surgery of 223 (73-346) nM, which was slightly higher than that of the Cholecystitis pre-surgery control at 210(82-290) nM. The median Peak values for these 4 groups showed no statistical significance when analysed further (p=0.078).

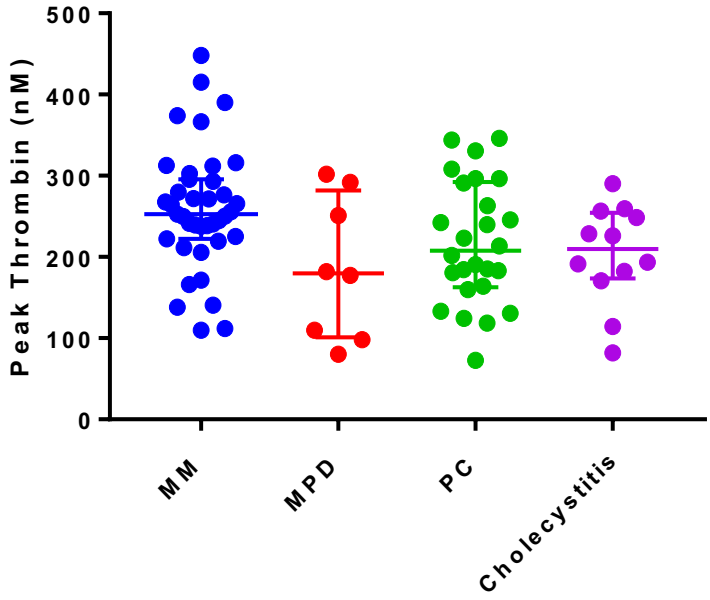


Figure 5.13 Peak thrombin comparison between PFP of patient groups.

Peak thrombin levels of MM patients (n=26) and MPD control (n=7) prior to treatment; PC patients (n=21) and Cholecystitis control (n=12) before surgery, measured by the CAT assay. The horizontal bar represents the median levels and the error bars represent the IQR.

5.9.2.4 Times-to-Peak (TTP)

The median (IQR) TTP of MM at T1 6.06 (3.56-13.83) minutes was similar to that in the MPD controls at 6 (4.56-10.17) minutes, which was in turn similar to that of PC before surgery 6.0 (4.11-16.33) and that of the Cholecystitis pre-surgery control 6.33(4.33-8.67) minutes), shown in figure 5.14. There were no statistically significant differences also observed upon further analysis (p=0.899).

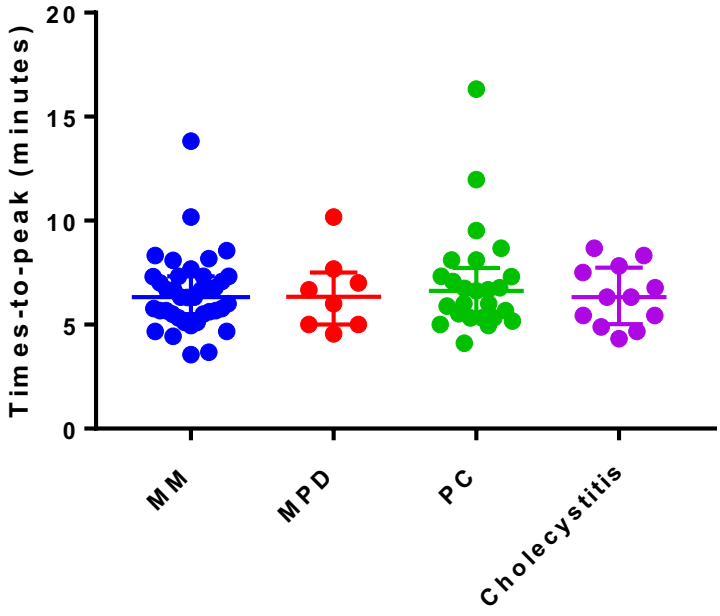


Figure 5.14 TTP comparison between PFP of patient groups.

TTP of MM patients (n=26) and MPD control (n=7) prior to treatment; PC patients (n=21) and Cholecystitis control (n=12) before surgery, measured by the CAT assay. The horizontal bar represents the median levels and the error bars represent the IQR.

5.10 Estimation of serum TF antigen (sCD142) measured in MM, localized PC patients and Healthy Volunteers (Normal control)

TF antigen (sCD142) levels was measured in serum by ELISA and for this assay, a healthy (Normal control) group was designated from the Cholecystitis group 8 weeks to 1 year after surgery. Out of the 12 cholecystitis patients, only 9 volunteered to return for post-surgery sample collections. For MM patients, 25 pre-treatment samples were analysed instead of 26 (one exception after cycle 2 for one patient as a serum-separating blood tube was not collected). 29 PC patient serum samples taken before surgery were available for use. Overall, the differences in TF (sCD142) measurements between the three groups were statistically significant (Kruskal-Wallis statistic = 10.037, df=2, p=0.007). Unexpectedly, sCD142 levels was found to be lower in PC pre-surgery patients than in Normal Control (p=0.013), as shown in table 5.11. The Normal control group have a significantly higher sCD142 median value of 53.92

(37.04-97.02) ng/ml than the PC pre-surgery group of 38.09 (15.91-69.22)ng/ml (Figure 5.15).

Table 5.11 TF (sCD142) levels found in serum of MM and PC before treatment and Healthy Volunteers (Normal control) group.

	Group 1: MM (n=25) pre-chemotherapy median (IQR)	Group 2: PC (n=29) pre-surgery median (IQR)	Group 3: (n=9) Normal Control median (IQR)	*P value between groups Group1/Group2 Group2/Group3 Group1/Group3
sCD142 (ng/ml)	47.24(25.65-113.17)	38.09(15.91 - 69.22)	53.92(37.04-97.02)	0.068 *0.013 0.672

Variances within groups were compared with one-way ANOVA tests (Kruskal-Wallis or KS). P values of <0.05 were assumed as statistically significant.

A)

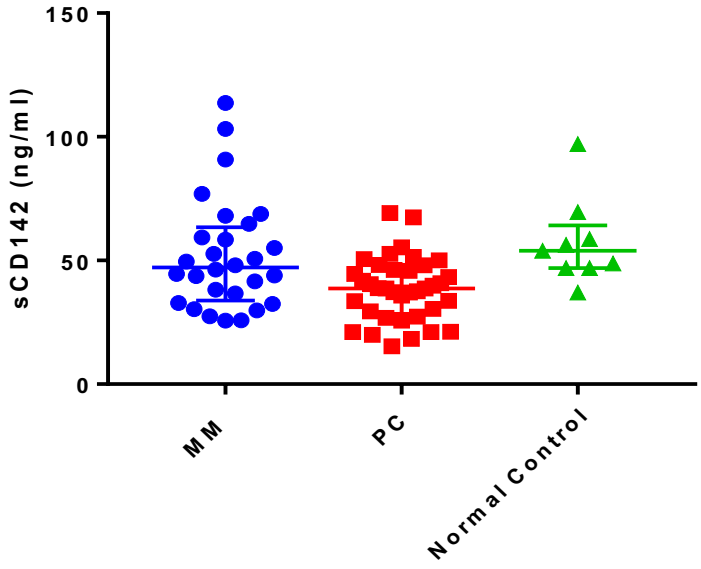


Figure 5.15 Serum analysis of TF (sCD142) levels in patient samples

Serum TF antigen (sCD142) measured by ELISA in MM (n=25) and PC (n=29) prior to treatment and Normal control (n=9) patient samples. The horizontal bar represents the median levels and the error bars represent the IQR.

5.11 Relationship between TG parameters in PFP with soluble TF antigen in serum in MM, localized PC patients and Healthy Volunteers (Normal control)

Correlations were sought between serum TF measured with ELISA (sCD142) and the TG variables in PFP measured by the CAT assay (Lag times, ETP, Peak and TTP) in MM, PC and Normal control samples. Overall, this TF (sCD142) marker mostly showed no significant associations between each group when compared with each TG parameter, except for a negative correlation with Peak thrombin in pre-treatment MM patients, which was significant (r=-0.41, p=0.04) such that TF antigen level increases in serum as Peak thrombin levels declines. In each group, although the correlations between variable pairs were different, however when Fisher's z-test was used to test the corresponding correlations, non-significant p-values (Table 5.12) were obtained in most of the parameters.

Table 5.12 Statistical correlation of serum TF (sCD142) levels measured by ELISA with TG parameters obtained by CAT assay.

1) MM before treatment

ELISA	**Patient numbers**	**Pearson Correlation coefficient (r)**	**P value (2-tailed)**
Lag times, sCD142	25	0.18	0.38
ETP, sCD142	25	-0.34	0.10
Peak, sCD142	25	-0.41	*0.04
TTP, sCD142	25	0.22	0.30

2.) PC before surgery

ELISA	Patient numbers	Pearson Correlation coefficient (r)	P value (2-tailed)
Lag times, sCD142	29	-0.02	0.93
ETP, sCD142	29	0.26	0.28
Peak, sCD142	29	0.09	0.72
TTP, sCD142	29	0.04	0.89

3.) Healthy volunteers (Cholecystitis post-surgery) control

ELISA	Patient numbers	Pearson Correlation coefficient (r)	P value (2-tailed)
Lag times, sCD142	9	-0.54	0.13
ETP, sCD142	9	-0.35	0.35
Peak, sCD142	9	-0.07	0.74
TTP, sCD142	9	-0.46	0.45

5.12 Comparison of the TG parameters in PC patients before and during chemotherapy

Of the nine patients studied for these four TG variables at baseline before chemotherapy and after week 8 of chemotherapy cycle, 2 patients provided data at week 12 and 2 at week 24, however these were not included in analysis. Furthermore, there were no statistically significant difference in any variable between baseline and week 8 of treatment (Table 5.13).

Table 5.13 Summary of median (IQR) TG parameters in PC on chemotherapy patient group before and at week 8 of treatment.

Time-points during chemotherapy	Patient numbers	Lag times-Median (IQR) /*P value	ETP-Median (IQR) /*P value	Peak-Median (IQR) /*P value	TTP-Median (IQR) /*P value
Baseline	9	2.4 (2.18-3.33) /0.34	1089 (937-1782) /0.34	278(153-346) /0.34	5.5(4.11-6) /0.11
Week 8	6	2.78(2.11-6.74)	1276(787-1746)	190(115-367)	6.76(4.67-10.41)

Variances within groups were compared with one-way ANOVA tests (Kruskal-Wallis or KS). P values of <0.05 were assumed as statistically significant.

