Study of the thrombogenicity induced by the cytotoxic treatment of malignant disease

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Abstract

Cancer and its treatment are frequently complicated by the development of venous thromboembolism (VTE). Interestingly, VTE incidence rates vary according to tumour type and the chemotherapy regimen administered. Yet, the precise mechanisms responsible for the increase in VTE in cancer patients remain unclear. Tissue factor (TF), the primary cellular initiator of the coagulation cascade, is over-expressed in many solid malignancies, particularly pancreatic cancer, and the number of circulating TF+ microparticles (MP) are increased in cancer patients. It is hypothesised that the increased risk of VTE in cancer patients generated by cytotoxic treatment may be partly attributed to the apoptotic process, involving the exposure of procoagulant phosphatidylserine (PS) on tumour cells or other chemotherapy damaged cells and increased release of TF+ MP into the circulation. The aim of this study was to explore how coagulation can be initiated in cancer cells and be potentiated by chemotherapy, with respects to the specific expression of TF and PS, and generation of MP.

Flow cytometry was used to evaluate expression of cell surface TF and level of apoptosis in untreated/doxorubicin (Dox)-treated cancer cell lines, and number of MP in platelet-free plasma (PFP) from untreated pancreatic cancer patients, myeloproliferative disorder (MPD) patients, and multiple myeloma (MM) patients before, during, and after chemotherapy. The cell proliferation of untreated/Dox-treated cancer cells was assessed by an MTS assay. Procoagulant activity (PCA) of untreated/Dox-treated cancer cells and their isolated cell-free supernatants containing MP, and also MM and MPD patient PFP was measured using a prothrombin time assay. Enzyme-linked immunosorbent assay quantified levels of TF in unfiltered/0.1 μm filtered cell-free supernatants isolated from cancer cell lines, and serum levels of soluble cell adhesion molecules CD106 and CD54 from MM patients.

Pancreatic (AsPC-1, CFPAC-1, MIA-PaCa-2), ovarian (A270, ES2, SKOV-3), colorectal (CaCo-2, LoVo), breast (MCF-7, MDA-MB-231, T47D), and haematological (JJN3, U937) cancer cells were found to support coagulation in a cell number-dependent manner, defined by a logarithmic relationship that was consistent across all cell lines. Furthermore, single cell clotting time (CT) was determined for each cancer cell line from the slope and y axis intercept of a logarithmically transformed data plot. A near linear relationship was observed between TF expression and single cell CT where a higher expression of TF results in a proportionally faster CT ($P = 0.01$). In addition, tumour cell-derived MP were shown to be procoagulant and the majority of procoagulant potential could be removed by passing isolated cell-free supernatants

through a 0.1 μm filter. A dose-dependent CT was observed with AsPC-1, CFPAC-1, ES2, SKOV-3, LoVo, and MDA-MB-231 cell-free supernatants.

The cytotoxic chemotherapeutic agent Dox was found to decrease the number of viable ovarian (ES2), breast (MDA-MB-231, T47D), and haematological (MM.1S, U937) cancer cells in a time- and dose-dependent manner, and cell death was shown to be induced by apoptosis and subsequently necrosis at higher drug concentrations. Cell surface expression of PS was found to increase following Dox treatment, while TF was not upregulated. Furthermore, Dox was shown to dose-dependently increase PCA in all cancer cells examined, although the effect of cell-free supernatants on PCA was less consistent; ES2, MDA-MB-231, and U937 cell-free supernatants isolated from Dox-treated cells demonstrated increased PCA at 0.01 and 0.1 μM concentrations. The Dox-induced increase in PCA of cancer cells and cell-free supernatants were found to correlate with tumour cell viability (r = 0.79 to 0.97, $P < 0.01$).

Numbers of TF+ MP were significantly higher in 35 untreated pancreatic cancer patients in comparison with 15 MM patients prior to chemotherapy ($P < 0.0005$). Furthermore, numbers of endothelial cell-derived MP (EMP), monocyte-derived MP, plasma cell-derived MP, and PS+ MP, were significantly higher in 6 MPD patients in comparison with 15 MM patients prior to chemotherapy ($P < 0.005$), but not levels of platelet-derived MP (PMP) or TF+ MP. Markers of endothelial dysfunction, including EMP and soluble cell adhesion molecules CD106 and CD54, were elevated after thalidomide (Thal)- or lenalidomide (Len)-based therapies in MM patients. Furthermore, PCA was significantly increased in MM patients after treatment ($P = 0.007$), and also levels of PMP, plasma cell-derived MP, and PS+ MP ($P < 0.05$), but not monocyte-derived MP ($P = 0.33$) and TF+ MP ($P = 0.41$).

In summary, this study shows that across a range of tumour sites a consistent relationship is seen between procoagulant potential and both cell number and TF cell surface expression. Dox can increase PCA of cancer cells through reduced cell viability that leads to PS exposure. Importantly, this Dox-induced procoagulant mechanism was not found to involve an upregulation of surface TF antigen on ovarian, breast, or haematological cancer cells. In MM patients treated with Thal- or Len-based therapies increased PCA was observed, which may be mediated by endothelial dysfunction and increased generation of MP.

Publications

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Poster presentations

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ASCO	American Society of Clinical Oncology
asTF	Alternatively spliced TF
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
bFGF	basic fibroblast growth factor
BMSC	Bone marrow stromal cells
Bor	Bortezomib
BSA	Bovine serum albumin
C of V	Coefficient of variation
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CD	Cluster of differentiation
CO_2	Carbon dioxide
CHH	Castle Hill Hospital
CRF	Case Report Form
CSF	Colony stimulating factor
CT	Clotting time
CTD	Cyclophosphamide, thalidomide, and dexamethasone
CTDa	Attenuated cyclophosphamide, thalidomide, and dexamethasone
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DVT	Deep vein thrombosis
E-selectin	Endothelial selectin
ECACC	European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGFRvIII	EGFR variant III
ELISA	Enzyme-linked immunosorbent assay
EMP	Endothelial cell-derived MP
ESMO	European Society for Medical Oncology

F	Clotting factor (suffix ‘a’ denotes activated factor)
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
flTF	Full-length TF
FSC	Forward scattered light
GLA	Glutamic acid
GP	Glycoprotein
h	Hours
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
H_3PO_4	Phosphoric acid
HEY	Hull and East Yorkshire
HGF	Hepatocyte growth factor
HRP	Horseradish Peroxidase
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
Il	Interleukin
IMiD	Immunomodulatory drugs
IMWG	International Myeloma Working Group
IQR	Interquartile range
ISS	International Staging System
ISTH	International Society of Thrombosis and Haemostasis
IVC	Inferior vena cava
kDa	Kilodaltons
KLF2	Kruppel-like factor 2
Len	Lenalidomide
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
min	Minutes

ml	Millilitres
mM	Milimolars
MM	Multiple myeloma
MMP	Matrix metalloproteinases
MP	Microparticles
MPD	Myeloproliferative disorder
mRNA	Messenger RNA
MUC-1	Mucin 1
NCCN	National Comprehensive Cancer Network
NET	Neutrophil extracellular DNA traps
NFκB	Nuclear factor κB
ng	Nanograms
nm	Nanometers
NSABP	National Surgical Adjuvant Breast and Bowel Project
NSCLC	Non-small cell lung cancer
P-selectin	Platelet selectin
PAD4	Peptidylarginine deiminase 4
PAI	Plasminogen activator inhibitors
PAR	Protease activated receptors
PBS	Phosphate buffered saline
PCA	Procoagulant activity
PE	Phycoerythrin
PFP	Platelet-free plasma
pg	Picograms
PML	Promyelocytic leukaemia
PMP	Platelet-derived MP
Pom	Pomalidomide
PPP	Platelet-poor plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
R&D	Research and Development
RAR	Retinoic acid receptor
RCDa	Attenuated lenalidomide, cyclophoshamide, and dexamethasone
RD	Reagent diluents

REC	Research Ethics Committee
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Seconds
sCD54	Soluble CD54
sCD106	Soluble CD106
SD	Standard deviation
SSC	Side scattered light
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
Thal	Thalidomide
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor-α
tPA	Tissue plasminogen activator
UFH	Unfractionated heparin
UK	United Kingdom
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
µg	Micrograms
µl	Microlitres
µm	Micrometers

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Author's declaration

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Chapter 1. General introduction

1.1 Cancer biology and haemostatic complications

The past few decades have seen many substantial advances in the field of cancer research. However, cancer still remains a leading cause of death in developed countries. In the United Kingdom (UK), approximately 157,000 cancer deaths and 323,000 new cancer cases were documented each year during the period of 2008 to 2010 (Office of National Statistics, 2012). While cancer defines a multitude of diseases, over 100 different types and subtypes, the most common types reported in England in 2011 were prostate cancer (in men) and breast cancer (in women), followed by lung and colorectal cancer (Office of National Statistics, 2013).

The sequential accumulation of genetic abnormalities and mutations drive the progressive malignant transformation of cells. Indeed Hanahan and Weinberg identified the following group of distinctive 'traits' that cancer cells develop enabling them to evade normal homeostatic mechanisms and support tumour growth; 'self-sufficiency in growth signals, evading apoptosis, insensitivity to anti-growth signals, sustained angiogenesis, tissue invasion and metastasis, limitless replicative potential, reprogramming of energy metabolism, and evading immune destruction' (Hanahan and Weinberg, 2011). Furthermore, increasing evidence suggests that procoagulant activity (PCA) mediated by tumours is not a mere epiphenomenom, but is likely to be instrumental in cancer progression. Malignant transformation, tumour angiogenesis and metastasis have all been linked to the haemostatic system, specifically through tissue factor (TF) expression and thrombin generation, mediated by signalling cascades that can be triggered in a clotting-dependent and clotting-independent manner (Rickles et al., 2003, Ruf et al., 2011), described in more detail in section 1.4.1 and 1.5.1, respectively. The spectrum of haemostatic complications noted in cancer patients ranges from laboratory alterations of coagulation factors in the absence of thrombotic symptoms to fatal thromboembolism (Rickles and Falanga, 2009). Thrombosis is the failure of proper haemostatic control and describes the formation of a clot within a blood vessel that reduces blood flow, which may cause infarction of tissues supplied by that vessel. Thrombotic episodes can present as various forms and combinations of venous, arterial, or microvascular thrombosis.

1.2 Cancer-associated venous thrombosis

Cancer-induced hypercoagulable states commonly manifest themselves as venous thrombosis; presenting as deep vein thrombosis (DVT) or pulmonary embolism that are collectively known as venous thromboembolism (VTE). The association between malignancy and VTE has been acknowledged for around 150 years and was originally described by the French physician Armand Trousseau in 1865. It is thought that 15% of all cancer patients at some stage develop symptomatic VTE, accounting for 20% of the total VTE burden (Caine et al., 2002). However, it is important to be aware that the magnitude of the problem is likely underestimated, as many cases are not often clinically apparent, and therefore may pose an even greater burden than official figures suggest. The finding of asymptomatic pulmonary embolism in patients undergoing imaging for cancer staging is an increasingly common occurrence, as exemplified in a prospective study that found undiagnosed or 'unsuspected' pulmonary embolism in 10 of 385 (2.6%) patients with malignancy who underwent a routine computed tomography scan of the chest (Sebastian and Paddon, 2006). In a large population-based study the duration between the diagnosis of cancer and the occurrence of VTE was determined with the highest risk of VTE being the first three months (Blom et al., 2005). Furthermore, risk-adjusted models have suggested that advanced, metastatic disease at the time of diagnosis in particular is associated with both a high incidence of thromboembolic complications, and substantial morbidity and mortality (Chew et al., 2006). Moreover, VTE is one of the major causes of death in cancer patients and the probability of recurrent thromboembolism is 2-fold higher in patients with cancer in comparison with healthy individuals (Levitan et al., 1999).

Conversely, VTE can also present as the first clinical manifestation of an underlying malignancy, particularly in hepatocellular carcinoma, pancreatic cancer, ovarian cancer and brain malignancies (Baron et al., 1998). Piccioli et al. extensively screened 99 patients with symptomatic idiopathic VTE for occult cancer and identified malignant disease in 13 patients (Piccioli et al., 2004). These results correspond with other studies that show there is an approximate 10% risk of clinically manifested malignancy after a thrombotic event (Prandoni et al., 1992, Ahmed and Mohyuddin, 1996, Sorensen et al., 1998). Furthermore, the diagnosis of cancer is highest shortly after an unprovoked VTE event occurs, the standardized incidence ratio for cancer in patients with VTE is 2.1 to 4.6 during the first year, and afterwards the risk steadily decreases (Baron et al., 1998, Sorensen et al., 1998,

Schulman and Lindmarker, 2000, Murchison et al., 2004). However, there is no evidence to suggest that intensive screening for cancer in patients with VTE improves prognosis, and therefore, is not generally warranted.

1.2.1 Risk factors for VTE in cancer

The risk of VTE in cancer is not equally distributed across different histological types (see Table 1). Although many histological types of cancer and their associated relative risk of VTE have been assessed, these data are inconsistent, as studies vary with regard to patient population, treatment regimen, duration of follow-up period, period of study, and method of detecting and reporting VTE. However, the general consensus, when adjusted for disease prevalence in the general population, is that the cancers most consistently associated with the highest VTE incidence are pancreatic, brain, stomach, ovarian, and lung, whereas a comparatively lower incidence is found with prostate, breast, and melanoma. Furthermore, high rates are reported among haematological malignancies, particularly in lymphoma and multiple myeloma (MM) (Blom et al., 2005). Of interest, tumour type has been included as a parameter in a recently developed risk scoring model to predict the development of VTE in cancer patients undergoing chemotherapy-based treatments (Khorana et al., 2008b).

Though cancer itself represents an independent risk factor, the probability of VTE development is further augmented by many other potential contributing factors that have been identified. For example, risk factors for VTE include, advanced age, obesity, prior or family history of VTE, prolonged immobility, and co-morbid conditions such as, acute infection, heart disease, and respiratory disease (Khorana, 2012). There are also several genetic risk factors that are associated with VTE and recurrent VTE. In particular, strong genetic risk factors include inherited deficiencies of antithrombin III, protein C, and protein S. Moderate genetic risk factors for VTE include; (1) Factor V (FV) Leiden; a variant of FV that is resistant to inactivation by activated protein C, (2) prothrombin G20210A; a polymorphism of the prothrombin gene that causes elevated plasma prothrombin levels, (3) fibrinogen C10034T; a C to T variant at position 10034 in the fibrinogen gamma chain that leads to reduced levels of the alternatively spliced form of the fibrinogen gamma-chain, and (4) non–type O blood; these individuals have increased levels of von Willebrand factor (vWF) and also FVIII (Rosendaal and Reitsma, 2009).

Table 1.1: Reported incidence rates of VTE events in cancers.

Study	Stage	Treatment	VTE (%)
Pancreas			
(Khorana et al., 2006)	NS	Chemotherapy	12.1
(Mandala et al., 2007)	Advanced (III/IV)	Surgery, chemotherapy	26
(Maraveyas et al., 2012)	Advanced	Chemotherapy	28
Brain			
(Brandes et al., 1997)	High-grade glioma (IV)	Surgery, chemotherapy, radiation	26
(Khorana et al., 2006)	NS	Chemotherapy	9.5
(Simanek et al., 2007)	High-grade glioma (III/IV)	Surgery, radiochemotherapy	24
(Streiff et al., 2004)	Glioma (II/III/IV)	Surgery, radiotherapy/chemotherapy	22.5
Lung			
(Alexander et al., 2014)	I-IV	Surgery, radiotherapy,	10.8
(Khorana et al., 2006)	NS	chemotherapy	7
(Numico et al., 2005)	III/IV	Chemotherapy	11.1
Ovarian			
(Khorana et al., 2006)	NS	Chemotherapy	6.5
(Otten et al., 2004)	III	NS	4
(Satoh et al., 2008)	I-V	NS	25
(Tateo et al., 2005)	I-IV	Surgery, chemotherapy	16.6
Gastroesophageal			
(Mandala et al., 2010)	NS	Chemotherapy	5.9
(Starling et al., 2009)	Advanced	Chemotherapy, (EOF/EOX)	7.6
		(ECF/ECX)	15.1
Stomach			
(Khorana et al., 2006)	NS	Chemotherapy	7.4
(Tesselaar et al., 2004)	I-IV	Chemotherapy	10
Colon			
(Alcalay et al., 2006)	I-IV	NS	3.1
(Khorana et al., 2006)	NS	Chemotherapy	6.8
(Mandala et al., 2009)	Advanced (IV)	Chemotherapy	10.2
(Otten et al., 2004)	Advanced (III/IV)	NS	15
Breast			
(Chew et al., 2007)	I-IV	NS	1.2
(Khorana et al., 2006)	NS	Chemotherapy	3.9
(Mandala et al., 2010)	NS	Chemotherapy	8.8
Prostate			
(Khorana et al., 2006)	NS	Chemotherapy	7.3
(Secin et al., 2008)	NS	Surgery	0.5

Abbreviations: ECF, epirubicin, cisplatin, and fluorouracil; ECX, epirubicin, cisplatin, and capecitabine; EOF, epirubicin, oxaliplatin, and fluorouracil; EOX, epirubicin, oxaliplatin, and capecitabine; NS, not specified.

In addition VTE risk factors specific to the malignant state have been described, such as tumour stage, placement of central venous catheters, and use of anticancer agents (described in more detail in section 1.7) (Otten et al., 2004, Khorana et al., 2006, Haddad

and Greeno, 2006, Falanga and Russo, 2012). Furthermore, major surgery is a well established risk factor of VTE. For example, post-operative DVT is estimated to be twice as likely in patients with cancer who undergo surgery than in those without malignancy (Prandoni et al., 1999), and post-operative fatal pulmonary embolism is increased 4-fold in cancer patients (Caine et al., 2002). In agreement with this, data from a large observational study indicates that cancer patients undergoing surgery have a 2-fold increased risk of post-operative VTE, and that this risk extends for a prolonged period after the procedure (up to 7 weeks), with 40% of all observed VTE events in this study occurring later than 21 days from surgery in cancer patients (Agnelli et al., 2006). Current clinical practice guidelines from cancer-specific panels such as the National Comprehensive Cancer Network (NCCN), American Society of Clinical Oncology (ASCO), and European Society for Medical Oncology (ESMO), recommend that thromboprophylaxis for patients undergoing surgery for cancer should be continued for at least 7 to 10 days post-operatively, and extended for up to 4 weeks post-operatively in patients undergoing major abdominal or pelvic surgery for cancer with low-molecular weight heparin (LMWH) (Khorana, 2007, Mandala et al., 2011, Lyman et al., 2013).

1.3 Venous thrombogenesis

The exact mechanisms that induce clotting in VTE have yet to be fully elucidated. Although arterial thrombosis is shown to be provoked by endothelial disruption, a frequent model described for DVT is venous stasis devoid of endothelial injury (except in thrombosis associated with surgery) (Sevitt, 1974, Lopez et al., 2004). The most common site for DVT initiation in humans is the valve pocket sinuses of the calf veins due to periods of blood stasis and low oxygen levels, and thereby predisposition to become hypoxic (Hamer et al., 1981). The endothelium is activated by hypoxia and/or inflammatory stimuli, leading to the expression of adhesion receptors such as platelet selectin (P-selectin), endothelial selectin (E-selectin) and vWF on the endothelium that facilitate the binding of circulating leukocytes, (specifically neutrophils and monocytes), platelets, and microparticles (MP) (Myers et al., 2003, Williams et al., 2011, Mackman, 2012). In contrast, the normal endothelium prevents thrombosis by inhibiting the attachment of cells and proteins necessary for clotting, expressing anticoagulants (including TF pathway inhibitor [TFPI], thrombomodulin, endothelial protein C receptor, and heparan sulphate proteoglycans), and releasing platelet inhibitors (nitric oxide and prostacyclin) (Watson, 2009, Esmon and

Esmon, 2011). However, following endothelial cell activation, thrombomodulin is downregulated while TF expression is increased on the cell surface (Moore et al., 1987), shifting the haemostatic balance towards a hypercoagulable state. In addition, the endothelial-bound leukocytes become activated and express TF, initiating the activation of the coagulation cascade, and inducing thrombosis (Manly et al., 2011, von Bruhl et al., 2012).

This proposed sequence of events is supported by a recent novel study employing a mouse inferior vena cava (IVC) stenosis model for DVT initiation and propagation (von Bruhl et al., 2012). The study found that in mice expressing minimal levels of TF on myeloid leukocytes the development of venous thrombosis was considerably reduced, implying that leukocyte-derived TF plays a key role in the initiation of thrombus formation in this model (von Bruhl et al., 2012). Additionally, von Brühl et al. confirm that platelets contribute to DVT propagation by binding to leukocytes and promoting secondary leukocyte recruitment at the thrombus site, and in agreement with others, suggested that neutrophils amplify DVT development by forming neutrophil extracellular DNA traps (NET), which trigger FXII-dependent coagulation (Fuchs et al., 2010, Massberg et al., 2010, Brill et al., 2012, von Bruhl et al., 2012). NET are formed by peptidylarginine deiminase 4 (PAD4)-mediated chromatin decondensation in the nucleus of neutrophils, a process that has been shown to be critical for venous thrombosis development in a stenosis mouse model of DVT, thus indicating that neutrophil activation, NET, and PAD4 may provide novel targets for thrombolysis in patients with DVT (Martinod et al., 2013). Furthermore, plasma DNA (a surrogate marker for NET) has been shown to be elevated in patients with DVT and correlated with biomarkers of DVT such as D-dimer (Diaz et al., 2013), also in another study, the presence of NET has recently been demonstrated in the thrombi of patients with VTE (Savchenko et al., 2014). Interestingly, a study found that tumour-induced neutrophils are more prone to the formation of NET than their normal counterparts in both leukaemia and solid tumour experimental models, thereby promoting a cancer-associated hypercoagulable state (Demers et al., 2012).

The pathogenesis of VTE in cancer patients is most likely a result of the interactions between cancer cells and host factors, which may be framed in the context of “Virchow's triad” (Rickles and Falanga, 2009). These elements include hypercoagulability, venous

stasis, and endothelial injury that can all simultaneously account for the prothrombotic state in the same cancer patient (Rickles and Falanga, 2009). For example, cancer-driven events found to specifically promote a hypercoagulable state include the increased activation of procoagulant factors with concomitant inhibition of anticoagulant mechanisms, impaired fibrinolysis, production and secretion of procoagulant substances and proinflammatory cytokines, increased platelet aggregation, and adhesive interactions among tumour cells, endothelium, and blood cells (Rickles and Falanga, 2009). One of the most notable of these is the activation of the TF coagulation pathway, via the characteristic upregulation of TF on the surface of tumour cells. In addition to hypercoagulability, venous stasis has been suggested to contribute to venous thrombosis in cancer that may manifest from immobility during a post-operative period or debility in advanced malignancy, extrinsic compression of blood vessels by the primary tumour or tumour invasion. Furthermore, slow venous blood flow can lead to the improper clearance of activated clotting factors causing hyperviscosity. Moreover, these accumulated clotting factors in stagnant blood could overwhelm local anticoagulant pathways, thus inducing a hypercoagulable state of malignancy that may trigger thrombosis. Furthermore, endothelial injury may promote thrombosis in cancer patients as a result of mechanical trauma, arising from tumour invasion or therapeutic interventions such as, surgery or placement of venous catheters for chemotherapy administration. In addition, endothelial dysfunction can result from antiangiogenic stimuli and the 'unselective' effects of some chemotherapeutic agents that are toxic to endothelial cells (discussed in more detail in section 1.7.1).

1.4 Haemostatic system and cancer

It is now well established that the haemostatic system is involved in the development of malignant tumours as cancer cells interact with various components of the haemostatic system for growth, angiogenesis and malignant dissemination. Normally, the coagulation cascade and fibrinolytic system sustain a fine balance between activation and inhibition of platelets, procoagulant factors, anticoagulant factors, and fibrinolytic factors as further described below. However, the coagulation system is inappropriately activated in cancer leading to a hypercoagulable state of malignancy (Rickles and Falanga, 2009). Consequently, cancer patients may exhibit a marked predisposition towards the development of thrombosis.

1.4.1 Primary and secondary haemostasis

Thrombus development is dependent on two essential components of the haemostatic system occurring simultaneously, namely the activation of platelets and conversion of fibrinogen to fibrin. The formation of a platelet plug to seal a vascular lesion is initiated by the adhesion of platelets to subendothelial vWF through the glycoprotein (GP)Ib receptor. This leads to platelets slowly rolling across the subendothelium and platelet adhesion to vWF and collagen resulting in platelet activation, and eventually platelet aggregation through the GPIIb/IIIa platelet receptors. Increasing evidence demonstrates a critical role for both platelets and vWF in cancer growth and dissemination. Increased levels of vWF have been shown in different human tumour types (Rohsig et al., 2001, Blann et al., 2001, Damin et al., 2002, Goldenberg et al., 2003) and levels correlate with tumour stage and metastases (Damin et al., 2002). Moreover, vWF facilitates the attachment of cancer cells to the endothelium via cancer cell-expressed GPIb receptor (Floyd et al., 1992), enabling dissemination into the surrounding tissue (Nierodzik et al., 1995). In addition, cancer cells have also been shown to present the GPIIb/IIIa receptor allowing them to bind to platelets, assisting cancer cells to evade immune detection, thereby providing a tumour survival benefit (Nieswandt et al., 1999). Studies have shown that defects in platelet activation can decrease the metastatic potential (Camerer et al., 2004, Palumbo et al., 2005).

Concurrent to platelet activation and aggregation, the sequential and surface-mediated activation of serine proteases (clotting factors) results in a fibrin network (Figure 1.1), stabilising the platelet plug and forming a thrombus. Upon vascular injury, exposed cell bound TF binds FVII. Very small amounts of FVII circulate in the blood in an active form (FVIIa), binding to TF; the TF-FVIIa complex can then activate surrounding TF-FVII complexes, thereby triggering the coagulation cascade by activating FX and FIX. This FXa binds with its cofactor Va to generate small quantities of thrombin from prothrombin in the initiation phase (Hoffman and Monroe, 2001). Thrombin then separates from its glutamic acid (GLA) domain, thereby enabling it to roam and promote clotting by activating platelets, FV, FVIII, and FXI in the amplification phase (Hoffman and Monroe, 2001). Additionally, thrombin provides negative feedback by binding to thrombomodulin on the endothelial cell surface, this complex then activates anticoagulant protein C. In the

propagation phase, FIXa with its cofactor VIIIa, activate additional FX on the surface of activated platelets in the presence of anionic phospholipids and calcium (Hoffman and Monroe, 2001). This FXa binds with its cofactor Va, generating large amounts of thrombin from prothrombin, resulting in the cleavage of fibrinogen to fibrin monomers that then polymerise, ultimately leading to the rapid formation of a fibrin clot and thus preventing blood loss.

Cancer cells may have a direct procoagulant effect by promoting the expression of TF (the primary initiator of the coagulation cascade) on the cell surface of tumour and/or stromal cells that can release circulating TF (Callander et al., 1992, Contrino et al., 1996, Shoji et al., 1998, Tesselaar et al., 2007, Tesselaar et al., 2009), and the potential production/secretion of cancer procoagulant (a cysteine proteinase), which activates FX (Falanga and Gordon, 1985). Furthermore, laboratory findings of increased coagulation factors such as FV, FVIII, FIX and FXI are frequently reported in malignancy (Minnema et al., 2003, Dogan et al., 2006, Auwerda et al., 2007, Battistelli et al., 2008, Yigit et al., 2008). Moreover, the activation of haemostasis in malignancy may hypothetically be a consequence of the leakage of procoagulant factors such as FVII into the perivascular space containing TF-bearing cells, due to the vascular endothelial growth factor (VEGF)-induced hyperpermeability of the tumour vasculature (Dutzmann et al., 2010). The haemostatic system is linked to multiple aspects of tumour angiogenesis by mechanisms involving coagulation proteases (particularly TF), fibrin, and platelets (Rickles et al., 2003, Ruf et al., 2011). Angiogenesis is defined as the formation of new blood vessels from the pre-existing vascular network, occurring primarily in embryonic development, wound healing, and in response to ovulation under physiological conditions. In addition, angiogenesis is a mechanism that nearly all clinically relevant tumours have pathologically undergone to sustain an appropriate blood supply for further tumour growth (Hanahan and Folkman, 1996).

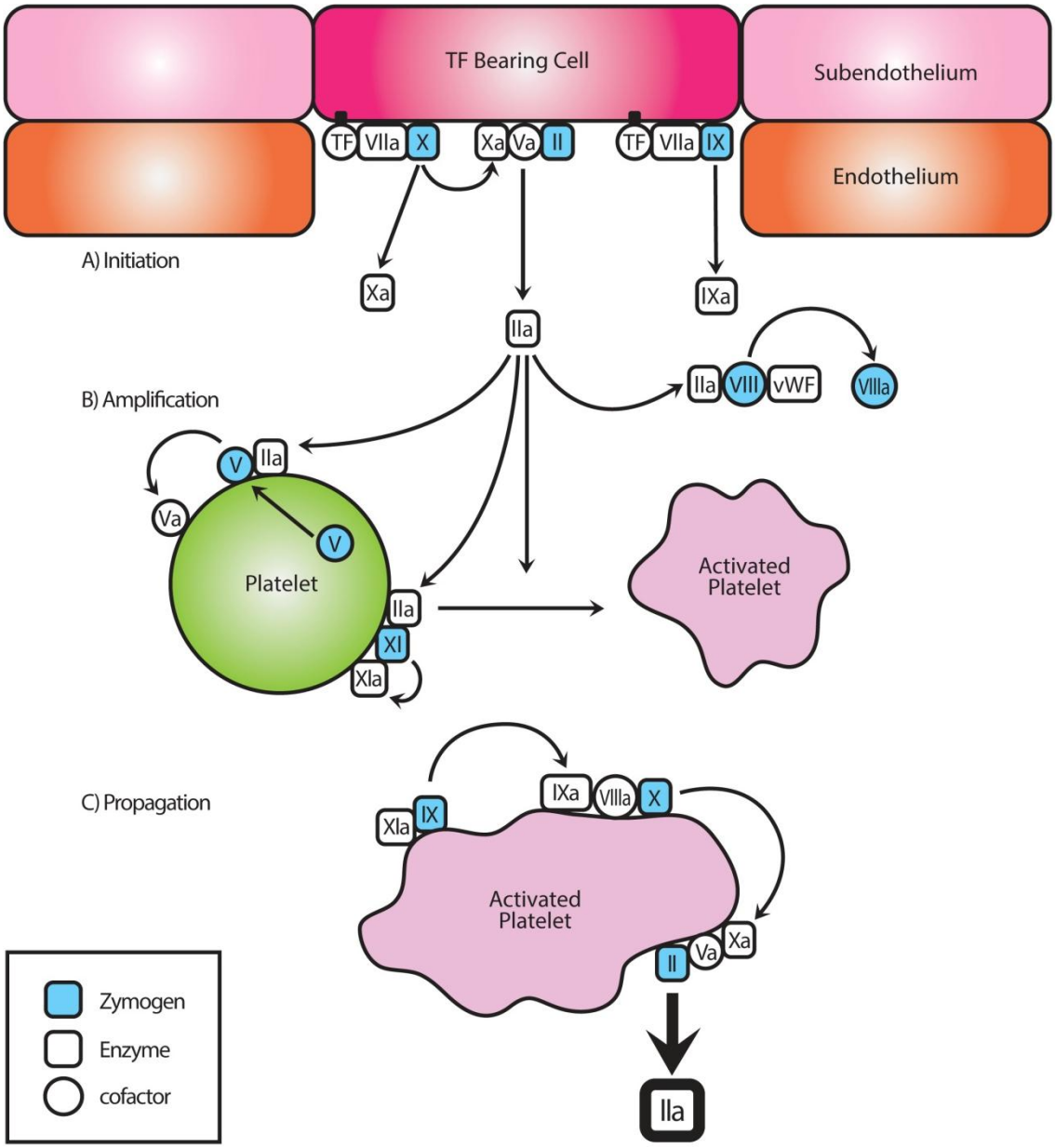


Figure 1.1: The cell-based model of coagulation.

In this model thrombin (IIa) generation occurs in overlapping phases and the contribution of various cell surfaces to fibrin generation is described. (A) The initiation phase occurs on a TF-bearing cell when injury exposes subendothelial TF to the flowing blood, which results in the generation of a small amount of FXa, FIXa and FIIa (thrombin). (B) In the amplification phase, thrombin that has diffused from the surface of the TF-bearing cell to the platelet surface results in platelet activation, vWF release, and activation of FV, FVIII and FXI. (C) Further FX is activated in the propagation phase on the procoagulant membrane surface of the activated platelet and ultimately a large burst of thrombin is generated, leading to fibrin formation.

Enhanced TF expression has been demonstrated in the angiogenic tumour-associated endothelium, and high TF antigen levels correlate with VEGF expression, increased angiogenesis, vascular density, and incidence of metastasis in a variety of human tumours (Contrino et al., 1996, Koomagi and Volm, 1998, Shigemori et al., 1998, Abdulkadir et al., 2000, Seto et al., 2000, Nakasaki et al., 2002, Ohta et al., 2002, Poon et al., 2003, Khorana et al., 2007a, Echrish et al., 2014). TF may indirectly induce angiogenesis by clotting-dependent downstream generation of thrombin that subsequently mediates the conversion of soluble fibrinogen to cross-linked fibrin (Rickles et al., 2003). Fibrinogen has been reported to be synthesised and over-expressed in human tumours (Sahni et al., 2008), and high plasma levels predict a poor prognosis in patients with ovarian (Polterauer et al., 2009), endometrial (Seebacher et al., 2010), colorectal (Tang et al., 2010), non-metastatic renal cell (Pichler et al., 2013), and urothelial cell carcinoma (Tanaka et al., 2013). In addition, fibrinogen and/or fibrin deposition in the tumour stroma provides a supportive scaffold for angiogenesis by binding promigratory and proangiogenic growth factors, particularly VEGF (Rickles et al., 2003). However, it has been shown that fibrinogen-deficient mice can permit growth and vascularisation of implanted tumours, but not tumour metastasis, thus indicating that the fibrin matrix is necessary for metastasis (Palumbo et al., 2002). Furthermore, fibrin is hypothesized to enable cancer cells to evade immune detection by forming a coating around tumour cells, thereby hiding tumour antigens (Palumbo et al., 2005). TF-FVIIa-driven thrombin generation further contributes to angiogenesis by directly stimulating the release of VEGF (and other proangiogenic factors) from the α-granules of activated platelets (Mohle et al., 1997, Maloney et al., 1998). In addition, enhanced tumour TF expression has been shown to further support angiogenesis and facilitate metastasis through clotting-independent protease activated receptors (PAR) signalling pathways (Rickles et al., 2003, Ruf et al., 2011), described in section 1.5.1.

1.4.2 Anticoagulant system

Normal haemostasis has mechanisms in place to prevent excessive clotting by the following anticoagulant factors; (1) TFPI that binds to the TF-FVIIa complex and FXa, (2) antithrombin III that inhibits the activity of FIXa, FXa, and thrombin, and (3) activated protein C, which together with its cofactor protein S, inhibits FVIIIa and FVa. Reduced levels of antithrombin III and protein C have been reported in cancer patients (Nand et al.,

1987), and the latter as a consequence of some chemotherapy regimens that could lead to activation of haemostasis (Mukherjee et al., 2010). Furthermore, acquired activated protein C resistance is a common finding among patients with cancer, representing a more important risk factor for VTE in cancer than in non-malignant conditions (Green et al., 1997, Haim et al., 2001, Nijziel et al., 2003, Soyer et al., 2012).

1.4.3 Fibrinolytic system

Fibrinolysis when activated, converts plasminogen to plasmin by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA). Plasmin then cleaves the fibrin network, releasing fibrin degradation products and D-dimers, and is subsequently inactivated by α-2-antiplasmin. Lastly, the plasminogen activator inhibitors (PAI) 1 and 2 inhibit tPA and uPA, thereby regulating plasmin generation. Cancer cells impair the fibrinolytic system, resulting in tumour invasion, cell proliferation, and metastasis. Plasma D-dimer levels reflecting local thrombin and fibrin formation are elevated in cancer patients, increasing with tumour stage (Nakashima et al., 1995, Blackwell et al., 2000, Kim et al., 2004), and adversely predict survival in patients with metastatic breast cancer and colorectal cancer (Dirix et al., 2002, Blackwell et al., 2004, Kilic et al., 2008). Furthermore, uPA may bind to the uPA-receptor expressed on cancer cells and monocytes converting plasminogen to plasmin, which can degrade the extracellular matrix, in addition to a fibrin clot, thereby facilitating cancer cell invasion to the surrounding tissues and metastasis (Schmitt et al., 1997). Finally, uPA and PAI-1 are both now suggested tumour markers for breast cancer and may be clinically utilized to guide the use of adjuvant chemotherapy for node-negative patients (Harris et al., 2007).

1.5 Tissue factor and cancer

TF is a 47-kDa transmembrane GP receptor for FVII and its activated form (FVIIa), permitting TF to act as the primary cellular initiator of the coagulation cascade (Nemerson, 1988). TF is exposed by subendothelial cells to the circulation as a consequence of vascular injury. Under normal physiological conditions, constitutive and high TF expression is restricted to extravascular cells, namely pericytes and adventitial fibroblasts in the tunica media and adventitia in blood vessels, epithelial cells enclosing organs and at body surfaces, and the parenchyma of extensively vascularised organs, such as the brain, heart,

lung, kidney, and placenta (Drake et al., 1989, Erlich et al., 1999, Pawlinski et al., 2002, Pedersen et al., 2005). Yet, very low levels of TF are present in the blood of healthy individuals associated with cells and MP (Giesen et al., 1999). Thus a paradox exists, where a coagulation initiator is exposed to plasma coagulation factors *in vivo* without initiating coagulation. This circulating TF is believed to be either encrypted or below the threshold required for activation of coagulation.

TF expression can be induced in a variety of cell types and recent evidence from *in vivo* studies suggests that not only arterial, but also venous thrombosis is initiated by intravascular TF, involving multi-cellular interactions at sites of ongoing thrombosis (Zhou et al., 2009, von Bruhl et al., 2012, Darbousset et al., 2012). In addition, cancer-associated thrombosis can frequently be attributed to high levels of TF expression, involving primarily the upregulation of TF on the surface of tumour cells and their migration into the vascular lumen during vascular invasion and metastasis, but also TF-bearing inflammatory and stromal cells within the tumour microenvironment, upregulated tumour vasculature TF expression by angiogenic or activated endothelial cells, and the release of TF+ MP into the blood circulation (Callander et al., 1992, Contrino et al., 1996, Shoji et al., 1998, Ueno et al., 2000, Tesselaar et al., 2007, Tesselaar et al., 2009).

TF is commonly upregulated in epithelial tumours (Callander et al., 1992), and in a study of 106 patients with solid tumours, its expression was reported to be 67% higher in cancer patients (Kakkar et al., 1995a). Moreover, TF expression was detected in almost all ovarian cancer cell lines and patient samples, yet was absent in non-transformed cells and normal ovarian tissue (Han et al., 2006, Cocco et al., 2011, Abu Saadeh et al., 2013). Similarly, TF was not detected in normal healthy pancreatic tissue, while high intratumoral TF has been demonstrated in pancreatic cancer, predicted to be an early event in malignant transformation and TF expression is seen to increase in concordance with progressive dedifferentiation of the tumour (Kakkar et al., 1995b, Khorana et al., 2007a, Thaler et al., 2013a). In colorectal cancer, TF antigen was positive in 57% of all specimens and the incidence of TF expression was 41%, 46%, 53%, 85%, and 81% in tumours from patients in clinical stages I, II, IIIA, IIIB, and IV, respectively (Nakasaki et al., 2002). Furthermore, increased TF mRNA and protein expression was shown to correlate with tumour grade in human glioma, while TF staining was not present in any normal brain specimens (Hamada

et al., 1996a, Hamada et al., 1996b, Guan et al., 2002). Furthermore, Chen et al. reported that the majority (91.3%) of oesophageal tumour samples expressed TF, but was absent in normal oesophageal tissues, and that the overall survival rate in patients with high TF expression was significantly poorer than those with low TF expression (Chen et al., 2010). Elevated TF expression is also a clinical indicator of poor prognosis in many other cancers, including breast cancer (Ueno et al., 2000), pancreatic cancer (Nitori et al., 2005), intestinal-type gastric carcinoma (Yamashita et al., 2007), bladder cancer (Patry et al., 2008), lung adenocarcinoma (de Meis et al., 2010), colorectal cancer (Rao et al., 2011), and ovarian cancer (Abu Saadeh et al., 2013).

1.5.1 Tissue factor in cancer angiogenesis and tumour progression

In addition to its central role in haemostasis and thrombosis, TF has also been implicated in tumour angiogenesis and metastasis via thrombin generation and the resulting activation of PAR signalling pathways (Rickles et al., 2003, Ruf et al., 2011). In particular, thrombin mechanisms independent of clot formation are mediated by the proteolytic cleavage of PAR-1, PAR-3, and PAR-4, but not PAR-2, on tumour, stromal, or endothelial cells. This results in the activation of G-protein–coupled signal transduction cascades (such as mitogen-activated protein kinase [MAPK]) that indirectly induce the transcriptional activation of a number of angiogenesis-related genes, including VEGF, VEGF receptors, basic fibroblast growth factor (bFGF), and matrix metalloproteinase-2, which promote cell adhesion, migration, proliferation, and metastasis (Rickles et al., 2003). In addition, thrombin cleaved PAR-1 can cross-activate PAR-2 (O'Brien et al., 2000), and the concurrent activation of both PAR-1 and PAR-2 are thought to be required for particular thrombin-induced responses in tumour, stromal, or endothelial cells (Shi et al., 2004, Lidington et al., 2005).

Direct signalling of the binary TF-VIIa complex, mediated via PAR-2 activation, upregulates proangiogenic chemokines and growth factors, including the chemokine ligand 1, interleukin (IL)-8, and VEGF, and immune regulators, such as granulocyte-macrophage colony stimulating factor (CSF) and macrophage CSF, that recruit innate immune cells to the tumour microenviroment for tumour progression and metastasis (Ruf et al., 2011). In the malignant state other proteases that are frequently over-expressed have been shown

to activate PAR-2, such as TMPRSS2 in prostate cancer (Wilson et al., 2005), and trypsin in a number of human gastrointestinal cancers, including oesophageal adenocarcinoma (Han et al., 2013), pancreatic (Shimamoto et al., 2004), gastric (Miyata et al., 2000), and colorectal cancer (Ducroc et al., 2002). TF-dependent signalling may also occur via the nascent product Xa in the ternary TF-VIIa-Xa complex that activates PAR-2, as well as PAR-1, or by the direct phosphorylation of the TF cytoplasmic domain, independent of thrombin generation/clot formation and ligand binding, which both result in signal transduction cascades and the subsequent transcriptional activation or inactivation of various genes (Ruf et al., 2011). Through these different signalling pathways, TF and the proangiogenic cytokine VEGF are shown to be involved in a shared cycle of coagulation activation and tumour growth, i.e. TF upregulates VEGF and downregulates the angiogenesis inhibitor thrombospondin-1, while VEGF increases further TF expression (Rickles et al., 2003). Indeed, decreased phosphatidyl 3-kinase activity with simultaneously increased p38 and Erk-1/2 MAPK activity is thought to induce positive regulation of TF expression via VEGF in tumour-associated endothelial cells (Blum et al., 2001).

1.5.2 Mechanisms of TF upregulation in cancer

Fundamental genetic alterations of the cell cycle machinery that contribute to cancer development include; 'gain-of-function mutations' in proto-oncogenes, which promote cell survival and proliferation, and 'loss-of-function mutations' in tumour suppressor genes that code for proteins which normally act as cell cycle checkpoint controls and promote DNA repair. Together these malignant transformation events have a cumulative synergistic effect in carcinogenesis, altering the expression profiles of various downstream effector genes. Consequently, the loss of equilibrium between cell replication and apoptosis is succeeded by the expansion of tumour tissue. Moreover, elevated TF expression has been linked with tumour-specific oncogenic events, several of which are described below.

TF expression in colorectal cancer cells is considered a consequence of two transformation events, activation of the K-ras oncogene and subsequent inactivation of the p53 tumour suppressor gene (Yu et al., 2005, Rao et al., 2011). While TF upregulation in glioblastoma has been ascribed mainly to PTEN tumour suppressor gene loss, but also to hypoxia and amplification of the epidermal growth factor receptor (EGFR) or its transforming mutant, EGFRvIII (Rong et al., 2005, Rong et al., 2009, Magnus et al., 2010). Additionally, mutations

in p53 and PTEN genes are associated with a significant increase in TF gene expression in non-small cell lung cancer (NSCLC) (Regina et al., 2009). Also, roles have been suggested in acute promyelocytic leukaemia (PML) for the PML and retinoic acid receptor (RAR) α fusion genes (Tallman et al., 2004, Guo et al., 2001), and for the activation of the c-MET oncogene in hepatocellular cancers and medulloblastoma (Boccaccio et al., 2005, Provencal et al., 2009).

Furthermore, the enhancement of TF expression could also involve other cancer-related processes such as changes in cellular differentiation status, entry into the epithelial-to-mesenchymal transition pathway, increased angiogenesis (see section 1.5.1) and formation of the cancer stem cell compartment (Hair et al., 1996, Milsom et al., 2007, Milsom et al., 2008). Interestingly, high TF expression in cancer cells often correlates with features of cellular aggressiveness, including primary tumour growth, invasion, and metastasis, while VTE occurrence is also thought to reflect the presence of cancers with a more biologically aggressive phenotype (D'Asti et al., 2014).

1.5.3 Tissue fator encryption and isoforms

In the aforementioned cases, oncogenic events appear to directly influence levels of TF gene expression. However, other reports suggest that, rather than an actual upregulation in either mRNA or protein expression of TF, PCA may be specifically augmented as a result of increased TF decryption (Wang et al., 2001, Swystun et al., 2009a, Swystun et al., 2011a, Boles et al., 2012). TF on the cell surface can be found in a latent (encrypted) or high procoagulant (decrypted) state (Chen and Hogg, 2013), and lipid rafts are thought to be required for the maintenance of cellular TF in an encrypted state (Dietzen et al., 2004). Evidence for TF decryption has been provided by many studies using various non-physiological activators of TF such as, calcium ionophores (e.g. ionomycin), endoplasmic reticulum calcium pump inhibitors (e.g. thapsigargin), detergents, and oxidants (Carson, 1996, Bach and Moldow, 1997, Wolberg et al., 1999, Caldwell et al., 2010). It is predicted that TF decryption involves both a post-translational modification (dithiol/disulfide switch) at the cysteine residues 186 and 209 in the cytoplasmic C-terminal domain of TF (Chen et al., 2006, Cho et al., 2008, Liang et al., 2011), and exposure of the membrane phospholipid, phosphatidylserine (PS) on the cell surface, triggered by an influx of calcium into the cytosol (Greeno et al., 1996, Bach and Moldow, 1997).

Under physiological conditions the majority of circulating TF is thought to be encrypted to prevent the inappropriate stimulation of haemostasis (Bach, 2006). However, an alternatively spliced isoform of TF (asTF) originally described in 2003 (Bogdanov et al., 2003) has also been detected in plasma, its solubility being dictated by its unique C-terminus. The TF gene (*F3*) encodes two variant proteins; a full-length transmembrane isoform of TF (flTF) consisting of a 219-amino acid extracellular domain, a 23-residue transmembrane domain, and a 21-residue intracellular domain, as well as a 206 amino acid soluble protein or asTF that lacks the C-terminal transmembrane and cytoplasmic domains (as shown in Figure 1.2). Establishing the precise individual roles of flTF and asTF is generally impeded by a lack of specific reagents to discriminate between them. Although, flTF in the circulation is known to have a systemic procoagulant effect, thought to directly contribute to increased VTE incidence, a procoagulant role for asTF has yet to be sufficiently elucidated. For example, the addition of recombinant asTF to platelet-poor plasma (PPP) shortened clotting time (CT), thus increasing PCA (Bogdanov et al., 2003), although in transfection studies in the absence of flTF, asTF seemed not to contribute to coagulation (Censarek et al., 2007, Boing et al., 2009). The circulating full-length isoform has been shown in an *ex vivo* model to possess thrombogenic properties (Giesen et al., 1999), and the accumulation of flTF in developing thrombi resulting in fibrin generation has been observed *in vivo* (Falati et al., 2003, Chou et al., 2004). Although asTF is also detectable in human thrombi, it demonstrates a more peripheral localisation, hinting at a role in thrombus propagation rather than actual thrombus formation (Bogdanov et al., 2003).

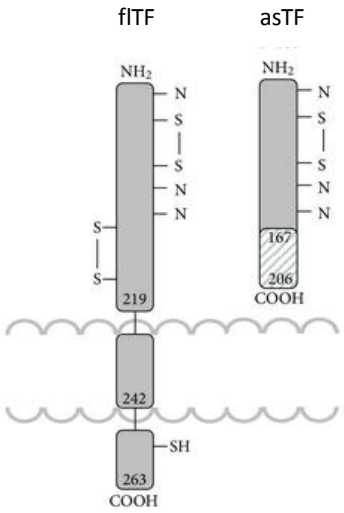


Figure 1.2: The structure of flTF and asTF.
TF is a 263 amino acid transmembrane protein containing 3 domains: (1) an extracellular domain (residues 1–219) representing the NH2-terminal part of the molecule involved in complex formation with FVIIa and increases, in a membrane dependent fashion, the activity of the protease toward its natural substrate FIX, FX, and FVII by several orders of magnitude; (2) a transmembrane domain (residues 220–242), which anchors TF to the membrane; and (3) a cytoplasmic COOH-terminal domain (residues 243–263), which is involved in signal transduction. The asTF variant is a 206 amino acid soluble protein that lacks the transmembrane and cyoplasmic domains; residues 1−166 are identical to the extracellular domain of TF, and residues 167−206 correspond to a unique COOH-terminal. Figure adapted from Butenas et al., 2007.

In cancer, accumulating studies have described the expression of asTF in both tumour cell lines and tissue specimens. Experiments in pancreatic cancer cell lines indicated that the procoagulant properties of the culture medium were attributed to presence of asTF, in addition to the release of flTF-bearing MP (Haas et al., 2006). Conversely, a study of colorectal cancer cell lines reported that flTF associated with MP, rather than asTF, may contribute to the prothrombotic state in cancer patients (Yu and Rak, 2004). More recently, studies suggest a role for asTF expression in tumour progression, rather than coagulation. For example, human pancreatic tumour cell lines were found to express both flTF and asTF, although the asTF detected in these cells did not confer PCA, while in an *in vivo* pancreatic cancer model, asTF was found to promote tumour growth and angiogenesis (Hobbs et al., 2007). Similarly, a recent study found that asTF, but not flTF, significantly associated with both tumour size and grade in breast cancer, by promoting tumour cell proliferation in an integrin-mediated manner (Kocaturk et al., 2013). In support of this, high asTF levels have been correlated with poor prognosis in NSCLC (Rollin et al., 2010).

In mice bearing tumours derived from human colorectal cancer cell lines, increased expression of TF on the surface of cancer cells has been demonstrated to correlate with higher circulating flTF antigen levels (Yu et al., 2005). Furthermore, this blood-borne TF was shown to be predominantly tumour cell-derived using a human TF-specific enzyme-linked immunosorbent assay (ELISA) (Yu et al., 2005). In addition, both tumour tissue TF expression and plasma flTF concentration are found to be elevated in breast cancer patients (Ueno et al., 2000), and flTF levels are also significantly elevated in the serum of ovarian cancer patients (Han et al., 2006).

1.5.4 Association of cancer cell TF expression *in vivo* with VTE

In pancreatic cancer patients, TF expression has been associated with VTE incidence in a study showing a symptomatic VTE rate of 26.3% in high TF-expressing pancreatic carcinomas compared with 4.5% in those with low expression (P=0.04) (Khorana et al., 2007a). In ovarian cancer, TF expression was found to be significantly higher in patients with VTE (n=10) than in patients without VTE (n=22; P=0.0003) (Uno et al., 2007), and similarly in a more recent study, both TF mRNA and antigen levels were increased in the ovarian tumours of patients who developed VTE (n=19) compared with matched patients without thrombosis (n=19; P<0.01) (Abu Saadeh et al., 2013). However, despite the presence of TF in 52% (n=22) of lung adenocarcinoma tumour samples (n=39), mostly in advanced stage (III and IV) tissue, no correlation was observed between the expression of TF and risk for thrombosis development (de Meis et al., 2010). Furthermore, recent evidence from a study in brain cancer patients receiving chemotherapy, radiotherapy and/or surgery indicated that while the vast majority of tumour samples were positive for TF (80.2%; n=77), although moderate in most cases (66.7%; n=64), there was no significant association between TF expression levels in brain tumours and VTE (Thaler et al., 2013b). Thus, further studies that are appropriately powered are necessary to establish a tangible link between VTE and the expression and/or activity of TF in cancer. Nevertheless, this relationship may be cancer type specific as evidence is emerging that indicates that the hypercoagulable state in patients with haematological malignancy may be TF-independent (Negaard et al., 2008, Cesarman-Maus et al., 2012, Cesarman-Maus et al., 2014).

Currently in the clinical setting, the role of TF in VTE is being extensively investigated, predominantly via the measurement of easily accessible circulating TF+ MP (including their

number and functional activity), isolated from the plasma of cancer patients. The levels of TF in the circulating blood could potentially have some utility as a disease biomarker, which could be reflective of not only the risk of cancer coagulopathy, but also others important parameters such as tumour burden and the extent of malignant transformation.

1.6 Microparticles

1.6.1 Function and formation of MP

The term microvesicles is broadly used to describe different types of phospholipid vesicles, namely exosomes, MP (the main focus of this study), and apoptotic bodies, which are heterogeneous in size and composition, but based on electron microscopy, they are generally depicted as being 50 to 100 nm, 0.1 to 1 µm, and 1 to 3 µm in diameter, respectively (Rak, 2010, van der Pol et al., 2012a). They are further distinguishable by their pathway of generation involving either, endocytosis-related formation of exosomes, release of MP by exocytic blebbing of the cell plasma membrane, or apoptotic body formation during the terminal stages of apoptotic cellular breakdown (Thery et al., 2001, Rak, 2010, van der Pol et al., 2012a).

The presence of procoagulant subcellular particles in plasma that could be removed by centrifugation was first indicated in the 1940s as the CT of PPP was demonstrated to be prolonged following high-speed centrifugation (Chargaff and West, 1946). Subsequently, in 1967, Wolf demonstrated the presence of fragments derived from activated platelets in human plasma; these fragments, originally described as “platelet dust” (Wolf, 1967) and now known as MP, were considered merely cellular debris for a long time. However, it is now proposed that many cells types (including platelets, endothelial cells, leukocytes, and erythrocytes) shed MP that act as vectors for the intercellular exchange of biological signals and information via interactions with target cells. Thus, establishing a communication network not only locally at their site of origin, but also at distant sites as MP constitutively circulate in the blood of healthy individuals (Mause and Weber, 2010) with platelet-derived MP (PMP) proposed to be the most abundant subset (Horstman and Ahn, 1999, Berckmans et al., 2001). MP are thought to originate at sites of specialized membrane microdomains known as lipid rafts on cell surfaces that physically concentrate receptors (Del Conde et al., 2005, Cai et al., 2012). Thus, MP membranes are selectively

enriched with receptors derived from the cell of origin, and as MP contain cell cytoplasm they can also harbour a diversity of cell-derived constituents such as, cytokines, mRNA and microRNA (Mause and Weber, 2010). Intercellular communication mediated by MP can occur via different pathways. Firstly, membrane receptors of MP may interact directly and specifically with ligands present on the surface of target cells, thereby inducing cell signalling, and secondly MP may transfer part of their cargo (e.g. proteins, bioactive lipids, mRNA or microRNA) by interacting with target cells through either fusion or internalisation, mediating cell activation, phenotypic modification, and reprogramming of cell function (Mause and Weber, 2010).

Importantly, the level of circulating MP in blood plasma can be significantly elevated in various pathological conditions including infection, cardiovascular disease, and malignancy among others (Rak, 2010, Tushuizen et al., 2011). Thus, MP could be utilized as markers of various diseases and have prognostic potential in some cases. For example, tumour cell-derived MP can facilitate cancer progression through various mechanisms such as; (1) confer chemoresistance to chemosensitive tumour cells (Shedden et al., 2003, Bebawy et al., 2009, Gong et al., 2012), (2) transfer onocogenes to target cells, thus promoting further malignant transformation (Al-Nedawi et al., 2008, Di Vizio et al., 2009), (3) evade immune recognition via the release of proapoptotic MP that induce immune cell death (Monleon et al., 2001, Andreola et al., 2002, Huber et al., 2005, Kim et al., 2005), and (4) initiate coagulation via the release of procoagulant MP, resulting in fibrin formation, which protects the tumour from immune-mediated attacks and provides a matrix to support angiogenesis (van Doormaal et al., 2009). Activated endothelial cells *in vitro* have been shown to release MP bearing matrix metalloproteinases (MMP), which are involved in cell invasion (through MMP-mediated proteolytic activity that degrades extracellular matrix) and capillary formation during angiogenesis (Taraboletti et al., 2002). In addition, PMP may transfer various proteins, such as platelet-endothelium attachment receptors (CD41, CD61 and CD62), G-protein receptors (CXCR4 and PAR-1), cytokine receptors, and ligands from their surface to surrounding cell membranes, including tumour cells, which benefit from PMP-induced angiogenesis, metastasis (Janowska-Wieczorek et al., 2005), and tumour cell invasiveness (Barry et al., 1997, Barry et al., 1998, Janowska-Wieczorek et al., 2006). Moreover, MP specifically expressing TF may be potential mediators of VTE (Geddings and Mackman, 2013).

Although exact mechanisms leading to the formation of MP have not been fully elucidated, they are proposed to be initiated as a consequence of activation or apoptotic signals by chemical (e.g. cytokines, thrombin, endotoxin) or physical (e.g. shear stress, hypoxia) stimuli, promoting active remodelling of the plasma membrane (Holme et al., 1997, Horstman and Ahn, 1999). In addition, the generation of MP may also arise during malignant transformation via the action of oncogenes such as, K-ras and EGFRvIII, and the loss of tumour suppressor p53 (Yu et al., 2005, Al-Nedawi et al., 2008, Di Vizio et al., 2009), likely in combination with other influences such as hypoxia. Recently, the generation of TF+ MP was proposed to be regulated by post-translational modifications of the cytoplasmic domain of TF, in particular phosphorylation of the serine residue 253 is thought to induce the incorporation and release of TF through PAR-2 activation, whereas the phosphorylation of the serine residue 258 by p38α MAPK acts to suppress the incorporation of TF into released MP through dephosphorylation of Ser^{253} (Collier and Ettelaie, 2011, Ettelaie et al., 2013).

Under resting conditions, aminophospholipids PS and phosphatidylethanolamine are predominantly localized to the internal leaflet of the cell surface membrane, while the external leaflet is enriched in choline phospholipids sphingomyelin, and phosphatidylcholine. During cell activation, the release of MP is crucially dependent on the translocation of the procoagulant phospholipid PS from internal to external leaflet, in response to an increase in cytosolic calcium that promotes inactivation of aminophospholipid translocase, and concurrent activation of scramblase and floppase activity (Bevers et al., 1999). PS exposure also occurs during the early stages of apoptosis, although whether it occurs through shared pathways with platelet activation involving calcium ions (described above) remains disputed (Morel et al., 2011). Nevertheless, the presence of PS on the outer surface membrane is critical to apoptosis as it supports macrophage clearance of apoptotic cells and cell fragments (Schroit et al., 1984).

In spite of the mechanism provoking spontaneous loss of membrane phospholipid asymmetry in either activated or apoptotic cells, the resulting imbalance is resolved through subsequent blebbing of the membrane (Morel et al., 2011), involving cytoskeletal protein reorganisation (Figure 1.3). In stimulated cells, an influx of extracellular calcium is

also associated with calpain and gelosin activation (the latter in activated platelets only), which promotes membrane blebbing via degradation of cytoskeletal proteins (Miyoshi et al., 1996). In contrast, during apoptosis, cytoskeletal rearrangement and membrane blebbing has been shown to be dependent on the caspase-mediated cleavage of the Rho-associated kinase (ROCK) 1 that induces its activation, thereby promoting the generation of actin–myosin force and cell contractility (Coleman et al., 2001). Interestingly, the quantity and phenotype of MP shed from activated and apoptotic endothelial cells have been shown to differ; for example, cell surface PS expression and release of PS enriched MP was higher in apoptotic cells in comparison with activated cells, as determined by flow cytometry annexin V binding (Jimenez et al., 2003). Thus, the proportion of procoagulant PS on the outer surface of MP can vary.

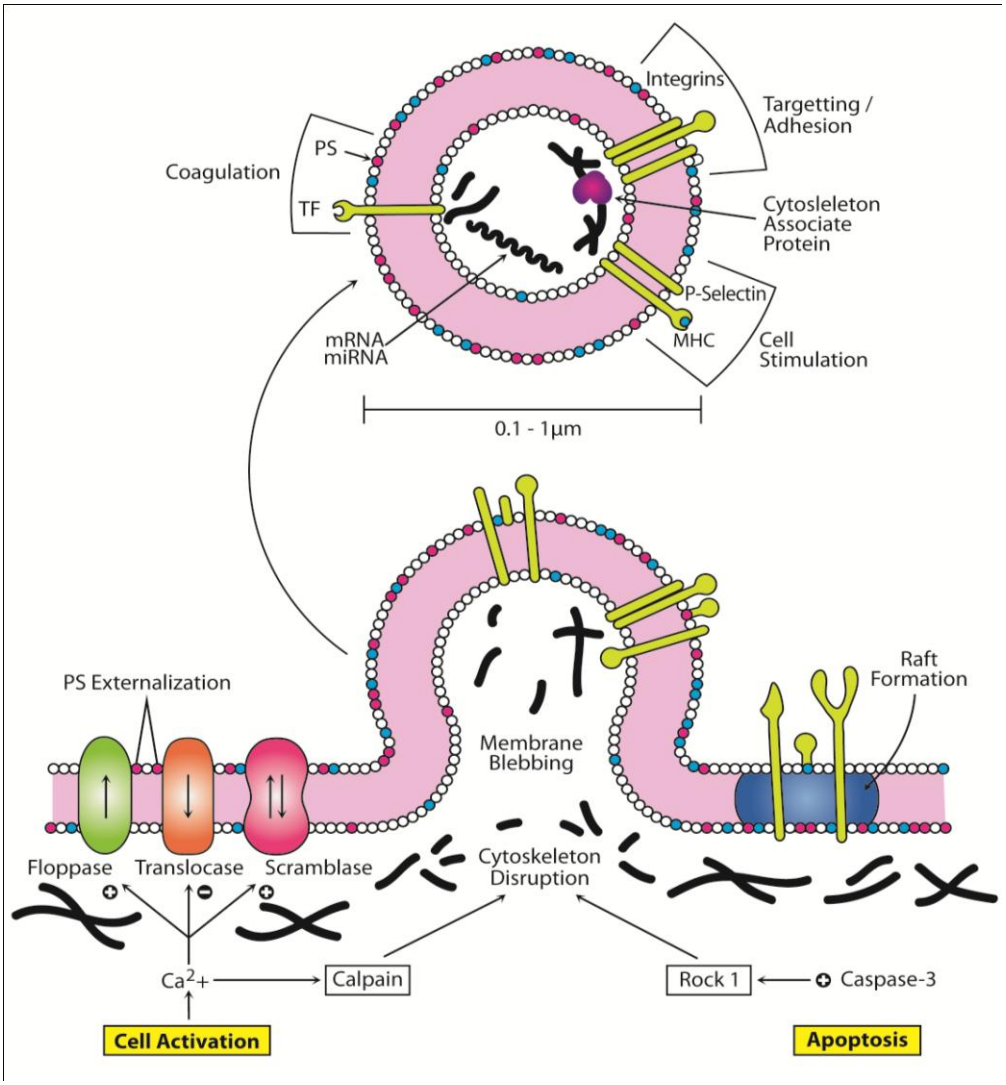


Figure 1.3: Basic schematic representation of the possible pathways leading to the formation of MP.

Resting cell membrane phospholipid symmetry is maintained by the activity of the following enzymes that catalyze the transport of phospholipids within the bilayer: an inward-directed ATP-dependent pump, specific for phosphatidylethanolamine and phoshatidylserine (PS), referred to as aminophospholipid translocase; an outward-directed ATP-dependent pump known as floppase; and a calcium-dependent non-specific lipid scramblase, which allows bidirectional lipid movement across the bilayer. During cell activation an increase in intracellular calcium induces externalization of PS (shown in pink) by stimulating scamblase and floppase activities, and concurrently inactivating aminophospholipid translocase. Calcium increase is also associated with calpain activation required for cytoskeleton disruption, which facilitates membrane blebbing and the release of MP. Lateral membrane reorganization also occurs following cell stimulation, leading to the formation of specialized raft domains by the inclusive and exclusive sorting of membrane constituents, and thereby the release of MP of a particular composition. Under apoptosis induction, cleavage of caspase-3 induces ROCK 1 activation, leading to cytoskeleton alteration and membrane blebbing. MP harbour a large repertoire of different membrane, cytoplasmic and nuclear constituents derived from the original cell, implicated in a variety of fundamental processes, and can vary depending on the stimulus leading to their production. Abbreviations: MHC, major-histocompatibility complex.

1.6.2 Procoagulant membrane surfaces

It is known that the externalization of PS has a profound impact on the procoagulant properties of membrane surfaces; PS acts synergistically as a cofactor with TF in the initiation of blood coagulation, thereby extending the available phospholipid surface area necessary for the assembly of enzyme complexes responsible for thrombin generation (Figure 1.4) (Zwaal et al., 1998). In agreement, an inherited defect in the inability of platelets to expose PS, or generate procoagulant MP, results in a rare bleeding disorder known as Scott syndrome (Piccin et al., 2007). Factors VII, IX, X, thrombin, protein C, and protein S all have vitamin K dependent positively charged γ-carboxy GLA domains on the amino terminus of the protein that enable calcium to bind to these proteins and change the conformation, allowing them to bind in turn, to a negatively charged phospholipid surface. It is currently thought that PS and phosphatidylethanolamine strongly synergize to bind GLA domains by providing "phospho-L-serine-specific" (involving the carboxylate on the headgroup of PS) and "phosphate-specific" interactions, respectively, thereby proposing PS is essential for coagulation to occur, but phosphatidylethanolamine can enhance the rate of these reactions (Tavoosi et al., 2011), a property regulated by its fatty acid chain length (Clark et al., 2013). In the described model of haemostasis, membrane-bound reactions of the coagulation system are thought to be primarily localized to the surface of TF-bearing cells and activated platelets. Interestingly, PMP surfaces have 50- to 100-fold higher PCA compared with the equivalent area on activated platelets (Sinauridze et al., 2007). As such, the intrinsic procoagulant properties of circulating MP may directly promote a prothrombotic state, a premise supported by a retrospective case-controlled study indicating a linear relationship between VTE risk and increasing numbers of MP in plasma (Bucciarelli et al., 2012). In this context, it has also been reported that plasma levels of MP are correlated with thrombin generation (Bidot et al., 2008).

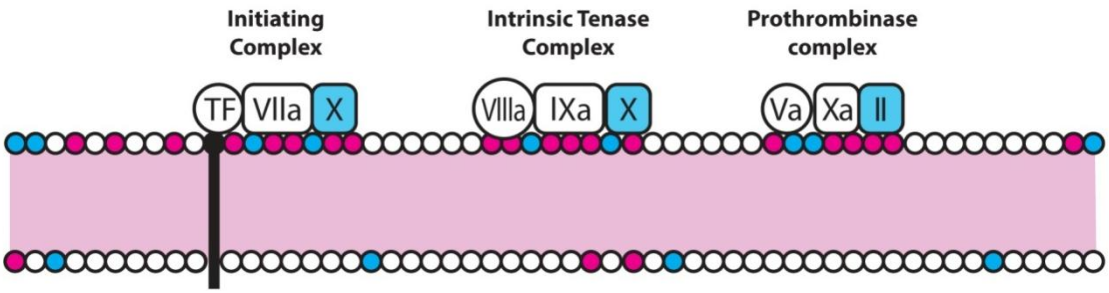


Figure 1.4: Assembly of coagulation complexes on a PS+ and phosphatidylethanolamine+ phospholipid membrane.
Coagulation complexes (TF-FVIIa; FVIIIa-FIXa; and FVa-FXa) are able to assemble on membrane surfaces that expose anionic phospholipids, such as PS (shown in pink) and the neutral phospholipid phosphatidylethanolamine (shown in blue). Thus, the presence of these phospholipids on the external membrane leaflet that are translocated from the internal leaflet during cell activation or apoptosis accelerates blood coagulation.

1.6.3 Procoagulant potential of TF+ MP

As mentioned, MP have various physiological functions and the ability to initiate coagulation is a result of PS and phosphatidylethanolamine surface exposure, and also due to the presence of active TF on their surface (Abid Hussein et al., 2008). For example, elevated levels of TF+ MP were shown to decrease the CT in mice with haemophilia, increasing PCA (Hrachovinova et al., 2003). *In vitro*, TF has been shown to localize to rafts in Lipopolysaccharide (LPS)-treated monocytes, which aid its release into the vasculature in the form of MP (Del Conde et al., 2005). These monocytic TF+ MP may then fuse with activated platelets, resulting in the transfer of TF to the platelet membrane, and subsequent initiation of coagulation (Del Conde et al., 2005). Similarly, Rauch et al. previously observed that activated human platelets acquired TF following incubation with monocytic cell line derived TF+ MP during thrombus formation (Rauch et al., 2000).

In agreement with this, real-time microscopy indicates in a laser-induced endothelial injury model that circulating haematopoietic cell-derived TF+ MP rapidly contribute to fibrin generation during arteriole thrombosis *in vivo* (Falati et al., 2003, Chou et al., 2004, Gross et al., 2005), with leukocyte MP rather than leukocytes themselves initially delivering TF to the developing thrombus (Gross et al., 2005). Furthermore, this accumulation of TF+ MP into the thrombus was shown to be mediated by the expression of P-selectin GP ligand-1 (PSGL-1) on MP that interacts with P-selectin on the surface of activated platelets (Falati et al., 2003). Although the delivery of MP to the site of injury is impeded by IVC ligation, a

frequently used model to study venous thrombosis, the injection of human MP isolated from the pericardial blood of cardiac surgery patients into an IVC rat model was shown to increase thrombus formation compared with venous blood of healthy controls (Biro et al., 2003). Importantly, this thrombogencity significantly correlated with TF exposure on MP (Biro et al., 2003). Similarly, Ramacciotti et al. demonstrated that the injection of MP positively impacts the weight of the thrombus formed in IVC ligated mice (Ramacciotti et al., 2009). The significance of TF+ MP accumulation in developing thrombi is further demonstrated by a study of TF localization in mouse models of thrombosis and skin wounding, which revealed by immunostaining that TF was present throughout thrombotic clots, while only found at the edges of haemostatic clots, thus implying that circulating TF plays a major role in propagating thrombosis (Hoffman et al., 2006).

1.6.4 Role of TF+ MP in cancer

An early study made the initial observation of tumour cell-derived MP being associated with the hypercoagulable state of cancer (Dvorak et al., 1981), which was later found to be TF-dependent *in vitro* (Dvorak et al., 1983, Bastida et al., 1984). Since then, tumour cell-derived MP have been shown to exhibit strong TF-dependent PCA both *in vitro* by one-stage clotting and thrombin generation assays, and *in vivo* in cell-free plasma from nude mice bearing orthotopically injected human cancer cells (Davila et al., 2008). Also, the concentration of TF present in cell-free plasma has been reported to correlate with the total tumour burden (Davila et al., 2008, Yu et al., 2005). In mice harbouring human pancreatic tumours, Thomas et al. demonstrate that tumour cell-derived MP, but not their parent cells, bind to injured vessels in a ferric chloride-induced vascular injury model by P-selectin-dependent interactions and enhance thrombus growth (Thomas et al., 2009). Interestingly, this study also reported that active TF is highly concentrated (> 100-fold) on the membrane of tumour cell-derived MP in comparison with parent cells *in vitro*, suggesting that cancer cells may be a major source of procoagulant TF+ MP circulating in blood and that such MP may exert systemic vascular effects (Thomas et al., 2009). Similarly, elevated levels of tumour cell-derived TF+ MP, as determined by flow cytometry, were associated with venous thrombosis in a mouse IVC stenosis model of human pancreatic cancer, despite < 5% of human TF protein in plasma from these tumour-bearing mice being associated with MP (Wang et al., 2012).

Furthermore, Yu et al. demonstrate both *in vitro* and *in vivo* that tumour cell-derived TF+ MP mediate the intercellular trafficking of TF from adjacent cancer cells to the tumour-associated endothelium, thereby potentially enhancing its procoagulant potential (Yu et al., 2008). More recently, tumour cell-derived TF+ MP were shown to be internalised by endothelial cells *in vitro*, resulting in the transfer of the TF receptor to the host cell surface at two distinct peaks (30 and 180-240 min) post-incubation, although high TF activity was exclusively associated with the second peak, presumably due to the concurrent exposure of PS on the endothelial cell surface at 90 min following incubation with MP (Collier et al., 2013).

Many studies have examined levels of circulating MP and/or their procoagulant potential in the plasma of cancer patients via an array of techniques for the detection of MP and enumeration or measurement of their functional activity (summarized in Table 1.2) (Key, 2010, Yuana et al., 2011). Consequently, measurements reported in the literature vary widely, but are also related to variations in pre-analytical conditions in plasma and the preparation of MP during blood collection, plasma isolation, and storage, all which have not yet been adequately standardized (Yuana et al., 2011). Flow cytometry is the most commonly used method for the detection of MP in clinical studies, arguably the current gold standard, as it can confer both quantitative and qualitative information, including the number and phenotype of MP by staining with fluorescently labelled antibodies directly in plasma, although it is not without limitations (see Table 1.2). In addition, functional assays detect the procoagulant potential of MP isolated from plasma by either a one-stage clotting assay that measures PCA or a two-stage, chromogenic, FXa generation assay (in the presence or absence of an inhibitory anti-TF antibody) that specifically determines the TF activity associated with MP. Recently, two commercial assays have been developed, the Zymuphen MP-Activity and MP-TF kits from Hyphen BioMed, that specifically capture MP from platelet-free plasma (PFP) using annexin V as a binding probe for exposed PS on the surface of MP or a polyclonal antibody specific for the extracellular domain of TF that does not interfere with TF activity, and subsequently determine the PCA or the TF activity associated with MP by either a thrombin generation, chromogenic assay or a two-stage FXa generation, chromogenic assay, respectively.

Table 1.2: Analytical methods used for the measurement of MP extracted from the blood of cancer patients.

Method	Quantification	Size	Cell origin	Limitations	References
Electron microscopy	Limited	Subjective*	Limited (only by immunoelectron microscopy)	Artefacts + alteration of morphology due to sample preparation	(Toth et al., 2008, Lima et al., 2009, Baran et al., 2010, Gercel-Taylor et al., 2012, Ma et al., 2013)
Atomic force microscopy	Limited	Subjective*	Limited (requires development of antibody-coated surfaces)	Artefacts due to abundance of cell debris + plasma proteins	(Baran et al., 2010, Yuana et al., 2010)
Confocal microscopy	Limited	Subjective*	Yes, allows identification of multiple antigens	Artefacts due to abundance of cell debris + plasma proteins	(Tesselaar et al., 2007, Ma et al., 2013)
Light scattering techniques: DLS and NTA	Yes	Yes	No **	Artefacts due to abundance of cell debris + plasma proteins	(Baran et al., 2010, Gercel-Taylor et al., 2012)
Light scatter-based flow cytometry	Yes	Limited	Yes, allows identification of multiple antigens	Lower detection limit of conventional flow cytometers is 300–500 nm	(Andreola et al., 2002, Kalinkovich et al., 2006, Hron et al., 2007, Al-Nedawi et al., 2008, Toth et al., 2008, Campello et al., 2011, Fleitas et al., 2012, Amirkhosravi et al., 2013, Papageorgiou et al., 2013, Reynes et al., 2013, Echrish et al., 2014)
Impedance-based flow cytometry	Yes	Limited	Yes, allows identification of multiple antigens	Not widely available + lower detection limit of 300 nm	(Zwicker et al., 2009, Zwicker et al., 2013)
Capture assays + functional assays: PCA, TF activity, thrombin generation, and ELISA-based tests	Limited (only capture-based assays)	No	Limited (only capture-based assays)	Only information on procoagulant, TF, or thrombin generating activity + presence of soluble antigen may affect results	(Kalinkovich et al., 2006, Tesselaar et al., 2007, Khorana et al., 2008a, Tilley et al., 2008, Tesselaar et al., 2009, Manly et al., 2010, Fleitas et al., 2012, Thaler et al., 2012, van Doormaal et al., 2012, Amirkhosravi et al., 2013, Papageorgiou et al., 2013, Reynes et al., 2013, Thaler et al., 2013a)

Abbreviations: DLS, dynamic light scattering; NTA, nanoparticle tracking analysis. * Due to limited number of measurements. ** Exception of custom modified NTA that allows limited fluorescent measurements.

In cancer patients, elevated levels of TF+ MP determined by flow cytometry (Hron et al., 2007, Campello et al., 2011, Ma et al., 2013), and also higher TF activity associated with MP (Tesselaar et al., 2007, Auwerda et al., 2011, Thaler et al., 2013a) have been reported in comparison with healthy controls. Numerous lines of evidence (discussed below) indicate that these elevated levels of circulating TF+ MP in malignancy are derived from the underlying tumour. Indeed, it can be assumed that cancer cells expressing high levels of TF can give rise to TF+ MP (Del Conde et al., 2005) since the composition of MP typically reflects the antigenic profile of the parent cell from which they originate. In agreement with this, animal models have demonstrated that circulating human TF antigen is detected in plasma samples from mice subcutaneously injected with colorectal cancer cells (Yu et al., 2005), and those orthotopically injected with pancreatic cancer cells (Davila et al., 2008). Furthermore, in a large proportion of cancer patients, phenotypic analysis of MP shed by tumour cells in two clinical studies revealed the expression of the tumour cell antigen, mucin-1 (MUC-1), a transmembrane GP, which is frequently over-expressed in epithelial malignancies (Tesselaar et al., 2007, Zwicker et al., 2009). In addition, Zwicker et al. also show that approximately 50% of the TF+ MP in pancreatic cancer patients were positive for MUC-1, and these levels subsequently declined following radical pancreatectomy in three patients (Zwicker et al., 2009). More recently, it was demonstrated in pancreaticobiliary adenocarcinoma patients (n=18) that the number of TF+ MP decreased following surgical tumour resection, and although this difference was found not to be statistically significant, the PCA of PFP measured by a one-stage clotting assay and the PCA associated with MP measured by the Zymuphen MP-Activity kit (as previously described), were both shown to be significantly reduced after surgery (Echrish et al., 2014).