All TG parameters; Lag times, ETP, TTP with the exception of Peak thrombin seemed slightly elevated after week 8 of chemotherapy. Peak thrombin in PC patients reduced by approximately 33% from 278(153-346) to 190(115-367) nM after 8 weeks in the chemotherapy cycle. However, none of these changes reached significance during analysis.

5.13 Summary of results:

- Changes within time in MM patients' sample under chemotherapy treatment showed that Lag times became progressively slower from 3.12 minutes at T1 to 3.67 minutes at T3 after which it returned to pre-treatment values at T4 at the end of chemotherapy courses.
- TTP (Times to reach peak thrombin height) also became progressively slower, similar to Lag times, from 6.06 minutes at T1 to 6.85 minutes at T3, before declining below baseline levels of 5.84 minutes at T4.

- However, only the difference between T3 and T4 in both Lag times and TTP were statistically significant (p=0.04 and p=0.03 respectively), once all data was fitted into a mixed modelling statistical model that allowed for missing values.
- Lag times and TTP results showed a very strong positive correlation (r>0.9)to each other in MM patients PFP during treatment.
- Median ETP obtained in MM samples pre-treatment at T1 (1429(676-2843) nM/minute) was higher than that of the other groups, although only the difference relative to its MPD control was significant (p=0.025).
- Overall, comparisons between the ELISA sCD142 measurements of MM, PC and healthy volunteers (Normal control) showed a statistically significant difference between the three groups (Kruskal-Wallis statistic = 10.037, df=2, p=0.007). Here, the amount of serum TF (sCD142) found in PC before surgery was lesser than in the healthy volunteers' Normal controls (p=0.013).
- In the MM pre-chemotherapy treatment group, peak thrombin correlated negatively with serum TF (sCD142) antigen levels (r=-0.41, p=0.04).

5.14 Discussion

MM and PC are cancers of different origins and aetiology. Although the existing clinical data in the literature reveal a high incidence of thrombosis in both malignancies in patients whilst receiving antineoplastic treatment, there are distinct differences that are apparent and can be highlighted. Firstly, the type of antineoplastic treatments used are different, with chemotherapy consisting of classical cytotoxics (GEM) being the mainstay of treatment for PC while MM is treated with a combination of immunomodulatory (IMiD) agents (Thal, LEN or BOR), chemotherapy (alkylating agents Cyclophosphamide or Mephalan) and hormonal therapies (DEX or Prednisolone). Secondly, the impact of these antineoplastic agents is much more pronounced upon commencement in MM than in PC, as PC patients often present with thrombotic signs in the absence of systemic treatments and even

before cancer diagnosis is made. Furthermore, the thrombotic effects of increments in the chemotherapy used in PC is also rather modest; whereas the classical baseline of VTE incidence in MM before IMiD-containing combinations became standard was typically recorded as approximately 5%(Carrier et al., 2011), but may increase by 17% to 75% based on the combinations used during treatment(Rajkumar et al., 2006) (Zonder et al., 2006). Thirdly, the treatment response rate in these two malignancies varies widely. In MM over 70% response rates occur on current 1st line options such as Thal/DEX combination therapy(Cavo et al., 2005; Rajkumar et al., 2006; Rajkumar et al., 2008) and there has been about 50% improvement in median survival in the past decade due to advent of IMiD-based therapies(Kumar et al., 2008). On the other hand, in PC there are practically no complete treatment responses (between 7-12%) and even partial responses are limited to a 1-year survival rate of 18% with the typical agent used-GEM (Burris et al., 1997). Therefore, the malignancy-related thrombophilic drivers from MM abate early and substantially, when compared to PC where they tend to be continuously present and contribute to a more aggressive malignancy.

As MM and PC are both known to have high VTE risks, any differences or similarities that exist may be distinguishable through products and substrates of the coagulation pathway that may highlight potential thrombotic markers. However, few studies have successfully linked VTE risk to the increase of any MV-associated coagulation biomarker, even though numerous cancers exhibit them, except for PC where the release of TFMVs are seen to be specifically associated with coagulation activation and thrombotic enhancement(Wang et al., 2012). Although VTE occurrence is known to depend on various factors such as imbalance between prothrombotic agents and antithrombotic deficiencies (Hisada et al., 2015), and use of chemotherapy, thrombin formation underpins clot development processes and therefore could be a valid means of comparatively assessing VTE pathways in cancer patients. In a large multicentre prospective study of 254 patients, high values of TG parameters (Lag times, ETP and Peak thrombin) were useful in the identification of patients at increased risk of recurrence after a symptomatic VTE episode (Tripodi et al., 2008). In this study however, the results obtained from TG were not compared to VTE risk

as patients involved in the study received thromboprophylaxis and no symptomatic VTE events were recorded before, during and 8 weeks post-chemotherapy.

In this chapter, while most changes measured by the assays in MM and PC did not reach significance, it is notable that of the few that did, Lag times and TTP were among the CAT parameters that showed prominence. In assessing the difference between MM PFP thrombin produced over time, both parameters highlighted higher values in T3 when compared to T4. This interesting result shows a significant (p=0.04; Table 5.6, Figure 5.7), progressive increase (lengthening) of lag times of the CAT assay as the time it takes to start the thrombin burst in MM patients across the chemotherapy cycles. A marked increase is also observed in the TTP parameter (p=0.03; Table 5.9, Figure 5.10), which means that it takes a progressively longer time to reach peak height of thrombin production from T1 toT3. One possibility from the resulting change in these TG parameters may be due to the compilation of damages to the endothelium from cycles of chemotherapy, which returns to pre-treatment values once chemotherapy treatment ended. An explanation for this observation may be that it is due to a concerted rise in endothelial MVs (EMVs) and other MVs in MM patients under treatment. This has been suggested by past data from our laboratory that showed a 2.4 fold EMVs increase at the third cycle of chemotherapy compared to baseline values in MM samples, although this remained elevated at T4(Hall et al., 2016). A damaged endothelium may actively prevent clot formation and TG by production of anti-thrombotic particles, including Nitric Oxide (NO) and prostacyclin and prevent fibrin deposition by production of tissue-type plasminogen activator (t-PA)(van Hinsbergh, 2012; Yau et al., 2015). An opposing view can also be argued that a damaged endothelium may likely release more apoptotic vesicles and procoagulant phospholipids into circulation resulting in faster thrombin clot formation and therefore cause Lag times and TTP to shorten. Further studies would be needed to explore these views, which reflect the functional heterogeneity of the endothelium during chemotherapy treatment, and to verify the TG kinetics that exist in these patients, although it is also important to note that there exists a flaw in this study whereby all MM patients unlike PC (5 patients only) were routinely placed on thromboprophylaxis (the LMWH Dalteparin) which may contribute to the prolonged

Lag times and TTP. Dalteparin acts by potentiating the actions of antithrombin III, which leads to the active inhibition of thrombin (through prevention of the propagation and growth of thrombi) and factor Xa and ultimately results in prolonged clotting times(Nutescu et al., 2016). It is these prolonged clotting times that may be reflected in the elongation of the Lag times and TTP observed in this chapter.

In previous studies in literature such as (Marchetti et al., 2012), as well as in chapter 4, Lag times and TTP have demonstrated marked differences in various established cancer lines *in vitro*, pointing to marked variability in mechanisms underlying coagulation pathways in PC and MM. In both the *in vitro* work and the *in vivo* study here in this chapter, both TG parameters show a strong correlation ($r>0.9$) to each other. A possibility may exist that since these 2 CAT assay parameters are wholly time-dependent, both may also be more sensitive to longitudinal studies that involve a measure of time, as seen in this chapter when the changes in TG was measured over the duration of chemotherapy cycles administered to MM patients. Here, changes in time to the thrombin burst and to reach maximum peak production, rather than the actual amount of thrombin formed may be more important. However, none of the published studies in literature have yet to report this observation, therefore the implications of these speculation remain to be seen and many studies are required to divulge additional insight and clarity.

In this chapter, apart from the ETP (total amount of thrombin producible over time) of MM being higher than the other patient groups especially the MPD control (Figure 5.12), there were no other significant differences between CAT parameters for PC and MM and the normal controls chosen for each. This ETP result suggests that there is a higher TG potential in MM, which may reflect the summary in chapter 4 that MM cells have a larger, intrinsic non-TF pathway reserve than PC cells (from their ability to generate thrombin during TF-FVII inhibition in the absence of factor VII from plasma) and therefore may be able to exhibit thrombotic features indefinitely. A recent 2017 study by Leiba et al has shown that in MM patients, those that have VTE during treatment are preceded by a rise in ETP of statistical significance (2896 nM/minute; $p<0.001$) which is higher than an ETP of 2028 nM/minute in those

without thromboembolic events (Leiba et al., 2017). Furthermore, ETP has been identified to be a potential predictive marker for VTE in metastatic patients, in a large prospective multicentre trial (n=831)(Falanga et al., 2015). Before treatment it would be expected that PC as a solid cancer with more of a TF-driven thrombin production pathway, would have shorter lag times (time to initiation of thrombin burst) and be generally faster as seen in the solid cancer cell lines in the chapter 4. However, this was not the case here, as there were no other significant differences in TG parameters obtained between MM and PC patient groups.

ETP has been useful in several studies in published literature. As main parameters of the CAT assay, ETP and peak thrombin have been reported in normal healthy individuals where they have been shown to be useful markers of TG ability(Dielis et al., 2008) and they also increase through pregnancy(McLean et al., 2012). In people with genetic susceptibilities to VTE, both are linked to single nucleotide polymorphisms and corresponding proteins such as F5 Leiden, F2 G20210A(Segers et al., 2010), indicating their sensitivity to genetic determinants and usefulness potential as screening tools. In relation to VTE occurrence, a few studies have reported weak associations with risk of first occurrence VTE; the Leiden Thrombophilia Study (LETS) associated ETP produced with a 1.5 fold increased risk of a first time DVT, although no link could be demonstrated with risk of recurrence (van Hylckama Vlieg et al., 2007). Another study associated peak thrombin of 349.2 (+/-108) nM with reduced risk of recurrence when compared to higher peaks of 419.5 (+/-110.5) nM (Hron et al., 2006). Presumably, it may be possible that these main parameters of the CAT assay have various analytical strengths that varies with substrates used; as suggested by one study that found higher sensitivity of ETP and Peak thrombin in whole blood analysis of patients with historical VTE than in their PFP or PRP samples (Tappenden et al., 2007).