Moreover, in two separate studies plasma levels of D-dimer, a coagulation activation marker, have been significantly correlated to the number of TF+ MP in advanced colorectal cancer patients (Hron et al., 2007), and the TF activity associated with MP in early-stage prostate cancer patients (Haubold et al., 2009). Furthermore, patients with pancreatic cancer were shown to have the highest plasma D-dimer level in comparison with brain, stomach or colorectal cancer patients, indicating strong activation of the haemostatic system, and a correlation between D-dimer level and TF activity associated with MP was only found in pancreatic cancer patients (Thaler et al., 2012). In addition, D-dimer levels

correlated with plasma levels of TF+ MP, but not TF activity associated with MP in patients with glioblastoma (Sartori et al., 2011, Sartori et al., 2013). Furthermore, Del Conde et al. report the case of an individual patient with giant cell lung carcinoma who experienced a high rate of thromboembolic events, and plasma TF antigen levels associated with MP that were 41 times higher than those of 16 healthy individuals (Del Conde et al., 2007).

1.6.5 Potential contribution of TF+ MP to VTE in cancer

As discussed, tumour cells are well known to express TF, and also spontaneously release TF+ MP that have demonstrated PCA and contribute to thrombus development in animal studies. It is therefore possible that tumour cell-derived TF+ MP may be deemed responsible, at least in part, for the increased risk of venous thrombosis in cancer patients. Initial *in vivo* evidence, provided by Tesselaar et al. in a retrospective study suggests that TF activity associated with MP correlates with the development of VTE in cancer patients, specifically by the extrinsic TF-mediated pathway, which was measured using a chromogenic assay that determined FVII-dependent FXa generation in the plasma of metastatic breast cancer and pancreatic cancer patients (Tesselaar et al., 2007). Khorana et al. subsequently evaluated this hypothesis in a prospective small cohort of patients with locally advanced or metastatic pancreatic cancer receiving chemotherapy, and found two patients later developed thrombotic complications following an increase in plasma TF antigen levels and TF PCA associated with MP (Khorana et al., 2008a). This is the first study to demonstrate that elevated levels of TF activity associated with MP precede thrombosis in some cases, and provides evidence to support the predictive role for TF+ MP as a biomarker for the development of thromboembolic complications in cancer (Khorana et al., 2008a). In a case-controlled study, Zwicker et al. reported that the levels of TF+ MP in the plasma of advanced pancreatic cancer, breast cancer, colorectal cancer, ovarian cancer, and NSCLC patients, determined by non-conventional (impedance-based) flow cytometry, were elevated in 60% (18 out of 30) of those cancer patients with VTE compared to 27% (16 out of 60) of those cancer patients without VTE, and predicted an approximate 4-fold increased risk of VTE in cancer patients associated with TF+ MP (Zwicker et al., 2009).

Furthermore, in two retrospective studies, the measured TF activity associated with MP in various unselected cancer patients who presented with VTE was significantly higher than

in those cancer patients without VTE (Tesselaar et al., 2009, Manly et al., 2010). In addition, TF activity associated with MP was also found to correlate with decreased survival, although the patients did not appear to exhibit increased numbers of MP (Tesselaar et al., 2009). In a separate study, even though the levels of TF+ MP in the plasma of cancer patients who developed VTE were significantly higher than in those cancer patients without VTE, these elevated levels of TF+ MP failed to be specifically linked to VTE by multivariate analysis (Campello et al., 2011). In a prospective study, van Doormaal et al. found that TF activity associated with MP in cancer patients correlated with the development of VTE, although no correlation was seen using light-scatter flow cytometry to detect the levels of TF+ MP (van Doormaal et al., 2012). Moreover, Thaler et al. failed to demonstrate a statistically significant association of TF activity with VTE in pancreatic, gastric, colorectal, and brain cancer patients (Thaler et al., 2012), while in a large cohort of 728 cancer patients with different tumour entities, although the total level of MP were raised in these patients, elevated procoagulant MP were not found to be predictive of VTE (Thaler et al., 2011).

More recently, the baseline plasma level of TF activity associated with MP in pancreaticobiliary cancer patients taken at the time of cancer diagnosis (n=117) correlated with the future development of VTE and decreased survival (Bharthuar et al., 2013). Additionally, another recent prospective study also found an association between the TF activity associated with MP and mortality in 252 cancer patients, but not with future VTE development (Hernandez et al., 2013). It is possible that these discrepancies reported in the literature regarding the association between the elevated levels of TF+ MP and VTE in cancer patients may, at least in part, relate to the relative difficulty in detecting MP and their associated TF activity, and may be caused by methodological inconsistencies that limit the sensitivity of the measurements. Further factors that may influence the findings of the above mentioned studies is the small patient cohort analyzed in the majority of these studies, and patient selection as attention to tumour stage/extent of malignancy and treatment status is rarely recorded or taken into consideration for statistical analyses.

In a second study by Zwicker et al. using the same novel and more sensitive impedance-based flow cytometry method to detect plasma TF+ MP, cancer patients undergoing chemotherapy were assigned to study arms based on their concentration of TF+ MP in this

recent prospective randomized phase II trial (Zwicker et al., 2013). In the two observational groups of this study, patients were not receiving primary thromboprophylaxis and after a 2 month follow up a 27.2% cumulative incidence of VTE was observed in cancer patients with high levels of TF+ MP (n=11) compared with 7.2% in those cancer patients with low levels of TF+ MP (n=32) at 2 months, thus confirming the potential prognostic utility of TF+ MP for the development of VTE in cancer (Zwicker et al., 2013). However, it is important to note that bilateral lower extremity ultrasound evaluations were performed following randomization and at follow up (60 $\pm$ 7 days) in this study to assess for the presence of an incidental proximal DVT, consequently the majority (5 out of 6; 83.3%) of the venous thromboembolic complications observed among all study groups were incidental and diagnosed by protocol-mandated lower extremity ultrasound evaluation, which would have otherwise remained undetected (Zwicker et al., 2013).

1.7 Chemotherapy increases risk of VTE

In cancer patients, the hypercoagulable state may be further amplified by the effects of chemotherapeutic agents. Combined study data indicates that chemotherapy, as either primary or adjuvant therapy, is associated with between a 2- to 7-fold increased risk of VTE compared to the general population, especially in the first 3 to 6 months of treatment (Heit et al., 2000, Khorana et al., 2005, Blom et al., 2006, Khorana et al., 2007b). However, chemotherapy-associated thrombosis is believed to be an underestimated phenomenon due to the presence of confounding factors that may further elevate the risk, such as surgery, use of intravenous catheters, and immobilisation (Otten et al., 2004). An increased risk of VTE has emerged as a frequent complication of many chemotherapy regimens, observed amongst those cancer types naturally associated with a high VTE incidence, such as malignant glioma and pancreatic cancer, where rates are elevated up to 28% (Brandes et al., 1997, Mandala et al., 2007, Simanek et al., 2007). Furthermore, even in those cancers associated with lower VTE incidence, for example in breast cancer patients, chemotherapy is reported to increase VTE incidence from around 3% (Khorana et al., 2006) to 9% (Mandala et al., 2010). Also, increases from around 3% (Zangari et al., 2002b, Klein et al., 2009) up to 35%, are observed in MM patients receiving immunomodulatory drugs (IMiD) in combination with other drugs in the absence of anticoagulation (Zangari et al., 2004, Cavo et al., 2005, Barlogie et al., 2006, Palumbo et al., 2006), discussed in more detail in section 1.8.3.

Furthermore, it is likely that VTE incidence varies with the type of chemotherapy administered, and specific combinations of drugs may elicit a particularly thrombogenic response. It is often difficult to assess the direct impact of an individual drug, as most are commonly administered as part of a multidrug regimen. In particular, platinum-based regimens have been shown to be associated with VTE (Kroger et al., 2006), although the risk of thromboembolism was higher in gastroesophageal patients treated with cisplatin-based therapy compared to oxaliplatin-based therapy in two prospective studies (Al-Batran et al., 2008, Starling et al., 2009). Furthermore, a prospective analysis of thromboembolism in NSCLC patients treated with cisplatin and gemcitabine found an incidence rate of 18% (Numico et al., 2005). More recently, the high incidence rate of thromboembolic events (16.1%) in patients treated with cisplatin-based chemotherapy for a variety of cancers was confirmed by a large retrospective study (Moore et al., 2011). In addition, platinum-based regimens were shown to be responsible for the induction of an acquired thrombophilic condition i.e. activated protein C resistance, and represent a predictor for VTE in cancer patients (Roselli et al., 2013).

In addition, VTE incidence during chemotherapy treatment may also be further modified by the inclusion of additional agents, such as hormonal therapies, IMiD, and antiangiogenic agents (Falanga and Marchetti, 2012a). For example, a systematic review of clinical studies in breast cancer revealed that hormone therapy using the selective oestrogen receptor modulator tamoxifen, in comparison with placebo or no treatment increases the risk of VTE by approximately 2- to 3-fold (Deitcher and Gomes, 2004), with the greatest risk seen in older, post-menopausal women (Saphner et al., 1991). In the cooperative National Surgical Adjuvant Breast and Bowel Project (NSABP) B14 trial investigating treatment modalities for oestrogen receptor-positive lymph node-negative breast cancer, women received tamoxifen or placebo for 5 years; the rates of VTE were 0.9% and 0.2%, respectively (Fisher et al., 1989). While in the follow-up NSABP B20 trial, the observed incidence rates of VTE were 0.8% and 4.2% when the same study population was treated with tamoxifen alone or tamoxifen plus chemotherapy, respectively, indicating that the increased risk of VTE seen in this study is chemotherapy-related (Fisher et al., 1997).

The SENDO (Southern Europe New Drugs Organization) phase I studies conducted between 2000 and 2010 identified a statistically significant increase of VTE in advanced cancer patients treated with a combination of various antiangiogenic agents and cytotoxic drugs (Mandala et al., 2012). The antiangiogenic agent semaxanib (SU5416) in combination with cisplatin and gemcitabine chemotherapy was associated with serious coagulatory complications during a phase I clinical trial in patients with solid tumours; 9 thromboembolic events occurred in 8 out of 19 patients (Kuenen et al., 2002b). In metastatic colon cancer, the addition of the VEGF inhibitor, bevacizumab, to a fluorouracil-based chemotherapy regimen increased the reported incidence rates of venous and arterial thrombotic events to 19% compared to controls in two clinical studies (Kabbinavar et al., 2003, Hurwitz et al., 2004). A meta-analysis of 5 randomized controlled trials including a total of 1745 patients with metastatic solid tumours found that combination treatment with bevacizumab and chemotherapy, compared with chemotherapy alone, was associated with an increased risk of arterial thromboembolism but not VTE (Scappaticci et al., 2007). In contrast, a meta-analysis of 15 randomized controlled trials studying a variety of advanced solid tumours concluded that bevacizumab combined with standard chemotherapy is associated with a significant increase in the riskof VTE by 33% (Nalluri et al., 2008). Similarly, a meta-analysis of 13 studies (7611 patients) concluded that anti-epidermal growth factor receptor agents, predominantly cetuximab and panitumumab, are also associated with a significant increase in the risk of VTE by 32%, but not of arterial thromboembolism, in patients receiving chemotherapy (particularly in combination with platinum-based chemotherapy) for advanced solid tumours (Petrelli et al., 2012).

1.7.1 Possible mechanisms of chemotherapeutic agents increasing VTE in cancer

The exact mechanisms by which chemotherapeutic agents increase VTE risk are predominantly undefined, both *in vitro* and *in vivo*. However, several mechanisms through which chemotherapy is capable of inducing a prothrombotic phenotype have been postulated (shown in Table 1.3) including; (1) the release of procoagulants and cytokines from damaged malignant cells, (2) downregulation of endogenous anticoagulant proteins such as protein C and protein S, (3) direct drug toxicity on vascular endothelium, and (4) direct induction of monocyte, endothelial, or tumour cell TF expression/activity (Furie and

Furie, 2006, Haddad and Greeno, 2006, Sousou and Khorana, 2009, Falanga and Marchetti, 2012a). In addition, chemotherapy drugs have been shown to induce platelet activation, affecting primary haemostasis (Togna et al., 2000) and the release of procoagulant MP (Date et al., 2013).

Table 1.3: Prothrombotic mechanisms of chemotherapy.

Study	Mechanism	Factor(s) altered	Examples
Endothelial Damage	Mechanical damage	Free Radical generation	**Fluorouracil** (Kinhult et al., 2003), **Dox** (Swystun et al., 2009b), **VEGFR inhibitors** (Vaklavas et al., 2010).
		Endothelial growth inhibition leading to exposure of sub-endothelium	**Gemcitabine** (Bruns et al., 2000), **Dox, bleomycin** (Mailloux et al., 2001), **corticosteroids** (Kerachian et al., 2009).
	Modulation of endothelial coagulant factors	↑Inflammatory cytokines (such as IL-1, IL-2, IL-6 and TNF-α), which in turn, facilitate adhesion molecule expression on endothelial cell surfaces (such as ICAM-1, VCAM-1 and E-selectin)	**CEF** (Bertomeu et al., 1990), **cisplatin** (Pogrebniak et al., 1991), **bleomycin** (Micallef, 1993), **anthracyclins** (Mills et al., 2004), **high-dose Dex** (Kerachian et al., 2009), **VEGFR inhibitors** (Vaklavas et al., 2010, Kuenen et al., 2002a), **Len** (Aue et al., 2011).
		↑TF expression/activity and/or PS exposure/release of MP	**Rapamycin** (Guba et al., 2005), **cisplatin** (Lechner et al., 2007), **Dox** (Swystun et al., 2009a, Hoshi et al., 2011), **epirubicin** (Swystun et al., 2009a), **daunorubicin** (Fu et al., 2010), **Dex** (Kerachian et al., 2009, Hoshi et al., 2011, Isozumi et al., 2013), **Thal/Dox, Thal/Dex** (Hoshi et al., 2011), **arsenic trioxide** (Zhou et al., 2011), **Len/Dex, Len/Dex/bortezomib** (Isozumi et al., 2013).
		↓natural anticoagulant proteins (such as TFPI, antithrombin III, protein C and protein S)/their functional activity (e.g. acquired protein C deficiency)/their endothelial cell surface receptors (e.g. EPCR)	**CMF** (Rogers et al., 1988, Feffer et al., 1989), **tamoxifen** (Pemberton et al., 1993, Mannucci et al., 1996, Erman et al., 2004), **Dox** (Woodley-Cook et al., 2006, Mukherjee et al., 2010), **CMF, epirubicin** (Mukherjee et al., 2010), **platinum compounds** (Roselli et al., 2013).
		↓Prostacyclin production	**Mitomycin** (Zakarija and Bennett, 2005).
Platelet activation	Effect on prostanoids	↑ Thromboxane A_2 generation	**Gefitinib** (Lynch et al., 2011).
		Activation of platelet phospholipase A_2	**Cisplatin** (Togna et al., 2000).
	Endothelial dysfunction	↑ vWF	**Cisplatin** (Licciardello et al., 1985), **high-dose Dex** (Kerachian et al., 2009).
	Modulation of platelet coagulant factors	↑ PS exposure and generation of PS+ MP	**Dox** (Kim et al., 2011).
Changes in Blood Flow	Hypertension (thrombotic microangiopathy)	↑ production of Endothelin-1 (vasoconstrictor)	**Corticosteroids** (Nickel et al., 2009).
		↓ NO and/or PGI_2 synthesis by arterial endothelial cells leading to increased vascular tone	**VEGFR inhibitors** (Sane et al., 2004, Eremina et al., 2008, Bollee et al., 2009).
Genetic predisposition	Heterozygous FV Leiden	Activated protein C resistance	**Prednisone** (Nowak-Gottl et al., 2003), **tamoxifen** (Garber et al., 2010).

Abbreviations: CEF, cyclophosphamide, epirubicin, and fluorouracil; CMF, cyclophosphamide, methotrexate, and fluorouracil; Dex, dexamethasone; Dox, doxorubicin; EPCR, endothelial cell protein C receptor; ICAM-1, intercellular adhesion molecule-1; Len, lenalidomide; NO, nitric oxide; PGI_2, prostaglandin I_2; Thal, thalidomide; TNF-α, tumour necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1; VEGFR, vascular endothelial growth factor receptor. ↑ and ↓indicate increase and decrease,respectively.

The majority of cytotoxic agents used in chemotherapeutic regimes target nuclear and mitochondrial DNA, which directly induces apoptosis in susceptible cells (Bhosle and Hall, 2009). Programmed cell death known as apoptosis is characterized by distinct morphological cellular changes such as nuclear condensation, loss of cell membrane asymmetry, membrane blebbing, and generation of MP (as previously described) (Hengartner, 2000). Apoptosis involves the activation of caspases, a family of cysteine acid proteases, which are central regulators of apoptosis (Hengartner, 2000). Once activated, these caspases cleave and activate downstream effector caspases that induce cell death (Hengartner, 2000). Apoptosis proceeds via two major cell death pathways: the intrinsic pathway that involves the mitochondria and the extrinsic pathway, initiated by signalling from cell surface death receptors (Hengartner, 2000). The specific mechanism of apoptosis induction may vary according to the type of drug administered (Fulda and Debatin, 2006), and may differentially determine the associated VTE risk.

A significant correlation between apoptosis and increased TF PCA has been proposed by Greeno et al. who observed a markedly increased cell surface TF activity following the induction of apoptosis (Greeno et al., 1996), as did Ma et al. who treated endothelial cells with cisplatin or gemcitabine (Ma et al., 2005). Similarly, doxorubicin (Dox) and epirubicin increased PS exposure and enhanced TF activity on endothelial cells, potentially through enhanced decryption of latent TF (Swystun et al., 2009a). Such TF activity, as observed in etoposide-, methotrexate- and vincristine-treated T24/83 human transitional bladder carcinoma cells, as well as in camptothecin treatment of human tumour cell lines, can be directly linked to an increase in cell surface thrombin generation (Paredes et al., 2003, Wang et al., 2001). Moreover, Wang et al. found a statistical correlation between TF activity and degree of apoptosis induced by campotothecin in NB4 and HL-60 leukaemia cell lines (Wang et al., 2001).

1.7.2 Chemotherapy and MP

Since the shedding of MP is a prominent feature of apoptosis, it has been hypothesized that cytotoxic chemotherapy may promote an increased risk of VTE by triggering the release of procoagulant MP bearing TF into the peripheral circulation from apoptotic tumour cells and vascular endothelial cells, as a result of tumour cell lysis and vascular injury, respectively. Indeed the release of MP has been specifically correlated with the

degree of apoptosis induced by oxysterols (Aupeix et al., 1997). Furthermore, proapoptotic stimulation promoted the release of MP by the topoisomerase I inhibitor, camptothecin, in human umbilical vein endothelial cells (HUVEC) (Simak et al., 2002), Jurkat T-cell leukaemia, and HL-60 promyelocytic cell lines (Reich and Pisetsky, 2009). Lechner et al., reported *in vitro* that cisplatin treatment induced apoptosis in HUVEC and human dermal microvascular endothelial cells, which was accompanied by an increased release of procoagulant endothelial cell-derived MP (EMP) (Lechner et al., 2007). Importantly, Boles et al. recently described an increase in the number of TF+ MP following apoptosis in response to daunorubicin and Dox treatment of THP-1 human leukaemia cells (Boles et al., 2012). However, significant *in vivo* evidence in support of this hypothesis is currently somewhat lacking.

In pancreatic cancer, Khorana et al. reported a progressive increase in plasma TF, measured either by TF antigen levels or PCA associated with MP, in 2 of the 11 patients who developed VTE during chemotherapy (Khorana et al., 2008a), while Maraveyas et al. observed elevated circulating TF antigen levels in those patients who received gemcitabine chemotherapy alone compared with gemcitabine plus LMWH (Maraveyas et al., 2010). In a study by Tesselaar et al., the TF activity associated with MP was surprisingly only elevated in 16.7% of cancer patients (4 out of the 24) who presented with VTE during the course of chemotherapy, as compared to healthy subjects (Tesselaar et al., 2009). Whilst Campello et al. demonstrated higher levels of TF+ MP in the plasma of patients undergoing chemotherapy compared with cancer patients not on chemotherapy; however this difference was not statistically significant (Campello et al., 2011). Furthermore, levels of PS+ MP in NSCLC patients did not significantly change after the administration of a first line course of platinum-based chemotherapy with or without bevacizumab (Fleitas et al., 2012), although these results were determined by only a pre-treatment and post-treatment measurement, thus a transient shift of MP during the initial cycles of chemotherapy cannot be excluded in this study. Also, TF activity associated with MP was found to be unaffected by chemotherapy in metastatic testicular cancer patients (van den Hengel et al., 2013). Once more, there is a possibility that these inconclusive and conflicting findings may potentially be a result of inaccurate estimations due to specific methodologies utilised or inappropriate timing of sample acquisition. Thus, the role MP are hypothesized to play in thrombosis development during chemotherapy remains

elusive, and requires further investigation in different cancer patients at different time points after the administration of chemotherapy. Furthermore, the specific type of chemotherapy may induce more or less TF+ MP or TF activity, depending on its mechanism of action.

In contrast, it is also possible that the variability of thrombotic risk in patients undergoing chemotherapy may be largely determined by tumour type rather than differences in cytotoxic drugs applied. In breast cancer, Mukherjee et al. were unable to detect significant changes in MP-associated TF activity following chemotherapy, despite observing increased thrombin generation (Mukherjee et al., 2010). However, it is important to note that breast cancer is generally associated with a relatively low thrombotic risk. In addition, chemotherapy was administered as an adjuvant or neoadjuvant therapy in this study, and as such, tumours have already been removed or are small and localised. Thus, chemotherapy-associated effects measured in the blood may arise as a result of the adverse effects of chemotherapeutic drugs on the haemostatic properties of normal blood cell populations. In MM, the absence of TF expression by neoplastic plasma cells, both in human MM cell lines and tumour samples from MM patients was recently reported (Cesarman-Maus et al., 2012), despite the fact that significant increases in VTE are noted in MM patients in response to chemotherapy. Moreover, Auwerda et al. measured TF activity associated with MP in 122 newly diagnosed MM patients before and after chemotherapy. Interestingly, the TF activity was higher in MM patients before chemotherapy compared to controls and remained elevated after chemotherapy only in patients with VTE during follow-up, although this association between increased TF activity levels associated with MP and future VTE in MM patients was found not to be statistically significant (Auwerda et al., 2011).

1.7.3 Thromboprophylaxis in ambulatory cancer patients receiving chemotherapy

VTE is principally a preventable disease; primary prophylaxis is applied in various clinical settings via anticoagulant therapy. Traditionally, cancer-associated VTE has commonly been observed in hospitalized patients admitted for surgery or acute medical illness (Stein et al., 2006). However, cancer therapy is now primarily administered in the outpatient environment. Subsequently, recent data has demonstrated that there is a high occurrence

of VTE events in ambulatory cancer patients (12.6%) (Khorana et al., 2013). Common anticoagulants in clinical use for the treatment and prophylaxis of VTE, including indirect thrombin inhibitors (unfractionated heparin [UFH] and LWMH), indirect FXa inhibitors (fondaparinux) and vitamin K antagonists (warfarin), are described in Table 1.4, although there is an increased risk of bleeding with all anticoagulant therapies, which may be particularly pronounced in patients with cancer (Prandoni et al., 2002). LMWH is currently the initial standard of care for the treatment of established VTE in cancer patients, as recommended by the NCCN, ASCO, ESMO and other consensus guidelines for typically the first 3 to 6 months, although UFH or fondaparinux can also be used (Lee et al., 2003, Khorana, 2007, Lyman et al., 2007, Mandala et al., 2011, Farge et al., 2013). In addition, present data is insufficient to recommend the routine use of newer oral anticoagulants, including direct thrombin inhibitors (dabigatran, lepirudin, and argatroban), direct FXa inhibitors (rivaroxaban and apixaban) and defibrotide, as therapies for VTE in patients with cancer (Lee and Carrier, 2014).

In recent years the safety and efficacy of ambulatory thromboprophylaxis has been evaluated in several randomized controlled trials, due to the lack of cancer-specific data regarding primary thromboprophylaxis. The PROTECHT study investigated anticoagulant prophylaxis in patients with cancer, including those with locally advanced or metastatic lung, gastrointestinal, pancreatic, breast, ovarian, and head and neck cancers actively undergoing chemotherapy. Overall, thomboembolic events were reported in 2% (15 out of 769) of patients receiving nadroparin, a LMWH, and 4% (15 out of 381) of patients in the placebo group (Agnelli et al., 2009). Subsequently, SAVE-ONCO, the current largest cancer thromboprophylaxis study, demonstrates a risk reduction of VTE in patients with locally advanced or metastatic solid tumours (lung, pancreas, stomach, colorectal, bladder, or ovary) receiving semuloparin (a novel ultra-LMWH; 1.2% versus 3.4%) compared with placebo (Agnelli et al., 2012). In both of the above trials, prophylaxis was applied to a broad, heterogeneous population, and as a result, thomboembolic event rates are low and the clinical impact of thromboprophylaxis is also low.

Table 1.4: Pharmacologic and clinical properties of anticoagulants commonly used to treat VTE.

Property	UFH	LMWH	Indirect FXa inhibitors	Vitamin K antagonists
Medications	Heparin	Dalteparin Enoxaparin Tinzaparin Nadroparin	Fondaparinux	Warfarin
Source	Extracted from porcine intestinal mucosa or bovine lung	Chemical or enzymatic depolymerisation of UFH	Chemical synthesis of a molecule based on the native pentasaccharide sequence in heparin	Chemical synthesis of a coumarin derivative, a chemical found naturally in many plants
MW (Daltons)	3000 – 30000	1000 – 10000	1728	1000
Mechanism of action	Binds to antithrombin III, equally accelerating its anti-Xa and anti-IIa activity	Similar action to UFH, but less pentasaccharide chains are sufficiently long enough to bridge antithrombin III to FIIa, resulting in a 2:1 to 4:1 acceleration ratio of anti-Xa: anti-IIa activity	Indirectly inactivates FXa by binding to antithrombin III and selectively accelerates its interaction with FXa, but not FIIa	Inhibits vitamin K epoxide reductase, an enzyme that recycles oxidized vitamin K to its reduced form after it has participated in the carboxylation of the GLA residues on several coagulation factors, mainly FII and FVII
Administration	Intravenous or subcutaneous	Subcutaneous	Subcutaneous	Oral
Half-life	30 – 150 min (dose dependent)	3 – 4 h	17 – 21 h	2.5 days
Monitoring	aPTT or anti-Xa assay	Anti-Xa assay	Anti-Xa assay	Prothrombin time and INR
Elimination	Enzymatic degradation at low doses and renal at higher doses	Primarily renal	Renal	Hepatic metabolism
Comments	Binds to other proteins resulting in short half-life, although potentially safer than LMWH in patients with renal insufficiency	Less non-specific binding to proteins, a more predictable dose response, a longer-half life, and may be associated with a lower risk of bleeding than UFH	Dose adjustments are necessary in patients with severe renal insufficiency	Dosing of warfarin to maintain therapeutic INR may be more difficult in cancer patients than in those without cancer and potential interactions with some chemotherapeutic agents. May be associated with a higher risk of bleeding than LMWH

Abbreviations: aPTT, activated partial thromboplastin time; INR, international normalized ratio.

Conversely, studies that have focused on single high-risk cancer sites have demonstrated considerable clinical benefit of thromboprophylaxis concurrent with chemotherapy. For example in advanced pancreatic cancer, significant reductions of observed VTE incidence rates were achieved with LMWH administration simultaneous to chemotherapy in the FRAGEM and CONKO-004 trials (23% no anticoagulation versus 3% treated with weight-adjusted dalteparin and 15% no anticoagulation versus 5% treated with enoxaparin, respectively) (Maraveyas et al., 2012, Riess et al., 2010). The preliminary results from FRAGMATIC, a large open label, multi-centre, phase III randomized controlled trial investigating the effect of long term LMWH in lung cancer patients has confirmed that prophylactic dalteparin reduces the risk of VTE events without a significant increase in major bleeding (Macbeth et al., 2013). Overall, confirmed VTE events were reported in 7.1% of patients in the control group, and 4.1% of patients in the LMWH group; a relative risk reduction of 40% (Macbeth et al., 2013). However, there was no significant difference observed in overall survival at 1 year (Macbeth et al., 2013). Therefore, these results do not support the implementation of routine prophylactic anticoagulation for all lung cancer patients undergoing chemotherapy.

Results from the trials discussed above have shown that VTE prophylaxis is safe, feasible, and to a certain extent, effective in selected outpatients with various malignancies who are receiving chemotherapy. However, thromboprophylaxis in ambulatory cancer patients requires individual and careful consideration of the balance of benefits and risks, as evidence suggests that a benefit in terms of reduction of VTE is accompanied by a potential risk of increased bleeding in cancer patients. A risk assessment model has been developed from a cohort of 2,701 patients, and then validated in an independent cohort of 1,365 patients from a prospective registry to predict the probability of VTE among cancer patients undergoing chemotherapy (Khorana et al., 2008b). This risk assessment tool uses a combination of easily available clinical and laboratory measures, including site of cancer, platelet count, haemoglobin and/or use of erythropoiesis-stimulating agents, leukocyte count, and body mass index (Khorana et al., 2008b). The VTE incident rates in the development and validation cohorts after a follow-up period of 2.5 months were 0.8% and 0.3% in the low-risk category, 1.8% and 2% in the intermediate-risk category and 7.1% and 6.7% in the high-risk category, respectively (Khorana et al., 2008b). Subsequently, this risk assessment model has been further validated in several retrospective and prospective

studies (Kearney et al., 2009, Khorana et al., 2010, Moore et al., 2011, Mandala et al., 2012). More recently, an expansion of the original risk score was introduced that incorporates two additional biomarkers, specifically soluble P-selectin and D-dimer (Ay et al., 2010). In comparison with the original risk score, the expanded risk model has considerably enhanced the specificity from 26% to 54% of patients correctly identified who developed VTE in the Vienna CATS study in 819 cancer patients, although it requires further validation in other studies (Ay et al., 2010).

Furthermore, the Microtec study randomized patients undergoing chemotherapy for advanced solid tumours of the pancreas, colon, lung, ovary, or stomach to receive prophylaxis by evaluating levels of circulating TF+ MP, in a novel biomarker risk stratification approach (Zwicker et al., 2013). Patients with lower levels of TF+ MP did not receive thromboprophylaxis, and those with higher levels of TF+ MP were randomized to LMWH (enoxaparin) or observation. Interestingly, this study shows in the higher TF+ MP group randomized to LMWH (n=23) the rate of VTE was 6% after a 2 month follow-up, whereas the higher TF+ MP group randomized to observation (n=11) reported VTE rate was 27%; primary thromboprophylaxis resulted in an 80% risk reduction (Zwicker et al., 2013). Furthermore, the observed rate of VTE in the low TF+ MP arm was 7% (n=32) (Zwicker et al., 2013). Although the results were underpowered and not statistically significant, this risk stratification approach warrants further investigation by phase III clinical trials evaluating TF+ MP as a biomarker-driven anticoagulation strategy in cancer patients (Zwicker et al., 2013). However, the impedance-based flow cytometry methodology used in this study is not widely available and assays for TF+ MP would need to be appropriately standardized before translation into clinical practice.

Clinical practice guidelines for VTE prevention in cancer patients recommend that hospitalized patients with cancer and cancer patients undergoing surgery receive prophylactic anticoagulation (Lyman et al., 2007, Mandala et al., 2011, Farge et al., 2013, Lyman et al., 2013). However, they do not recommend routine thromboprophylaxis for ambulatory cancer patients undergoing chemotherapy, except for treatment of MM with thalidomide (Thal) or lenalidomide (Len) in combination with either chemotherapy or dexamethasone (Dex) discussed further in section 1.8.4. In light of data from the above mentioned randomized controlled trials, recently revised guidelines from the ASCO, in agreement with the ESMO, advise that clinicians may consider LMWH prophylaxis on a

case-by-case basis in selected outpatients with solid tumours receiving chemotherapy (Mandala et al., 2011, Lyman et al., 2013). Furthermore, international clinical practice guidelines state that primary prophylaxis of VTE may be indicated in patients specifically with locally advanced or metastatic pancreatic or lung cancer treated with chemotherapy and having a low risk of bleeding (Farge et al., 2013). The current guidelines endorse the use of risk assessment tools, and also stipulate for further research to identify biomarkers that could improve the positive predictive value of risk scores, and help clinicians select patients for thromboprophylaxis. The identification of which chemotherapy drugs, or combination regimens, increase the thrombotic risk in cancer patients, and understanding the specific mechanism(s) through which they elicit this effect is of great interest.

1.8 Multiple myeloma introduction and pathogenesis

MM is a plasma cell disorder characterised by the malignant transformation and clonal proliferation of plasma cells in the bone marrow, monoclonal paraprotein, osteolytic bone lesions, and renal disease. Plasma cells are derived from post-germinal-center B cells. They are the primary mediators of humoral immunity, secreting antigen specific immunoglobulin (Ig) composed of two identical light chains (either kappa or lambda) attached to two heavy chains. The majority of patients with MM (approximately 82%) have elevated paraprotein or M-protein levels on serum electrophoresis (Kyle et al., 2003), which may arise from the monoclonal secretion of any of the five distinct classes of Ig, namely IgG, IgA, IgM, IgE, and IgD, in the form of intact Ig or Ig fragments by myeloma cells. However, some patients may have slightly increased or normal serum Ig levels, but have elevated urine excretion of monoclonal light chains, known as Bence Jones protein. This excess of monoclonal paraprotein secreted by myeloma cells can lead to increased blood viscosity (Mehta and Singhal, 2003), and renal failure as a result of damage caused to renal tubules by free light chain deposition (known as cast nephropathy, or “myeloma kidney”) (Haubitz and Peest, 2006). Various other nephrotoxic processes may precipitate or exacerbate this damage such as, dehydration, hypercalcaemia, nephrotoxic drugs, and infection (Clark et al., 1999, Haubitz and Peest, 2006, Penfield, 2006).

The molecular pathogenesis of MM is not fully understood, but recurrent chromosomal abnormalities, both numerical (i.e. hyperdiploidy or hypodiploidy) and structural (i.e. gene rearrangements such as translocations involving the Ig heavy chain locus at 14q32), are

frequent in virtually all myeloma cells (Sawyer, 2011). Secondary chromosomal aberrations may occur in advanced disease including translocations and/or amplifications of the oncogene *MYC* at locus 8q24, the loss or deletion of chromosome 13, deletions and/or amplifications of chromosome 1, and deletion of chromosome 17p13 (Sawyer, 2011). Increasing evidence has established that the complex interactions among myeloma cells and the bone marrow microenvironment are critical to myeloma cell migration, proliferation, survival, and resistance to chemotherapy (Caligaris-Cappio et al., 1991, Damiano et al., 1999, Roodman, 2002, Nefedova et al., 2003, Abe et al., 2004, Dalton et al., 2004, De Raeve and Vanderkerken, 2005, Tanaka et al., 2007).

The tumour microenvironment consists of myeloma cells, extracellular matrix, and bone marrow stromal cells (BMSC), including fibroblastic stromal cells, osteoblasts, osteoclasts, vascular endothelial cells, and lymphocytes (Roodman, 2002). The myeloma cells can interact either directly with extracellular matrix proteins and the BMSC, or indirectly by secretion of soluble cytokines, growth factors, and adhesion molecules (Mitsiades et al., 2006). In particular, adhesion-mediated or tumour necrosis factor-α (TNF-α)-induced activation of the nuclear factor κB (NFκB) signalling pathway upregulates adhesion molecules such as CD54 (also known as intercellular adhesion molecule-1 [ICAM-1]) and CD106 (also known as vascular cell adhesion molecule-1 [VCAM-1]) on both myeloma cells and BMSC, thereby further increasing the binding of myeloma cells to BMSC and ensuing production of cytokines, such as IL-6 (a potent myeloma growth and survival factor) (Chauhan et al., 1996, Hideshima et al., 2001, Mitsiades et al., 2006). In addition, the secretion of angiogenic factors, such as VEGF, bFGF, and hepatocyte growth factor (HGF), from myeloma cells and BMSC stimulates angiogenesis (Jakob et al., 2006). Importantly, the microvessel density of bone marrow in MM patients has been shown to correlate with disease progression and/or poor prognosis (Vacca et al., 1994, Vacca et al., 1995, Rajkumar et al., 2000, Munshi and Wilson, 2001). Moreover, given the fact that myeloma cells are in close proximity to sites of bone resorption, it is believed that cross-talk between myeloma cells and BMSC is involved in the pathogenesis of osteolytic lesions through mechanisms involving; (1) osteoblastogenesis inhibition by the secretion of IL-3 and Dickkopf 1 from myeloma cells and HGF from BMSC, and (2) osteoclastogenesis activation by receptor activator of NFκB ligand (RANKL) produced by BMSC, and macrophage inflammatory protein-1α produced by myeloma cells (Roodman, 2001, Edwards et al., 2008). The result

is tumour-induced excessive bone destruction mediated by osteoclasts and exacerbated by a reduction in osteoblastic bone formation, which may lead to hypercalcaemia, the primary metabolic complication observed in myeloma patients (Roodman, 2001, Edwards et al., 2008).

1.8.1 Clinical presentation and classification of MM

The median age at presentation of MM is approximately 70 years, and only 15% of myeloma patients are aged less than 60 years (Bird et al., 2011). Furthermore, MM has a higher incidence in Afro-Caribbean ethnic groups than in Caucasians but there are few other distinctive epidemiological features (Bird et al., 2011). Many patients with MM initially present with bone disease, either focal or diffuse osteolytic lesions, that can result in pain (particularly backache) and pathological fractures/spinal cord compression (Coleman, 1997). Other presenting clinical manifestations of MM include anaemia, renal insufficiency, hypercalcaemia, hyperviscosity, neuropathy, and immunodeficiency (Mehta and Singhal, 2003, Talamo et al., 2010). The diagnostic criteria for symptomatic MM is defined by an increased number of plasma cells in the bone marrow (bone marrow aspirate, trephine biopsy showing more than 10% clonal plasma cells), elevated M-protein (serum or urine), and evidence of myeloma-related end-organ damage (including hypercalcaemia, renal failure, anaemia, and/or osteolytic lesions) (Group., 2003).

Traditionally symptomatic MM is classified using the Durie-Salmon staging system that has been in use for nearly 40 years (Durie and Salmon, 1975), although the International Staging System (ISS) introduced by the International Myeloma Working Group (IMWG) in 2005 (Greipp et al., 2005) is now more frequently used (Table 1.5). In particular, the ISS has been shown to be very sensitive in predicting prognosis and guiding treatment of MM as levels of serum β_2-microglobulin reflect the tumour burden and renal impairment (Greipp et al., 2005). Moreover, high serum levels of β_2-microglobulin and low levels of albumin confer a poor prognosis in MM patients. Furthermore, it is now generally accepted that the Ig heavy chain gene translocations t(4;14), t(14;16), and t(14;20) as well as the copy number changes 1q gain and 17p deletion, demonstrated by fluorescence *in situ* hybridisation, confer an adverse outcome in MM (Fonseca et al., 2009, Sawyer, 2011). Thus, it has been proposed that these cytogenetic abnormalities define 'high-risk' myeloma, and it is now international consensus that they should be specifically tested for

at diagnosis in all patients as they provide important prognostic information (Fonseca et al., 2009, Ross et al., 2012, Pratt et al., 2014), although their role in directing therapy needs further evaluation in prospective clinical trials.

Table 1.5: Staging of MM.