In the results of this chapter, PFP peak thrombin correlated significantly with TF antigen(sCD142) measured in MM pre-treatment serum with ELISA, indicating that as TF levels increases the peak thrombin proportionally reduces. This may mean that although TF is present, it may exist in a different configuration in serum, such that

the amount of thrombin produced in relation is significantly reduced in MM patients, at least in peak height. This conflicting result may be understood in view of several reports in literature that shows that TF exists in various configurations and cryptic isoforms in circulation, and not all of these may be functional in different conditions (Bogdanov et al., 2003; Bach, 2006; Chen & Hogg, 2013). Yates et al, have also suggested that freely soluble TF may have limited activity in cell-free media from PC cell lines (Yates et al., 2011). Furthermore, some methods of measuring TF in substrates do not necessarily correlate with each other (van Doormaal et al., 2012; Tatsumi et al., 2014). For instance, studies using TF antigen and TFMV activity have been shown to not only contradict each other but also confound assessments of thrombosis risk in patients (Zwicker et al., 2013), an observation that contributes to difficulties in measurement and also to making comparisons across various studies, and may confound the results described here.

The view of different TF configurations may also explain the observation in this chapter that serum TF (sCD142) in localized PC patients was significantly less ($p=0.013$) than that measured in Normal controls from healthy volunteers. This may be because TF isoforms that exist in malignancies with established inflammatory component such as PC may be different to those in normal individuals. However, this result is in marked contrast to studies in literature that have found higher TF levels in PC than in controls, and attribute this to be associated with the high VTE incidents of this solid malignancy in particular. For example, the CATCH (Comparison of Acute Treatments in Cancer Hemostasis) study was a large randomized, multicenter trial that analysed about 900 patients and successfully showed that the highest VTE reoccurrence was found amongst patients with solid tumours such as PC that have the largest circulating serum TF levels, as measured by the same ELISA assay used in this study (Khorana et al., 2017). In addition, Maraveyas et al used a similar ELISA method in the FRAGEM study to demonstrate that the level of soluble circulating TF in PC patients on chemotherapy before administration of VTE prophylaxis correlated with the level of serum-induced invasion of PC cells (Maraveyas et al., 2010). However, none of these studies included a normal control group, as used in this chapter, as both had different end points. Khorana et al compared soluble TF levels

in serum to recurrent VTE events in the CATCH study, while Maraveyas et al studied the effects of LMWH on levels of serum TF before and after commencing anticoagulants compared to a group that did not receive anticoagulants in the FRAGEM study. Furthermore, it has been suggested by Echrish et al that most of TF activity is found associated with MVs (TFMVs) in plasma(Echrish et al., 2014), and is a component that may have clotted out from plasma given the process of obtaining serum. These may explain the observations in this chapter; although another possibility could be the varying durations of storage of the samples used (for example some had been stored for over 7 years). However, the CATCH study also recruited over a period of 3-4 years and the FRAGEM study samples were also accrued over a recruitment period of 7 years. It is unknown how TF antigen in serum may degrade over time, or under prolonged storage conditions as occurred in this project. The process of its disassembly and the type of conditions that could induce biodegradation in different substrates have not yet been studied extensively, and could be an interesting addition to published research.

The other correlations between TG parameters measured in PFP by the CAT assay and TF values (sCD142) in serum measured by ELISA in this project were mostly weak or insignificant, a fact that may be due to the use of serum as ELISA substrate. Ideally, patients plasma, which contains the full complement of clotting factors as was used in the CAT assay according to the thrombinoscope manufacturer's instructions (Hemker et al., 2006; Giesen, 2012), should also be utilized in the ELISA assay for consistency and to align with measurements from plasma. However, plasma volumes available were low and serum was therefore used for ELISA, as studies such as the FRAGEM and CATCH trials discussed above have already shown that ELISA assays on serum samples could significantly distinguish between patient groups with thrombotic issues. Moreover, in studies of other tumours, TF measured in serum can also be useful as biomarkers for the presence of cancers such as renal cell carcinoma(Silva et al., 2017) and as prognostic indicators for ovarian cancer(Han et al., 2006).

There are weaknesses that exist in this study, the main one being the low numbers of patients particularly for the localized PC study where chemotherapy was administered. This PC clinical project has been designed to study two groups of non-metastasized patients; one with PC *in situ* (non-resected) and the other post-pancreatectomy surgery (resected) to shed light on the impact of the tumour presence but also 'isolate' the effect of chemotherapy alone (without the tumour present). Unfortunately, the chemotherapy arm of the PC study was still on going at completion of the experimental work of this thesis, hence the numbers of patients were too low for meaningful analysis during this period and comparative evaluation with MM during treatment time points could not be achieved. As a compromise the data for this section was analysed for all patients with PC at baseline and week 8 of chemotherapy cycle, which may explain the lack of any meaningful conclusion at this point in time.

Chapter:6 General Discussion

A better understanding of the molecular mechanisms that underlie cancer-related venous thromboembolism (VTE) is of vital importance for the development of better screening, treatment and prevention strategies. The mechanisms through which cancer induces or influences prothrombotic activity are many and varied and still not well understood. There are some biomarkers of this activity including Tissue Factor (TF), the selectins, D-dimers (Ay et al., 2009; Pabinger & Ay, 2009) that have been shown to demonstrate variable degrees of clinical correlation with CAT but robust markers and causative stimuli are yet to be identified and developed. This study concentrated on thrombin generation (TG), a key constituent of coagulation, and it was demonstrated that thrombin production can potentially show haemostatic differences in cancer cells of varied aetiology *in vitro* as well as in blood obtained from patients in the clinical setting. This project has also contributed to the understanding of thrombin activity under various conditions, including the presence of freely soluble TF, circulating TF microvesicles (MVs) and coagulation factors of the intrinsic (factor XII) and extrinsic (factor VII) pathways. In addition, it has shown the acquisition of procoagulant characteristics in the endothelium through prothrombotic mechanisms such as TFMV uptake, TF expression and thrombin generation.

It is known that the vascular endothelium is prominently involved in clot formation, and can contribute to clinical VTE complications including DVT in malignancies(Blom et al., 2005). Several reports have described increased levels of procoagulant molecules including active TF produced from disruption of the endothelial wall (Moore et al., 1987; Mackman, 2009; 2012). Increasing TF levels in endothelial cells, delivered through uptake of TFMVs (often circulating at increased levels in cancer

patients) can lead to endothelial transformation through activation of PAR-2 and induction of a pro-apoptotic state that leads to cumulative damage and denudation of the endothelial layer. This endothelial dysfunction results in a decrease in production of anti-coagulant factors such as TF pathway inhibitor [TFPI], thrombomodulin (Moore et al., 1987) compounding the pathologic conditions that can initiate thrombosis clinically. However, endothelial deterioration is likely to be a much more complex event driven by a number of inflammatory, mechanical and molecular procoagulant mechanisms operative in the tumour micro and macro environment. As described in chapter 3, this thesis has found changes in endothelial behaviour positively linked to the production of TF in association with MVs (TFMVs) from cancer cells (Table 3.2). Here, increased TF antigen levels, were correlated with endothelial acquisition of procoagulant qualities such as TG in significant quantities, measured through parameters of the calibrated automated thrombography (CAT) assay. The endothelial changes also include decreased (faster) clotting times from previously non-clotting endothelial cells, presence of tumour MVs attachment to the endothelial cell membrane surface. This procoagulant activity (PCA) is a crucial example of endothelial heterogeneity displayed through its ability in conforming to different functional needs at a cellular level(Aird, 2012). It has been shown in published literature that TFMVs from other malignancies can have the same effect on human dermal blood endothelial cells (HDBECs), and a mechanism of how this could be induced has been proposed by Collier et al through internalization and recycling of circulating TF to the cell surface and associated PS exposure(Collier et al., 2013). There are a few other studies that support this hypothesis; for example human glioblastoma cancer cells have been found to cause platelet aggregation and thus increase hypercoagulability through TFMVs shed in patients' blood(Bastida et al., 1984). In addition, Maiolo et al have previously described an increase in TF-like PCA found in tumour cell-endothelial cell co-cultures- localized on endothelial cells(Maiolo et al., 2002). These variations that exist in hypercoagulability of the endothelium may reveal therapeutically targetable ways that may be explored in cancer development. Potentially, anti-thrombotic strategies for VTE prevention may also be developed that focus on protecting the endothelium from such prothrombotic transformation, by reducing the PCA response to malignancies.

A novel finding in this study is that HUVECs that displayed new PCA from tumour MVs association also displayed similar TG characteristics to the parent tumour from which the MVs were derived. This may mean that the endothelial cells inherit similar TG abilities transferred by cancer MVs, which is a novel example of cross-talk between the tumour environment and cancer. This finding may have significant implications, as it provides an understanding of how tumours force not only their micro- but also macro-environment to be remodelled after them. Although beyond the scope of this study, it would be interesting to conduct intracellular studies of these endothelial cells using PCR techniques and proteomic assays to detect any genetic transcription and translation molecules that are produced as result of the change in endothelial phenotype reported in this thesis. A most recent discovery has been made by Ettelaie et al, regarding a possible post-translational modification of TF that enables its continued exposure on primary endothelial cells, and its release within cell-derived MVs(Ettelaie et al., 2018). This modification occurs in the cytoplasmic domain of TF, where phosphorylation of the serine 258 by protein kinase C and the ubiquitination of lysine 255 residues, are protected by peptidy-prolyl trans/cis isomerase 1 (Pin1), a multifunctional and diverse enzyme, with eventual prevention of TF release. Additional studies are however needed to further the understanding on the intracellular regulation of TF endometrial PCA.