Stage	Durie-Salmon criteria	ISS criteria
I	All of the following: Haemoglobin > 10 g/dl Serum calcium value normal or <10.5mg/dl Normal bone structure or solitary plasmacytoma only Low M-protein: • IgG <5 g/dl • IgA <3 g/dl • Bence Jones protein <4g/24h (Low plasma cell mass: 600 billion MM cells/m^2)	Serum β_2-microglobulin <3.5mg/l and serum albumin ≥3.5g/dl
II	Neither stage I nor stage III (Intermediate cell mass: 600 to 1,200 billion MM cells/m^2)	Neither stage I nor stage III
III	One or more of the following: Haemoglobin <8.5 g/dl Serum calcium >12 mg/dl ≥3 lytic bone lesions High M-protein: • IgG >7 g/dl • IgA >5 g/dl • Bence Jones protein: >12 g/24h (High cell mass: > 1,200 billion MM cells/m^2)	Serum β_2-microglobulin ≥5.5 mg/l
Subclassification (either A or B)	A: Normal renal function (serum creatinine level: <2.0 mg/dl) B: Abnormal renal function (serum creatinine level: ≥2.0 mg/dl)	N/A

Although MM can occur *de novo*, MM is frequently preceded by a premalignant plasma cell disorder known as monoclonal gammopathy of undetermined clinical significance (MGUS) (Landgren et al., 2009); these patients can be safely observed without chemotherapy. Approximately 1% of individuals with MGUS progress to active MM per year (Kyle et al., 2002), while this annual risk of progression is increased to 10% in individuals with asymptomatic MM in the first five years, but interestingly, declines in subsequent years (Kyle et al., 2007). In 2010, there were approximately 4700 new cases of MM diagnosed in the UK, accounting for 1.4% of all new cancer cases and 10 to 15% of haematological malignancies (Cancer Research UK, 2014). MM remains an incurable condition for virtually all patients, due to the effects of minimal residual disease after treatment and the emergence of drug resistance. The average survival of MM patients is 4 to 6 years, although survival can vary from months to more than 10 years after diagnosis.

Factors affecting survival and outcome include tumour burden, extent of end-organ damage, type of cytogenetic abnormality, age, performance status, and response to treatment. With the introduction of novel agents in the management of MM including the IMiD Thal and Len, and the proteasome inhibitor bortezomib (Bor), response rates and clinical outcomes have been significantly improved (Kumar et al., 2008). Consequently, novel agent-based regimens are now widely considered standard of care in the treatment of MM patients, although additional prospective clinical trials are required to determine the best sequence and combinations of therapies in both the newly diagnosed and relapsed/refractory setting.

1.8.2 Multiple myeloma treatment

The broad aims of treatment in myeloma are to control disease, enhance quality of life, and extend survival. This may be accomplished by a combination of specific tumour-targeted therapy, and supportive care to treat the symptoms associated with MM. Typically, newly diagnosed MM patients receive a combination of three anti-myeloma agents including chemotherapy with an alkylating agent (such as melphalan or cyclophosphamide), a corticosteroid (such as prednisolone or Dex), and a novel agent (such as Thal or Bor). The aim of this first-line therapy is to achieve a period of stable disease (plateau phase) for as long as possible. In the UK the standard induction therapy for young (usually ≤ 60 years) and/or healthy individuals is cyclophosphamide, Thal, and Dex (CTD) (Bird et al., 2011) following the demonstration that it can be safely and effectively administered in a large, multi-centre clinical trial conducted by the Medical Research Council (Morgan et al., 2012). Induction therapy for this group of individuals is typically followed by high-dose melphalan and autologous stem cell transplantation (Bird et al., 2011). Importantly, stem cell mobilization and harvesting are not adversely affected by the use of Thal-containing regimens (Kumar et al., 2009). In the older and/or less fit group unable to undergo the intensive treatment pathway involving high-dose therapy and autologous stem cell transplantation, the initial treatment is commonly a Thal-containing regimen in combination with an alkylating agent and a corticosteroid such as melphalan, prednisolone, and Thal or an attenuated dose of CTD (CTDa). Alternatively Bor in combination with melphalan and prednisolone may be administered in some cases (Bird et al., 2011).

Following initial treatment, the majority of patients typically experience a period of remission, although almost all relapse eventually, and some have disease that does not respond or is 'refractory' to treatment. While maintenance therapy is not currently standard practice in the UK, some patients may participate in clinical trials in this setting. The use of Thal-, Bor-, and Len-based regimens as treatment modalities at first and subsequent relapse is supported by extensive clinical trial data (Richardson et al., 2002, Weber et al., 2003, Offidani et al., 2004, Jagannath et al., 2005, Weber et al., 2007, Richardson et al., 2005, Jagannath et al., 2006, Dimopoulos et al., 2007, Morgan et al., 2007, Wang et al., 2008, Palumbo et al., 2010b, Richardson et al., 2014). Factors such as the timing of relapse, efficacy and toxicity of drugs used in prior therapy, age, bone marrow and renal function, co-morbidities, and patient preference should be taken into consideration when determining treatment regimens in the relapsed/refractory setting (Bird et al., 2011).

1.8.3 Venous thromboembolism associated with MM and IMiD

One of the major complications unexpectedly reported in many clinical studies of MM patients treated with regimens including IMiD was the development of thrombosis (Carrier et al., 2011). Thal and its analog Len have various mechanisms of action in myeloma including (1) immunomodulatory effects by inducing T cell and Natural Killer cell anti-myeloma immunity, (2) modulation of proinflammatory cytokine levels (e.g. TNF-α and IL-6) within the bone marrow microenvironment, (3) direct induction of apoptosis of malignant plasma cells through caspase 8-mediated cell death pathway (4) modulation of the expression of cell surface adhesion molecules (e.g. CD54, CD106, and E-selectin) that facilitate the binding of myeloma cells to BMSC, and (6) an antiangiogenic effect through reduction in the levels of VEGF and bFGF (Anderson, 2005, Kotla et al., 2009). Nevertheless, it remains unclear which of these effects may lead to the activation of coagulation and thrombus formation in MM patients treated with IMiD.

Furthermore, the exact role of combination therapy with Thal or Len in assessing thrombotic risk is confounded by the concurrent presence of many disease-specific prothrombotic factors in MM patients. For example, high levels of circulating paraprotein in MM can result in hyperviscosity and may interfere with fibrin formation, thereby leading to impaired fibrinolysis (Gabriel et al., 1983, Robert et al., 1993, Carr et al., 1996, Zangari

et al., 2003). In addition, the paraprotein may act as a procoagulant autoantibody: antiphospholipid (including lupus anticoagulant), antithrombin, and antiprotien S antibodies have been detected in myeloma patients (Yasin et al., 1999, Thiagarajan et al., 2000, Zangari et al., 2003). Other proposed mechanisms of hypercoagulablity in MM include the cytokine-mediated upregulation of vWF, FVIII, fibrinogen, and PAI-1, and downregulation of thrombomodulin, endothelial cell protein C receptor, activated protein C, and protein S, resulting in an increased activation of coagulation, dysfunction of endogenous anticoagulation, and impaired fibrinolysis (Amrani, 1990, Minnema et al., 2003, Yagci et al., 2003, Elice et al., 2006, Auwerda et al., 2007, van Marion et al., 2008). Furthermore, a higher incidence of acquired activated protein C resistance has been observed in MM patients (Zangari et al., 2002a, Sarig et al., 2005, Elice et al., 2006).

Intriguingly, the increased risk of VTE associated with IMiD appears to be a synergistic effect when Thal and/or Len are given in combination with other drugs, and not as a single-agent therapy for which the incidence remains below 5% in both newly diagnosed and relapsed/refractory patients (shown in Tables 1.6 and 1.7, respectively). In particular, Thal or Len with concurrent Dex has been evidently shown to increase the risk of VTE. For example, in a randomized controlled trial that compared the use of Thal in combination with Dex to Dex alone in newly diagnosed MM patients, a DVT incidence of 17% was reported in the Thal/Dex arm, as compared to 3% in the control arm when similar doses of Dex and no anticoagulants were mandated by the protocol (Rajkumar et al., 2006). Moreover, an extremely high rate of thrombosis was initially observed in one trial comparing Dex alone versus Dex plus Len in newly diagnosed patients; 9 out of the first 12 patients (75%) enrolled in the Len/Dex arm experienced thromboembolic events (including one ischemic stroke) in the absence of thromboprophylaxis, while no events were observed in the control arm (n=9) (Zonder et al., 2006). Subsequently, Zonder et al. prescribed a prophylactic dose of aspirin (325 mg daily) to patients recruited in this study thereafter, which reduced the rate of thrombosis to 15%.

Table 1.6: Reported incidence rates of VTE in newly diagnosed myeloma patients using different treatment regimens, with and without routine thromboprophylaxis.

Study	Patient no.	Regimen	Thromboprophylaxis	VTE (%)
Single agent				
(Rajkumar et al., 2006)	102	Dex	No	3
Multi-agent chemotherapy				
(Barlogie et al., 2006)	337	DCEP/CAD/DPACE	No	17
(Cavo et al., 2005)	100	VAD	No	2
(Minnema et al., 2004)	201	VAD	No	5
(Palumbo et al., 2006)	126	Melphalan + prednisone	No	2
(Palumbo et al., 2010a)	253	VMP	No	2
(Zangari et al., 2001)	50	VAD/DCEP/CAD	No	4
(Zangari et al., 2004)	196	VAD/DCEP/CAD	No	14
IMiD single agent				
(Rajkumar et al., 2003)	31	Thal	No	3
IMiD + Dex				
(Cavo et al., 2005)	19	Thal + Dex	No	26
(Cavo et al., 2005)	81	Thal + Dex	Low-dose warfarin	12
(Rajkumar et al., 2002)	50	Thal + Dex	LMWH	12
(Rajkumar et al., 2005)	34	Len + Dex	Aspirin	3
(Rajkumar et al., 2006)	102	Thal + Dex	No	17
(Rajkumar et al., 2008)	234	Thal + Dex	No	18
(Wang et al., 2005)	26	Thal + Dex	Warfarin/LMWH	8
(Weber et al., 2003)	40	Thal + Dex	Warfarin	15
IMiD-based chemotherapy				
(Kumar et al., 2012)	48	VRDC	Aspirin/warfarin/LMWH	2
(Ludwig et al., 2013)	98	VTD/VTDC	Aspirin/LMWH	2
(Palumbo et al., 2006)	65	Thal, melphalan + prednisone	No	17
(Palumbo et al., 2006)	64	Thal, melphalan + prednisone	LMWH	3
(Palumbo et al., 2010a)	250	VMPT-VT	Aspirin/warfarin/LMWH	5
IMiD-based chemotherapy with Dox				
(Barlogie et al., 2006)	162	Thal + DCEP/CAD/DPACE	No	34
(Barlogie et al., 2006)	152	Thal + DCEP/CAD/DPACE	LMWH	24
(Baz et al., 2014)	57	RDD	Aspirin/warfarin/LMWH	9
(Jakubowiak et al., 2011)	72	VRDD	Aspirin/warfarin/LMWH	6
(Minnema et al., 2004)	211	TDD	LMWH	9
(Zangari et al., 2001)	50	Thal + VAD/DCEP/CAD	No	28
(Zangari et al., 2004)	87	Thal + VAD/DCEP/CAD	No	35
(Zangari et al., 2004)	35	Thal + VAD/DCEP/CAD	Low-dose warfarin	31
(Zangari et al., 2004)	68	Thal + VAD/DCEP/CAD	LMWH	15

Abbreviations: CAD, cyclophosphamide, doxorubicin, and dexamethsone; DCEP; dexamethasone, cyclophosphamide, etoposide, and cisplatin; Dex, dexamethasone; DPACE, dexamethasone, cisplatin, doxorubicin, cyclophosphamide, and etoposide; RDD, lenalidomide, dexamethasone, and liposomal doxorubicin; TDD, thalidomide, dexamethasone, and doxorubicin; VAD, vincristine, doxorubicin, and dexamethasone; VMP, bortezomib, melphalan, and prednisone; VMPT-VT, bortezomib, melphalan, prednisone, and thalidomide followed by maintenance therapy with bortezomib and thalidomide; VRDD, bortezomib, lenalidomide, dexamethasone, and liposomal doxorubicin; VTD, bortezomib, thalidomide, and dexamethasone; VTDC, bortezomib, thalidomide, dexamethasone, and cyclophosphamide.

Table 1.7: Reported incidence rates of VTE in relapsed/refractory myeloma patients using different treatment regimens, with and without routine thromboprophylaxis.

Study	Patient no.	Regimen	Thromboprophylaxis	VTE (%)
Single agent				
(Dimopoulos et al., 2007)	175	Dex	No	5
(Jagannath et al., 2005)	256	Bortezomib	No	<1
IMiD single agent				
(Barlogie et al., 2001)	169	Thal	No	2
(Offidani et al., 2004)	23	Thal	No	4
(Richardson et al., 2002)	27	Len	No	0
IMiD + Dex				
(Dimopoulos et al., 2007)	176	Len + Dex	No	11
(Klein et al., 2009)	45	Len + Dex	No	2
(Weber et al., 2007)	353	Len + Dex	No	15
IMiD-based chemotherapy				
(Morgan et al., 2007)	21	RCD	No	14
(Offidani et al., 2004)	27	Thal + melphalan	No	11
(Palumbo et al., 2010b)	44	RMPT	Aspirin	0
(Richardson et al., 2014)	64	VRD	Aspirin/warfarin/LMWH	3
(Zangari et al., 2002b)	40	Thal + DCEP	No	3
IMiD-based chemotherapy with Dox				
(Zangari et al., 2002b)	192	Thal + DPACE	No	16

Abbreviations: DCEP; dexamethasone, cyclophosphamide, etoposide, and cisplatin; Dex, dexamethasone; DPACE, dexamethasone, cisplatin, doxorubicin, cyclophosphamide, and etoposide; RCD, lenalidomide, cyclophosphamide, and dexamethasone; RMPT, lenalidomide, melphalan, prednisone, and thalidomide; VRD, bortezomib, lenalidomide, and dexamethasone.

In addition, the dose of Dex given in combination with Len has been shown to further potentiate the risk of VTE; the incidence of VTE in newly diagnosed MM patients randomized to Len plus high-dose Dex was 26% (and was also associated with increased early mortality), compared to only 12% in those patients treated with Len plus low-dose Dex (Rajkumar et al., 2010). Similarly, a retrospective analysis of patients with relapsed or refractory MM revealed that a Dex dose reduction lowered VTE incidence from 11.6% to 7.1% (Harousseau et al., 2010). Dex induces apoptosis in MM cells through glucocorticoid response element transactivation (Sharma and Lichtenstein, 2008). Early studies demonstrated increased LPS-induced TF expression by Dex on monocytes through increasing the stability of TF mRNA (Bottles and Morrissey, 1993, Reddy et al., 2004), and more recently Dex treatment of monocytes and endothelial cells *in vitro* increased TF expression, which may induce PCA (Hoshi et al., 2011, Isozumi et al., 2013). Furthermore, Kerachian et al. showed that high-dose Dex also enhanced the expression of TF, as well as cellular adhesion molecules (CD54, CD106, and E-selectin) and vWF, while downregulating

thrombomodulin on endothelial cells (Kerachian et al., 2009). Additionally, enhanced plasma antigen levels of PAI-1 and decreased levels of tPA were observed in rats after Dex treatment, resulting in reduced fibrinolytic activity (van Giezen and Jansen, 1992, van Giezen et al., 1994). It has been proposed by Jilma et al. that high-dose Dex may contribute to adverse vascular events by increasing platelet activation (through the elevation of soluble P-selectin) and vWF-dependent thrombosis (Jilma et al., 2005).

Among the different chemotherapy agents given in combination with IMiD, such as alkylating agents or anthracyclines, Dox (either liposomal or not) was shown to carry the highest risk of VTE (see Table 1.6 and 1.7). Following the introduction of prophylaxis with LMWH in newly diagnosed MM patients receiving Thal plus multi-agent chemotherapy containing Dox, there was a significant reduction of VTE incidence from 35% to 15% and 34% to 24%, reported by Zangari et al. and Barlogie et al. respectively, although the risk still remains unacceptably high (Zangari et al., 2004, Barlogie et al., 2006). In all of these studies the major risk of thrombosis is generally within the first three months after the initiation of combination therapy with IMiD, when the tumour load is maximal. Thus, this complication may possibly be related to the release of thrombogenic factors from apoptotic myeloma cells or a direct action of Thal on endothelial cells previously damaged by chemotherapy, rather than to cumulative drug exposure. Kaushal et al. demonstrated that the addition of Thal to intact human coronary artery endothelial cells *in vitro* did not cause any appreciable change, whereas Thal added to Dox-injured endothelial cells resulted in endothelial dysfunction by altering the expression of the thrombin receptor PAR-1 in the injured endothelium, which may explain hypercoagulability in MM patients treated with chemotherapy followed by Thal (Kaushal et al., 2004). Furthermore, modifications in the levels of markers of endothelium activation such as vWF (Baz et al., 2005, van Marion et al., 2008) and thrombomodulin (Corso et al., 2004, Aue et al., 2011) in patients receiving chemotherapy with IMiD indicate endothelial dysfunction.

Additionally, some studies have found evidence of platelet aggregation (Baz et al., 2005) and activation (Dunkley and Gaudry, 2007, Abdullah et al., 2013) caused by Thal, which is also abrogated by aspirin (Dunkley and Gaudry, 2007). While, Len has been shown to upregulate cathepsin G a potent platelet aggregation agonist *in vitro* and in patients treated with Len-based regimens (Pal et al., 2010). Furthermore, there is some *in vitro*

evidence that suggest IMiD may enhance TF expression in host cells, specifically endothelial cells and/or monocytes (Hoshi et al., 2011, Valsami et al., 2011, Isozumi et al., 2013, Kornberg et al., 2013). Prothrombotic coagulation abnormalities including increases in FVIII and fibrinogen have been observed in MM patients during Thal-based induction therapy, although these were not deemed to be associated with the development of VTE (van Marion et al., 2008). Interestingly, genetic risk factors that may predispose for VTE in MM patients receiving combination therapy with IMiD have been identified. For example, in a study of 1966 MM patients treated with Thal-based regimens several single-nucleotide polymorphisms in genes associated with inflammatory response, DNA damage repair, and endothelial activation correlated with an increased risk of VTE (Johnson et al., 2008), and more recently, genetic variants of genes that are involved directly or indirectly in inflammatory response were shown to be linked with increased VTE in 200 MM patients treated with Len-based regimens (Bagratuni et al., 2013).

In contrast to IMiD, the novel agent Bor alone or in combination with Dex and/or chemotherapy is associated with a low VTE risk in MM patients. Intriguingly, the use of Bor potentially has a protective effect in combination with regimens including IMiD against elevated VTE risk (Shen et al., 2011, Zangari et al., 2011), and although the mechanism is not well understood, some authors have suggested that Bor may inhibit platelet aggregation (Avcu et al., 2008, Shen et al., 2011). While others have recently indicated that Bor induces Kruppel-like factor 2 (KLF2) mRNA and decreases TF mRNA in endothelial and haematopoietic cells, and in an experimental model, KLF2 deletion is associated with a prothrombotic phenotype, whereas KLF2 overexpression prolongs time to carotid artery thrombosis (Nayak et al., 2014). However, further research is needed to explore the mechanisms for the treatment-related effects on the risk of VTE with all of these novel agents, and understanding the impact of treatment on the thrombotic risk of these patients is of vital importance to allow a more superior strategy for their thromboprophylaxis.

1.8.4 Thromboprophylaxis in MM patients receiving IMiD

A number of different thromboprophylatic strategies have been utilized in myeloma patients receiving combination therapy with IMiD including, aspirin, LMWH, or warfarin. A randomized controlled trial on optimal thromboprophylaxis in low-risk newly diagnosed

MM patients treated with Thal-based regimens randomized patients to LMWH (enoxaparin), aspirin, or warfarin, and the reported rates of VTE after follow-up were 5.0%, 6.4%, and 8.2%, respectively (Palumbo et al., 2011). The authors concluded that LMWH, aspirin, and warfarin are likely to be similarly effective prophylactic regimens, except in elderly patients, in whom warfarin showed less efficacy than LMWH. In another study, 342 low-risk of VTE MM patients were treated with Len-based therapy and randomized to either low-dose aspirin or LMWH (enoxaparin); the reported rates of VTE after follow-up were 2.3% and 1.2%, respectively (Larocca et al., 2012). Given its relative efficacy, the authors suggested that aspirin could be an effective alternative to LMWH as prophylaxis in this low-risk setting. Nonetheless, which agent is optimal for preventing VTE in MM patients with more than one risk factor for VTE requires further investigation.

Routine thomboprophylaxis is recommended by the IMWG, ESMO, and ASCO for patients with MM who are receiving combination therapy with IMiD, and as with other areas of thromboprophylaxis, a risk stratified approach is appropriate (Palumbo et al., 2008, Mandala et al., 2011, Lyman et al., 2013). In MM patients receiving Thal- or Len-containing therapy, aspirin (75 to 325 mg) may be considered as VTE prophylaxis in low risk patients only (i.e. without risk factor present), unless contraindicated (Snowden et al., 2011). Moreover, patients receiving Thal or Len in addition to combination chemotherapy/anthracyclines/high-dose corticosteroids, or those with two or more myeloma-related risk factors (e.g. disease burden, hyperviscosity) and individual risk factors (e.g. previous VTE, obesity, co-morbidities) should be offered prophylaxis with LMWH (high risk prophylactic dose) or dose-adjusted therapeutic warfarin (Snowden et al., 2011).

1.9 Aims and Objectives

VTE events are frequently encountered in patients with cancer, which pose a significant health burden due to the associated high morbidity and mortality rates. However, the precise pathophysiological mechanisms underlying the development of VTE in cancer patients remain unclear. TF, the primary cellular initiator of the coagulation cascade, is over-expressed in many malignancies and the number of circulating TF+ MP are increased in cancer patients (Geddings and Mackman, 2013). The potential utility of TF and especially TF+ MP in indentifying patients with cancer who are at high risk of thrombosis is

currently under intense investigation (Geddings and Mackman, 2013). In addition, the risk of VTE is found to be further amplified by the cytotoxic treatment of malignant disease, and this too is thought to be mediated by an increase in TF activity (Falanga and Marchetti, 2012a). It is hypothesised that anticancer agents, in eliciting cancer cell apoptosis, result in an increase in the release of tumour cell-derived TF+ MP (Date et al., 2013), despite little supporting evidence. However, treatment-related thrombosis is also reported in MM, a haematological malignancy in which the neoplastic plasma cells do not express TF (Cesarman-Maus et al., 2012). Thus, it is hypothesised that in this clinical situation the thrombogenicity is elicited through a host response to treatment, which may include an upregulation of vascular cell TF expression or activity, endothelial dysfunction, platelet activation, and/or release of procoagulant MP with exposed PS. In order to develop an efficient strategy for the prevention of VTE in cancer patients receiving chemotherapy or improve those that are already in place for MM patients receiving IMiD in combination with chemotherapy agents and/or Dex, it is important to understand exactly how these anti-cancer agents elicit this prothrombotic effect.

Thus, the aim of this thesis was to investigate the specific roles of the TF antigen and MP in the thrombogenicity associated with cancer and its treatment, with the intent to elucidate the underlying mechanisms. Initial work focussed on determining the *in vitro* procoagulant potential of a wide range of solid and haematological tumour cells and tumour-derived MP by characterizing their expression of TF and measuring their fibrin clot formation time in human plasma. The *in vitro* work was further extended to investigate the effect of the cytotoxic chemotherapy agent Dox on tumour cell viability, PS and TF expression, and ultimately the PCA of tumour cells and tumour-derived MP in selected ovarian, breast, and haematological cancer cell lines. Furthermore, the clinical project aimed to evaluate and compare the number of TF+ MP determined using flow cytometry in untreated pancreatic cancer patients (whose high incidence of VTE has been associated with TF), MPD patients (that represent a group of haematological neoplasms with a known propensity to develop thromboses irrespective of treatment), and MM patients prior to the administration of treatment (whose risk of thrombosis is particularly elevated with Thal- or Len-based therapies). Finally, the main aims of the clinical study were to determine the PCA (by measuring fibrin clot formation time) and the number of various subtypes of MP (including, EMP, PMP, monocyte-derived MP, plasma cell-derived MP, TF+ MP, and PS+

MP) determined using flow cytometry before, during, and after treatment with Thal- or Len-based therapies in MM patients.

Chapter 2: Materials and methods

2.1 Cell culture

2.1.1 Maintenance of cancer cell lines

All cell medium preparation, tissue culture, and cell maintenance was conducted in a class II sterile laminar flow cabinet under aseptic working conditions to avoid contamination and infection. All equipment, including the tissue culture hood, water bath, and incubator was cleaned methodically at regular intervals with Virkon disinfectant (Scientific Laboratory Supplies Limited, Hessle, UK) and 70% alcohol, and all equipment was sprayed thoroughly with 70% alcohol before placing in the tissue culture hood. Furthermore, all cell lines have been regularly tested for mycoplasma contamination (MycoProbe Assay, R&D Systems, Abingdon, UK).

Cell cultures were grown in 75cm^2 tissue culture flasks (Sarstedt, Leicestershire, UK) and stored in a humidified incubator at 37°C under an atmosphere of 5% CO_2 (except MDA-MB-231, atmosphere of 100% air). All cells were subcultured in the log phase when 70-80% confluent to maintain the optimal density for continued growth and the complete cell growth medium was refreshed three times per week, pre-warmed at 37°C in a water bath for 30 min prior to use. Cell viability was controlled via the trypan blue exclusion test, described in section 2.2.

The majority of adherent cells were physically detached from the surface of a flask using a cell scraper (Sarstedt) and were pelleted by centrifugation at 300 x *g* for 5 min. The supernatant was discarded and cells were resuspended in an appropriate volume of fresh growth medium and transferred to a new flask. Conversely, trypsinisation was used to release ES2 and MDA-MB-231 cells from the surface of a flask as cell scraping negatively influenced the viability of these two cell lines. These cells were washed with phosphate buffered saline (PBS) and subsequently incubated with 3-4 ml trypsin ethylenediaminetetraacetic acid (EDTA; PAA, Yeovil, UK) for 5-10 min at 37°C to allow cell dissociation. Cells were recovered in an excess of complete growth medium to neutralise the trypsin reaction, and pelleted by centrifugation at 300 x *g* for 5 min. Cells were

resuspended in an appropriate volume of fresh growth medium and transferred to a new flask.

Suspension cells were maintained by withdrawing a portion of the cells from the culture flask and diluting the remaining cells with fresh medium to the recommended seeding density for individual cell lines.

2.1.2 Cryopreservation

Cells at a low passage number were harvested in the log phase of growth and brought into suspension for adherent cells as described previously. A small aliquot of cells was removed (10 µl) and a cell count performed (see section 2.2) to ensure cell viability in excess of 90% for good recovery after freezing. The cell cultures were centrifuged at 300 x *g* for 5 min and resuspended in freezing medium (90% fetal bovine serum [FBS; BioSera, Ringmer, UK], 10% dimethyl sulfoxide [DMSO; Sigma-Aldrich, Poole, UK]) at a high cell concentration (2-4 x 10^6 cells/ml of freezing media). The cryoprotective agent DMSO prevents ice crystal formation during freezing and lowers solute concentration, thus reducing ionic stress. The cells were aliquoted into 1 ml cryovials and frozen slowly in a -80°C freezer by reducing the temperature at approximately 1°C per min in a cryo-freezing container ("Mr. Frosty" Nalgene®, from Sigma-Aldrich) filled with isopropyl alcohol. The frozen cryovials were transferred to a liquid nitrogen storage vessel the following day.

Cryopreserved cells were recovered by rapid thawing in a 37°C water bath for 1-2 min with constant agitation. Once thawed, all cryovials were sprayed with 70% ethanol and wiped before transferring to the tissue culture hood. The cells were slowly diluted in a 10-fold volume of pre-warmed cell medium in a 50 ml sterile tube and spun at 300 x *g* for 5 min to pellet the cells, thus allowing separation from the freezing media as DMSO is toxic to cells above 4°C. The supernatant was discarded and cells were appropriately diluted in their respective culture medium and transferred to tissue culture flasks.

2.1.3 Pancreatic cancer cell lines

AsPC-1, CFPAC-1, and MIA-PaCa-2 were purchased from the American Type Culture Collection (ATCC). AsPC-1 is a metastatic pancreatic adenocarcinoma derived from ascites

of a 62 year old female (Chen et al., 1982), and CFPAC-1 is a metastatic ductal pancreatic adenocarinoma obtained from the liver of a 26 year old male with cystic fibrosis (Goldman-Leikin et al., 1989), whereas MIA-PaCa-2 is derived from tumour tissue of the pancreas taken from a 65 year old male (Yunis et al., 1977). AsPC-1 was maintained in RPMI 1640 medium (PAA) supplemented with 10% (v/v) FBS, 1% penicillin/streptomycin (10,000 units/ml), 1% L-glutamine, 1% HEPES buffer, and 1% sodium pyruvate (all PAA). CFPAC-1 and MIA-PaCa-2 cells were maintained in Iscove's Modified Dulbecco's Medium (ATCC), supplemented with 1% penicillin/streptomycin, 1% $_{L}$-glutamine, 10% FBS, and 2.5% (v/v) horse serum from Gibco® (Life Technologies Ltd, Paisley, UK**;** for MIA-PaCa-2 cells only).

2.1.4 Ovarian cancer cell lines

A2780 and SKOV-3 were purchased from the European Collection of Cell Cultures (ECACC), while ES2 was purchased from ATCC. The A2780 human ovarian cancer cell line was established from the tumour tissue of an untreated patient (Behrens et al., 1987). Whereas, SKOV-3 is a metastatic ovarian adenocarcinoma taken from the ascites of a 64 year old female (Fogh et al., 1977), and ES2 was established from the tumour tissue of a 47 year old female with poorly differentiated ovarian clear cell carcinoma (Lau et al., 1991). A2780 cells were maintained in RPMI 1640 medium supplemented with 1% penicillin/streptomycin, 1% $_{L}$-glutamine, and 10% (v/v) FBS. ES2 and SKOV-3 were maintained in McCoy's 5A medium (PAA) supplemented with, 1% $_{L}$-glutamine, 1% penicillin/streptomycin, and 10% (v/v) FBS for ES2 and 15% (v/v) FBS for SKOV-3.

2.1.5 Breast cancer cell lines

MCF-7, MDA-MB-231, and T47D breast cancer cell lines were purchased from ATCC. MCF-7 and MDA-MB-231 are adenocarcinomas of the breast established from malignant cells of pleural effusions from a 69 year old female and 51 year old female, respectively (Soule et al., 1973, Cailleau et al., 1974). Whereas, T47D is established from the pleural effusion of a ductal carcinoma of the breast of a 54 year old female (Keydar et al., 1979). MCF-7 and T47D cells were maintained in RPMI 1640 medium supplemented with 1% penicillin/streptomycin, 1% $_{L}$-glutamine, and 10% (v/v) FBS. MDA-MB-231 cells were

maintained in Leibovitz's L-15 medium (PAA) supplemented with 1% penicillin/streptomycin, 1% $_{L}$-glutamine, and 10% (v/v) FBS.

2.1.6 Colorectal cancer cell lines

CaCo-2, Colo320, and LoVo were purchased from ATCC. Colo320 is a moderately undifferentiated human colon adenocarcinoma established from the tumour mass of a 55 year old female (Quinn et al., 1979), and CaCo-2 was established from a primary colonic tumour in a 72 year old male (Fogh et al., 1977). While, LoVo cells were derived from a metastatic tumour in the left supraclavicular region of a 56 year old male with adenocarcinoma of the colon (Drewinko et al., 1978). Colo320 and LoVo cells were maintained in RPMI 1640 medium supplemented with 1% penicillin/streptomycin, 1% $_{L}$-glutamine, and 10% (v/v) FBS. CaCo-2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; PAA) supplemented with 1% penicillin/streptomycin, 1% $_{L}$-glutamine, and 10% (v/v) FBS.

2.1.7 Haematological cell lines

U937 and MM.1S were obtained from ATCC, while H929, JJN3, and U266 were a kind gift from Dr Guy Pratt (School of Cancer Sciences, University of Birmingham, Birmingham, UK). U937 was derived from malignant cells of a pleural effusion of a 37 year old male with diffuse histiocytic lymphoma and displays many monocytic characteristics (Sundstrom and Nilsson, 1976). H929 was established from a malignant pleural effusion in a patient with myeloma; the myeloma cells produce IgA kappa light chains (Gazdar et al., 1986). JJN3 is a subclone of the parental cell line JJN1 that was established from the bone marrow of a 57 year old woman with plasma cell leukaemia; the myeloma cells produce IgA kappa light chains (Jackson et al., 1989). MM.1S was established from the parent cell line, MM.1, taken from peripheral blood of a 42 year old female MM patient who was sensitive to steroid-based therapy; the myeloma cells produce IgA lambda light chains (Goldman-Leikin et al., 1989). U266 was established from the peripheral blood of an IgE producing myeloma patient (Nilsson et al., 1970). All cell lines were maintained in ATCC-formulated RPMI 1640 medium (1% $_{L}$-glutamine and 1% HEPES buffer and 1% sodium pyruvate) supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin.

2.2 Cell count and trypan blue exclusion test

Cell suspensions of both adherent and non-adherent cells (10 µl) were transferred into 0.5 ml polypropylene tubes and equal amounts of 0.4% trypan blue (Sigma-Aldrich) was added to ascertain the cell viability since the dye cannot permeate the cell membrane of viable cells, whereas dead cells with damaged membranes stain blue. The cells and dye solution were gently mixed to attain a homogeneous cell suspension and applied to the counting chamber on the Hawksley haemocytometer (improved Neubauer, depth 0.1mm 1/400mm^2) with the area under the cover slip filling by capillary action. The viable and dead cells were visualised under a 10X objective of a phase contrast microscope and counted in the large central gridded square (1 mm^2) (see Figure 2.1) to determine the percentage viability of the sample. Dilution and volume corrections were applied to the count, which was expressed as cells/ml. Cells were then diluted to the desired count depending on the experiment.

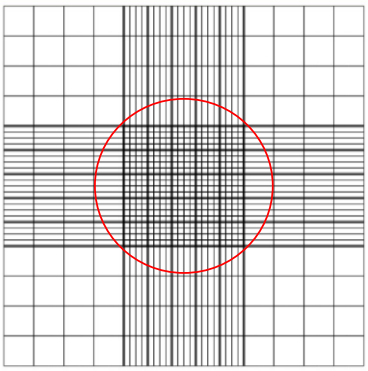


Figure 2.1: Illustration of haemocytometer gridlines used to count cells.
Cells were visualised and counted in the circled gridded square (1 mm^2) of a haemocytometer under the microscope.

2.3 Chemotherapeutic agents

Reconstituted stocks of 10 mM Dox (Sigma-Aldrich) were prepared in ultra pure water (18 megohm-cm; dH_2O), stored in 100 µl aliquots at -20°C and working concentrations were diluted in the appropriate cell medium, once defrosted stored at 4°C for up to 2 weeks. Dox was used to assess the antiproliferative effect of this cytotoxic chemotherapeutic agent with various cancer cell lines (described in section 2.4), and also the cell surface TF expression (section 2.5.1), the percentage of apoptotic cells (section 2.5.2), and the CT of

cells and cell-free supernatants (section 2.6.1), following 48 h incubation, was simultaneously determined using the same untreated and Dox-treated tumour cells.

Furthermore, 10 mM reconstituted stocks of Len (Cambridge Bioscience Ltd., Cambridge, UK) and pomalidomide (Pom; Sigma-Aldrich) were prepared in DMSO, stored in 100 µl aliquots at -20°C and working concentrations were diluted in the appropriate cell medium. Any thawed surplus was stored at 4°C for up to 2 weeks. The final concentration of DMSO in cell cultures was ≤0.1%, and all treatments were compared with vehicle controls. Len and Pom were used to determine the antiproliferative effects of these IMiD with various haematological cancer cell lines (described in section 2.4).

2.4 Cell proliferation assay

Cells (2 x 10^4 cells/well except MM cell lines 1 x 10^5 cells/well) were seeded into sterile 96-well flat bottomed tissue culture plates (Sarstedt) with various concentrations of Dox, Len, and Pom (0-100 µmol/l) in media (200 µl). Fresh sterile media was added to the outermost wells to prevent the plate from drying out during incubation. After 24, 48, and 72 h incubation (37°C, 5% CO_2), cell titre aqueous one reagent (Promega, Southampton, UK) was added (20 µl per well) and plates incubated for 1 to 4 h (37°C, 5% CO_2). This assay assesses metabolic activity by the use of a tetrazolium compound (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium), known as MTS, that is reduced by the inherent mitochrondrial dehydrogenase activities of metabolically active cells to form brightly coloured formazan products that are soluble in tissue culture medium and can be measured by absorbance. The amount of formazan dye produced is directly proportional to the number of metabolically active cells that are assumed to be the total viable cell population. Each dilution was performed in quadruplicate and the mean absorbance was determined at 490 nm using a plate reader (BioTek, Bedfordshire, UK).

2.5 Flow cytometry

The method of flow cytometry (described in Figure 2.2) can be used to simultaneously measure multiple parameters on cells or particles in a fluid suspension. Briefly, a loaded

sample is drawn up into the fluidic system of a flow cytometer, and pumped to the flow chamber where samples are hydrodynamically focused into a single file stream. This fluid stream (with the sample at the core) transports cells and particles to the laser beam(s). At the interrogation point, the laser light intersects individual cells or particles (exciting any fluorochromes present) causing light to be scattered in all directions. Emitted scattered light and any fluorescence present are collected by lenses and filters that route the light signals to the appropriate detectors. The electronic system converts the light signals to electrical signals, which are subsequently transformed by an external computer into a digital signal. The acquired data can then be analysed to characterize the cells or particles based on their properties of light scatter. In particular, the forward scattered light (FSC) correlates with single cell or particle relative size, and side scattered light (SSC) with its internal complexity. While the measured fluorescence intensity of cells or particles incubated with fluorochrome conjugated antibodies to specific cell antigens prior to sample acquisition, correlates with the expression level of the antigen of interest on single cells or particles. Subpopulations can be further studied using gates and various different plotting options such as, histograms and dot plots.

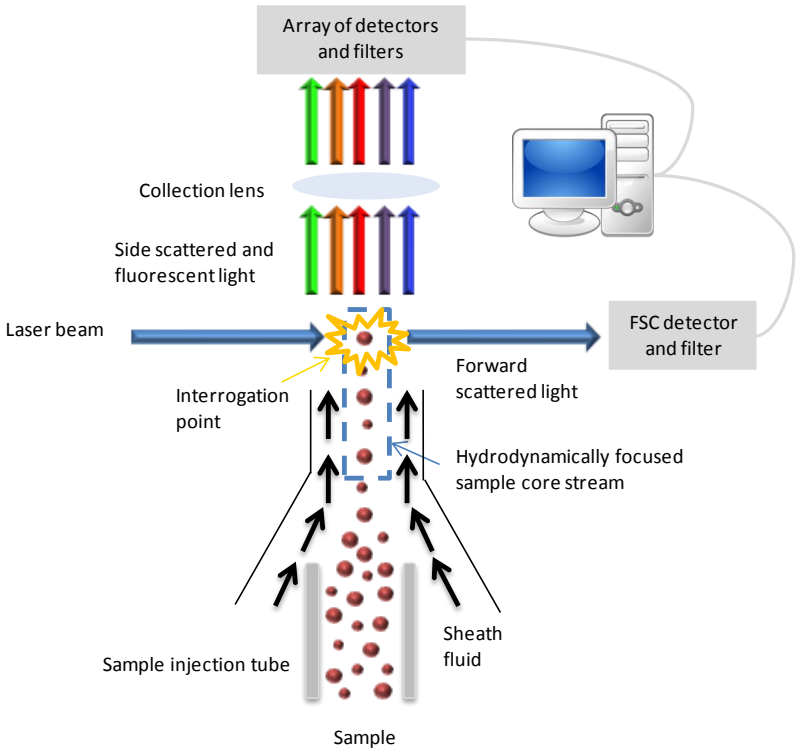


Figure 2.2: Schematic diagram illustrating the basic components of a flow cytometer.

The sample is transported in a single file stream to the laser so that cells or particles individually pass through the interrogation point, resulting in the scattering of light in all directions. Collection lens in the forward and side (90 degrees) position route specific wavelengths to corresponding detectors. The light signals are subsequently converted into an electrical current and the digitized data is visualised on a computer screen.

In this study, all flow cytometry experiments were performed on a Becton Dickinson FACS Calibur instrument (BD Biosciences, Oxford, UK), which is cleaned, serviced and calibrated on a regular basis. Furthermore, the acquired data was analysed on an Apple Macintosh G5 computer, equipped with BD CellQuest Pro v.6.0 software (BD Biosciences).

2.5.1 Flow cytometric evaluation of cell surface TF expression

To study the TF expression levels of all cancer cell lines examined in this study, harvested cancer cell suspensions were pelleted at 300 x *g* for 5 min and the supernatant discarded. The cells were then washed (300 x *g* for 5 min) and subsequently resuspended in PBS (1 x 10^6 cells/ml). Furthermore, the TF expression level was examined in haematological (MM.1S, U937), ovarian (ES2), and breast (MDA-MB-231, T47D) cancer cells that had been untreated and pre-treated with various concentrations of Dox (0-1 µmol/l) in duplicate 25

cm^2 cell culture flasks (1 x 10^5 cells/ml) for 48 h. These cells were washed twice in PBS at 300 x *g* for 5 min and subsequently resuspended in PBS (1 x 10^6 cells/ml).

All untreated and Dox-treated cancer cell suspensions (50 µl) were transferred to 5 ml polypropylene tubes and 5 µl of a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal IgG1 isotype control antibody (AbDSerotec, Kidlington, UK) and 5 µl of a FITC-conjugated mouse monoclonal anti-human CD142/TF antibody (1 mg/ml; AbDSerotec) were added simultaneously to respective tubes. These tubes were then incubated in the dark at room temperature for 30 min. During this incubation period, the fluorochrome conjugated antibodies bind directly to their complementary epitopes on the cell surface antigen of interest. Thus, the fluorochrome allows the indirect visualisation of the antigen of interest on the flow cytometer as the amount of fluorescent signal detected is proportional to the number of fluorochrome molecules conjugated to anti-TF on each cell, and in turn, this relates to the level of TF antigen expressed on the surface of individual cells within the acquired sample. Following incubation, the cells were washed and pelleted at 300 x *g* for 5 min, removing any unbound antibody. Cells were resuspended in 350 µl PBS and analysed by flow cytometry.

Each polypropylene tube containing the cell suspension was acquired individually with a minimum of 10,000 events counted. TF expression was determined relative to mean fluorescence intensity (MFI) observed within cells incubated with the isotype and concentration matched negative control antibody, and data are presented as mean fluorescence ratio of TF/negative control (to account for changes in autofluorescence between cell lines).

2.5.2 Flow cytometric evaluation of apoptosis and cell viability

FITC-conjugated annexin V apoptosis detection kit (BD Biosciences) was used to quantitatively establish the percentage of cells within a population that are actively undergoing apoptosis, thus determining cell viability. Haematological (MM.1S, U937), ovarian (ES2), and breast (MDA-MB-231, T47D) cancer cells that had been untreated and pre-treated with various concentrations of Dox (0-1 µmol/l) in duplicate 25 cm^2 cell culture flasks (1 x 10^5 cells/ml) for 48 h were washed twice in PBS at 300 x *g* for 5 min, and 1 x 10^6

cells were resuspended in binding buffer (that contains the optimal concentration of calcium required for annexin V to bind to PS on the cell surface). Cell suspensions (100 µl) were then transferred to 5 ml polypropylene tubes, one tube was left unstained and 5 µl of FITC-conjugated annexin V and/or 5 µl of propidium iodide were added simultaneously to the tubes for each sample, these were then incubated in the dark at room temperature for 15 min. Following the incubation period, 350 µl of binding buffer was added to each tube and the unstained, FITC annexin V stained, propidium iodide stained, and dual stained tubes (annexin V and propidium iodide) were immediately analysed using flow cytometry. The controls, specifically unstained cells, cells stained with FITC annexin V alone (no propidium iodide), and propidium iodide alone (no FITC annexin V), were used to set up flow cytometry compensation and quadrants. A minimum of 10,000 events were counted for each FITC annexin V and propidium iodide dual stained sample.

Cell membrane asymmetry is lost in the early phases of apoptosis and the membrane phospholipid PS is translocated from the inner leaflet of the plasma membrane to the outer leaflet in apoptotic cells. Thereby, cells that stain positive for FITC-conjugated annexin V and negative for propidium iodide are undergoing apoptosis, since annexin V is a calcium-dependent phospholipid-binding protein that has a high affinity for PS, thus identifying cells with exposed PS. Cells with intact membranes exclude the universal viability probe propidium iodide, while the membranes of dead and damaged cells are permeable to propidium iodide. Cells that stain positive for both FITC annexin V and propidium iodide are either in the end stage of apoptosis, or undergoing necrosis, although this assay cannot explicitly differentiate between these two cell death pathways. Cells that stain negative for both FITC annexin V and propidium iodide are viable and not undergoing measurable apoptosis.

2.5.3 Flow cytometric evaluation of MP

For immunophenotyping of MP in blood samples collected from MM patients before, during, and after chemotherapy, and myeloproliferative disorder (MPD) patients (clinical studies will be described in section 5.2), 5 µl of FITC-conjugated mouse monoclonal IgG1 isotype control antibody (AbDSerotec) or 5 µl of FITC-conjugated mouse monoclonal IgG1 anti-TF; anti-CD138; anti-CD42b; anti-CD14; anti-CD54; anti-CD105; anti-CD106 (all

purchased from AbDSerotec); and anti-CD144 (BD Biosciences) antibodies, were added to 25 µl of processed PFP (see section 5.2.2) in separate polypropylene tubes and incubated in the dark at room temperature for 30 min. Following incubation, these tubes were resuspended in 350 µl filtered (0.1 µm) PBS. Furthermore, 5 µl of phycoerythrin (PE)-conjugated annexin V (BD Biosciences) was added to two 25 µl PFP samples in separate 5 ml polypropylene tubes, and were incubated in the dark at room temperature for 15 min. Following incubation, one tube was resuspended in 350 µl filtered (0.1 µm) binding buffer, and the other in 350 µl filtered (0.1 µm) PBS, for PE-labelled annexin V unspecific control.

Subsequently, 25 µl of counting beads with an established concentration close to 1000 beads/µl (Caltag Laboratories, Buckingham, UK) were added to each tube immediately prior to analysis by flow cytometry. A size gate for MP detection was established using Megamix beads (Biocytex, Marseille, France) according to the current International Society of Thrombosis and Haemostasis (ISTH) protocol (Lacroix et al., 2010), and 25,000 events were counted, with positive MP being defined as the difference in labelling between cell-specific FITC-labelled monoclonal antibodies and their respective isotype-matched control antibodies. The absolute number of MP ≥ 0.5 µm was quantified in relation to counting beads according to manufacturer's instructions (and will be described in section 5.6). All samples were treated identically to ensure comparable results, and all analyses of MP were carried out on fresh PFP.

Furthermore, blood samples collected from pancreatic cancer patients prior to surgical resection of the tumour and the cell-free supernatants were analysed for TF+ MP. These cell-free supernatants (containing suspensions of MP) were isolated from the conditioned cell culture media (24 h minimum) of various cancer cell lines by double centrifugation; initially at 300 x *g* for 5 min to remove tumour cells, and then the extracted supernatant was spun again at 1000 x *g* for 5 min in 1.5 ml polypropylene tubes to remove larger cell debris. These isolated supernatant samples were subsequently confirmed cell-free by light microscopy. Specifically, 5 µl of FITC-conjugated mouse monoclonal IgG1 isotype control antibody or 5 µl of FITC-conjugated mouse monoclonal IgG1 anti-TF were added to 25 µl of processed PFP (see section 5.2.2) or 25 µl cell-free supernatants in separate polypropylene tubes and incubated in the dark at room temperature for 30 min. These samples were resuspended in 350 µl filtered (0.1 µm) PBS and 25 µl of counting beads were added to

each tube immediately prior to analysis by flow cytometry. Each sample was acquired and analysed by flow cytometry as described above and further in section 5.6.

2.6 Clotting Time assay

In this study, the CT was determined by a prothrombin time-based assay using a Thrombotrack SOLO coagulometer (Alere, Stockport, UK); a semi-automated, mechanical clotting end point method. Once the timer is initiated a small magnetic field is generated by the rotation of a magnetic sensor causing the steel ball in the fluid plasma sample to move. The formation of a solid fibrin clot pulls the steel ball out of the detection range of the magnetic sensor stopping the timer and this time is recorded as the CT. All samples were performed in triplicate when possible (duplicate at minimum) and the mean ± standard deviation (SD) calculated.

Conventionally, the one-stage prothrombin time assay is based on the time required for a fibrin clot to form after the addition of lipidated TF and 25 mM $CaCl_2$ to decalcified , PPP, to detect inherited or acquired coagulation defects in the extrinsic and common pathway (Figure 2.3), specifically factors VII, X, V, prothrombin and fibrinogen. Modifications were made to the traditional prothrombin time assay (described above) in this study by incubating normal human plasma (source of coagulation factors within physiological ranges) with tumour cells/tumour cell-derived supernatants (potential source of TF and phospholipids) at 37°C for 2 min prior to the addition of 25 mM $CaCl_2$ which initiates clotting (as described in section 2.6.1), thereby allowing the measurement of the tumour cell/tumour cell-supernatent dependent CT. This modified prothrombin time method has been used similarly by other groups (Silberberg et al., 1989, Ogiichi et al., 2000, Hobbs et al., 2007, Berny-Lang et al., 2011, Tormoen et al., 2011, Tormoen et al., 2013, Thaler et al., 2014). Furthermore, the prothrombin time assay for patient PPP sample CT determination was also modified by omitting the addition of a known concentration of lipidated TF (clinically used to speed up the reaction; method described in section 2.6.2), which was thereby only influenced by TF and phospholipids present in the patient sample.

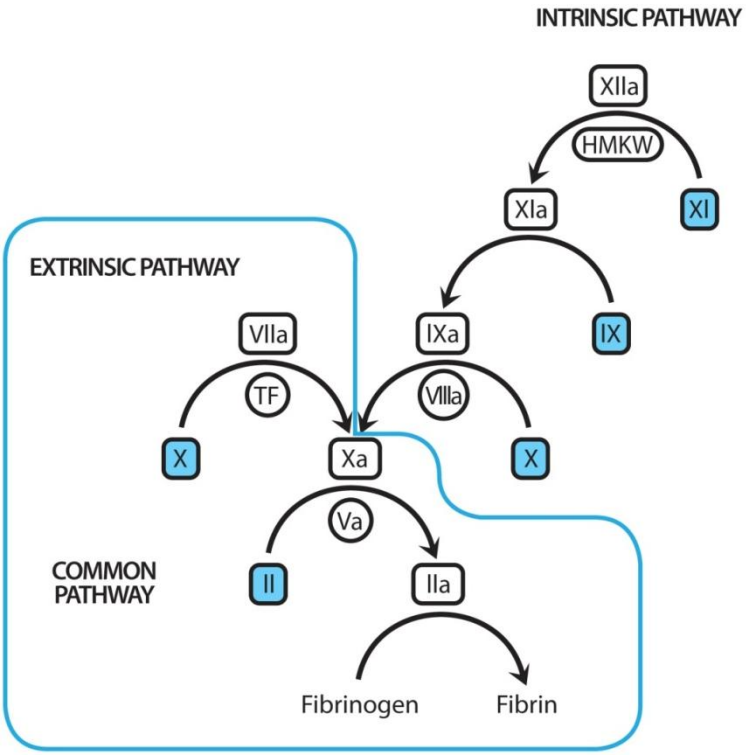


Figure 2.3: Classical cascade model of coagulation.
This model illustrates the redundant, extrinsic and intrinsic pathways of the coagulation system, either of which can generate FXa; TF triggers the extrinsic pathway, activating FVII, and leads to the activation of FX, whereas the intrinsic pathway is triggered by contact activation, acting via FXII, high molecular weight kininogen (HMWK), and prekallikrein, which activate FXI, and in turn, FIX and FVIII, leading to activation of FX. The common pathway results in the generation of FIIa (thrombin) and subsequent cleavage of fibrinogen to fibrin.

2.6.1 Cells and cell-free supernatant CT assessment

Harvested cells from various cancer cell lines were washed in PBS (300 x *g* for 5 min) and quantified as described in section 2.2. For cell count experiments, cells were resuspended in PBS at a concentration of 3 x 10^5 cells/100 µl and subsequent serial dilutions of cell suspensions were prepared using PBS for each cell line prior to CT assessment. Furthermore, cells (1 x 10^5 cells/100 µl PBS) were incubated in the presence and absence of 10 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 neutralize cell surface TF. In addition, several serial dilutions of this anti-TF antibody were simultaneously added to 1 x 10^5 cells/100 µl in separate 1.5 ml polypropylene tubes and also incubated for 30 min at 37°C. All cell suspensions were then washed in 1 ml PBS (300 x *g* for 5 min) and adjusted for cell count (as described in section 2.2) so that 1 x 10^5 cells were resuspended in 100 µl of PBS prior to CT assessment. Furthermore, haematological (MM.1S, U937), ovarian (ES2),

and breast (MDA-MB-231, T47D) cancer cells (5 x 10^5) were seeded into duplicate 25 cm^2 cell culture flasks with various concentrations of Dox (0-1 μmol/l) in the appropriate cell line medium (5 ml). After 24, 48, and 72 h incubation time points, cells were washed twice in PBS at 300 x *g* for 5 min and adjusted for cell count (as described in section 2.2) so that 1 x 10^5 cells were resuspended in 100 μl of PBS (3 x 10^5 cells/100 μl for MM.1S cell line) prior to CT assessment. For determination of the CT, enumerated cell suspensions (100 μl) were incubated with 100 μl normal plasma control (Helena Biosciences, Gateshead, UK) for 2 min at 37°C in the centre cuvette and clotting was initiated by the addition of 25 mM $CaCl_2$ (100 μl).

For experiments involving the dilution of MP, serial dilutions of cell-free supernatants were prepared using fresh cell culture media for each cell line prior to CT assessment. In selected experiments, cell-free supernatants were filtered through 1.2, 0.45, and 0.1 μm syringe filters (Sartorius AG, Göttingen, Germany) to sequentially remove fractions of MP prior to CT assessment. Furthermore, the cell-free supernatant was isolated from haematological (MM.1S, U937), ovarian (ES2), and breast (MDA-MB-231, T47D) cancer cells (5 x 10^5) that had been incubated for 24, 48, and 72 h in duplicate 25 cm^2 cell culture flasks with various concentrations of Dox (0-1 μmol/l) in the appropriate cell line medium (5 ml) prior to CT assessment. For determination of the CT, cell-free supernatants (100 μl) were incubated with 100 μl normal plasma control for 2 min at 37°C in the centre cuvette and clotting was initiated by the addition of 25 mM $CaCl_2$ (100 μl). Samples that did not clot within 10 min were deemed as non-clotting. Normal control plasma, vehicle controls (PBS for cells and media for cell-free supernatants), or Dox alone did not support coagulation.

2.6.2 Clotting time assessment of patient samples

PFP was processed from sodium citrate-anticoagulated whole blood as described in section 5.2.2. For determination of the CT, 100 μl of PFP was incubated for 2 min at 37°C in the centre cuvette and clotting was initiated by the addition of 25 mM $CaCl_2$ (100 μl). Samples that did not clot within 999.9 s were deemed as non-clotting.

2.7 Enzyme-linked immunosorbent assay

2.7.1 Determination of TF concentration in cell-free supernatant

Solutions:
Wash buffer: 0.05% Tween® 20 (Sigma-Aldrich) in PBS, pH 7.4.

Reagent diluent (RD): 1% Bovine serum albumin (BSA; Sigma-Aldrich) dissolved in PBS, pH 7.4, sterilised through a 0.2 µm pore filter and stored in 50 ml aliquots at 4°C for up to one week.

Substrate solution: Colour Reagent A (stabilised H_2O_2) and Colour Reagent B (tetramethylbenzidine [TMB]; both R&D Systems), mixed together in equal volumes at room temperature and protected from light, 15 min prior to use.

Reagent stock solutions:
Capture antibody: 720 µg/ml mouse anti-human TF (R&D Systems) reconstituted stock was prepared in 1 ml PBS, stored in 50 µl aliquots at -80°C up to 6 months.

Detection antibody: 36 µg/ml biotinylated goat anti-human TF (R&D Systems) reconstituted stocks were prepared in 1 ml RD, stored in 50 µl aliquots at -80°C up to 6 months.

Standard: Each 90 ng/ml recombinant human TF (R&D Systems) vial was reconstituted in 500 µl RD, allowed to sit for 15 min with gentle agitation and stored in 50 µl aliquots at -80°C up to 2 months.

The TF antigen concentration of cell-free supernatants isolated from various cancer cell lines was measured using the commercially available TF DuoSet ELISA development kit (R&D systems, UK) and samples were processed according to manufacturer's instructions. Prior to use, all required solutions were freshly prepared, also reagents and samples were brought to room temperature and then diluted to specified working concentrations.

Nunc MaxiSorp® 96-well microplates were coated with a mouse antibody specific to human TF (diluted in PBS; 4 µg/ml, 100 µl per well) overnight and subsequently washed three times with wash buffer (400 µl per well) to remove any unbound antibody. All unbound sites were then blocked by applying 300 µl of RD per well to prevent non-specific binding and thus false positive results. 100 µl of cell-free supernatants derived from various cancer cell lines (undiluted) and human recombinant TF standards (high standard of 500 pg/ml serially diluted 2-fold in RD to construct a seven point standard curve) were added per well, plates were covered and incubated for 2 h at room temperature. Afterwards, any unbound material was washed away as previously described. Biotinylated

goat anti-human TF (detection antibody) diluted in RD (200 ng/ml, 100 µl) was loaded into each well and the plates were further incubated for 2 h at room temperature and then washed three times.

Streptavidin-Horseradish Peroxidase (HRP; 100 µl per well) was applied to each plate and incubated for 20 min at room temperature in the dark to allow the HRP to bind to the detection antibody in the sandwich immunoassay and then washed as aforementioned. TMB substrate solution (100 µl) was dispensed to the wells and plates were incubated for 20 min at room temperature in the dark whilst a blue colour developed in proportion to the amount of TF present in the sample. 50 µl of 1 M H_2SO_4 was added per well to stop the action of HRP on TMB substrate solution, turning the colour in the wells to yellow. Each sample or standard was performed in duplicate and the optical density of each well was immediately measured using a microplate reader set at 450 nm with a 540 nm wavelength correction.

2.7.2 Determination of soluble cell adhesion molecules in serum

Serum was processed from whole blood as will be described in section 5.2.2. The soluble CD54 (sCD54) and soluble CD106 (sCD106) antigen concentrations in serum samples from all MM patients taken before, during, and after chemotherapy was measured using the commercially available human sCD54 and sCD106 Platinum ELISA kits (eBioscience, Hatfield, UK) and samples were processed according to manufacturer's instructions. Prior to use, all required solutions were freshly prepared, also reagents and samples were brought to room temperature and then diluted to specified working concentrations.

Wash buffer (20 ml of PBS with 1% Tween®) was diluted in dH_2O to prepare a total volume of 1000 ml, and assay buffer (5 ml of PBS with 1% Tween® and 10% BSA) was diluted in dH_2O to prepare a total volume of 100 ml. Monoclonal antibody against human sCD54 conjugated to HRP and the conjugate mixture containing biotin-conjugate anti-human sCD106 monoclonal antibody mixed with HRP were both individually diluted 1:100 with assay buffer prior to use.

sCD54 ELISA: The microwell plate (coated with monoclonal antibody to human sCD54) provided by the manufacturer was washed twice with wash buffer (400 µl per well) prior

to use. 100 µl of serially diluted human sCD54 standards (ranging from 100 to 3.1 ng/ml) were added to the standard wells and assay buffer served as a blank. Serum samples were diluted 1:10 with assay buffer and 100 µl added to sample wells, followed by the addition of 50 µl of HRP-conjugated anti-human sCD54 monoclocal antibody to all wells. Plates were covered and incubated for 1 h at room temperature on a microplate shaker and then washed three times. TMB substrate solution (100 µl) was dispensed to all wells and the plate was incubated for 10 min at room temperature in the dark whilst a blue colour developed in proportion to the amount of sCD54 present in the sample. Subsequently, 100 µl of 1 M H_3PO_4 stop solution was added per well, turning the colour in the wells to yellow. Each sample or standard was performed in duplicate and the optical density of each well was immediately measured using a microplate reader set at 450 nm.

sCD106 ELISA: the methodology was similar to above except serum samples were diluted 1:50 with assay buffer and a conjugate mixture containing biotin-conjugated anti-human sCD106 monoclonal antibody mixed with HRP was used, which was incubated for 2 h before washing.

2.8 Statistical analysis

Data are expressed as mean (± SD) or median and interquartile range (IQR) according to the normality of distribution. The coefficient of variation (C of V) was used as an alternative to SD in some cases for quantitative variables with normal distribution, which represents the ratio of the SD to the mean, thereby showing the extent of variability in relation to mean of the population. Quantitative variables with non-normal distribution were transformed using the logarithm function (to either the natural base e or common base of 10) to achieve normal distribution before correlation analysis. Pearson's correlation coefficient (*r*) was used to describe correlations and the associated two-tailed test was used for statistical significance. P values < 0.05 were considered to be statistically significant. All statistical analyses were performed with SPSS computer software, version 20.0 for Windows (IBM Corp., Armonk, New York, USA).

Chapter 3: Tissue factor and the procoagulant potential of solid and haematological malignant cells and tumour cell-derived MP

3.1 Introduction

VTE is one of the most common complications reported in cancer patients and significantly contributes to morbidity and mortality. Even in the absence of overt coagulation disorders, cancer patients frequently have abnormalities in laboratory coagulation tests specifically associated with tumour onset that typically worsen during tumour progression (Rickles and Falanga, 2009). Many different histological types of cancer have been studied and their associated relative risk of VTE assessed. In summary, malignancies of the breast, colon, and lung are most commonly associated with VTE, although when adjusted for disease prevalence, cancers of the pancreas, ovary, and brain possess the highest risk of VTE (Lee and Levine, 2003).

The upregulation of TF within the tumour microenvironment as a result of oncogenic transformation and angiogenesis contributes to the prothrombotic phenotype in tumour cells, and plays a key role in the hypercoagulable state in cancer (Falanga et al., 2009, Yu et al., 2010, Ruf et al., 2011). Activation of blood coagulation occurs by direct contact of either TF-bearing tumour cells or tumour cell-derived TF+ MP with circulating FVII and its activated form (Zwicker et al., 2009, Wang et al., 2012, Geddings and Mackman, 2013), resulting in thrombin generation and subsequently fibrin clot formation. In addition to their roles in coagulation, TF and thrombin are able to stimulate tumour cell proliferation and proangiogenic cytokine release involved in cancer progression (Rickles et al., 2003, Ruf et al., 2011). Understanding the aberrant expression of TF and the generation of thrombin by tumour cells may provide potential targets for the prevention of thrombosis and for cancer therapy.

In normal human tissues, TF expression is limited to extravascular cells, including the vascular adventitia and organ capsules, where it acts mainly as a haemostatic barrier to prevent blood loss during trauma (Drake et al., 1989). However, relatively high TF expression has been observed in predominantly solid malignancies such as, NSCLC (Koomagi and Volm, 1998, Goldin-Lang et al., 2008) and cancers of the pancreas (Kakkar et

al., 1995b, Haas et al., 2006, Khorana et al., 2007a, Thaler et al., 2013a), brain (Thaler et al., 2013b), ovary (Han et al., 2006, Cocco et al., 2011, Abu Saadeh et al., 2013), colon (Nakasaki et al., 2002), and breast (Contrino et al., 1996). Furthermore, TF may be an independent predictive indicator of prognosis and overall survival in various solid malignancies (Koomagi and Volm, 1998, Ueno et al., 2000, Nitori et al., 2005, Yamashita et al., 2007, Patry et al., 2008, de Meis et al., 2010, Rao et al., 2011, Abu Saadeh et al., 2013). Ultimately, these observations suggest that the variability of cancer-induced thrombotic risk might be, at least in part, related to quantitative differences of TF expression by cancer cells of different histology.

Previously in this laboratory, prothrombin time-based coagulation assays were carried out on pancreatic cancer cells *in vitro* with tumour cell lines expressing a range of TF cell surface levels, specifically low (MIA-PaCa-2), moderate (AsPC-1), and high (CFPAC-1), suggesting a link between TF cell surface expression and PCA (Yates et al., 2011). Here, the focus of the work has been to expand upon this existing observation by investigating the relationship between TF expression on cancer cells across a range of histological tumour types and their respective PCA. To extend this, particular emphasis has also been placed on determining the PCA of cell-free supernatants isolated from various cancer cell lines to determine the contribution of MP to coagulation. Therefore, *in vitro* experiments were designed to determine the PCA of various cancer cells and their respective supernatants, examined by a modified version of the prothrombin time assay described in section 2.6.1.

In a healthy individual, approximately 100 to 150 pg/ml of TF circulates within the blood (Koyama et al., 1994, Fareed et al., 1995, Zumbach et al., 1997), a proportion of which is known to be associated with cellular MP (Giesen et al., 1999). As previously discussed in section 1.6.1, MP are membrane vesicles derived from activated or apoptotic cells (Mause and Weber, 2010). Increased levels of MP are associated with tumour development implying this increase is tumour cell-derived (Tesselaar et al., 2007, Zwicker et al., 2009). Previously, tumour cell-derived MP have been shown to express active human TF both *in vitro* in conditioned cell-free media from cancer cell lines and *in vivo* in cell-free plasma from mice injected with human cancer cells (Yu et al., 2005, Davila et al., 2008, Thomas et al., 2009, Wang et al., 2012). Additionally, tumour cell-derived MP have been shown to enhance thrombus development in experimental models (Thomas et al., 2009, Wang et al.,

2012). Importantly, thrombosis occurrence in cancer patients and the level of TF activity associated with MP or number of TF+ MP have been shown to be linked in several studies (Tesselaar et al., 2007, Khorana et al., 2008a, Tesselaar et al., 2009, Zwicker et al., 2009, Manly et al., 2010, van Doormaal et al., 2012, Bharthuar et al., 2013).

The aim of this chapter was to identify the procoagulant potential of human pancreatic (AsPC-1, CFPAC-1, MIA PaCa-2), ovarian (A2780, ES2, SKOV-3), colorectal (CaCo-2, Colo320, LoVo), breast (MCF-7, MDA-MB-231, T47D), and haematological (H929, JJN3, MM.1S, U266, U937) cancer cell lines by characterizing the expression of TF and measuring the time to fibrin clot formation in human plasma by all cancer cells and their respective cell-free supernatants.

3.2 Determination of TF expression in human cancer cells

Cell culture growth and harvesting of seventeen cancer cell lines, specifically pancreatic (AsPC-1, CFPAC-1, MIA-PaCa-2), ovarian (A2780, ES2, SKOV-3), colorectal (CaCo-2, Colo320, LoVo), breast (MCF-7, MDA-MB-231, T47D), and haematological (H929, JJN3, MM.1S, U266, U937) cells (detailed in section 2.1), were screened for the cell surface expression of TF (described in section 2.5.1). Briefly, cells were stained with FITC-conjugated anti-TF antibody, washed and the MFI was determined via flow cytometry. In this study, eleven tumour cell lines, namely MIA-PaCa-2, A2780, CaCo-2, Colo320, MCF-7, T47D, H929, JJN3, MM.1S, U266, and U937, were determined to express little or no detectable TF (between 1.0 and 1.3 MFI [relative to isotype control]; data not shown) although they did support coagulation, but to a far lesser extent than the other cell lines. Of the remaining tumour cell lines, pancreatic CFPAC-1, breast MDA-MB-231, and ovarian SKOV-3 cells had a high relative expression of surface TF (between 17.5 and 38.8 MFI [relative to isotype control]), while pancreatic ASPC-1, ovarian ES2, and colorectal LoVo cells had moderate TF expression levels (between 2.8 and 6.5 MFI [relative to isotype control]) (Figure 3.1).

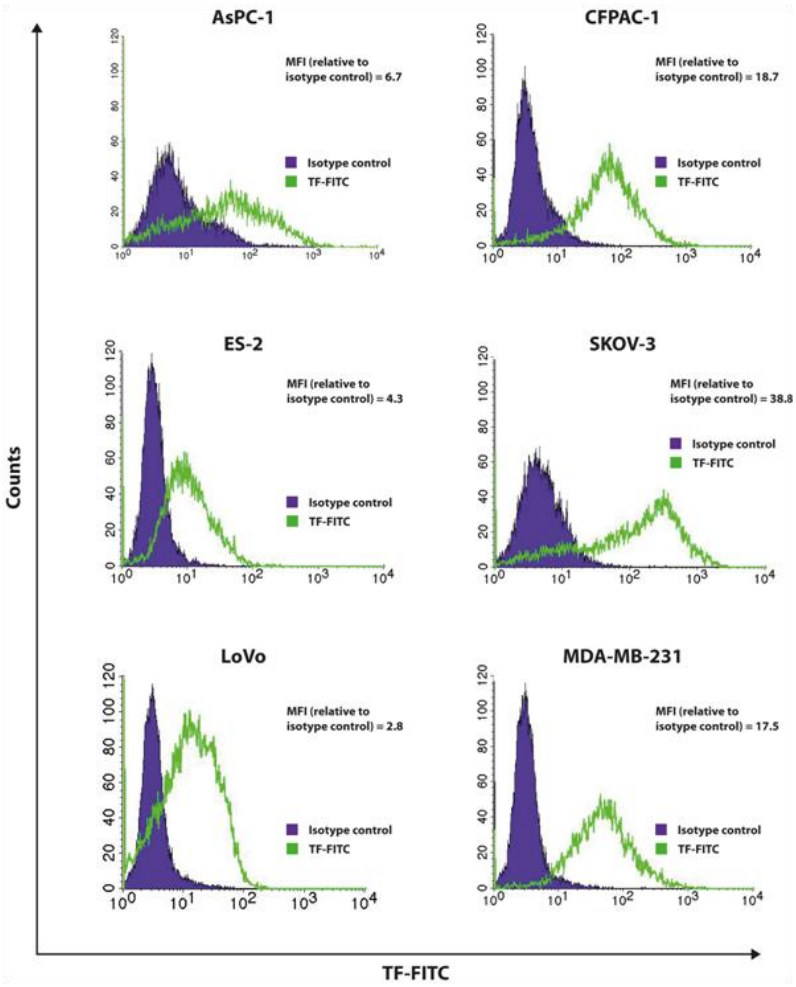


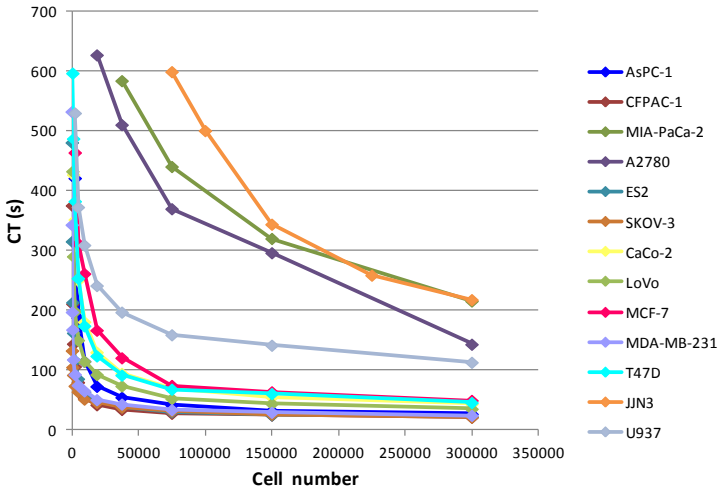
Figure 3.1: The TF expression levels on cancer cell lines.
Flow cytometric analysis of surface TF (green line) on AsPC-1, CFPAC-1, ES2, SKOV-3, LoVo, and MDA-MB-231 tumour cell lines compared to isotype-matched negative control antibody (filled purple). Representative histograms from two or more independent experiments are shown.

3.3 Characterization of cancer cell surface-supported coagulation

Initial *in vitro* experiments were conducted to measure the PCA of various cancer cells using a one-stage clotting assay described in section 2.6.1. Briefly, enumerated cancer cell suspensions in PBS were mixed with equal parts of human plasma for 2 min at 37°C on a Thrombotrack SOLO coagulometer. Following recalcification (25 mM $CaCl_2$), the time for the plasma to clot (if less than 10 min) was recorded in duplicate and repeated independently, three times. It is important to note that the reconstituted normal human plasma used in this assay does not clot without the addition of exogenous TF. Thus, all cancer cell lines with detectable cell surface TF expression supported coagulation in cell count experiments. As would be expected, the results show for all cell lines tested that the

higher the number of cells per assay, the faster clot formation occurred (Figure 3.2 A). Moreover, the observed PCA across all tumour sites tested was shown to be inversely dependent upon the cell count in a power relationship using logarithmic transformations of data to the base of 10 (log_{10}), which was statistically significant for all cell lines examined (Pearson's correlation coefficient r = -0.97 to -0.99, $P < 0.005$) on a log_{10}/log_{10} plot (Figure 3.2 B).

(A)



(B)

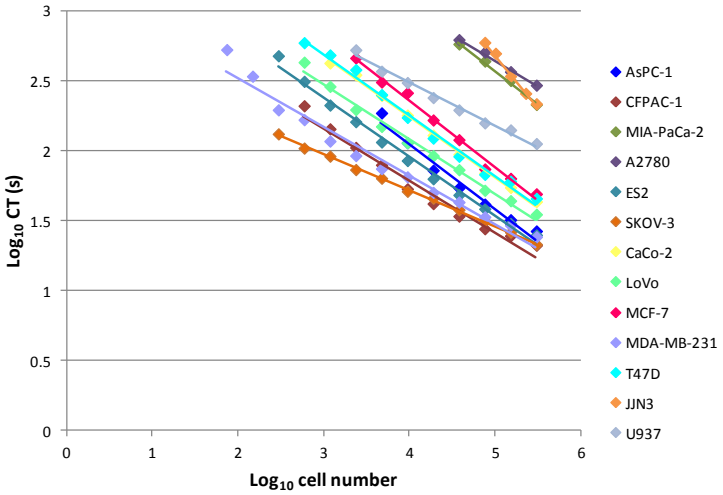
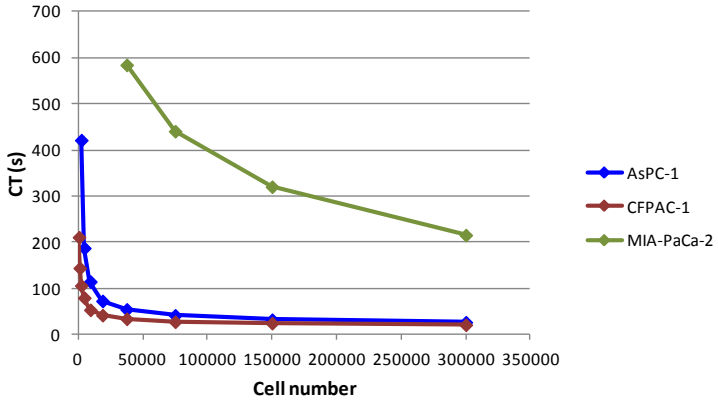


Figure 3.2: Characterization of the PCA of human cancer cells.
(A) Pancreatic (AsPC-1, CFPAC-1, MIA-PaCa-2), ovarian (A2780, ES2, SKOV-3), colorectal (CaCo-2, LoVo), breast (MCF-7, MDA-MB-231, T47D), and haematological (JJN3, U937) cancer cell lines were procoagulant in a cell number-dependent manner, and (B) the variation in CT (s) with cell number on log_{10} transformed data. CT was measured using a one-stage clotting assay. Data are the average of three independent measurements performed in duplicate (C of V < 5%).

Of the three pancreatic cancer cell lines examined, CFPAC-1 had the fastest CT (or greatest PCA) and MIA-PaCa-2 the slowest CT (or least PCA) at the same cell concentrations (Figure 3.3). While, SKOV-3 had the fastest CT and A2780 the slowest of the three ovarian cancer cell lines tested (Figure 3.4). Additionally, in the three colorectal cell lines investigated, LoVo demonstrated the fastest CT followed by CaCo-2 (Figure 3.5), while Colo320 cells did not support coagulation (over 600 s; data not shown) so were not examined any further. Of the three breast cancer cell lines, MDA-MB-231 had the fastest CT, with both MCF-7 and T47D having similar and notably slower CT (Figure 3.6). Lastly, U937 had the fastest CT of the haematological cell lines, followed by slow clotting JJN3 MM cells (Figure 3.7). The average CT (± SD) of H929, MM.1S, and U266 myeloma cells (3×10^5 per assay) were 443.0 ± 38.5, 555.1 ± 36.4, and 591.0 ± 25.0 s, respectively, and clot formation within 600 s ceased following one serial dilution of each cell line. Furthermore, the PCA of the haematological cell lines tested was typically slower than the majority of solid malignant cell lines in this study. Overall, the cell lines that showed the highest expression of TF (CFPAC-1, SKOV-3, and MDA-MB-231) have the fastest CT at the same cell concentrations, whereas the cell lines with little or no detectable TF (MIA-PaCa-2, A2780, CaCo-2, Colo320, MCF-7, T47D, H929, JJN3, MM.1S, U266, and U937) have extremely slow CT in comparison or do not support coagulation.

(A)



(B)

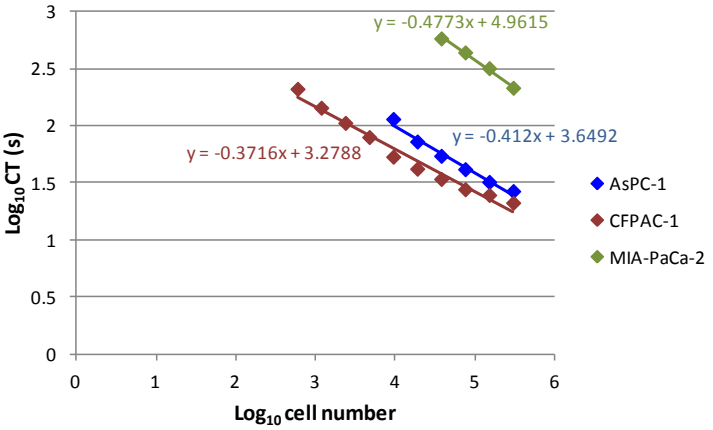
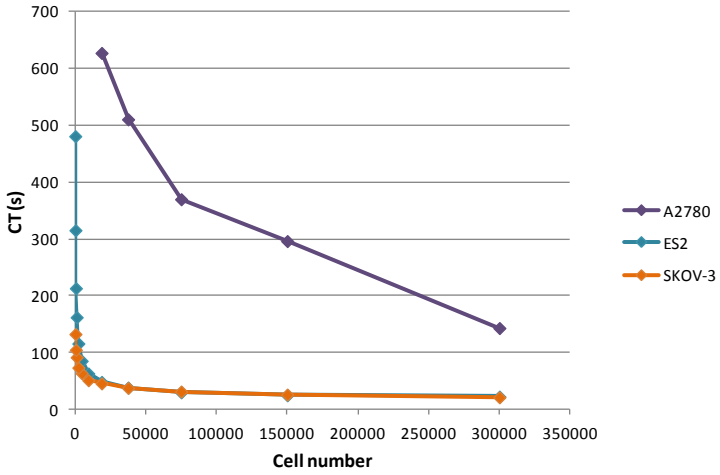


Figure 3.3: Characterization of the PCA of pancreatic cancer cells.