The CAT assay has been used on numerous studies to study TG mostly *in vitro*, and while informative, these studies have yet to give complete representations of clinical thrombotic mechanisms and possible biomarkers. Correlation of a procoagulant state in suspected VTE patients with TG has been shown to various degrees; one recent study in particular which compared *in vivo* and *ex vivo* characteristics using urine and plasma substrates suggested that higher levels of prothrombin 1+2 fragments in urine correlated with increased Lag times, times-to-peak (TTP) and endogenous thrombin potential (ETP) in diagnosed VTE cases(Wexels et al., 2017). However, the issue of acceptable substrates to use and the consistency of the results is also a factor preventing wide uptake of CAT assays. While thrombin can be generated in virtually all substrates including whole blood(Ninivaggi et al., 2012), cord blood(Rice et al.,

2016), synovial fluid(Chang et al., 1995), urine and plasma(Wexels et al., 2017), not all substrates generate consistently recordable CAT parameters with the available Thrombinoscope calibrator, which creates potential sources of discrepancy in VTE assessment. Calibrator variability also occurs amongst laboratories, compounding the technical issues that exist(van Veen et al., 2008). In chapter 5, platelet-free plasma (PFP) was used, as recommended by the assay protocol developed by Hemker et al. As varied as these methods of assessing VTE through TG are, however inter-laboratory variability of results has been a major deterrent to routine use in clinical practice. Also there are chances of high inter-individual differences in thrombin recorded per patient as seen in some studies(Wegert et al., 2005), which was not seen in this study. An attempt at standardization of TG assays with a large multicentre study was proposed in 2012, and although this study reported minor variability in acceptable limits (Dargaud et al., 2012), the study population was too small (6 patients per centre) to be reliably utilized. Therefore, at present the CAT results obtained in this project are not generalizable or strictly comparable to other studies performed with various substrates on the assay, necessitating the need for more work to develop better fully standardized protocols.

Several studies as discussed in the introduction section have already described the coagulation cascade and its numerous clotting factors involved in thrombus formation (Davie & Ratnoff, 1964; Macfarlane, 1964; Hoffman & Monroe, 2001; Ferreira et al., 2010). Thrombin is a key protease that has a multifunctional role in the cascade. Despite the understanding of its key importance and necessity for clot formation, thrombin production requires further studies to establish it as an independent predictor of symptomatic VTE in clinical settings. Although VTE is now known to be driven by anti-cancer treatments, in the context of the two malignancies (Multiple myeloma (MM) and Pancreatic cancer (PC)) that were the subject of this work, not many studies have investigated the thrombin generation kinetics, or explored the effect of treatment such as chemotherapy on its production. It was shown in the *in vitro* studies in chapter 4, through plasma deficient (of specific coagulation factors) conditions that the coagulation pathways (intrinsic and contact) can have variable impact on the TG parameters, which are affected significantly but

also variably in different cancer cells. The cytological pathology of cancer (for example adenocarcinoma of PC vs plasma cell malignancy of MM) therefore matters in TG as it was shown in chapter 4 that solid cancer cell lines of PC have a TF-dependent thrombotic propensity over haematological lines such as MM. This propensity was exhibited in faster TG times and overall higher amount of thrombin produced in PC cell lines than MM, although MM cells may have a larger intrinsic pathway TG reserve than PC cells. The intrinsic reserve may result from the ability of MM cells to generate thrombin in both factor XII and VII absence in plasma, unlike in PC cells where TG was significantly reduced in factor VII absence but not factor XII. The observation here implies differential contributions of both cancer types to TG, and has also been suggested in a recent study by Rice et al(Rice et al., 2016), where MM may have the ability of persistent continuous, subclinical clot formation(Duchemin et al., 2008). Furthermore, the results in chapter 3 showed that tumour MVs produce similar TG patterns as their parent cell sources, which further implicates MVs as an important thrombotic facilitator that enhances TF PCA and a source of TG. The different pro-thrombotic qualities measured by CAT assay gives an insight into how these malignancies may behave dissimilarly inside the host by portrayal of differing thrombotic profiles. This assay has also proven insightful in a few other studies on clinical VTE. For example, an ETP of 30% or greater has been found in one study to reflect the severity of a bleeding tendency(Al Dieri et al., 2002). In the clinical setting, this could be therapeutically advantageous, as currently anti-thrombotic medications used to prevent VTE occurrences are not tailored. Anti-TF therapy through TF silencing can be developed for cancers with TF-dependent thrombotic pathways, while inhibition of coagulation Factor XII by selective depletion in non-TF dependent cancer(Revenko et al., 2011), might represent an important therapeutic goal in cancer thrombosis management(Marchetti et al., 2012). In this regard, personalized VTE therapy or prophylaxis would be of utmost benefit as a future goal, and the determination of thrombotic phenotypes using TG may have a major impact on mortality from thromboembolic incidents, as has been potentially suggested for TF as a tumour-type specific biomarker for VTE recurrence by Fonseca et al (Fonseca et al., 2017) in their response to the CATCH study(Khorana et al., 2017).

These very interesting *in vitro* findings generated the hypothesis that similar differences may underlie the VTE phenotype of these two malignancies *in vivo* as well and that the study of TF levels and TG in human tissue (PFP in this instance) could mirror or parallel these findings, and also that cancer treatment could have differential effects on these coagulation parameters. Unfortunately, by the completion time of the fieldwork for this thesis not enough patients had accrued into the pancreatic cancer on chemotherapy cohort of this study for this comparison to be made, although this study is ongoing. However, in chapter 5 some interesting observations could be made, of the MM patient group in particular, and a few tentative observations could also be made across the two cohorts of cancer patients. In chapter 5 part A, it was shown that the paraprotein levels of MM patients progressively reduced across the cycles of chemotherapy treatment up to 8 weeks after end of treatment. This result reflects a treatment response which signifies a reduction in plasma viscosity that did not impact TG measurements as none of the TG parameters in chapter 5 part B showed a similar trend of progressive decline during treatment similar to the paraprotein levels. Chapter 5 part B also demonstrated that in MM patients the peak thrombin was the TG parameter that significantly correlated ($r=-0.41$, $p=0.04$) with TF antigen levels (sCD142 measured by ELISA) in patient samples at baseline before treatment commenced. This may signify that the actual amount of peak thrombin produced reduces as TF antigen levels increase. These correlations, though modest and at first appearing contradictory, may indicate the importance of studies of TF antigen configurations, as although TF may be available in plasma in association with MVs, in serum it may exist in a different isoform, one whose function decreases in relation to the amount of TF present. Several studies of various TF isoforms and encryptions in literature have implied that not all TF configurations have functional ability (Bogdanov et al., 2003; Bach, 2006; Chen & Hogg, 2013). Detailed studies of the mechanisms that influences and regulate TF isoforms in circulation with MVs, and in concert with thrombin production are desirable in the future to further the understanding of hypercoagulability in the cancer patients during treatment.

Another interesting result observed in chapter 5 part B, was that MM had the highest ETP (1429nM/minute) amongst the 4 patient groups measured for TG including PC. This result, and those presented in chapter 4, further highlighted the above-mentioned intrinsic pathway reserve possible in MM, presumably due to a residual capacity of these cells to induce TG after TF-FVII inhibition in FVII-deficient plasma as previously suggested by Marchetti et al(Marchetti et al., 2012). Thus, it may be theorized that MM may have a larger haemostatic ability or procoagulant potential than previously known in literature, which may be responsible for the larger thrombin potential observed. Interestingly, TF antigen levels measured in PC serum were less than that in MM and healthy controls. This apparent paradox can be understood in view of the type and conditions of the serum substrate used, instead of plasma in which most of the TF and TFMV activity is known to be carried. Studies of TF isoforms have not extensively detailed the types found in different substrates, and approximations from *in vitro* studies may not accurately translate to sample obtained directly from patients themselves. Moreover, differing lengths of storage and freeze-thawing cycles may have significant effects on TF solubility and attachments to MVs in serum, although this has yet to be studied in detail in literature. In our laboratory group, Echrish et al, found that both TF antigen and associated MV activity of some PC cases investigated showed no significant variations from the control groups when measured by ELISA in frozen patient serum, inviting the suggestion that TFMV activity as well as reliable TF antigen detection might be affected by lengths of storage (unpublished work; 2008). Another possibility could be due at least in part, to the inhibitory effect of current thromboprophylaxis treatment in cancer patients. In particular, Maraveyas et al has previously described that Low molecular weight heparin (Dalteparin) can affect the circulating levels of TF in PC patient serum and serum-induced cell-invasion(Maraveyas et al., 2010), although no such knowledge currently exist in literature on this observation in MM patients. In the Maraveyas et al study, PC patients studied on Dalteparin treatment (n=39), serum TF antigen reduced from 336 pg/ml to 303 pg/ml as compared to controls (p=0.005). It would be interesting to explore this result further in view of the results from this chapter that showed that serum TF levels are higher in MM patients in comparison to PC. Therefore, more prospective studies are necessary to explore TF antigen and

TFMV activity in various cancers under treatment and to fully evaluate the premises of prolonged storage on the TF isoforms in different substrates, and the practical implications in assay selections.

In PC, clots are known to form through highly TF-dependent mechanisms, unlike in MM. Several studies using various assays have measured high levels of TF in PC patients before treatment and correlated this to VTE risks. In the CAT assay used for this thesis, two TG parameters were of prominence in chapter 4; Lag times and TTP, which both showed consistent results differentiating TG in the solid cancers such as PC from the haematological ones such as MM in cell lines *in vitro*. Chapter 5 also showed that these two parameters were also of significance in highlighting differences seen in MM patient samples during chemotherapy treatment, although assessing their relationship to VTE risk was beyond the scope of this project. Specifically, both Lag times and TTP illustrated a progressive delay in thrombin formation that reached its peak at the third cycle of chemotherapy administration but recovered after the end of chemotherapy and returned to pre-treatment values. From these results, it may be hypothesized that the delay may be related to the concurrent administration of LMWH as anti-thrombotic in MM patients, or a rise in cumulative endothelial damage from chemotherapy which may be marked by a rise in MVs of endothelial origins that has been previously observed in a clinical study prior to this project(Hall et al., 2016). In support of this hypothesis is a study by Auwerda et al, which has identified increased levels of TFMVs in MM patients that develop VTE after prolonged chemotherapy treatment, compared to those that do not (Auwerda et al., 2011). Elevated MVs have also been recognized to reflect alterations in endothelial states in cancer patients from treatment(Burger & Touyz, 2012; Hall et al., 2016), and this damage has been associated with increased cancer VTE(Kuenen et al., 2002). These observations all serve to strengthen the relevance of endothelial injury as a probable mechanism of chemotherapy-induced hypercoagulability in cancer patients.