(A) Pancreatic (AsPC-1, CFPAC-1, MIA-PaCa-2) cancer cell lines were procoagulant in a cell number-dependent manner, and (B) the variation in CT with cell number on $\log_{10}$ transformed data. CT was measured using a one-stage clotting assay. Data are the average of three independent measurements performed in duplicate (C of V < 5%).

(A)



(B)

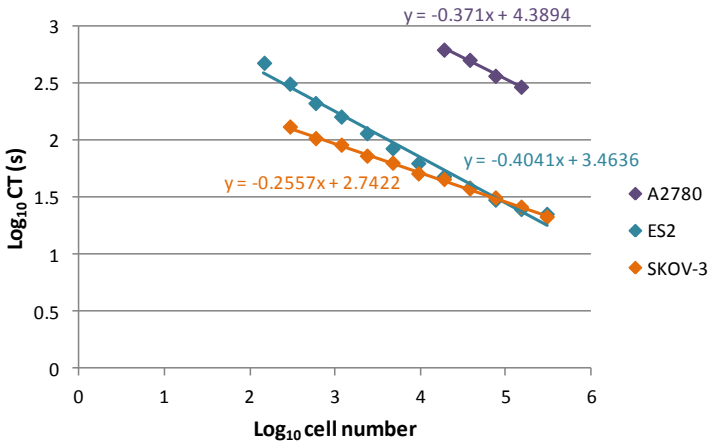
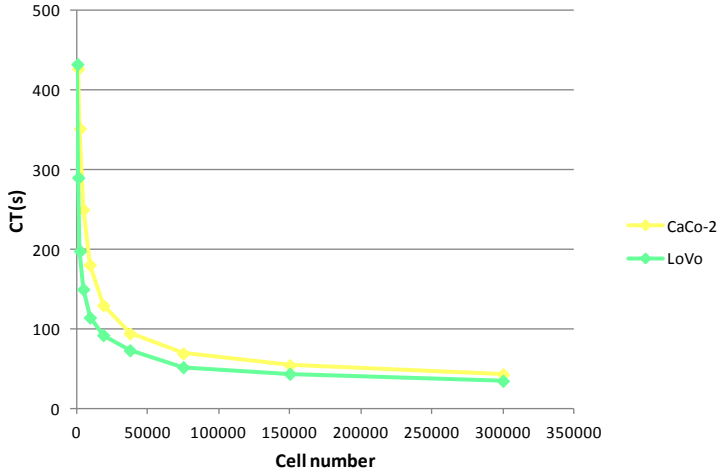


Figure 3.4: Characterization of the PCA of ovarian cancer cells.

(A) Ovarian (A2780, ES2, SKOV-3) cancer cell lines were procoagulant in a cell number-dependent manner and (B) the variation in CT with cell number on $\log_{10}$ transformed data. CT was measured using a one-stage clotting assay. Data are the average of three independent measurements performed in duplicate (C of V < 5%).

(A)



(B)

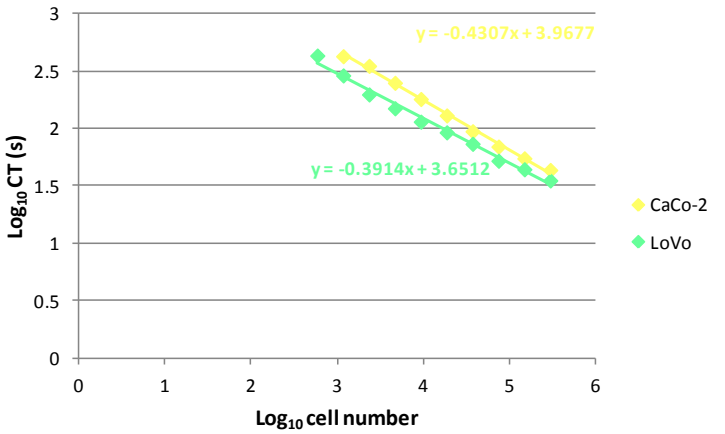
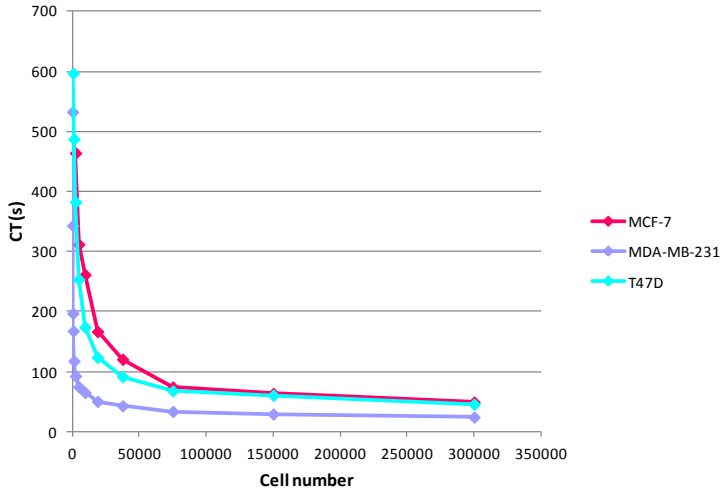


Figure 3.5: Characterization of the PCA of colorectal cancer cells.

(A) Colorectal (CaCo-2, LoVo) cancer cell lines were procoagulant in a cell number-dependent manner and (B) the variation in CT with cell number on $\log_{10}$ transformed data. CT was measured using a one-stage clotting assay. Data are the average of three independent measurements performed in duplicate (C of V < 5%).

(A)



(B)

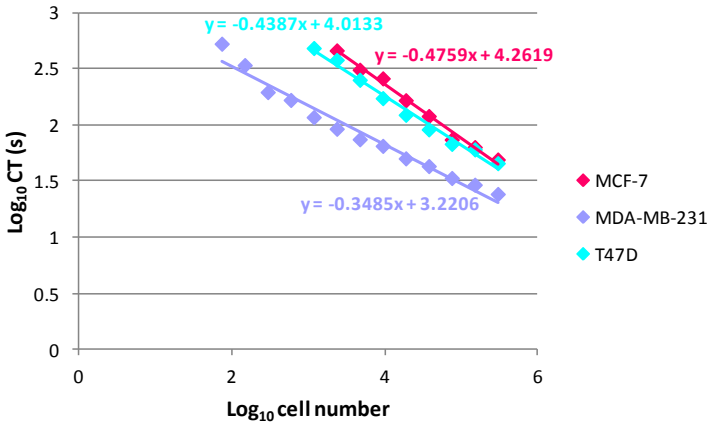
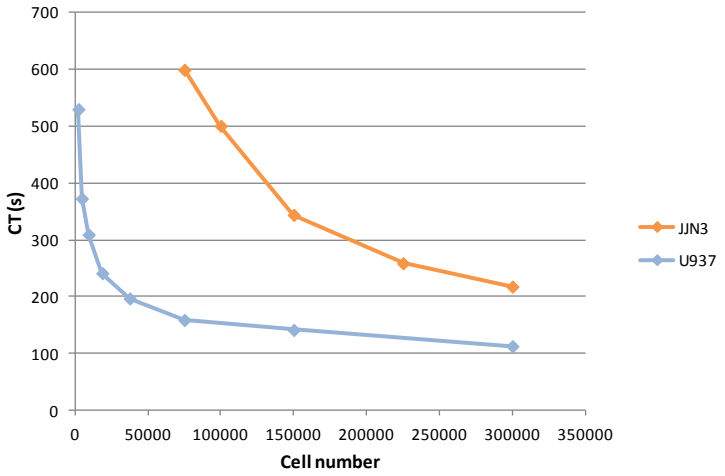


Figure 3.6: Characterization of the PCA of breast cancer cells.

(A) Breast (MCF-7, MDA-MB-231, T47D) cancer cell lines were procoagulant in a cell number-dependent manner and (B) the variation in CT with cell number on $\log_{10}$ transformed data. CT was measured using a one-stage clotting assay. Data are the average of three independent measurements performed in duplicate (C of V < 5%).

(A)



(B)

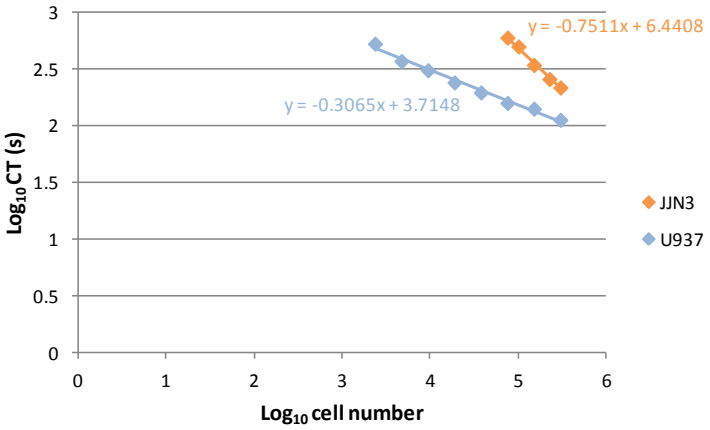


Figure 3.7: Characterization of the PCA of haematological cancer cells.
(A) Haematological (JJN3, U937) cancer cell lines were procoagulant in a cell number-dependent manner and (B) the variation in CT with cell number on $\log_{10}$ transformed data. CT was measured using a one-stage clotting assay. Data are the average of three independent measurements performed in duplicate (C of V < 5%).

3.3.1 Determination of single cancer cell procoagulant potential

The PCA of a single cell for each cell line was individually calculated using the slope (m) and y axis intercept (c) of the best fit lines obtained on the $\log_{10}/\log_{10}$ plots (Figures 3.3 B to 3.7 B), respectively. Initially, the $\log_{10}$CT of a single cell was determined using equation (3.1) where y represents CT and x represents cell number.

$$\log_{10} y = m \log_{10}(x) + c \qquad \text{(Equation 3.1)}$$

A representative example is shown below in equations (3.2) to (3.4) for the pancreatic cancer cell line AsPC-1 following the appropriate substitution of equation (3.1) for AsPC-1 values (see Figure 3.3 B).

$$\text{Log}_{10}\,\text{CT} = -0.412 \times (\log_{10}\ 1) + 3.6492 \qquad \text{(Equation 3.2)}$$

$$\text{Log}_{10}\,\text{CT} = (-0.412 \times 0) + 3.6492 \qquad \text{(Equation 3.3)}$$

$$\text{Log}_{10}\,\text{CT} = 3.6492 \qquad \text{(Equation 3.4)}$$

Subsequently, the antilog of this number was then calculated in equation (3.5) to give the single cell CT for AsPC-1.

$$\text{CT} = 10\text{^}3.6492 \qquad \text{(Equation 3.5)}$$

CT for one AsPC-1 cell = 4459 s.

The single cell CT calculated for each TF expressing cell line examined in this study are shown in Table 3.1. As expected, the cell lines which were previously shown to have the highest PCA also had the fastest single cell CT.

Table 3.1: The TF expression (MFI) and single cell CT (s) of pancreatic (AsPC-1, CFPAC-1), ovarian (ES2, SKOV-3), colorectal (CaCo-2, LoVo), and breast (MCF-7, MDA-MB-231, T47D) tumour cells.

Tumour cell line	TF expression (MFI; relative to isotype control)	Single cell CT (s)
AsPC-1	6.7	4459
CFPAC-1	18.7	1900
ES2	4.3	2908
SKOV-3	38.8	552
LoVo	2.8	4479
CaCo-2	1.3	9283
MCF-7	1.1	18277
MDA-MB-231	17.5	1662
T47D	1.2	10311

The single cell CT measurements for all six moderate to high TF expression cell lines, explicitly AsPC-1, CFPAC-1, ES2, SKOV-3, LoVo, and MDA-MB-231 (see Figure 3.8), were plotted as a function of the TF expression (MFI; relative to isotype control), previously determined by flow cytometry in section 3.2. There was a significant negative correlation (Pearson's correlation coefficient r = -0.88, P = 0.01) between TF expression and single cell CT, where the higher the TF expression, the faster the observed CT for the same number of cells. In support of this, recombinant TF of varying concentrations was also shown to result in a similar power relationship (Pearson's correlation coefficient r = -0.99, P = 0.0009) between TF concentration and CT (Figure 3.9).

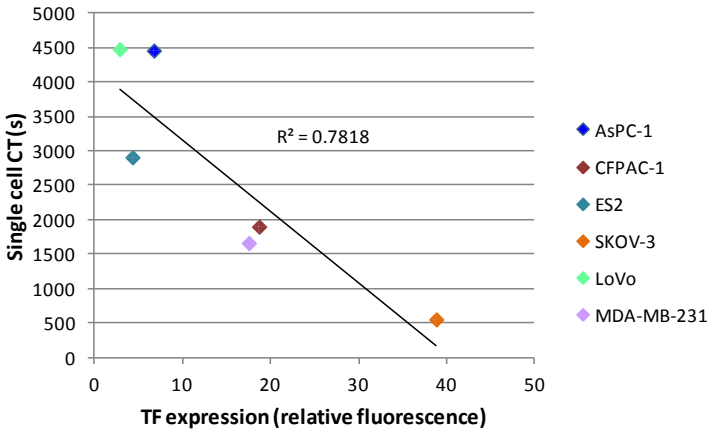
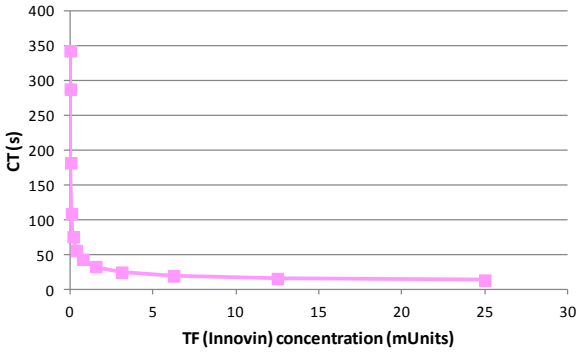


Figure 3.8: Relationship between single cell CT of tumour cells and their relative cell surface TF expression.
Single cell CT of pancreatic (AsPC-1, CFPAC-1), ovarian (ES2, SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cell lines plotted against their TF MFI (relative to isotype control).

(A)



(B)

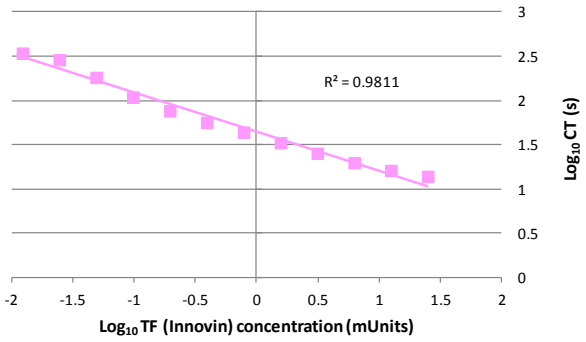


Figure 3.9: Characterization of the PCA of various concentrations of recombinant TF.
(A) The CT is dependent upon the concentration of TF and (B) the variation in CT with the concentration of recombinant TF (Innovin) on $\log_{10}$ transformed data. CT was measured using a one-stage clotting assay. Data are the average of three independent measurements performed in duplicate (C of V < 5%).

3.3.2 The effect of neutralizing cell exposed procoagulant TF on PCA

Since the previous findings confirm that there is a direct association between surface TF expression and CT in cancer cells, the contribution of TF was further examined by measuring cell-associated PCA following the neutralization of TF on these cell lines. Briefly, cancer cell suspensions (1 x 10^5 cells/100 µl PBS) were pre-treated with an anti-TF antibody (5 µg/ml) and several subsequent serial dilutions for 30 min at 37°C. The time for cell and plasma mixtures to clot in the presence of calcium (if less than 10 min) was recorded in triplicate, as detailed in section 2.6.1. As may be expected, this resulted in an antibody dose-dependent increase in CT (or decrease in the coagulability) of nine TF-expressing tumour cell lines, namely AsPC-1, CFPAC-1, ES2, SKOV-3, CaCo-2, LoVo, MCF-7, MDA-MB-231, and T47D cells (Figure 3.10), and prevented clot formation in those tumour cells with extremely low TF levels that were undetectable by flow cytometry; MIA-PaCa-2, A2780, H929, JJN3, MM.1S, U266, and U937.

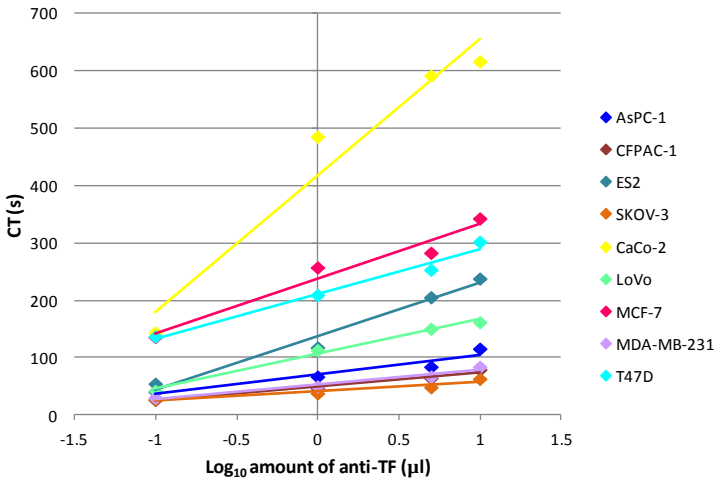


Figure 3.10: The effect of neutralizing TF on the PCA of various human cancer cells.
The effect of TF blocking on CT of pancreatic (AsPC-1, CFPAC-1), ovarian (ES2, SKOV-3), colorectal (CaCo-2, LoVo), and breast (MCF-7, MDA-MB-231, T47D) cancer cells (1 x 10^5 per assay) with varying amounts of antibody (0.1-10 µl of 5 mg/ml anti-TF). CT was measured using a one-stage clotting assay. Data are the average of three measurments (C of V < 5%).

Furthermore, the slopes of the individual best fit lines shown in Figure 3.10 were then plotted against the respective cell line TF MFI (relative to isotype control), as previously determined by flow cytometry in section 3.2, demonstrating that the rate of the decrease in PCA or increase in CT caused by blocking TF with an anti-TF antibody directly relates to the level of TF expressed on the cell surface for each of the nine cell lines examined (Figure 3.11). In other terms, the lower the cell surface expression of TF, the more prominent the inhibitory effect of an anti-TF antibody is on cell-mediated PCA.

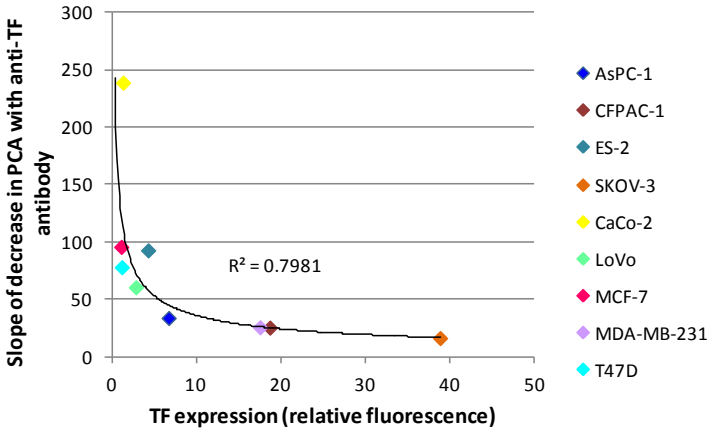


Figure 3.11: Relationship between the neutralization of TF on tumour cells and their relative cell surface TF expression.

The slope of the decrease in PCA (or increase in CT) with anti-TF antibody of pancreatic (AsPC-1, CFPAC-1), ovarian (ES2, SKOV-3), colorectal (CaCo-2, LoVo), and breast (MCF-7, MDA-MB-231, T47D) cancer cells plotted against their TF MFI (relative to isotype control).

3.4 Characterization of the procoagulant potential of tumour cell-derived MP

To determine the PCA of tumour cell-derived MP *in vitro* a modified version of the prothrombin time assay described in section 2.6.1 was used. Briefly, cell-free supernatants containing suspensions of MP and secreted particles < 0.1 µm were removed from tumour cells via double centrifugation; initially at 300 x *g* for 5 min to remove tumour cells, and then the extracted supernatant was spun again at 1000 x *g* for 5 min to remove larger cell fragments. These isolated cell-free supernatants were then filtered in selected experiments to sequentially remove fractions of MP. The cell-free supernatants were initially mixed with equal parts of human plasma for 2 min at 37°C on a Thrombotrack SOLO coagulometer. Following recalcification (25 mM $CaCl_2$), the time for the plasma to clot (if less than 10 min) was recorded in duplicate and repeated independently, three times. Media alone did not support coagulation. The procoagulant potential of cell-free supernatants was assessed for all cell lines and the majority showed no or diminutive PCA (MIA-PaCa-2, A2780, CaCo-2, MCF-7, T47D, H929, JJN3, MM.1S, U266, and U937). In contrast, the cell-free supernatants extracted from the moderate to high TF expressing cancer cells (as previously determined by flow cytometry and detailed in section 3.2), specifically pancreatic (AsPC-1, CFPAC-1), ovarian (ES2, SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cell lines, consistently resulted in clot formation and

subsequent serial dilutions showed that there was a CT/supernatant dose-response on a $\log_{10}/\log_{10}$ plot (Figure 3.12). However, no relationship was found between the cell surface TF MFI (previously determined in section 3.2) and the CT of the cell-free supernatants isolated from these 6 tumour cell lines with procoagulant potential (data not shown).

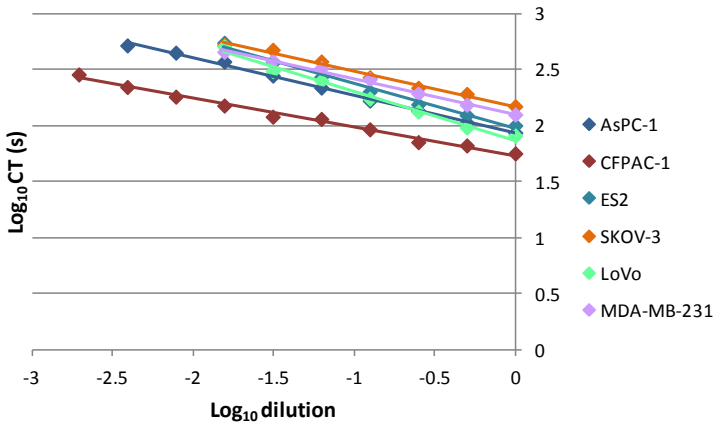


Figure 3.12: Characterization of the PCA of cell-free supernatants isolated from human cancer cells. Cell-free supernatants of pancreatic (AsPC-1, CFPAC-1), ovarian (ES2, SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cells containing suspensions of MP were procoagulant in a dilution-dependent manner. CT was measured using a one-stage clotting assay. Data are the average of three independent measurements performed in duplicate (C of V < 10%).

Further assessment of the PCA associated with MP was made via filtering cell-free supernatants through specific pore sizes of 1.2, 0.45, and 0.1 µm and this resulted in a progressive increase in CT (or decrease in the coagulability) for the six cell lines examined (Figure 3.13), suggesting that the majority of the PCA of cell-free supernatants was in fact associated with MP as a certain proportion of MP were presumably removed through each successive filtration step. The dose-response of CT with serial dilutions of filtered cell-free supernatants also showed a strict correlation on a $\log_{10}/\log_{10}$ plot for pancreatic (AsPC-1, CFPAC-1), ovarian (ES2, SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cell lines (Figure 3.14 A to F, respectively).

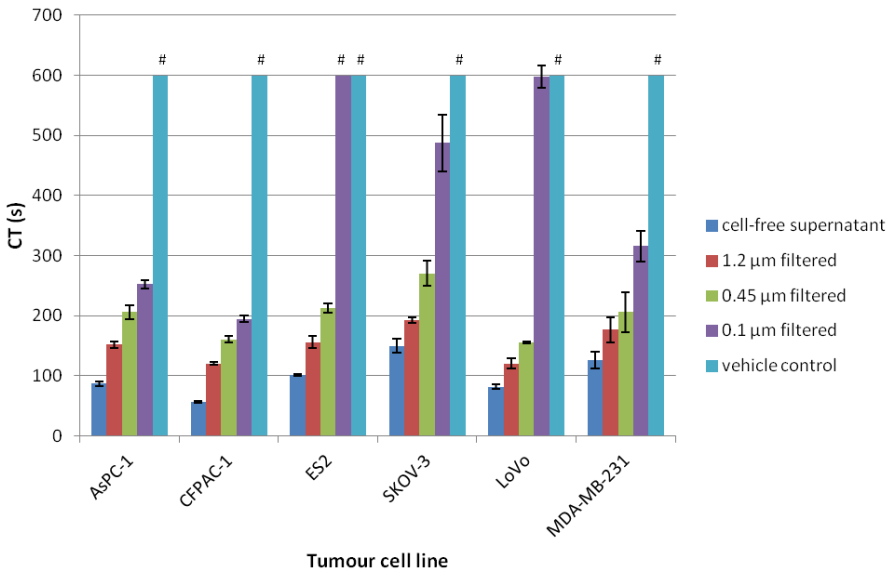
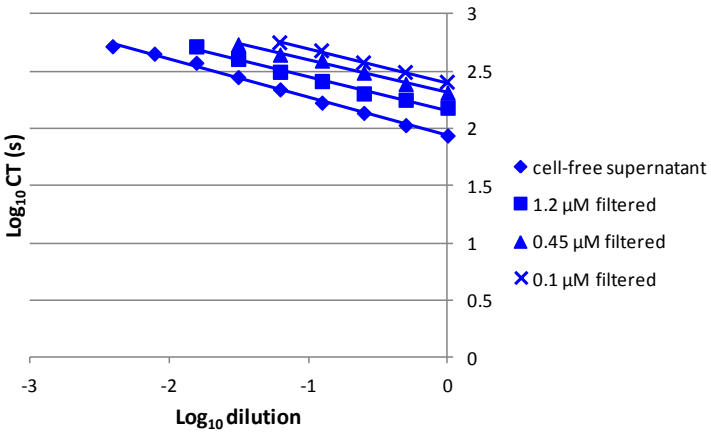


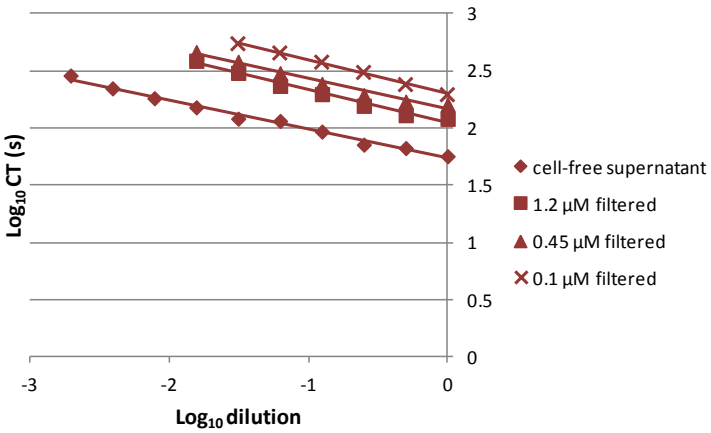
Figure 3.13: The effect of cell-free supernatant sequential filtration on the PCA to determine the contribution of tumour cell-derived MP to coagulation.

The CT of pancreatic (AsPC-1, CFPAC-1), ovarian (ES2, SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cell-free supernatants increases following 1.2, 0.45, and 0.1 µm filtration. CT was measured using a one-stage clotting assay. Data are the mean ± SD of three independent measurements performed in duplicate. # indicates sample did not clot.

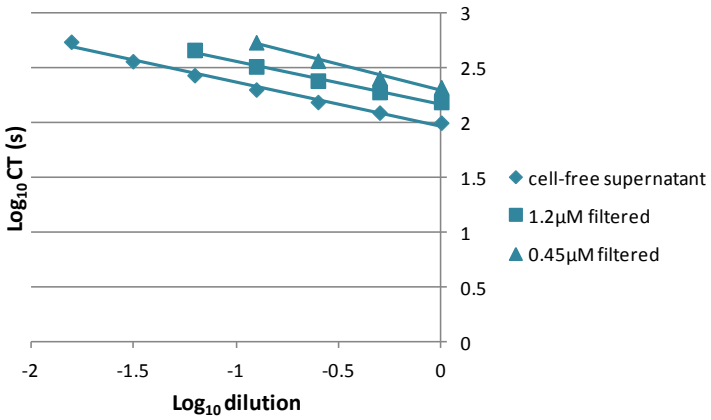
(A) AsPC-1



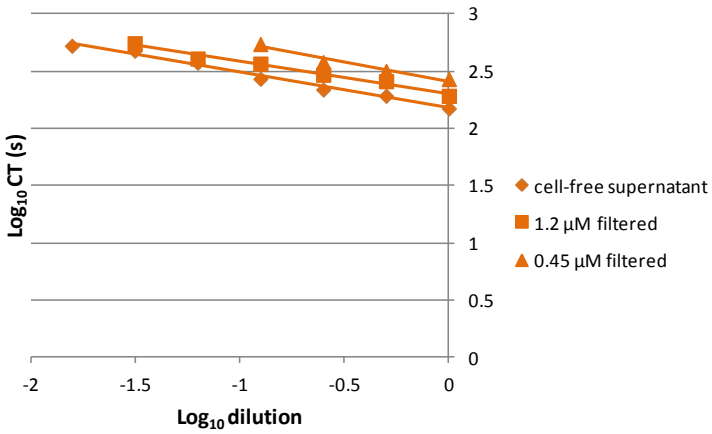
(B) CFPAC-1



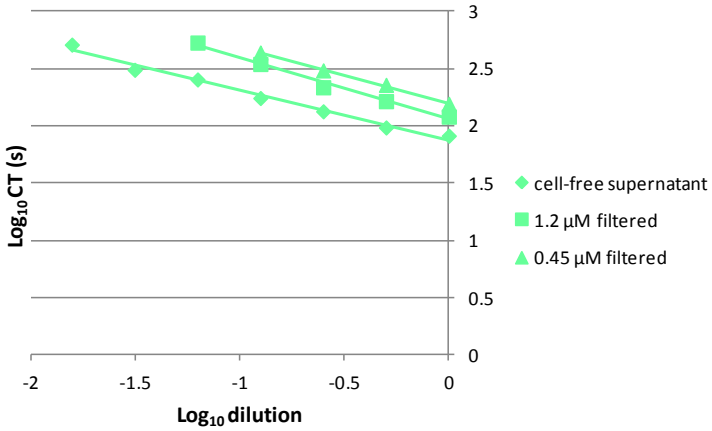
(C) ES2



(D) SKOV-3



(E) LoVo



(F) MDA-MB-231

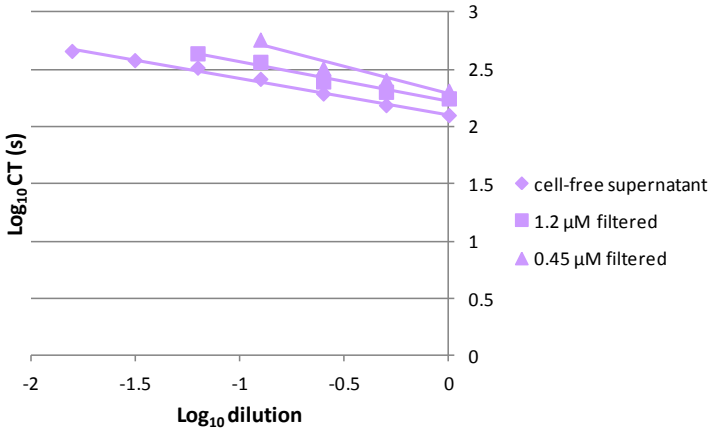


Figure 3.14: Characterization of the PCA of unfiltered and filtered cell-free supernatants isolated from human cancer cells.

Unfiltered and filtered cell-free supernatants of (A) AsPC-1, (B) CFPAC-1, (C) ES2, (D) SKOV-3, (E) LoVo, and (F) MDA-MB-231 cancer cell conditioned media were procoagulant in a dilution-dependent manner. CT was measured using a one-stage clotting assay. Data are the average of three independent measurements performed in duplicate (C of V < 10%).

3.4.1 Effect of the concentration of cell-free supernatant TF isolated from cancer cells on the PCA

Initially, cell-free supernatants derived from various cancer cell lines were examined by flow cytometry, yet this method failed to detect sufficient numbers of TF+ MP for analysis (data not shown). Therefore, to distinguish the contribution of TF+ MP from that of TF associated with smaller microvesicles (< 1 μm) and soluble TF in coagulation, TF antigen was quantified by ELISA in the conditioned cell-free supernatant (both unfiltered and 0.1 μm filtered) of all cell lines (with the exception of the ovarian cell line ES2 due to low cell viability) as detailed in section 2.7.1, and the CT measured (prior to freezing). For each cell line the medium was replenished every 24 h across four days of incubation for the same flask of cells. There was no quantifiable human TF detected by ELISA in the cell-free supernatants extracted from the following tumour cell lines; MIA-PaCa-2, A2780, CaCo-2, MCF-7, T47D, H929, JJN3, MM.1S, U266, and U937, or in any of the cell line-specific controls (complete cell growth medium alone). While, TF was detected in the conditioned cell-free supernatant of pancreatic (AsPC-1, CFPAC-1), ovarian (SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cell lines. However, the total quantity of TF removed by filtration through a 0.1 μm filter pore was relatively low (Figure 3.15).

Furthermore, the total generation of TF by cancer cells and release into the media as either TF+ MP and/or < 0.1 μm forms (i.e. TF-bearing exosomes and soluble TF) in the cell-free supernatants varied not only between cells of different histology, but also for the same cell line across four days of cell incubation. For AsPC-1, the highest concentration of TF was detected in the unfiltered cell-free supernatant following the first 24 h of cell culture (47.9 pg/ml) that was subsequently followed by a decrease, although at days two to four there was a sequential increase in TF (18.2 to 45.9 pg/ml) (Figure 3.16). The highest concentration of TF for CFPAC-1 was also detected in the first 24 h (124.5 pg/ml) and the TF generated and measured in the cell-free media during the following days was considerably lower (ranging from 1.4 to 9.7 pg/ml) (Figure 3.16). The TF concentration measured in unfiltered cell-free supernatant sequentially decreased over the four day culture period (ranging from 15.8 to 7.8 pg/ml) for SKOV-3 (Figure 3.16), while the TF concentration generated every 24 h in culture remained consistent across the four day period for LoVo (ranging between 9.8 and 10.4 pg/ml) (Figure 3.16). For MDA-MB-231, the unfiltered cell-free supernatant TF concentration was lower in the first and second day

(25.8 and 25.6 pg/ml, respectively) in comparison to third and fourth day (60.8 and 45.6 pg/ml, respectively) in culture (Figure 3.16).

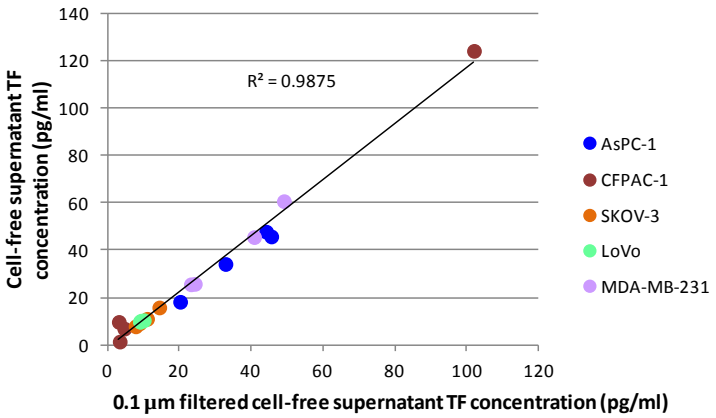


Figure 3.15: Relationship between the TF concentration of unfiltered cell-free supernatants isolated from human cancer cells and their relative 0.1 µm filtered TF concentration.
The TF concentration (pg/ml) of unfiltered cell-free supernatants of pancreatic (AsPC-1, CFPAC-1), ovarian (SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cells plotted against their relative 0.1 µm filtered TF concentration (pg/ml) following 24, 48, 72, and 96 h cell culture, quantified by ELISA. Data are the average of duplicate measurements.

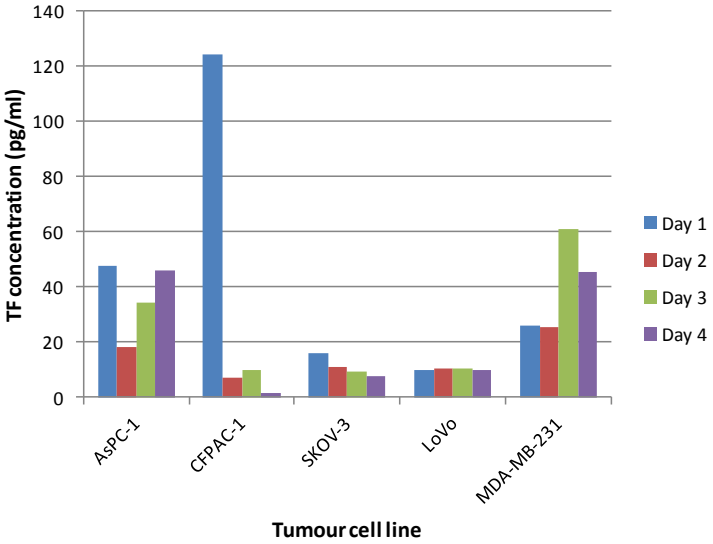


Figure 3.16: The variation of TF concentration of cell-free supernatants isolated from human cancer cells over time.
The TF concentration (pg/ml) of the unfiltered cell-free supernatants of pancreatic (AsPC-1, CFPAC-1), ovarian (SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cells over a four day incubation period, quantified by ELISA. Data are the average of duplicate measurements.

In contrast to the TF concentration (that does not markedly alter between unfiltered and 0.1 μm filtered cell-free supernatants), the CT of 0.1 μm filtered cell-free supernatants from AsPC-1, CFPAC-1, SKOV-3, LoVo, and MDA-MB-231 were considerably increased compared to the matched unfiltered samples (Figure 3.17). Additionally, there are significant negative correlations between the TF concentration and CT of both unfiltered and 0.1 μm filtered conditioned cell-free supernatants for all cell lines combined shown in Figure 3.17 (r = -0.86, $P < 0.0005$ and r = -0.76, $P < 0.0005$, respectively). Lastly, the increase in CT observed in the 0.1 μm filtered cell-free supernatants was shown to be directly proportional to the PCA or CT of the original (unfiltered) cell-free supernatants in this study (Figure 3.18). Thus, suggesting that a relative proportion of PCA is diminished following filtration through a 0.1 μm filter pore for all five cell lines examined, and in turn, that the MP generated by these cell lines possess comparable procoagulant potential.

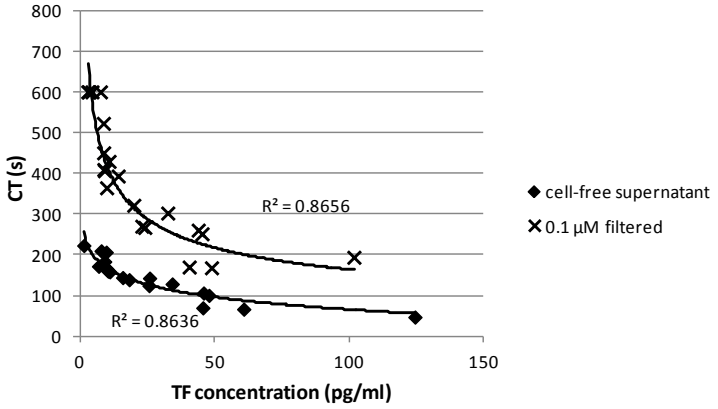


Figure 3.17: Relationship between the TF concentration of unfiltered and 0.1 μm filtered cell-free supernatants isolated from human cancer cells and their relative PCA.

The TF concentration (pg/ml) of unfiltered and 0.1 μm filtered cell-free supernatants of pancreatic (AsPC-1, CFPAC-1), ovarian (SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cells following 24, 48, 72, and 96 h cell culture was quantified by ELISA and plotted against their relative CT (s), measured by a one-stage clotting assay. Any cell-free supernatant sample that did not clot was given a value of 600 s. Data are the average of duplicate measurements.

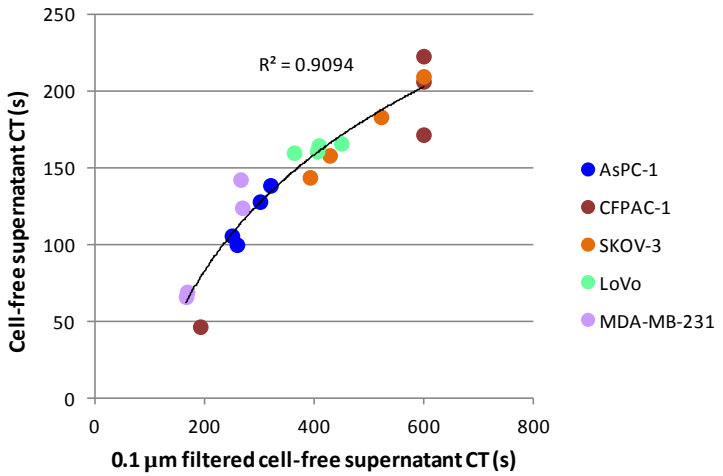


Figure 3.18: Relationship between the PCA of unfiltered cell-free supernatants isolated from human cancer cells and their relative 0.1 µm filtered PCA.

The CT (s) of unfiltered cell-free supernatants of pancreatic (AsPC-1, CFPAC-1), ovarian (SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cells plotted against their relative 0.1 µm filtered CT (s) following 24, 48, 72, and 96 h cell culture, measured by a one-stage clotting assay. Any cell-free supernatant sample that did not clot was given a value of 600 s. Data are the average of duplicate measurements.

3.5 Discussion

Most tumours carry an increased risk of patients developing VTE, which is thought to be linked to TF expression/activity on tumour cells (Khorana et al., 2007a, Uno et al., 2007, Abu Saadeh et al., 2013) and tumour cell-derived MP (Tesselaar et al., 2007, Thomas et al., 2009, Zwicker et al., 2009, Wang et al., 2012, Echrish et al., 2014) as TF is the primary cellular initiator of coagulation. Thus, the initial aim of this study was to characterize TF expression levels on the surface of various solid and haematological tumour cells that could potentially affect the coagulation kinetics of these cells *in vitro*. Specifically, TF antigen levels, quantified by flow cytometry, were found to be highly variable among all established cell lines from the same or different histological types of cancer examined. Indeed, previous studies have observed a correlation between high tumour TF expression in human surgical specimens and advanced tumour stage (Kakkar et al., 1995b, Nakasaki et al., 2002, Khorana et al., 2007a). These results are consistent with the *in vitro* findings in this study, that show TF is expressed by various solid carcinoma cell lines with high metastatic potential in animal models, including pancreatic (AsPC-1, CFPAC-1), ovarian (SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cells and poorly differentiated ovarian (ES2) cancer cells, while in contrast, the less aggressive, pancreatic

(MIA-PaCa-2), ovarian (A2780), colorectal (CaCo-2, Colo320), and breast (MCF-7, T47D) cancer cells typically derived from primary tumours or have low growth rates and metastatic potential in animal models, express lower cell surface TF in comparison, or none detectable by flow cytometry.

However, the expression of TF on the broad range of haematological malignancies is less clearly defined. Previously, TF mRNA levels in leukocytes have been shown to be significantly elevated in patients with acute myeloid leukaemia and lymphoma (Sase et al., 2004, Sase et al., 2005). While in contrast, TF protein expression determined by immunohistochemistry was not found in precursor or mature B- or T-cell lineage lymphoma and leukaemia patient samples (n=90), and also TF gene (*F3)* expression was absent in all lymphoid cell lines analyzed (n=114) in comparison to 74.2% of the 856 solid cancer cell lines examined that expressed *F3* (Cesarman-Maus et al., 2014). Thus, indicating that the pathophysiology of thrombosis in patients with haematological malignancies may differ from that of patients with solid carcinomas. The U937 haematological cancer cell line, that was established from a diffuse histiocytic lymphoma, did not express any detectable cell surface TF antigen by flow cytometry in this study, although previous studies have demonstrated that TF expression can be induced on these monocyte-like cells in response to a variety of stimuli, such as LPS, phorbol ester, and TNF-α (Rana et al., 1988, Tanaka, 1989). Furthermore, plasma levels of TF antigen and also TF mRNA levels measured in peripheral blood mononuclear cells were not found to be elevated in a small number of MM patients (Negaard et al., 2008). Recently, Cesarman-Maus et al. extensively confirmed the absence of TF on myeloma cells, examining 55 human myeloma cell lines and 239 MM tumour samples (Cesarman-Maus et al., 2012), which correlates with the negative TF results obtained for all 4 myeloma cell lines used in this study (H929, JJN3, MM.1S, and U266), as characterized by flow cytometry.

Subsequently, the PCA of various tumour cells was assessed in an *in vitro* one-stage clotting assay following their addition to recalcified normal human plasma and these results were further analysed to determine whether the tumour-mediated PCA correlated with TF. Cancer cells in the presence of recalcified human plasma consistently shortened the CT in a cell number-dependent manner in various pancreatic (AsPC-1, CFPAC-1, MIA-PaCa-2), ovarian (A2780, ES2, SKOV-3), colorectal (CaCo-2, LoVo), breast (MCF-7, MDA-

MB-231, T47D), and haematological (JJN3, U937) cancer cell lines examined. As expected, the cells with the highest TF expression (SKOV-3, CFPAC-1, MDA-MB-231) also showed the fastest CT, or greatest PCA at the same cell concentrations, while tumour cells with relatively little or no TF expression were shown to have low PCA, and so it may be presumed that in the total absence of cell surface TF expression, for example, on noncancerous cells, that coagulation would not be supported.

Intriguingly, this study establishes an explicit link between cell surface TF expression and the PCA of various solid carcinomas, specifically pancreatic (AsPC-1, CFPAC-1), ovarian (ES2, SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) tumour cells, whereby a higher number of TF molecules present on the surface of a single cancer cell is associated with increased PCA. In particular, the single cell CT of colorectal (CaCo-2) and breast (MCF-7, T47D) tumour cells with little detectable surface TF was calculated to range between 9.3 x 10^3 and 1.9 x 10^4 s and they consequently supported slow coagulation. Whereas, the other 6 tumour cells lines examined with moderate to high TF expression (AsPC-1, CFPAC-1, ES2, SKOV-3, LoVo, and MDA-MB-231) all had a single cell CT below 4.5 x 10^3 s. This implies that once above a TF concentration threshold a near linear relationship exists with the tumour cell-mediated PCA as shown in Figure 3.8. Additionally, the cell surface mediated PCA was further shown to be dependent on TF activity, as blocking TF in the presence of a neutralizing antibody to TF greatly prolonged the CT in 9 TF-bearing tumour cell lines (AsPC-1, CFPAC-1, ES2, SKOV-3, CaCo-2, LoVo, MCF-7, MDA-MB-231, and T47D) or ceased PCA in the remaining tumour cells studied that express relatively low levels of TF, which are undetectable by flow cytometry. The lack of complete PCA inhibition in the higher TF expressing cell lines by anti-TF may potentially be a result of not all binding sites being fully saturated due to insufficient antibody titration, although this is unlikely as high concentrations were used. An alternative explanation is that this result may be due to poor antibody kinetics. Specifically, all antigen-antibody interactions are non-covalent and reversible, thus antibodies have an association and dissociation rate, which is influenced by various factors including the ambient temperature and in particular, lower binding affinities may occur at higher temperatures such as 37°C in comparison with 4°C (Berzofsky et al., 2008).

Moreover, other studies have reported similar findings to those described here using the same one-stage clotting assay; specifically the CT was shown to be cell number- and TF-dependent (via TF neutralizing antibody) in metastatic (MDA-MB-231) and non-metastatic (MCF-10A) breast cancer cells (Berny-Lang et al., 2011), U937 cells stimulated with LPS to induce TF expression (Tormoen et al., 2011), and acute myeloid leukaemia cell lines (NB4, HL60, AML14) (Tormoen et al., 2013). Although the data presented in the current study are based on *in vitro* cultured cancer cells and therefore translation into the *in vivo* human condition remains to be determined, the results may suggest that cancer patients with a higher number of TF-bearing tumour cells are at risk of developing thrombotic complications. In particular, the clinical importance of tumour TF expression in cancer-associated thrombosis has been demonstrated in pancreatic cancer, whereby patients with high TF expression were more likely to develop VTE than those with low TF expression (Khorana et al., 2007a), and two other studies have shown in ovarian cancer patients with VTE that tumour TF expression was significantly higher than in patients without VTE (Uno et al., 2007, Abu Saadeh et al., 2013). Thus, TF may present a target to disrupt coagulation as well as an effective target for anticancer therapeutics, since in addition to its primary role in coagulation TF via TF-dependent signalling is also responsible for tumour growth, metastasis, and angiogenesis (Rickles et al., 2003, Ruf et al., 2011). Furthermore, targeting the initiation phase of coagulation may prevent thrombus formation more efficiently than targeting thrombin in cancer by providing broader suppression of coagulation proteases.

MP are generally characterized as cell-derived particles that are smaller than 1 µm, yet larger than 0.1 µm. The aim of this section was to characterize the PCA of various tumour cell-derived MP *in vitro*. Initially, cell-free supernatants isolated from various cancer cell lines were subjected to ultra high-speed centrifugation (100 000 × *g* for 60 min) to sediment MP, thereby allowing separation from particles smaller than 0.1 µm that remain in the supernatant. However, when the CT was recorded in duplicate for the same sample using the resuspended pellets from two separate thick-wall polycarbonate tubes (Beckman Coulter Ltd, High Wycombe, UK) the results were inconsistent (data not shown), suggesting that not all of the MP had been completely recovered from the tubes. This was subsequently confirmed using the protein stain Coomassie® Blue (Life Technologies Ltd) that demonstrated residual MP in some ultracentrifugation tubes (data not shown). Thus,

ultracentrifugation of cell-free supernatants isolated from cancer cells to selectively obtain MP for further analysis was considered an unfeasible option in this study, and consequently, filtration was used to determine the nature of the PCA mediated by cell-free supernatants (i.e. associated with MP or particles < 0.1 μm/soluble).

In this study, tumour cell-derived MP within the cell-free supernatants of pancreatic (AsPC-1, CFPAC-1), ovarian (ES2, SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cells with relatively high cell surface TF expressions were shown to support coagulation in a dose-dependent manner, as the sequential removal of these tumour cell-derived MP via filtration through 1.2, 0.45, and 0.1 μm pores and subsequent dilution in media resulted in prolonged CT. Whereas, the cell-free supernatants of the other cell lines examined in this study (MIA-PaCa-2, A2780, CaCo-2, Colo320, MCF-7, T47D, H929, JJN3, MM.1S, U266, and U937), that were previously demonstrated to possess little or no TF by flow cytometry, also exhibited little or no PCA. This may suggest that either these cancer cell lines do not shed adequate numbers of MP or that the MP that are released do not have sufficient TF to trigger coagulation.

Interestingly, around 46, 38, 28, 26, 17, and 14% of the PCA mediated by cell-free supernatants of CFPAC-1, MDA-MB-231, SKOV-3, AsPC-1, ES2, and Lovo, respectively, was associated with secreted particles smaller than 0.1 μm following filtration. Similarly, Davilia et al. filtered MDA-MB-231-derived MP through a 0.1 μm pore and measured CT via a one-stage clotting assay, although they report that only 23% of the PCA was recovered after filtration (Davila et al., 2008). In contrast, Gheldof et al. recently reported that the largest proportion of the cell-free supernatant PCA (74%) measured by a thrombin generation assay is associated with particles smaller than 0.1 μm following filtration of MDA-MB-231-derived MP (Gheldof et al., 2013). These discrepancies regarding the remaining PCA in MDA-MB-231-derived 0.1 μm filtered cell-free supernatants, may be, at least in part, attributed to the different protocols utilized for cell-free supernatant sample preparation (i.e. centrifugation speed) and the test used to determine PCA (i.e. one-stage clotting assay versus thrombin generation). It is possible that the PCA of this population of particles, smaller than 0.1 μm, corresponds to tumour cell-derived exosomes (50 to 100 nm) (Garnier et al., 2012) and/or soluble asTF antigen secreted by cancer cells (Haas et al.,

2006). However, the majority of studies suggest that asTF does not trigger coagulation (Yu and Rak, 2004, Hobbs et al., 2007, Censarek et al., 2007, Boing et al., 2009).

Davila et al. have previously demonstrated that tumour cell-derived MP exhibit strong TF-dependent PCA *in vitro* and *in vivo* (Davila et al., 2008), and other studies have associated tumour cell-derived TF+ MP with thrombosis in both experimental models (Thomas et al., 2009, Wang et al., 2012) and human cancer patients (Tesselaar et al., 2007, Zwicker et al., 2009). In this study, one of the aims was to characterize the TF expression of tumour cell-derived MP within the cell-free supernatants of all cancer cell lines examined. However, flow cytometry failed to detect sufficient numbers of MP in the cell-free supernatant of various cancer cell lines for analysis (data not shown). It is possible that the majority of cancer cell secreted MP may be below the detection limit of the flow cytometer (500 nm). For example, a recent sizing study of MP derived from various human cellular sources (including, plasma, cancer cells, and placental villi cells), stimulated by several methods (such as, calcium ionophore treatment or serum starvation *in vitro*) demonstrates that most MP are spherical having diameters from 150 to 400 nm by direct-imaging cryogenic-transmission electron microscopy, although each population of MP was heterogeneous, showing diverse morphologies (Issman et al., 2013). Similarly, another study using 3 different sizing techniques for MP, including transmission electron microscopy, atomic force microscopy, and dynamic light-scattering analysis, has shown that most plasma-derived MP have a diameter of between 80 and 400 nm. While, Gheldof et al. estimated by transmission electron microscopy that the mean (±SD) size of MDA-MB-231-derived breast cancer MP was 121 ± 54 nm with a majority of MP between 100 and 200 nm following 10,000 x *g* centrifugation of the cell-free supernatant.

Therefore in this study, the TF antigen levels were quantified via ELISA in unfiltered and 0.1 µm filtered cell-free supernatants derived from various tumour cell lines. TF expression was detected in cell-free supernatants derived from pancreatic (AsPC-1, CFPAC-1), ovarian (SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cells, but surprisingly, the TF concentration did not noticeably vary between unfiltered and 0.1 µm samples, suggesting that only minute quantities of TF is associated with tumour cell-derived MP. Although unfiltered cell-free supernatants containing suspensions of MP are associated with a considerably higher PCA in comparison to respective 0.1 µm filtered samples. TF is known

to circulate in active and encrypted forms, which may explain, at least in part, the discrepancy between the quantified TF antigen levels and PCA observed in the unfiltered and 0.1 µm filtered cell-free supernatants from the 5 cancer cell lines (AsPC-1, CFPAC-1, SKOV-3, LoVo, and MDA-MB-231) examined in this study, i.e. a larger proportion of TF expressed on tumour cell-derived MP may be active in comparison with the TF present in the 0.1 µm filtered cell-free supernatants. In addition, it is possible that this may be a result of other factors such as the PS content of MP that may be primarily contributing to the faster CT of unfiltered cell-free supernatants isolated from these cancer cells. Another possible explanation is that the quantity of TF in these samples has potentially been underestimated by this assay as TF+ MP may have blocked the smaller TF-expressing particles (< 0.1 µm) or asTF from binding to the 2D anti-TF antibody coated platform, which were subsequently able to bind following the removal of MP in the 0.1 µm filtered cell-free supernatant samples.

The highest TF concentration measured in the unfiltered cell-free supernatants containing suspensions of MP derived from pancreatic (AsPC-1, CFPAC-1), ovarian (SKOV-3), colorectal (LoVo), and breast (MDA-MB-231) cancer cells were 48, 124, 16, 10, and 61 pg/ml, respectively. While, Davila et al. have shown the presence of 130 pg/ml TF on L3.6pl pancreatic cell line-derived MP generated *in vitro* that was 5.5 times less TF than that measured in the plasma of pancreatic (L3.6pl) tumour-bearing mice with the highest concentration of circulating TF (Davila et al., 2008). Intriguingly, L3.6pl pancreatic cancer cells demonstrated higher PCA *in vitro* in comparison with the *in vivo* models (Davila et al., 2008). Thus, the *in vitro* one-stage clotting assay used in this study and other studies may not accurately reflect the *in vivo* situation, in which the hypercoagulable state is more complex. For example, in the tumour microenvironment TF expression may also be induced on other cell types via the secretion of various factors, such as tumour cell-derived TF+ MP that have been shown to transfer TF antigens to the surface of endothelial cells (Yu et al., 2008, Collier et al., 2013). These other sources of TF together with tumour cell-derived TF expression may contribute to the high plasma levels of TF reported in cancer patients. However, high TF expression *in vivo* may not necessarily equate to initiation of coagulation as TF activity is regulated by endothelial cell TFPI and TF encryption.

To put the levels of TF antigen described in this section into both physiological and pathological context, one study reported a mean (±SD) plasma circulating TF antigen level of 91 ± 62 pg/ml from 16 healthy individuals, and in comparison, the plasma TF antigen level of a lung cancer patient who experienced a high rate of thromboembolic events was 3764 pg/ml (aproximately 41-fold higher than the mean control value). This study, taken together with the relationship described here between the TF expression level of a single cancer cell and PCA, indicate that a critical TF concentration threshold must be reached to stimulate coagulation and increase the risk of thrombosis in cancer patients. Moreover, a recent study has demonstrated that elevated levels of circulating TF+ MP may serve as a novel biomarker for predicting the risk of thrombosis in high-risk populations, such as patients with advanced solid malignancies undergoing chemotherapy, and is useful in determining which patients should receive prophylactic anticoagulation (Zwicker et al., 2013).

In summary, this study offers evidence that cancer cells support coagulation in a cell number- and TF-dependent manner and that high TF expressing cancer cells secrete procoagulant MP in a controlled environment. In light of these findings, it is hypothesized that a high peripheral cell count of TF-expressing tumour cells and the presence of procoagulant tumour cell-derived MP in the circulation possess the potential to stimulate coagulation, and may therefore predict the risk of developing thrombosis in cancer patients. In this context, cancer cells expressing no or low TF would not be expected to increase the risk of thrombosis. Although it would be of interest to determine whether circulating levels of TF are induced/elevated in cancer patients in response to treatments known to provoke thrombogencity, such as chemotherapy and other anticancer therapies. TF and its link to the prothrombotic *in vivo* phenotype of cancer patients requires further investigation.

Chapter 4: Modulation of the procoagulant potential of solid and haematological malignant cells by doxorubicin

4.1 Introduction

As suggested in the previous chapter, the intrinsic properties of tumour cells, such as TF expression, may directly promote a prothrombotic state. Furthermore, the baseline hypercoagulable state in cancer patients may be further potentiated by the effects of chemotherapeutic agents, as has already been discussed in section 1.7. The precise mechanisms that induce chemotherapy-associated thrombosis are unknown, and are most likely to be complex and multifactorial. The cytotoxicity induced in both tumour cells and non-malignant cells by many chemotherapeutic and anticancer agents typically manifests as apoptosis. Indeed, many *in vitro* studies have demonstrated that apoptotic cells acquire procoagulant potential regardless of the stimulus used to induce such apoptosis and the cell type examined (Casciola-Rosen et al., 1996, Greeno et al., 1996, Bombeli et al., 1997, Wang et al., 2001, Ma et al., 2005, Stampfuss et al., 2008, Kim et al., 2011, Yates et al., 2011, Zhou et al., 2011, Boles et al., 2012). It is therefore hypothesised that the increased risk of VTE in cancer patients generated by chemotherapeutic treatment may be, at least partly, attributed to the apoptotic process involving exposure of procoagulant PS on tumour cells (or other chemotherapy damaged cells) and increased release of TF+ MP into the peripheral circulation. This effect may be specifically related to the level of TF expression on the tumour, tumour type, and also the chemosensitivity of the tumour. However, the definitive role of MP in the process of chemotherapy-associated thrombosis remains to be conclusively proven both *in vitro* and in clinical studies.

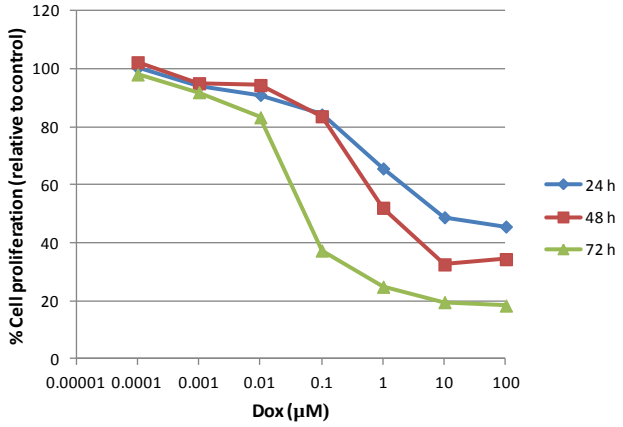
In this chapter the aim was to investigate the direct *in vitro* effect of anticancer agents that are known to increase the risk of VTE in cancer patients on the PCA of various solid and haematological cancer cells with high, moderate, and low TF cell surface expression, and the PCA of tumour cell-derived MP in isolated cell-free supernatants, in an attempt to elucidate the underlying prothrombotic mechanism.

4.2 Determination of chemotherapeutic drug-induced cytoxicity on human cancer cells

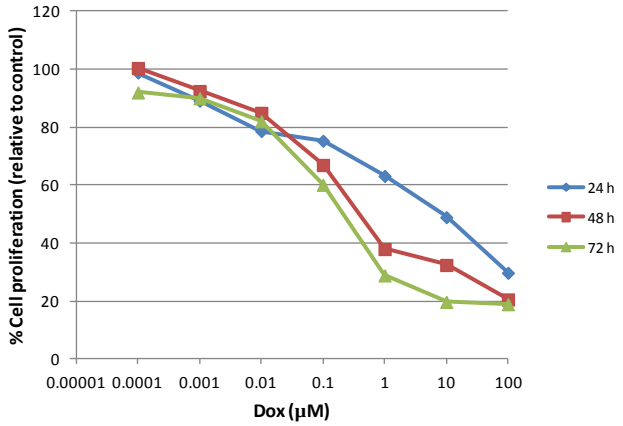
Initially, the antiproliferative effect of novel IMiD, namely Len and Pom (Thal analogues used in myeloma treatment), were assessed in a number of haematological cancer cell lines, including H929, JJN3, MM.1S, and U937. Briefly, a fixed number of cells (1 x 10^5 cells/well for MM cell lines and 2 x 10^4 cells/well for U937) were incubated with varying concentrations of Len and Pom (0-100 μM) and cultured for 24, 48, and 72 h in 96-well tissue culture plates at 37°C, and cell proliferation, relative to the controls (no drug), was determined by an MTS assay (see section 2.4 for methodology). However, direct antiproliferative effects of Len or Pom at concentrations up to 10 μM were not observed *in vitro* with any of the 4 cell lines tested (data not shown). Thus, the sensitivity to Dox, a more general and potent cytotoxic drug that is commonly used in MM combination chemotherapy regimens was assessed with these 4 haematological cell lines. In particular, there was a decrease in the number of viable MM.1S and U937 tumour cells in a time- and dose-dependent manner between 24 and 72 h, and 1 x 10^{-4} and 100 μM, respectively, shown in Figure 4.1 A and B, respectively.

Subsequently, the antiproliferative effect of Dox was assessed with various solid malignant cell lines, specifically ovarian (ES2, SKOV-3) and breast (MDA-MB-231, T47D) cancer cells. These cells (2 x 10^4 cells/well) were also incubated with varying concentrations of Dox (0-100 μM) and cultured for 24, 48, and 72 h in 96-well tissue culture plates at 37°C, and cell proliferation, relative to the controls (no Dox), was determined by an MTS assay, as described in section 2.4. Dox typically decreased the number of viable tumour cells in a time- and dose-dependent manner between 24 and 72 h, and 1 x 10^{-4} and 100 μM, respectively, in 3 of the 4 cell lines tested, specifically ES2 (Figure 4.1 C), MDA-MB-231 (Figure 4.1 D), and T47D (Figure 4.1 E). The 5 Dox sensitive cell lines ES2, MDA-MB-231, T47D, U937, and MM.1S were selected for further assessment.

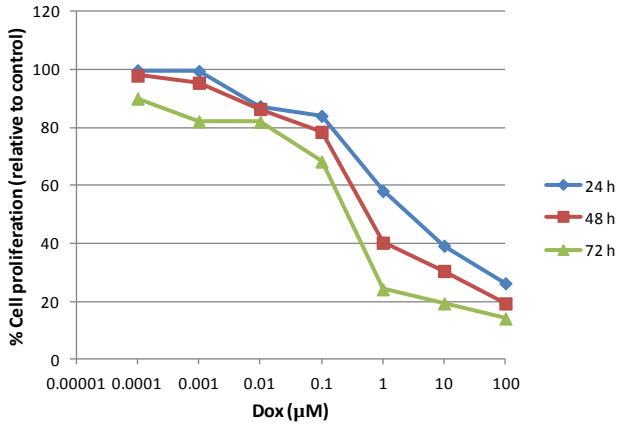
(A) MM.1S



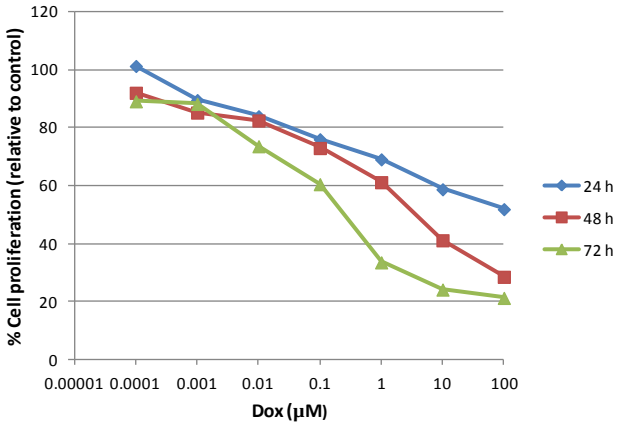
(B) U937



(C) ES2



(D) MDA-MB-231



(E) T47D

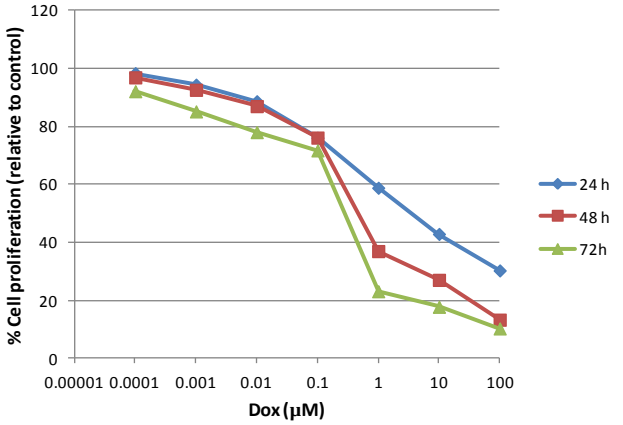


Figure 4.1: Effect of exposure time and concentration on the cytotoxicity of Dox against human cancer cells.

Cell proliferation as a percentage of control (no Dox) of (A) MM.1S, (B) U937, (C) ES2, (D) MDA-MB-231, and (E) T47D cancer cells with increasing concentrations of Dox (0-100 µM) over 24, 48, and 72 h incubation periods at 37°C, measured by MTS assay. Control samples were given the arbitrary value of 100. Data are the average of two independent measurements performed in quadruplicate (C of V < 5%).

4.3 Induction of apoptosis on human cancer cells by Dox

The most commonly used dose schedules of Dox are 60 to 75 mg/m^2 when administered as a single agent by intravenous injection at 21 day intervals, and 30 to 60 mg/m^2 when administered as a single intravenous injection in combination with other chemotherapy drugs every 21 to 28 days. Early pharmacokinetic studies reported that the maximal initial plasma concentration of chemotherapy patients receiving intravenous bolus administration of Dox between 15 and 90 mg/m^2 was approximately 5 µM (Greene et al., 1983, Brenner et al., 1985). However, initial plasma concentrations have typically been

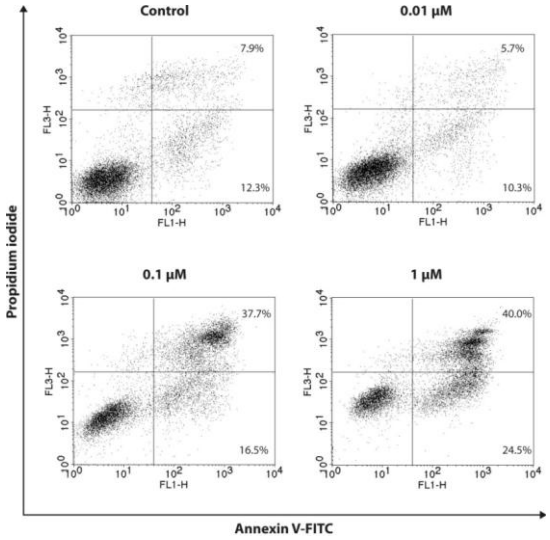
shown to range between 1 and 2 μM, which rapidly decline to approximately 0.025 to 0.25 μM 1 h after bolus injection (Speth et al., 1987, Muller et al., 1993). These levels are similar to the average steady-state concentration of Dox achieved by continuous infusion, around 0.05 μM within 72 h (Greene et al., 1983, Speth et al., 1987, Muller et al., 1993, Berg et al., 1994). Thus, 0.01 and 0.1 μM Dox were chosen as the most therapeutically relevant concentrations to evaluate Dox-induced apoptosis at 48 h, and a higher concentration of 1 μM was used to induce sufficient amounts of cell death for detection of apoptosis based on the Dox-mediated antiproliferative activity demonstrated against all 5 human cancer cells examined *in vitro* in section 4.2. For example, changes in cell proliferation mediated by Dox from 0.01 to 1 μM ranged from 94.4 to 52.1%; 84.9 to 37.9%; 86.4 to 40.2%; 82.6 to 61.3%; and 87.0 to 37.0% in MM.1S (Figure 4.1 A), U937 (Figure 4.1 B), ES2 (Figure 4.1 C), MDA-MB-231 (Figure 4.1 D), and T47D (Figure 4.1 E) tumour cells, respectively, after 48 h incubation. Specifically, haematological (MM.1S, U937), ovarian (ES2), and breast (MDA-MB-231, T47D) cancer cells were incubated with and without various concentrations of Dox (0-1 μM) for 48 h, prior to flow cytometry assessment. Subsequently, FITC-labelled annexin V and propidium iodide binding was measured to detect the extent of PS exposure on outer cell membranes and establish membrane integrity, respectively.

Viable cells expressing no PS with membrane integrity stain negative for both annexin V and propidium iodide and are seen in the lower left quadrants of the flow cytometric dot plots in Figure 4.2 A to E. However, with increasing concentrations of Dox there was a general shift towards PS exposure on the cell surface, as detected by FITC-labelled annexin V binding seen in the lower right quadrants of Figure 4.2 A to E, indicating apoptosis. At higher concentrations of Dox cells become necrotic, exposing PS and have lost membrane integrity, thereby binding annexin V and allowing propidium iodide to pass through to the nucleus, respectively; this dual staining can be seen in the upper right quadrants of Figure 4.2 A to E. The effect of Dox treatment on cell viability and specifically the induction of apoptosis and necrosis varied for each cell line with regards to drug concentration. For example, there was no negative impact of 0.01 μM Dox detected on the viability of MM.1S myeloma cells (83.3% viable) after 48 h incubation, in comparison to the control value (77.6% viable; Figure 4.2 A). In contrast, higher concentrations of Dox, specifically 0.1 and 1 μM, considerably reduced the cell viability from 77.6 (control) to 44.4 and 34.4%,

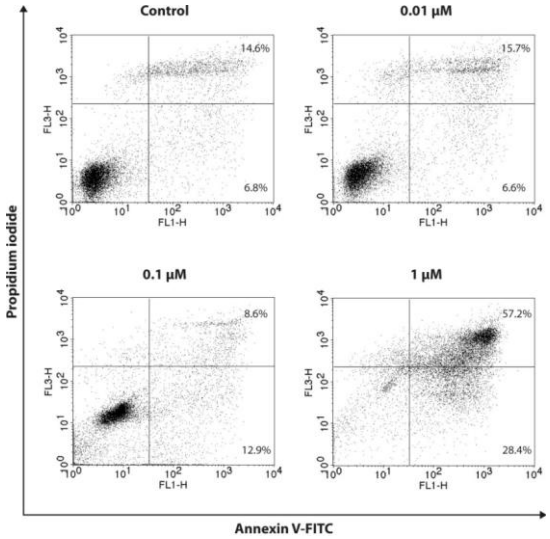
respectively, which was shown to be predominately a result of necrosis as 37.7 (0.1 μM) and 40.0% (1 μM) of necrotic cells were detected in proportion to 7.9% (control; Figure 4.2 A). In addition, an increased proportion of apoptotic cells, specifically 16.5 and 24.5%, were also detected compared with the control value of 12.3% for 0.1 and 1 μM Dox, respectively (Figure 4.2 A). Furthermore, the lower concentrations of Dox (0.01 and 0.1 μM) have little impact on the overall viability of U937 cancer cells (74.0 and 77.3% viable, respectively) compared to the control value (76.5% viable; Figure 4.2 B). However, at 0.1 μM the predominating cell death pathway induced by Dox is apoptosis, with 12.9% apoptotic cells detected in comparison with 6.8 and 6.6% apoptotic cells detected in the control and 0.01 μM Dox treated cells, respectively (Figure 4.2 B). While the proportion of U937 apopotic cells further increases in 1 μM Dox treated cells (28.4%), the majority of cells overall are necrotic (57.2%), relative to the control value (14.6% necrotic) as shown in Figure 4.2 B.

In addition, the lowest concentration of Dox (0.01 μM) had little impact on the viability of ES2 ovarian cancer cells (90.8% viable) compared with the control value (91.0%), but the proportion of apoptotic ES2 cells increased from 3.2% (control) to 15.8% in 0.1 μM Dox-treated cells and became predominately apoptotic (48.7%) at 1 μM, which also induced necrosis (16.9%) compared with the control value (3.7%; Figure 4.2 C). Furthermore, the proportion of MDA-MB-231 apoptotic cells within the population increased from 0.7 (control) to 2.4% 0.01 μM Dox-treated cells, and subsequently increased to 5.9 and 8.6% at higher concentrations of Dox (0.1 and 1 μM, respectively) as shown in Figure 4.2 D. Furthermore, the proportion of MDA-MB-231 necrotic cells increased from 1.6% (control) to 2.6, 8.0, and 9.6% following 0.01, 0.1, and 1 μM Dox treatment (Figure 4.2 D), respectively, although overall the majority of MDA-MB-231 breast cancer cells remained viable (91.1, 83.6, and 76.5%) throughout Dox treatment (0.01, 0.1, and 1 μM, respectively). Whereas, the proportion of T47D apoptotic breast cancer cells considerably increases to 18.7, 20.5, and 33.0% following Dox treatment at 0.01, 0.1, and 1 μM concentrations, respectively, compared to the control value (3.0%; Figure 4.2 E). Although cell death of T47D cells induced by Dox is predominantly apoptotic, there is also a noticeable increase in the proportion of necrotic cells, even at the lowest concentration of Dox (0.01 μM; 12.6%) and up to 22.9% at 1 μM, compared to 3.8% (control; Figure 4.2 E).

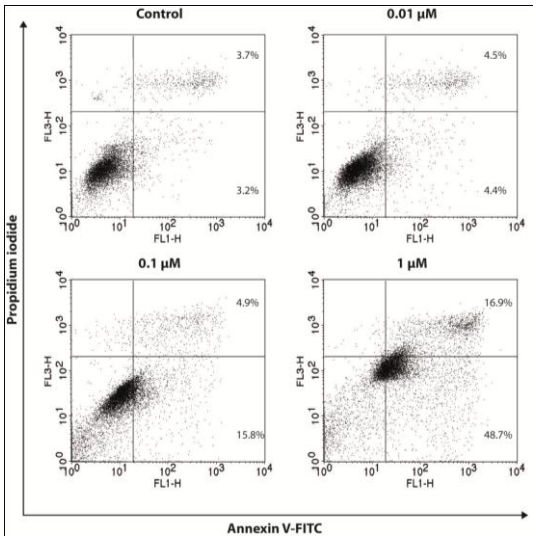
(A) MM.1S



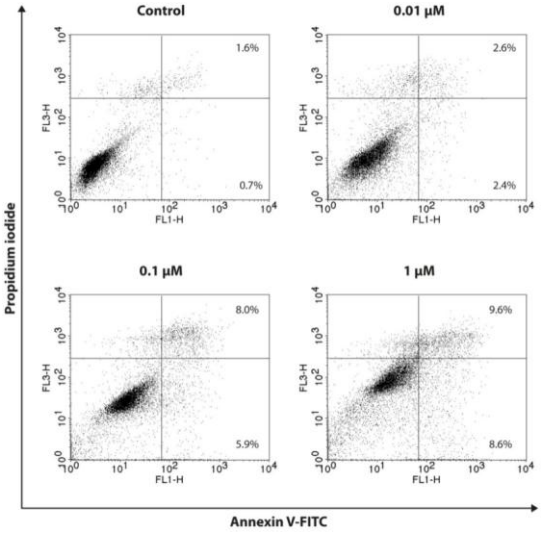
(B) U937



(C) ES2



(D) MDA-MB-231



(E) T47D

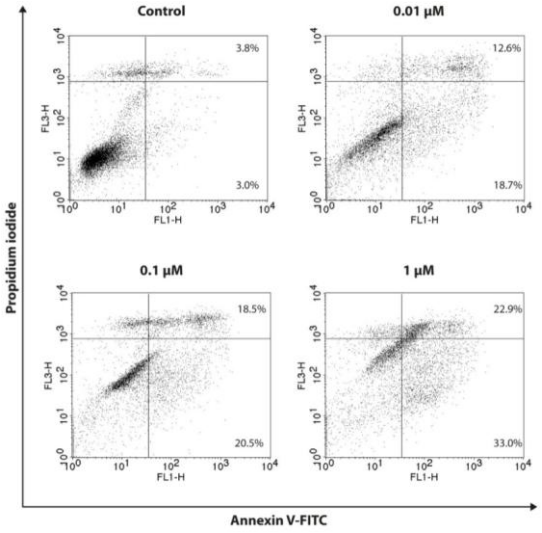


Figure 4.2: Induction of apoptosis and necrosis by Dox in human cancer cells.
Cell viability of (A) MM.1S, (B) U937, (C) ES2, (D) MDA-MB-231, and (E) T47D cancer cells were determined by co-staining with annenxin V:FITC and propidium iodide and analyzed by flow cytometry after 48 h exposure to Dox (0-1 µM). Dot plots depict viable (lower left quadrant), apoptotic (lower right quadrant), and necrotic (upper right quadrant) cells.