In summary, this thesis has provided data that has contributed to the understanding of thrombin and TF-associated prothrombotic mechanisms in cancers of different

aetiology. This project has expanded the present knowledge of endothelial involvement in cancer VTE development through TF and TFMV-dependent mechanisms, by demonstrating that procoagulant MVs shed by tumour cells induced a procoagulant effect in endothelial cells through increased clotting activity, TG and cell membrane surface expression of TF. The interplay of these main players in thrombus development during treatment may influence specific TG kinetics that may eventually aid in hospital identification of at-risk VTE patients and monitoring of thromboprophylaxis in cancer patients. In addition, the contribution of several lesser-known factors in the coagulation cascade may reveal new therapeutic targets. However, these hypotheses need to be explored further under various haemostatic conditions in several other cancer types and in different clinical settings. More studies are also needed that reflect the *in vivo* thrombotic processes in cancer patients to fully confirm the observations made in this project.

6.1 Future work

Future work recommended for this study include the extension of both clinical studies most especially the PC on chemotherapy arm to maximally recruit the desired patient numbers. This would remove the limitations of the data reported in this thesis by allowing full exploration of study aims; such as the detailed comparison of TG kinetics in the cancer groups- PC pre-surgery baseline and after surgery, MM relapse and newly diagnosed patients on chemotherapy, and effects of chemotherapy on TG in PC over the duration of treatment. Estimation of TG in the absence of other coagulation factors of the clotting pathway would also be beneficial in order to obtain a more comprehensive and holistic profile of the coagulation cascade in various malignancies. Furthermore, statistically analysis of the patterns observed in TFMV origins with changes in treatment should be assessed to provide more information on MV characteristics. The contribution of other cell types to the complex interactions during TG should also not be underestimated. For example, how activated neutrophils generate MVs and also how the avulsed extracellular DNA produced from these neutrophils (NETs)(von Bruhl et al., 2012) interplays with the

endothelium in coagulation processes *in vitro* and *in vivo* are worthy of further study. In addition, studies that utilize cell-sorting flow cytometry to reliably isolate and separate the endothelial cell populations with procoagulant capacity that are induced by tumour TFMVs should be conducted. This will enable selection of TF-expressing endothelial cells from non-TF expressing cells, which may then be transfected with TF siRNA to knockdown TF production and demonstrate inhibition of prothrombotic activity such as thrombin generation. Although beyond the scope of this study, it may be an intriguing way to characterize these procoagulant endothelial cells populations further, which may propel future investigations on the inner composition of the TF-adherent cells, the signalling mechanisms employed in PCA acquisition and the intracellular changes in mRNA/cDNA expression that occur as a result. It may also shed light on how these endothelial cells are chosen or selected for specific TFMVs attachment. Lastly, detailed studies should also be conducted with MVs from treated cancer cell lines to determine whether chemotherapy (or other treatment) effects can result in variable PCA transformation (TG kinetics) of the endothelium.

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APPENDIX A: Multiple Myeloma

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APPENDIX A1:

Patient information sheet for newly diagnosed Multiple Myeloma patients undergoing chemotherapy

Title

Study of apoptosis related changes and endothelial responses of multiple myeloma patients treated with chemotherapy.

Introduction

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1
What is the purpose of the study and why have I been chosen?

You have been diagnosed as having multiple myeloma, a type of bone cancer, which can hopefully be treated with chemotherapy. Chemotherapy is given to eliminate or reduce the size of the tumour in the bone marrow. Your doctor will have determined which combination of chemotherapy drugs is best for you. This will depend on the stage of the disease and your personal circumstances. Apart from the common symptom of persistent bone pain that this cancer causes, and which you may be experiencing, it can also have effects on the blood. These can include; increased thickness of the blood, reducucedproduction of red blood cells, white blood cells and platelets, and an increased capacity of the blood to clot. In addition, certain chemotherapy drugs such as, thalidomide and lenalidomide, which you may be receiving have been linked with clotting problems. To reduce this risk you may also receive low molecular weight heparins that thin the blood to prevent blood clots forming. It is currently unknown exactly how these drugs can increase the chance of blood clots, also known as thrombosis. However, one of the ways in which chemotherapy kills cancer cells, can cause the release tiny cell particles into the blood. These cell fragments, known as microparticles may contain factors that have the potential to cause thrombosis. To prove this is one of the mechanisms in which clotting can occur during chemotherapy, we have designed a clinical study of these microparticles with clotting potential before, during and after chemotherapy, in multiple myeloma patients. Furthermore, other factors involved in clotting (e.g. platelets) or those that suggest a predisposition to thrombosis (e.g. vessel damage) will also be assessed.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will **not** affect the standard of care you receive.

What will happen to me if I take part?

- Blood will be drawn for regular blood tests during the course of your treatment and at this point we would like to draw some more (about two-three tablespoons). There will be up to a maximum of five samples taken; one sample taken before the start of your chemotherapy course, three samples during and one at the end of chemotherapy.
- Also, a routine bone marrow biopsy will be taken at the time of diagnosis and we would like a small amount (approximately 1 ml) of this bone marrow aspirate, this will have absolutely **no** impact on the accuracy of your diagnosis.

Therefore, we would like to ask your permission for some of your blood and bone marrow to be used for research purposes. This will include samples for DNA analysis that will be processed and stored separately using DNA preservative (Allprotect

Tissue Reagent, QIAGEN), which will be done to identify whether any genes (eg factor V leiden) may have promoted the tendency to clot.

What do I have to do?
Attend all scheduled visits so blood samples can be taken at the set timepoints.

What are the side effects or risks involved when taking part?

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

What are the possible benefits of taking part?
No direct benefits to you are expected from this research. The information we get from this study may help us to improve the future treatment of patients with multiple myeloma.

What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?
Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

Contact details:
Dr. Anthony Maraveyas (Chief Investigator) Queens Centre for Oncology & Haematology Hull & East Yorkshire NHS Trust

Castle Hill Hospital
Tel: 01482 461318

This completes Part 1 of the Information Sheet.
If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What will happen if I don't want to carry on with the study?
If you withdraw from the study, we will destroy all your identifiable blood samples and any analysis that may have taken place.

What if there is a problem?

Complaints:
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (01482 461318). If you

remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.
Harm:

In the event that something does go wrong and you are harmed during the research, no special compensation arrangements exist. However, if you were harmed due to someone's negligence then you may have grounds for legal action against the NHS Trust. The normal NHS complaints mechanisms will still be available to you.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised.

What will happen to any samples I give?
The blood analysis may take place immediately after collection. Stored samples will be **coded anonymously** then locked securely in locked freezers. Access to these samples is restricted to the research members only.
After we complete our tests, we would like to save any left-over blood or bone marrow for other myeloma research projects, subject to appropriate approval. All samples will be destroyed after a period of five years.

What will happen to the results of the research study?
The results of this study will be kept completely confidential, and no personal details will be disclosed. These results will be analysed and written in a research degree thesis (Ph.D). The findings may be presented at learned societies or published in scientific journals. In such cases the information will only identify you with a number and **not** your name or other personal details.

Who is organising and funding the research?
Dr. Anthony Maraveyas is the Chief Investigator of this clinical trial that is sponsored by the NHS (Hull and East Yorkshire Hospitals NHS Trust).

Who has reviewed the study?
This study was given a favourable ethical opinion for conduct in the NHS by the local Research Ethics Committee.
We would like to thank you for your attention so far and hopefully for your participation in this study. Please feel free to ask your doctors any questions about the study or about any of the treatments described above. You will be given a copy of the information sheet and a signed consent form to keep if you choose to participate.

Appendix A2:

Patient initials and study number for this trial:

CONSENT FORM for newly diagnosed Multiple Myeloma patients undergoing chemotherapy

Title of Study: Study of apoptosis related changes and endothelial responses of multiple myeloma patients treated with chemotherapy.
Name of Researcher: Dr Anthony Maraveyas

Please write your initials in the boxes

1. I confirm that I have read and understood the information sheet dated *(insert date)* version *(insert version number)* for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that if I later decide to withdraw from the study, the blood samples taken from me and any analysis of these results will be destroyed and not included in the study. ☐
4. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the Academic Oncology Department, sponsors and from the regulatory authority, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
5. I agree to take part in the above study and give (*insert number*) sample/s of my blood for the purpose of this research study. ☐
6. I agree to genetic material being taken from my blood and bone marrow samples for analysis at a later date. ☐
7. I give permission for a small amount (only surplus material will be used) of my bone marrow (taken at the time of diagnosis) to be used for the purpose of this research study. ☐
8. I give permission for my sample and the information gathered about me to be stored by Dr Anthony Maraveyas at the Academic Oncology Department and the University of Hull for possible use in future myeloma studies over a period of five years. ☐

Name of Patient	Signature	Date
Name of Person taking consent	Signature	Date

CASE REPORT FORM

Newly diagnosed multiple myeloma/control group

STUDY TITLE

Study of apoptosis related changes and endothelial responses of multiple myeloma patients treated with chemotherapy

Study reference number:

CHIEF INVESTIGATOR:	**Dr A. Maraveyas**

Subject Initials:	
Trial number:	

Study arm:

Multiple myeloma	☐	**Myeloproliferative group (control)**	☐
Newly diagnosed myeloma		Chronic myelocytic leukemia	
		Polycythemia rubra vera	☐
		myelofibrosis	☐

Data entry reminder

- **Check accuracy when entering data.**
- **Always write in black.**
- **Put a single line through any mistake.**
- **Never occlude the original entry.**
- **Initial and date any alteration even if completing blank fields retrospectively.**

Case note reminders

- Stick study label on inside front cover of casenotes (alert notes).
- File patient information sheet, consent form, GP letter, copy of inclusion/exclusion criteria in plastic wallet with GCP sticker in casenotes.
- Record patient visit/telephone contacts in casenotes.
  Minimum details to record are:
  - Clearly written; date, brief study title/acronym and visit number.
  - Date patient given patient information sheet (PIS)
  - Date of consent
  - Date of screening
  - Relevant results .
  - Brief description of any AEs (onset & offset times/dates) including any change in blood/urine etc test results.
  - Any change in concomitant diseases and medication including study medication.
  - Any other relevant details.

Myeloma Inclusion Criteria

		Yes	No*
1	Is the subject aged 18 years or greater?	☐	☐
2	Has the subject willingly given written informed consent?	☐	☐
3	Does the subject have a confirmed diagnosis of symptomatic multiple myeloma based on the presence of a paraprotein in serum and/or urine, organ damage or symptoms considered by the clinician to be related to myeloma?	☐	☐
4	Does the subject require treatment for their myeloma either at presentation or at the time of relapse?	☐	☐

Myeloproliferative Inclusion Criteria

1	Is the subject aged 18 years or greater?	☐	☐
2	Has the subject willingly given written informed consent?	☐	☐
3	Subject has a diagnosed myeloproliferative disorder (Chronic myelocytic leukemia, polycythemia rubra vera or myelofibrosis)	☐	☐

***If any inclusion criteria are ticked no then the patient is not eligible for the study.**

Exclusion Criteria

		Yes*	No
1	Does the subject have an active infection?	☐	☐
2	Does the subject suffer from uncontrolled hypertension?	☐	☐
3	Is the subject suffering from diabetes mellitus with HBA1C indicative of poor diabetic control?	☐	☐
4	Has the subject had a myocardial infarction within the past 3 months?	☐	☐
5	Does the subject suffer from rheumatoid arthritis or other inflammatory process in active phase (e.g. psoriasis)?	☐	☐
6	Has the subject being previously treated with long term anti-coagulants (e.g. warfarin for DVT)?	☐	☐
7	Has the subject had a recent thrombosis and still on secondary prophylaxis	☐	☐

*** If any exclusion criteria are ticked yes then the patient is not eligible for the study.**

HAVE ALL INCLUSION AND EXCLUSION CRITERIA BEEN SATISFIED? Yes ☐ No ☐

HAS THE PATIENT READ AND UNDERSTOOD THE PIS? Yes ☐ No ☐

HAS THE PATIENT SIGNED AND DATED THE CONSENT FORM? Yes ☐ No ☐

Decision: ☐ Inclusion ☐ Exclusion

If excluded, specify reason .....

Study investigator:

Name	Signature	Date

VISIT 1 (Baseline)

Date: _____
DD MM YYYY

*Baseline taken at time of diagnosis for myeloma patients and time of study enrolment for controls.

DEMOGRAPHIC DATA

Age (yrs): ☐☐ Sex: Female ☐ Male ☐

MEDICATIONS TAKEN

Is the subject currently or previously taking any **anticoagulant medication**? Yes* ☐ No ☐

*If yes, please provide details below

Medication	Dose	Start date

PREVIOUS MEDICAL HISTORY

Is there any relevant medical history in the following systems?

Code	System	*Yes	No
1	Cardiovascular		
2	Haematological		
3	Neoplasia		

Code	System	*Yes	No
4	Immunological		
5	Renal		
6	Other		

*If *YES* for any of the above, enter the code for each condition in the boxes below, give further details (including dates of diagnosis) and state if the condition is currently or potentially active. Use a separate line for each condition.

		Currently Active?	
Code	Details (including dates)	Yes	No

LABORATORY ANALYSIS

Research study blood samples taken: Yes ☐ No ☐
Date and time or reason if not done: .....

Conventional blood test results:
Protein
electrophoresis*:.....
.....
*Please attach copy of redacted report

WBC: ..... GFR: ..... FBC: .....
Platelets: ..... Plasma viscosity:.....

CHEMOTHERAPY REGIMEN

Medication	Dose	Start date

Is the subject taking anticoagulant medication: Yes* ☐ No ☐
*If yes, please specify medication (e.g. LMWH) including dose and frequency:

BONE MARROW BIOPSY (myeloma patients only)

Has a bone marrow biopsy been taken: Yes ☐ No* ☐
*If no, reason why .....
Does biopsy confirm presence of myeloma cells: Yes ☐ No ☐
Comments:.....
.....
Please attach copy of redacted report

Patient's status: ☐ Ongoing ☐ Withdrawn*

*If withdrawn, please complete off study form at the end of CRF

Have there been any protocol deviations or violations?

If so, please specify and notify R&D monitor: .....

Any comments .....

Completed by:

Name	Signature	Date

Following pages only applicable for myeloma patient groups

VISIT 2 (day 1, cycle 2)

Date:_____
DD MM YYYY

HAS THE PATIENT CONFIRMED WILLINGNESS TO CONTINUE IN THE STUDY AND HAS THIS BEEN DOCUMENTED IN THE PATIENTS CASENOTES? Yes ☐ No☐

FOLLOW UP

Has the subject developed any condition that may exclude him/her from the study? (see exclusion criteria for details)

Yes* ☐ No ☐

*If yes, provide details and complete AE form in study working folder

Has the subject developed renal disease or chronic renal failure since the previous study visit?

Yes* ☐ No ☐

*If yes, provide details and complete AE form in study working folder

ADVERSE EVENTS SINCE PREVIOUS VISIT

Has the subject experienced any thrombus-related complications during treatment: Yes* ☐ No☐

***If yes, please complete the AE form. AE form has to be completed for each AE. The form is provided by R&D or available in the study working folder**

SERIOUS ADVERSE EVENTS SINCE PREVIOUS VISIT

Any SAEs must be reported within 24hrs to R&D using the initial and follow-up serious event report forms provided by R&D or available in the study working folder

LABORATORY ANALYSIS

Research study blood samples taken: Yes ☐ No ☐
Date and time or reason if not done: .....

Conventional blood test results:
Platelets: ..... WBC: ..... GFR: .....
FBC: ..... plasma viscosity:.....

Patient's status: ☐ Ongoing ☐ Withdrawn*
*If withdrawn, please complete off study form at the end of CRF
Have there been any protocol deviations or violations?
If so, please specify and notify R&D monitor: .....

Completed by:

Name	Signature	Date

VISIT 3 (day 1, cycle 3)

Date:_____
DD MM YYYY

HAS THE PATIENT CONFIRMED WILLINGNESS TO CONTINUE IN THE STUDY AND HAS THIS BEEN DOCUMENTED IN THE PATIENTS CASENOTES? Yes ☐ No☐

FOLLOW UP

Has the subject developed any condition that may exclude him/her from the study? (see exclusion criteria for details)

Yes* ☐ No ☐

*If yes, provide details and complete AE form in study working folder

Has the subject developed renal disease or chronic renal failure since the previous study visit?

Yes* ☐ No ☐

*If yes, provide details and complete AE form in study working folder

ADVERSE EVENTS SINCE PREVIOUS VISIT

Has the subject experienced any thrombus-related complications during treatment: Yes* ☐ No☐

***If yes, please complete the AE form. AE form has to be completed for each AE. The form is provided by R&D or available in the study working folder**

SERIOUS ADVERSE EVENTS SINCE PREVIOUS VISIT

Any SAEs must be reported <u>within 24hrs</u> to R&D using the initial and follow-up serious event report forms provided by R&D or available in the study working folder

LABORATORY ANALYSIS

Research study blood samples taken: Yes ☐ No ☐
Date and time or reason if not done: .....

Conventional blood test results:
Platelets: ..... WBC: ..... GFR: .....
FBC: ..... plasma viscosity:.....

Patient's status: ☐ Ongoing ☐ Withdrawn*
*If withdrawn, please complete off study form at the end of CRF
Have there been any protocol deviations or violations?
If so, please specify and notify R&D monitor: .....

Completed by:

Name	Signature	Date

VISIT 4 (8 weeks after end of chemo)

Date:_____
DD MM YYYY

HAS THE PATIENT CONFIRMED WILLINGNESS TO CONTINUE IN THE STUDY AND HAS THIS BEEN DOCUMENTED IN THE PATIENTS CASENOTES? Yes ☐ No☐

FOLLOW UP

Has the subject developed any condition that may exclude him/her from the study? (see exclusion criteria for details)

Yes* ☐ No ☐

*If yes, provide details and complete AE form in study working folder

Has the subject developed renal disease or chronic renal failure since the previous study visit?

Yes* ☐ No ☐

*If yes, provide details and complete AE form in study working folder

ADVERSE EVENTS SINCE PREVIOUS VISIT

Has the subject experienced any thrombus-related complications during treatment: Yes* ☐ No☐

***If yes, please complete the AE form. AE form has to be completed for each AE. The form is provided by R&D or available in the study working folder**

SERIOUS ADVERSE EVENTS SINCE PREVIOUS VISIT

Any SAEs must be reported within 24hrs to R&D using the initial and follow-up serious event report forms provided by R&D or available in the study working folder

LABORATORY ANALYSIS

Research study blood samples taken: Yes ☐ No ☐
Date and time or reason if not done: .....

Conventional blood test results:
Platelets: ..... WBC: ..... GFR: .....
FBC: ..... Plasma viscosity:.....

Patient's status: ☐ Ongoing ☐ Withdrawn*
*If withdrawn, please complete off study form at the end of CRF
Have there been any protocol deviations or violations?
If so, please specify and notify R&D monitor: .....

Completed by:

Name	Signature	Date

OFF STUDY FORM

Date Off Study: __ __ / __ __ / __ __ __ __

(MM/DD/YYYY)

Reason Off Study (Please mark only the primary reason. Reasons **other than Completed Study** require explanation next to the response)

- ☐ Completed study
- ☐ Lost to follow-up _____
- ☐ Non-compliant participant _____
- ☐ Concomitant medication _____
- ☐ Medical contraindication _____
- ☐ Withdraw consent _____
- ☐ Death **(complete SAE form)** _____
- ☐ Other _____

Chief/Principal Investigator

Patient`s status: ☐ Completed ☐ Withdrawn*

***If withdrawn, please specify reason:** .....

"I confirm that the contents of this CRF are accurate and complete"

Name	Signature	Date

APPENDIX B: Pancreatic Cancer

Hull and East Yorkshire Hospitals NHS
NHS Trust

Oncology and Haematology Centre
Castle Hill Hospital
Castle Road
Cottingham
HU16 5JQ

APPENDIX B1:

Patient Information Sheet of Pancreatic Cancer Patients undergoing Pancreatic Resection

Title

A Study of the effect of resection of localised Pancreatic Cancer on tissue-factor promoted pathways of Thrombosis and Angiogenesis markers

Introduction

You have been invited to take part in a research study. Before you decide it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Consumers for Ethics in Research (CERES) publish a leaflet called Medical Research and You.

This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy can be obtained from the doctor or nurse inviting you to take part or from CERES, PO Box 1365, London N16 OBW.

What is the purpose of the trial?

You have been diagnosed as having cancer of the pancreas, which is localised and can hopefully be removed. Your surgeon therefore has offered you surgery with the intention of removing the tumour in your pancreas. If removal is successful you may be offered more treatment called chemotherapy. This will depend on the stage of the tumour and will only be accurately known after the pathologist has had chance to study the tumour under the microscope. Chemotherapy is given to improve the effectiveness of the surgical treatment. Apart from the common symptoms of blockage of the gut and the bile system that this cancer causes, and which you may be experiencing, it can also have effects on the blood. We and others have found that it increases the capacity of the blood to clot and to provide nutrients for cancer blood vessels. All these effects promote the growth of secondary cancer and are collectively called 'cancer promoting factors'. We think that the planned removal of this cancer from your pancreas may provide benefit by removing these 'cancer promoting effects' from the blood as well as relieving the blockage on the gut.
To prove this we have designed a clinical study of these 'cancer promoting factors' before and after surgery and during and after chemotherapy.

What will happen to me during the trial?

We would want to study the concentration of these 'cancer promoting factors' in your blood before and after surgery and during and after

chemotherapy if it is offered to you. We would also like to correlate these factors with the appearances of the tumour that has been removed. We therefore would like to ask your permission for some of your blood and for a small portion of the tumour to be used for research purposes.

Before the operation, blood will be drawn for regular blood tests and at this point we would like to draw some more (about two-three tablespoons) for the study. The surgeon will undertake the operation as planned. The cancer will be sent for histological diagnosis and with the aid of the pathologist (the expert who looks at cancers under the microscope) we would like a small portion of this tumour to be removed for research of factors that promote cancer. We want the expert to do this so that the removal of this small portion has absolutely no impact on the accuracy of the staging of your cancer.

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

If you are offered chemotherapy a further three blood tests will be taken, one during chemotherapy, at 12 weeks, one at the end of chemotherapy, at 24 weeks, and one eight weeks after the conclusion of all the treatment. At this point, your involvement with the study finishes.

In the case, therefore, of you going on to chemotherapy, a total of five blood samples are required.

Are there any side effects associated with these treatments?

There should be no extra risks or side effects from the procedures as they will be part of the regularly planned tests that your doctors need before the

surgery and to monitor your progress after the surgery and during and after chemotherapy.

Are there any other extra procedures?

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

What are the possible benefits of taking part?

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

What is something goes wrong?

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

What if new information becomes available?

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

Will my taking part in this study be kept confidential?

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

Who is organising and funding the research?

This is a trial sponsored and funded by the NHS (HEYNHST) & The University of Hull.

Dr. Anthony Maraveyas is the Chief Investigator

What if I do not wish to take part or change my mind?

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

We would like to thank you for your cooperation, Please feel free to ask your doctors any questions about the study or about any of the treatments described above.

Please contact:
Dr. Anthony Maraveyas-Castle Hill Hospital . Tel: 01482 676703

Dr. Mufuliat Adeola Adesanya – ***Hull York Medical School, Hull University. Mobile Number: 07586203204.***

APPENDIX B2

Patient Information Sheet for Patients Undergoing Chemotherapy for resected primary pancreatic cancer (adjuvant).

A study of the effect of chemotherapy on the Tissue-factor promoted pathways of thrombosis and angiogenesis in resected (adjuvant) and non-resected localised pancreatic cancer patients

Introduction

We would like to invite you to take part in a research study. Before you decide whether you would like to participate, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information.

Take time to decide whether or not you wish to take part. Consumers for Ethics in Research (CERES) published a leaflet called Medical Research and You.

This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy can be obtained from the doctor or nurse inviting you to take part or from CERES, PO Box 1365, London N16 OBW.

What is the purpose of the trial?

You have been diagnosed as having cancer of the pancreas, which is localised and your surgeon was able to remove the cancer. Your surgeon has now referred you for chemotherapy to try and reduce the risk of this cancer coming back (relapsing).

We and others have found that chemotherapy can increase the capacity of the blood to clot. We have also found that the same factors that promote clot can also promote the growth of the cancer. Collectively we call these 'cancer promoting factors'. We know that the production of a clot can make patients unwell (swelling of the leg or cause chest symptoms such as shortness of breath and chest pain). We also suspect it may also signify worse cancer related outcomes. We would like to find out if there are groups of patients more likely to be affected by these 'cancer promoting factors'. Being able to predict this could lead to better cancer treatment and better preventive treatments of thrombosis (clots).

We are studying two major groups of pancreatic cancer patients, those in which the surgeon could not remove the tumour and those (like yourself) in whom the tumour was removable.

What will happen to me during the trial?

We would want to study the concentration of these 'cancer promoting factors' in your blood during and after chemotherapy, if it is offered to you and you decide to accept. We therefore would like to ask your permission to collect a blood sample from you at specific time-points during your treatment. The amount of blood that will be taken for the research is about 2 to 3 tablespoons each time.

If you decide to proceed with the chemotherapy your doctor has offered then we would require the blood samples just before you commence chemotherapy, and during some of your regular follow-up visits (at weeks 8-12 and 24).

At around 24 weeks of treatment you will have reached the planned completion of your chemotherapy and you are likely to be having further scans. After this point at week 32 (assuming your scans have shown no reappearance of the cancer) you will have completed your treatment with

chemotherapy and this will also be the date for the last sample for the study. We will not require any further samples for the study after this.

You will therefore be having four blood samples in total if you complete the planned chemotherapy course in full.

Are there any side effects associated with these treatments?

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

Are there any other extra procedures?

No extra procedures are planned.

What are the possible benefits of taking part?

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

What is something goes wrong?

If you were harmed by taking part in this study, no special compensation arrangements exist. However, if you were harmed due to someone's negligence, then you would have grounds for legal action.

Regardless of this, if you have any cause to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

What if new information becomes available?

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

Will my taking part in this study be kept confidential?

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

Who is organising and funding the research?

This is a trial sponsored and funded by the NHS (HEYNHST) & The University of Hull.

Professor Anthony Maraveyas is the Chief Investigator

What if I do not wish to take part or change my mind?

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

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

We would like to thank you for reading this information sheet.

Contact for further information

Professor Anthony Maraveyas- Chief Investigator, Castle Hill Hospital, Hull
Tel: 01482 461245, 07772717393
Dr Mufuliat A. Adesanya – Hull York Medical School, University of Hull.
Mobile: 07586203204

APPENDIX B3

Patient Information Sheet for Patients Undergoing Chemotherapy for non-resected primary pancreatic cancer.

A study of the effect of chemotherapy on the Tissue-factor promoted pathways of thrombosis and angiogenesis in resected (adjuvant) and non-resected localised pancreatic cancer patients.

Introduction

We would like to invite to take part in a research study. Before you decide whether you would like to participate, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information.

Take time to decide whether or not you wish to take part. Consumers for Ethics in Research (CERES) published a leaflet called Medical Research and You.

This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy can be obtained from the doctor or nurse inviting you to take part or from CERES, PO Box 1365, London N16 OBW.

What is the purpose of the trial?

You have been diagnosed as having cancer of the pancreas, which is localised but could not be removed. Your surgeon therefore has either attempted to remove the cancer unsuccessfully or has just biopsied the cancer and referred you for chemotherapy to relieve the symptoms and

delay the worsening of the cancer condition. The aim of chemotherapy is to improve symptoms and to prevent or delay further complications of the cancer on your gut, such as blockages. Apart from the common symptoms of blockage of the gut and the bile system that this cancer can cause, and which you may be experiencing, it can also have effects on the blood. We and others have found that it increases the capacity of the blood to clot and to provide nutrients for cancer blood vessels. All these effects promote the growth of secondary cancer and are collectively called 'cancer promoting factors'. To prove this we have designed a clinical study of these 'cancer promoting factors' before and during chemotherapy. We are studying two major groups of pancreatic cancer patients, those in which the surgeon has removed the tumour and those in whom this was not possible.

What will happen to me during the trial?

We would want to study the concentration of these 'cancer promoting factors' in your blood during and after chemotherapy, if it is offered to you. We therefore would like to ask your permission to collect a blood sample from you at specific time-points during your treatment. The amount of blood that will be taken for the research is about 2 to 3 tablespoons each time.

If you decide to proceed with the chemotherapy your doctor has offered then we would require the blood samples just before you commence chemotherapy, and during some of your regular follow-up visits, (weeks 8-12 and 24).

At around 12 weeks of chemotherapy you will also be due a CT scan to assess the response of the cancer. If your doctor decides to continue for a further 12 weeks then a further blood test will be taken at 24 weeks of treatment at which point you are likely to be having further scans. About eight weeks after this scan the final blood test will be required (at this point you may still be on chemotherapy or your doctor may have decided on a treatment break). After this last sample we will not require any further samples for the study.

You will therefore be having four blood samples in total if you complete the full planned chemotherapy course.

Are there any side effects associated with these treatments?

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

Are there any other extra procedures?

No extra procedures are planned.

What are the possible benefits of taking part?

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

What is something goes wrong?

If you were harmed by taking part in this study, no special compensation arrangements exist. However, if you were harmed due to someone's negligence, then you would have grounds for legal action.

Regardless of this, if you have any cause to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens,

your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Will my taking part in this study be kept confidential?

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

Who is organising and funding the research?

This is a trial sponsored and funded by the NHS (HEYNHST) & The University of Hull.

Professor Anthony Maraveyas is the Chief Investigator

What if I do not wish to take part or change my mind?

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

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

We would like to thank you for reading this information sheet.

Contact for further information

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Tel: 01482 461245, 07772717393
Dr Mufuliat A. Adesanya- Hull York Medical School, University of Hull.
Mobile: 07586203204

APPENDIX B4:

Patient Initials ☐ Trial Number ☐ Hospital No ☐

Pancreatectomy, Chemotherapy and Tissue Factor
Data Collection Form

Chemotherapy

Gender: M ☐ F ☐ Date of Birth ☐

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

Present Clinical History:

Respiratory system:

Cardiac system:

Hypertension: Y ☐ N ☐

Myocardial Infarct: Y ☐ N ☐

If Yes give Date of infarct ☐

DVT/VTE: Y ☐ N ☐

Date (s) of VTE ☐ ☐

Type of VTE ☐

Recurrent VTE Y ☐ N ☐

Alimentary system:

History of diabetes mellitus Y ☐ N ☐

History of Jaundice Y ☐ N ☐

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

Are there signs of Infection? Y ☐ N ☐

Does the patient still have a T-tube? Y ☐ N ☐

Has the patient had a line (CVC) inserted Y ☐ N ☐

Date: ☐

Is the patient receiving thromboprophylaxis Y ☐ N ☐

If answer Yes, What type? _____

When stopped: [ ]

Concurrent Medication including anticoagulant and dose:

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Haematological test

Date: [ ]

Hb [ ]

WBC [ ]

Platelet [ ]

Neutrophils [ ]

Biochemical test

Date: [ ]

CRP [ ]

Bilirubin [ ]

AST [ ]

Alkaline Phosphatase [ ]

Total serum protein [ ]

Albumin [ ]

Chemotherapy:

Type of Chemotherapy: ☐

Date of Chemotherapy: ☐ Cycle: ☐ Week: ☐

Dates of venesections:

Pre-Chemotherapy ☐

Week 8 or 12 ☐

Week 24 ☐

Post Chemotherapy ☐ (8 weeks after end of chemotherapy)

Samples complete: Yes ☐ No ☐

Pathological staging and margins (for the patients that have resected tumour)

Histopathology:

Specimen made available Y ☐ N ☐

Date: ☐

Assays done: (please detail with dates)

TF- activity ☐

Angiogenesis ☐

Invasion ☐

Thrombin generation ☐

TF-MP-FACS ☐

Investigator signature: _____ Date: _____

APPENDIX B5:

Consent Form for Pancreatic Cancer Patients Undergoing Pancreatic Resection.

Title of Project: **A study of the effect of resection of localised pancreatic cancer on Tissue-factor promoted pathways of thrombosis and angiogenesis markers.**

Name of Researcher: _____

Please initial box

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

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

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

4) I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from Academic Oncology Department, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my record. ☐

5). I agree to take part in the above study and give samples of my blood for the purpose of the research as outlined in the information sheet. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking Consent (if different from Investigator)	Date	Signature
_____	_____	_____
Investigator	Date	Signature

1 Copy for the Patients, 1 Copy for the Investigator file, 1 Copy for the Patient's Hospital notes.

Consent Form for Patients undergoing Chemotherapy.

Title of Project: **A study of the effect of chemotherapy on the Tissue-factor promoted pathways of thrombosis and angiogenesis in resected (adjuvant) and non-resected localised pancreatic cancer patients**

Name of Researcher: _____

Please initial box

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

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

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

4) I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from Academic Oncology Department, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my record. ☐

5). I agree to take part in the above study and give samples of my blood for the purpose of the research as outlined in the information sheet. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking Consent (if different from Investigator)	Date	Signature
_____	_____	_____
Investigator	Date	Signature

1 Copy for the Patients, 1 Copy for the Investigator file, 1 Copy for the Patient's Hospital notes.

APPENDIX C: TFMVs in Multiple Myeloma and Pancreatic Cancer

As a one-off experiment, TFMVs were measured by flow cytometry in MM (n=25) and PC (n=29) serum samples (serum obtained as described in section 2.8.3). In 5ml polypropylene tubes, 5 µl of FITC-conjugated mouse IgG_1 isotype control antibody (AbD Serotec) or 5µl of FITC-conjugated mouse anti-human monoclonal anti-TF were added to 25 µl of serum before incubation in the dark for 30 minutes to allow binding.

Although not benchmarked for serum studies, a well-known protocol for identifying MV subpopulations as detailed by the International Society on Thrombosis and Haemostasis (ISTH) Scientific and Standardization Subcommittee in Vascular Biology was utilized(Lacroix et al., 2010), was used here. This method allows the reproducible isolation of MVs with different scatter properties on a flow cytometer by addition of an antibody which detects specific membrane surface antigen (Robert et al., 2009) in plasma. In this study, the cell surface TF antigen (sCD142), expressed on membrane surfaces was used to detect TFMVs in serum. TFMVs from a region between 0.1-0.9 µm in size were gated by addition of Megamix beads (Biocytex, Marseille, France) which contains different-sized beads of 0.5, 0.9, and 3.0 µm diameter. TFMVs are enumerated by AccuCheck counting beads (Invitrogen Ltd, Paisley, UK) which are fluorescent-labelled and contains quantities of latex beads (A and B) of known concentration from the supplier (approximately 1000 beads/µl).

Following incubation, 350 µl of PBS was added to resuspend the antibody in the tubes, after which 25 µl of the counting bead mixture were added immediately prior to flow cytometric evaluation. On the cytometer 25,000 positive events were counted, where positive MV events were denoted as the difference in labelling between cell-specific FITC-labelled TF (sCD142) antibodies and the isotype control

antibody. A differential relation to the standard bead events counted by the cytometer allows the estimation of an absolute count of MVs in the substrate found in the fixed size gated region. This was calculated as:

MVs Absolute count (MVs/ µl) = Number of MVs count/ Total number of beads counted (A + B) x known concentration of beads as supplied by manufacturer (1000 beads µl)

For example, if the number of TFMVs count in a sample tube is 500, and the total number of latex beads counted for A is 650 while B is 600, then the equation gives the Absolute TFMVs count as:

500/ (650+600) x 1000 = 400 TFMVs present.

Results:

In this lone experiment, there were no significant differences (p=0.3419) in the median (IQR) sTFMVs in MM (2196 (842.7-11340)) and PC (1714 (593.9-13623))/µl of serum (Figure A.0.1) Correlations of serum TF (sCD142) measured by ELISA with serum TFMVs in MM and PC patients also showed no association (Figure A.0.2 and A.0.3 respectively).

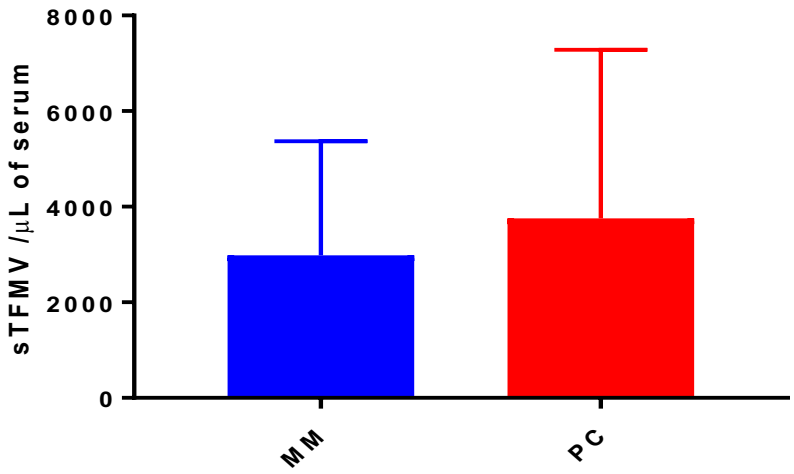

Figure A.0.1 Serum TFMVs measured by Flow cytometry in MM and PC patients

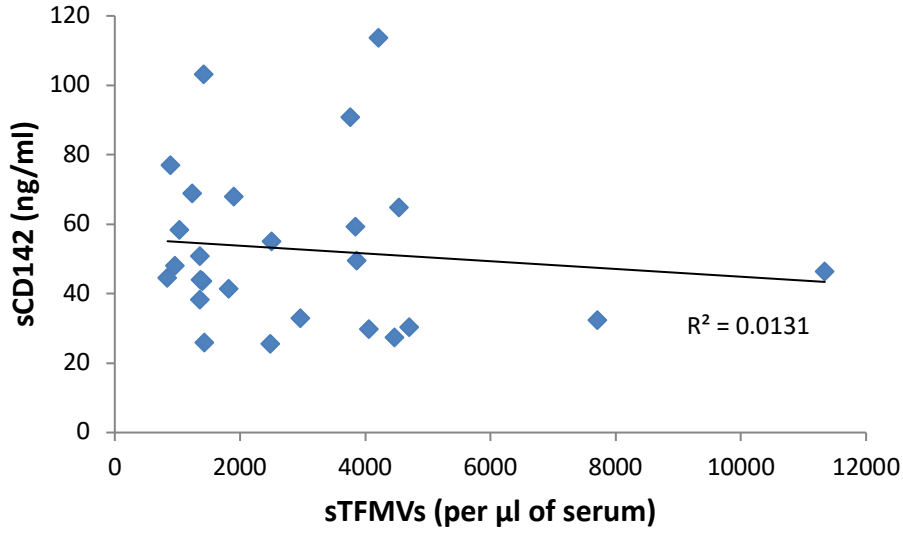

Figure A.0.2 Correlation of serum TF (sCD142) with serum TFMVs in MM patients

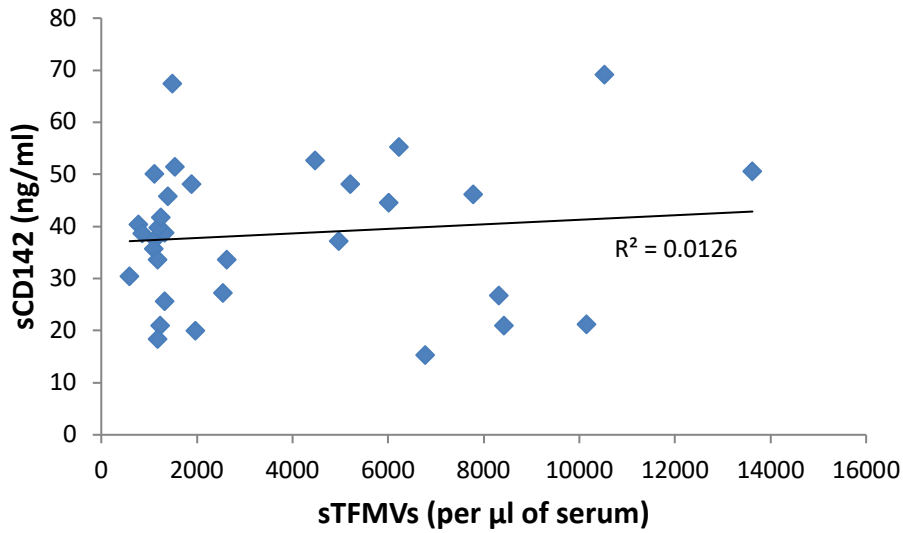

Figure A.0.3 Correlation of serum TF (sCD142) with serum TFMVs in PC patients