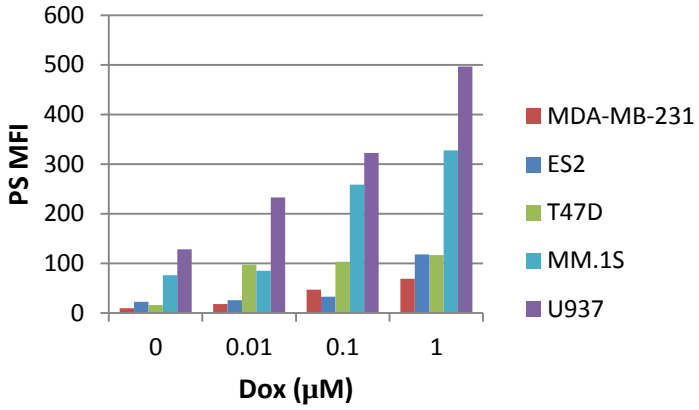
Overall, incubation of haematological (MM.1S, U937), ovarian (ES2), and breast (MDA-MB-231, T47D) cancer cells with various concentrations of Dox (0-1 µM) for 48 h in 25 cm^2 tissue culture flasks, resulted in an induction of apoptosis and subsequently necrosis at higher concentrations. However, necrosis is presumably secondary to apoptosis in cell culture as apoptotic cells are not cleared *in vitro* (Figure 4.2 A to E). These results are

consistent with those described in section 4.2, showing that Dox dose-dependently reduces the cell viability of cancer cells.

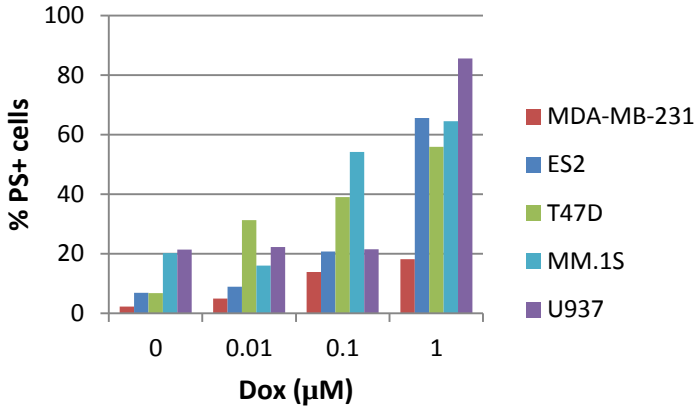
4.4 Effect of Dox on cancer cell surface PS exposure

FITC-labelled annexin V-binding that relates to cell surface PS exposure typically increased relative to controls (no Dox) on haematological (MM.1S, U937), ovarian (ES2), and breast (MDA-MB-231, T47D) cancer cells following Dox treatment (0-1 μM) for 48 h, as demonstrated by the PS MFI in Figure 4.3 A and the total percentage of PS-positive (PS+) cells in Figure 4.3 B. Moreover, the percentage of PS+ cell surface exposure was shown to increase up to 18.6, 55.9, 64.5, 65.6, and 85.6% in MDA-MB-231, T47D, MM.1S, ES2, and U937 tumour cells, respectively after incubation with the highest concentration of Dox used (1 μM) in Figure 4.3 B. In addition, there were 3.2-, 4.0-, 7.9-, 8.2-, and 9.5-fold increases in the total PS expression induced by 1 μM Dox (relative to the controls) in MM.1S, U937, MDA-MB-231, T47D, and ES2 cells, respectively. Results shown in Figure 4.2 A to E suggest that the increase in PS, which is paralleled by increasing concentrations of Dox, is initially caused by the induction of apoptosis. Yet the degree of apoptosis induced by Dox varied with the different cell lines examined. For example, the range of spontaneous apoptosis under baseline control conditions (no Dox) for all cell lines was between 0.7 and 12.3% after 48 h incubation (Figure 4.3 C), while under drug-treated conditions, Dox-induced apoptosis ranged between 2.4 and 18.7% at 0.01 μM; 5.9 and 20.5% at 0.1 μM; and 8.6 and 48.7% at 1 μM concentrations following 48 h incubation (Figure 4.3 C). Overall, there were 2.0-, 4.2-, 11.0-, 12.3-, and 15.2-fold increases in the degree of apoptosis induced by 1 μM Dox (relative to the controls) in MM.1S, U937, T47D, MDA-MB-231, and ES2 cells, respectively.

(A)



(B)



(C)

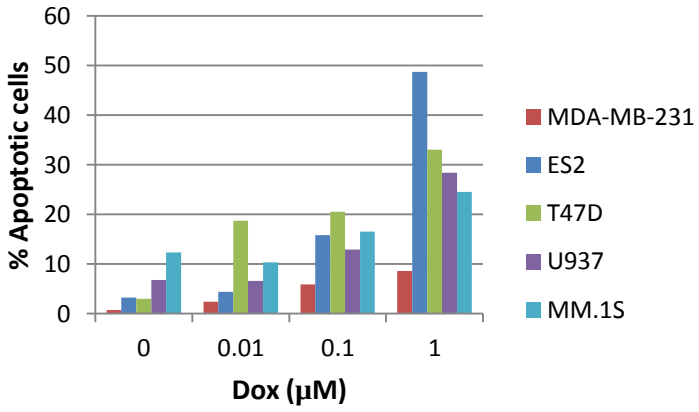


Figure 4.3: The effect of Dox on the enhancement of PS and percentage of apoptotic cells exposure in human cancer cells.

Haematological (MM.1S, U937), ovarian (ES2), and breast (MDA-MB-231, T47D) cancer cells were each incubated in the presence and absence of Dox (0-1 µM) for 48 h and cell surface expression of PS was analyzed by flow cytometry. PS was detected by FITC-labelled annexin V; A) MFI of annexin V-binding cells, B) total percentage of annexin V-binding cells, and C) percentage of apoptotic cells (determined by annexin V-binding and negative propidium iodide staining).

4.5 Effect of Dox on cancer cell surface TF antigen expression

Anti-TF antibody conjuagated to FITC was utilized to measure the degree of cell surface TF expression by flow cytometry, which is responsible for the initial generation of FXa by forming TF-FVII(a) complexes. Dox induces a dose-dependent decrease in cell surface TF expression on those cell lines that have detectable TF by flow cytometry, specifically ES2 and MDA-MB-231 tumour cells (4.4 to 1.4 MFI and 18.2 to 11.7 MFI by 0 to 1 µM concentrations of Dox, respectively; Figure 4.4). Whereas, the baseline level of TF (1.1 MFI) on T47D cells was reduced to undetectable levels following 48 h incubation with 0.01, 0.1, and 1 µM Dox (data not shown). In addition, TF expression was not observed on MM.1S and U937 cells nor affected by Dox (0.01, 0.1, and 1 µM, 48 h incubation; data not shown).

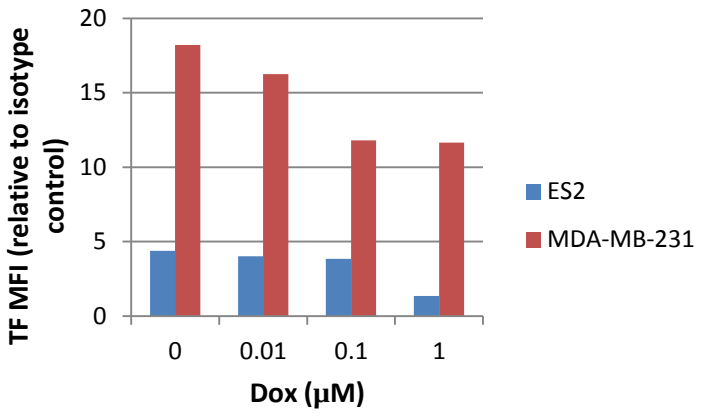


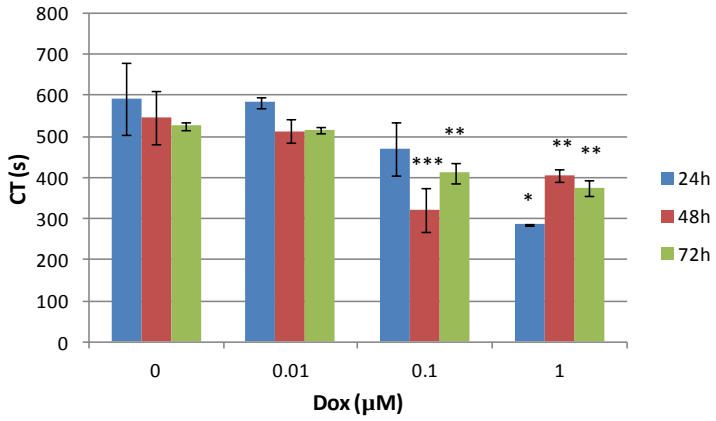
Figure 4.4: The effect of Dox on TF expression in human cancer cells.
After 48 h treatment with various concentrations of Dox (0-1 µM) there was a dose-dependent reduction of TF expression by ovarian (ES2) and breast (MDA-MB-231) cancer cells, determined by flow cytometry.

4.6 Effect of Dox on cancer cell surface-mediated PCA

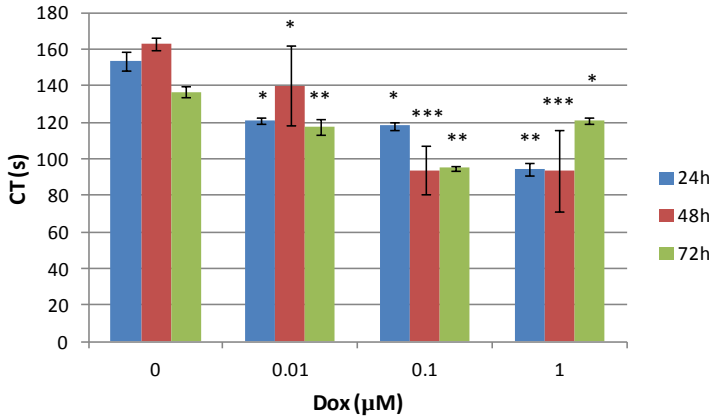
Given the decrease in cell viability mediated via the induction of apoptosis, and subsequent increased cell surface PS exposure in the 5 cancer cell lines tested with increasing levels of Dox, the ability of drug-treated cancer cells to generate fibrin in recalcified plasma was subsequently measured, as PS exposed on cell membranes provides a site for the assembly of coagulation complexes (Zwaal et al., 1998). Briefly, cancer cells were treated with various concentrations of Dox (0.01, 0.1, and 1 µM) similar to therapeutic levels for 24, 48, and 72 h, which was then removed via washing the cells in

PBS twice. Subsequently the resuspended cells were incubated with recalcified plasma and the CT assessed in comparison with untreated cells, as described in section 2.6.1. Dox was typically shown to dose-dependently decrease the CT (thus enhancing coagulability) in all cancer cells examined (MM.1S, U937, ES2, MDA-MB-231, T47D) in comparison to negative controls (no Dox) shown in Figure 4.5, except at the highest concentration (1 μM) for ES2 and MDA-MB-231 cells, whereby the CT was significantly increased (Figure 4.5 C and D, respectively). This decrease in coagulability by ES2 and MDA-MB-231 tumour cells following incubation with 1 μM Dox may be explained by the 3.1- and 1.6-fold decrease in TF cell surface expression observed at 48 h, respectively, in comparison with untreated controls (seen in Figure 4.4). Thus, the level of TF may have fallen below the critical amount required to sustain the baseline PCA, despite the increased exposure of procoagulant PS on the cell surface of these cells.

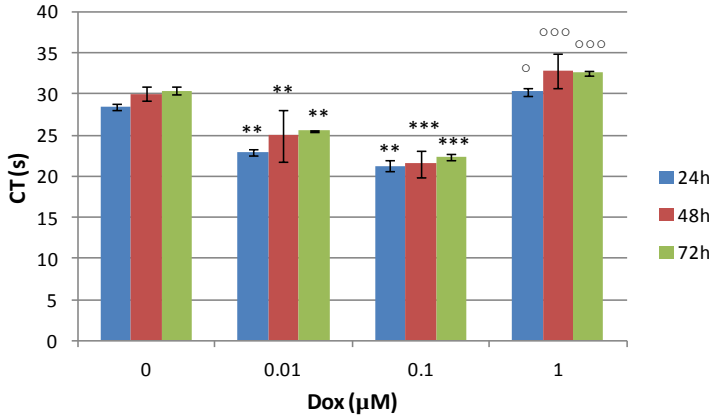
(A) MM.1S



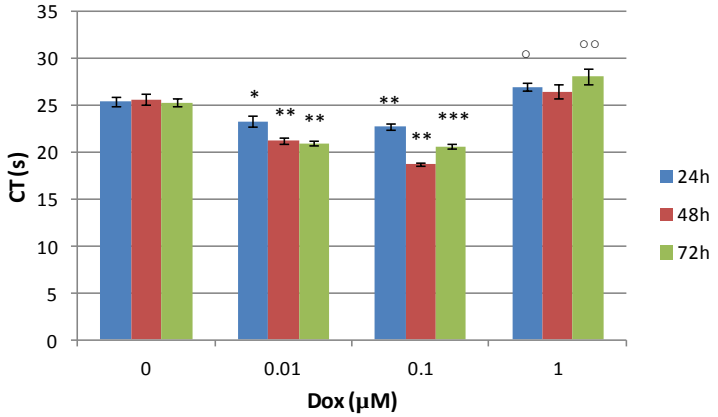
(B) U937



(C) ES2



(D) MDA-MB-231



(E) T47D

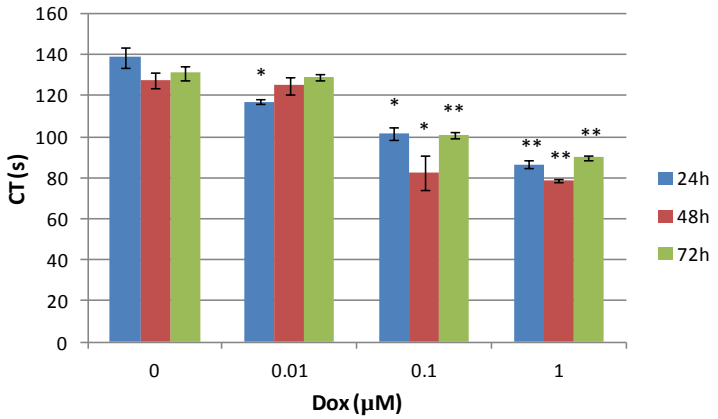
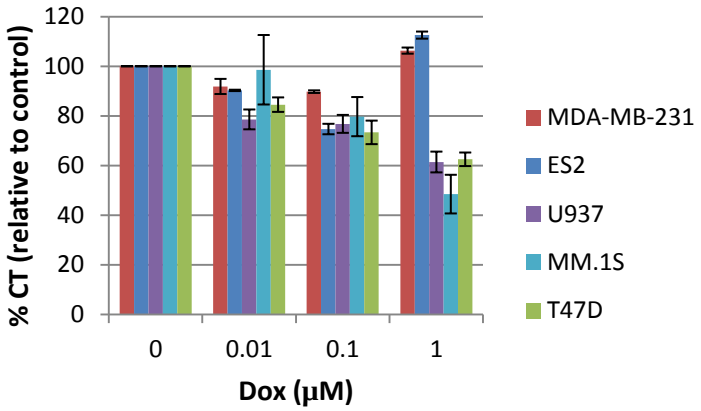


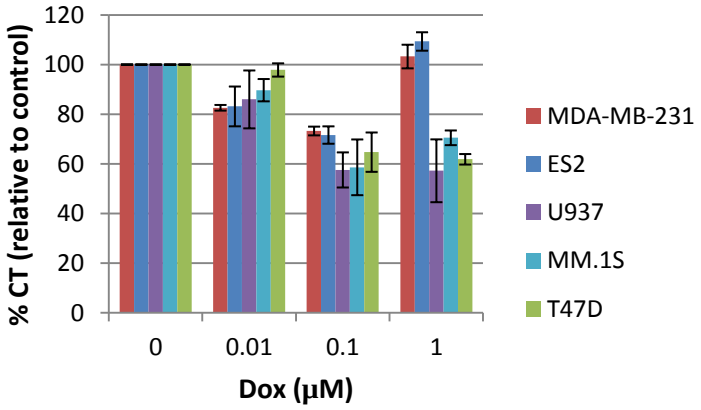
Figure 4.5: Effects of Dox on human cancer cell surface PCA.
(A) MM.1S, (B) U937, (C)ES2, (D) MDA-MB-231 and (E) T47D cancer cells were each incubated in the presence and absence of Dox (0-1 µM) for 24, 48, and 72 h. CT was measured using a one-stage clotting assay. Data are the mean ± SD of two independent measurements performed in triplicate. The paired Student's t test was used to evaluate the level of difference in the CT of Dox-treated cells relative to untreated cells. Significant decreases and increases with $P < 0.05$ are indicated by * or ○ compared to controls (no Dox), respectively. Whereas, ** or ○○ indicate significant decreases and increases with $P < 0.01$, respectively, and *** or ○○○ with $P < 0.001$ compared to controls (no Dox), respectively.

Furthermore, the percentage change in CT relative to the control (no Dox) for each cell line following Dox treatment was highly variable with regards to drug dose and incubation time (Figure 4.6 A to C). However, the CT was considerably reduced (or the coagulability increased) for all cell lines at 0.1 µM Dox following 48 h incubation.

(A) 24 h



(B) 48 h



(C) 72 h

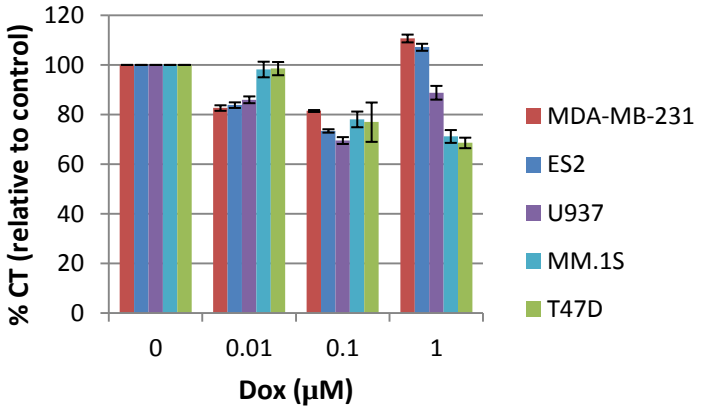
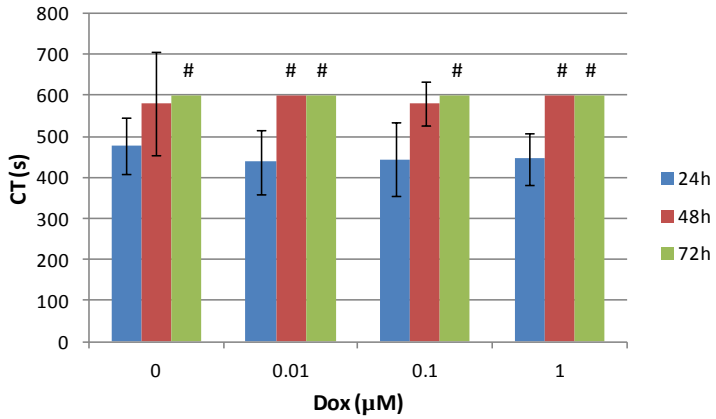


Figure 4.6: Percentage change in human cancer cell surface PCA following Dox treatment.
Haematological (MM.1S, U937), ovarian (ES2), and breast (MDA-MB-231, T47D) cancer cells were each incubated in the presence and absence of Dox (0-1 µM) for (A) 24 h, (B) 48 h, and (C) 72 h. CT was measured using a one-stage clotting assay. Data represent the mean percentage change of CT calculated for each cell line relative to controls (no Dox; where controls are CT adjusted to 100%) ± SD of two independent measurements performed in triplicate.

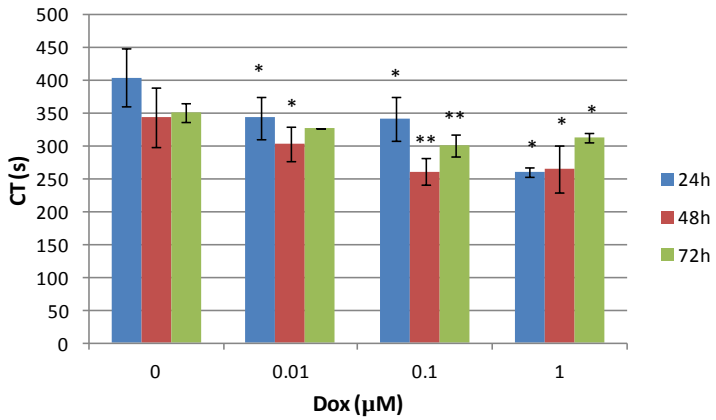
4.7 Effect of Dox on the PCA of tumour cell-derived MP

The procoagulant potential of cell-free supernatants isolated from the 5 cancer lines examined in this study were assessed in the absence and presence of Dox as Dox-associated reduction of cell viability was induced initially by apoptosis, which may lead to an increased release of procoagulant tumour cell-derived MP into the conditioned cell culture media. Briefly, cells were incubated with Dox (0-1 μM) for 24, 48, and 72 h, and cell-free supernatants were collected via double centrifugation; initially at 300 x *g* for 5 min to remove tumour cells, and then the extracted supernatant was spun again at 1000 x *g* for 5 min to remove larger cell fragments. Subsequently, the CT of untreated and Dox-treated cell-free supernatants was assessed using a one-stage clotting assay described in section 2.6.1. The effect of Dox on the cell-free supernatant CT was not consistent across all 5 cancer cell lines tested. For example, the CT of U937 isolated suspensions of MP significantly decreased across all Dox concentrations (0.01, 0.1, and 1 μM) at 24, 48, and 72 h ($P < 0.05$, paired Student's *t* test), shown in Figure 4.7 A. Furthermore, suspensions of MP derived from ES2 and MDA-MB-231 cells were previously shown to possess strong procoagulant properties in section 3.4, and the cell-free supernatants of these two cell lines treated with 0.01 and 0.1 μM Dox significantly reduced the CT ($P < 0.01$, paired Student's *t* test), thus increasing the coagulability, in comparison to untreated control cells across 24, 48, and 72 h incubation periods (Figure 4.7 C and D, respectively). However, at 1 μM Dox the cell-free supernatant CT of ES2 and MDA-MB-231 is significantly increased ($P < 0.05$, paired Student's *t* test), thus decreasing the coagulability, across 24, 48, and 72 h (Figure 4.7 C and D, respectively). Furthermore, the effect of Dox on the cell-free supernatant PCA of those cell lines previously shown to possess little PCA associated with MP, specifically MM.1S and T47D, was minimal with no significant changes shown relative to the controls in Figure 4.7 B and E, respectively), except for T47D following 24 h incubation with 0.01 μM Dox where the CT was found to significantly decrease ($P = 0.035$, paired Student's *t* test; Figure 4.7 E).

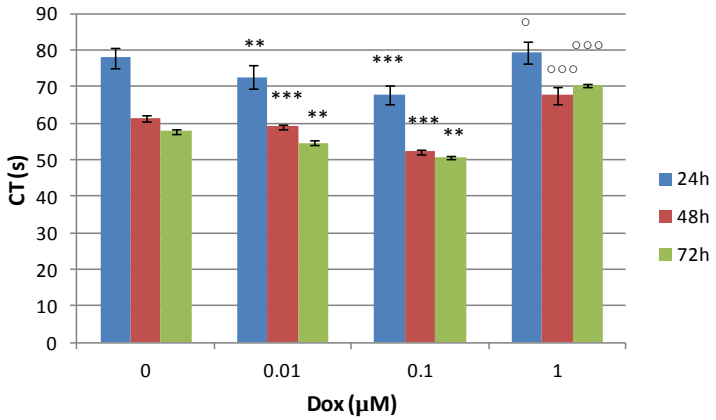
(A) MM.1S



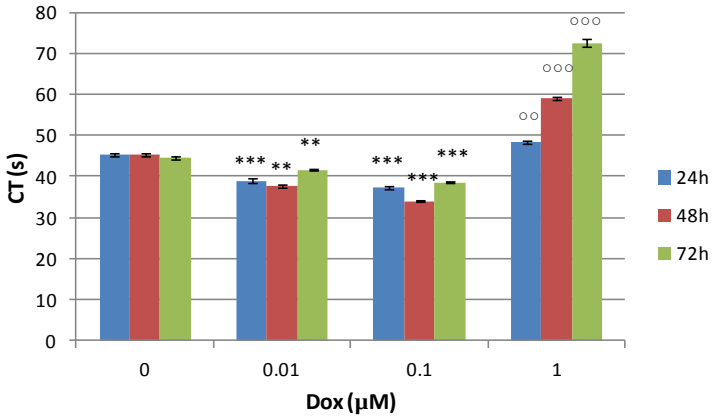
(B) U937



(C) ES2



(D) MDA-MB-231



(E) T47D

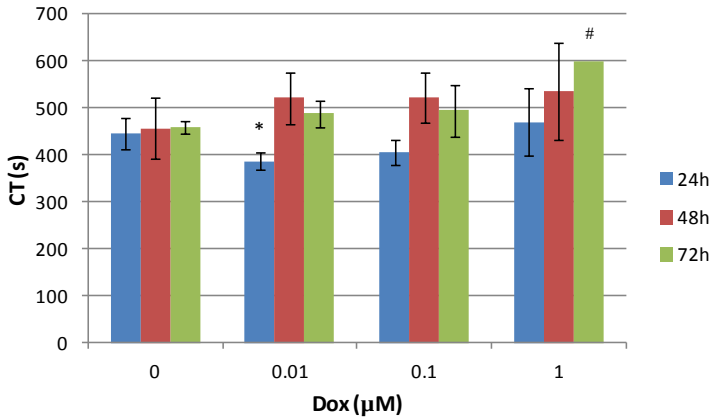
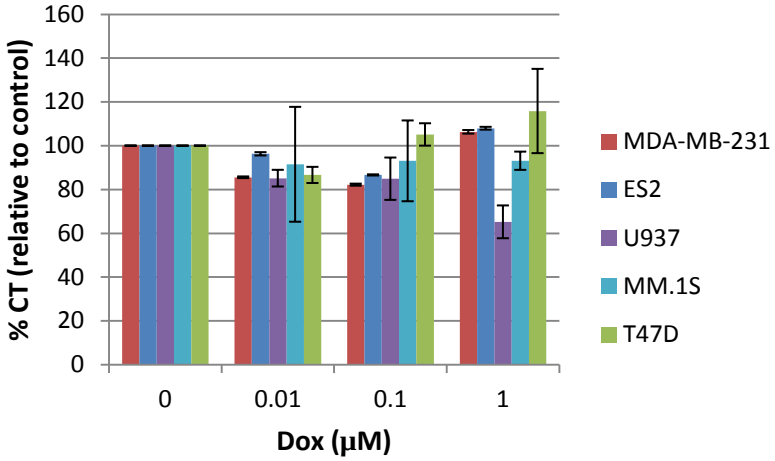


Figure 4.7: Effects of Dox on cell-free supernatant PCA isolated from human cancer cells
(A) MM.1S, (B) U937, (C)ES2, (D) MDA-MB-231, and (E) T47D cancer cells were each incubated in the presence and absence of Dox (0-1 µM) for 24, 48, and 72 h. The cell-free supernatant was isolated from each cell line and CT was measured using a one-stage clotting assay. Data are the mean ± SD of two independent measurements performed in triplicate. The paired Student's t test was used to evaluate the level of difference in the CT of the cell-free supernatant isolated from Dox-treated cells relative to the cell-free supernatant isolated from untreated cells. Significant decreases and increases with $P < 0.05$ are indicated by * or ○ compared to controls (no Dox), respectively. Whereas, ** or ○○ indicate significant decreases and increases with $P < 0.01$, respectively, and *** or ○○○ with $P < 0.001$ compared to controls (no Dox), respectively. # indicates sample did not clot.

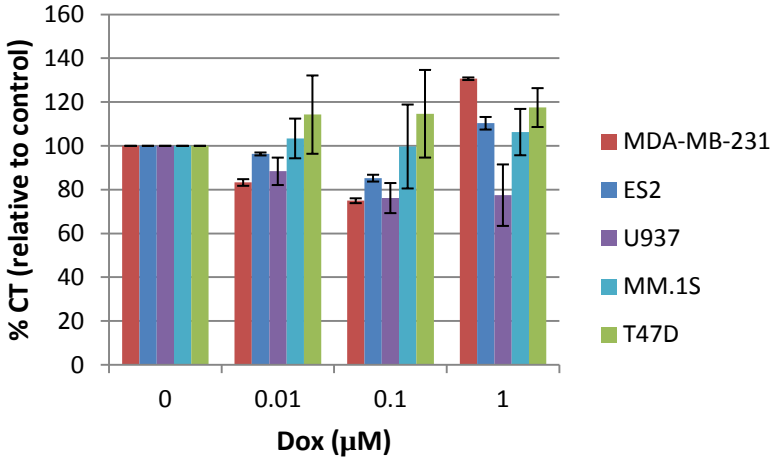
Similar to the cell-mediated PCA, the percentage change in the cell-free supernatant CT relative to the control (no Dox) for each cell line following Dox treatment was highly variable with regards to drug dose and incubation time (Figure 4.6 A to C). However, the CT was noticeably reduced by approximately 15 to 20% in those cell lines that demonstrate PCA associated with MP, specifically ES2, MDA-MB-231, and U937 at 0.1 µM Dox following 24, 48, and 72 h incubation (Figure 4.8 A to C). Furthermore, the effect of Dox on the cell-free supernatant PCA was found to be associated with MP since the CT of

the cell-free supernatants, isolated from all 5 cancer cell lines, that were treated with various concentrations of Dox (0-1 μM) considerably increased, thus the coagulability decreased, following 0.1 μm filtration; representative graphs for ovarian ES2 tumour cells following 24, 48, and 72 h incubation are shown in Figure 4.9 A to C, respectively.

(A) 24 h



(B) 48 h



(C) 72 h

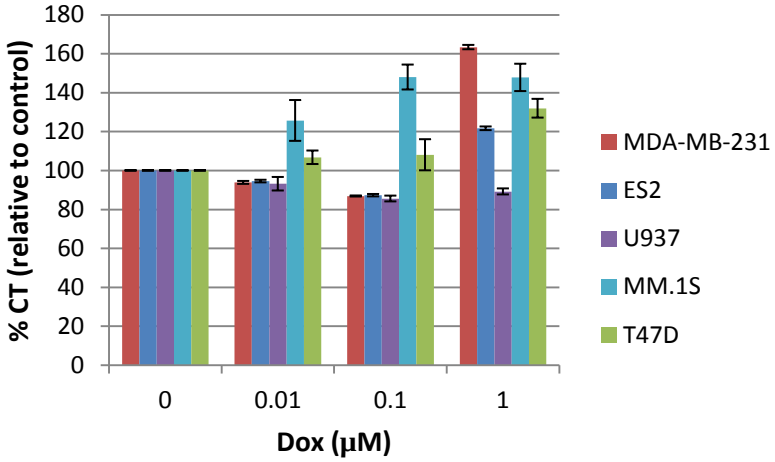
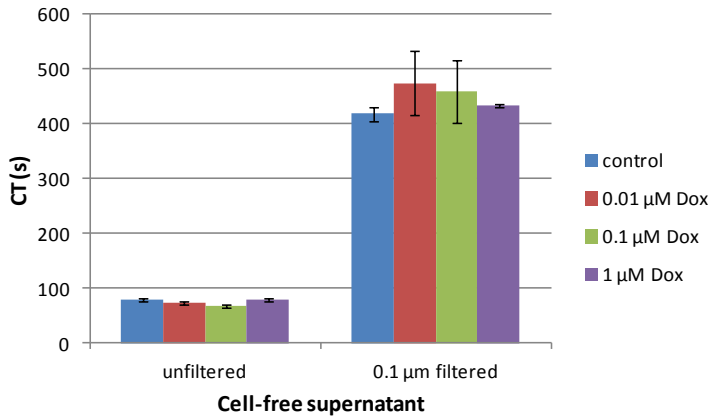


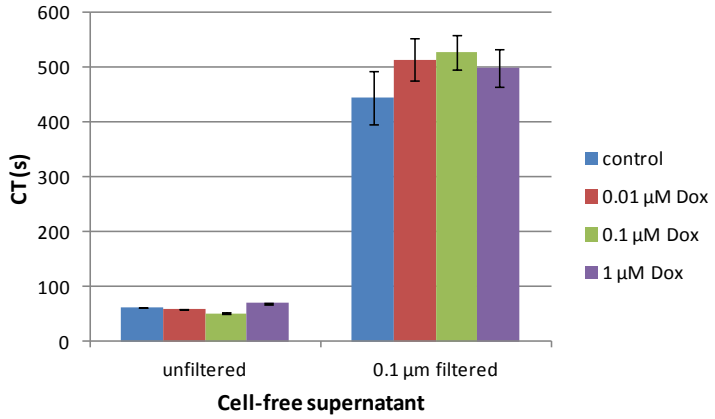
Figure 4.8: Percentage change in cell-free supernatant PCA isolated from human cancer cells following Dox treatment.

Ovarian (ES2), breast (MDA-MB-231, T47D), and haematological (MM.1S, U937) cancer cells were each incubated in the presence and absence of Dox (0-1 µM) for (A) 24 h, (B) 48 h, and (C) 72 h. The cell-free supernatant was isolated from each cell line and CT was measured using a one-stage clotting assay. Data represent the mean percentage change of CT calculated for each cell line relative to controls (no Dox; where control is CT adujusted to 100%) ± SD of two independent measurements performed in triplicate.

(A) ES2 24 h



(B) ES2 48 h



(C) ES2 72 h

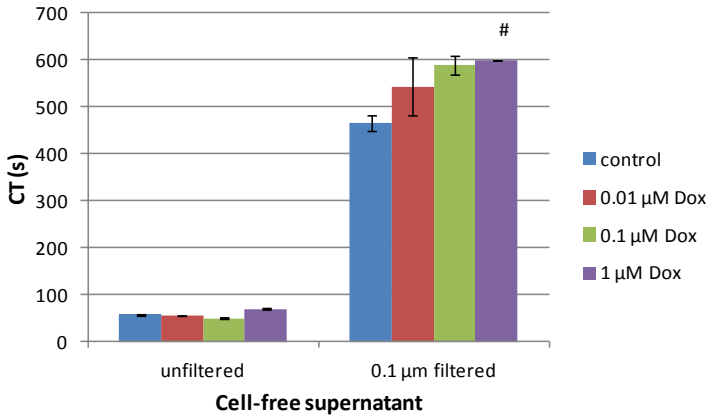


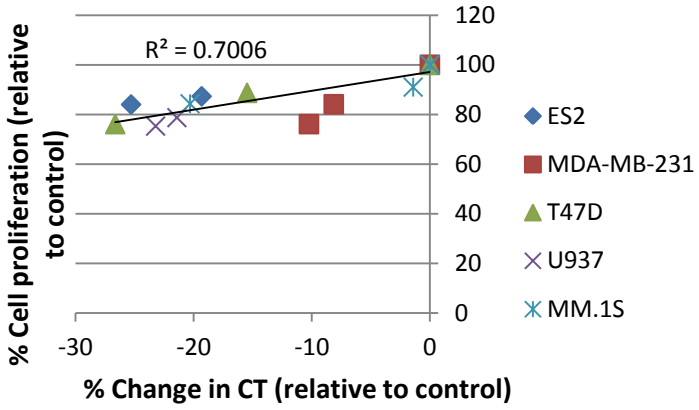
Figure 4.9: Effect of cell-free supernatant 0.1 µm filtration on the PCA to determine the contribution of tumour cell-derived MP to Dox-induced coagulation.

Ovarian ES2 cancer cells were incubated in the presence and absence of Dox (0-1 µM) for (A) 24 h, (B) 48 h, and (C) 72 h. The cell-free supernatant was isolated and CT was assessed using unfiltered and 0.1 µm filtered (to remove MP) samples by a one-stage clotting assay. Data are the mean ± SD of two independent measurements performed in triplicate. # indicates sample did not clot.

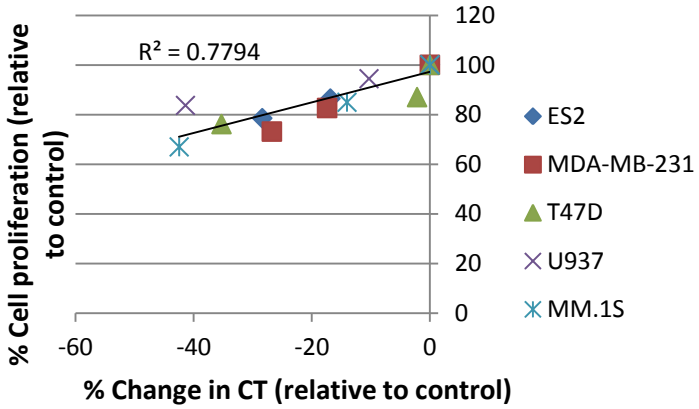
4.8 Effect of cell viability on cancer cell-mediated and cell-free supernatant-mediated PCA

To determine whether or not the increase in drug-induced PCA of ES2, MDA-MB-231, T47D, U937, and MM.1S cells corresponds with the tumour cell viability, 0.01 and 0.1 μM Dox concentrations were selected for further study as these are the most therapeutically relevant concentrations. The percentage change in CT (relative to controls) induced by 0.01 and 0.1 μM Dox (seen in section 4.6) were plotted against the matched cell viability, (previously determined by an MTS assay in section 4.2), for all cell lines examined at 24, 48, and 72 h (Figure 4.10 A to C, respectively). Statistical correlations (Pearson's correlation coefficient r = 0.79 to 0.88, $P < 0.01$) were found to exist between enhanced PCA and reduced cell viability for all cell lines tested at 24, 48, and 72 h, respectively, in terms of observed decreases in cell-mediated CT with decreasing cell proliferation.

(A) 24 h



(B) 48 h



(C) 72 h

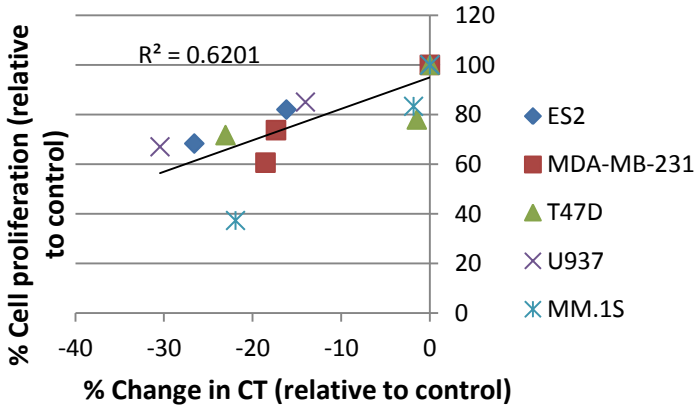


Figure 4.10: The Dox-enhanced cancer cell surface-mediated PCA is related to cell viability.
The percentage of cell proliferation (relative to control) plotted against the percentage change in CT induced by Dox treatment (0.01 and 0.1 µM) after (A) 24 h (B) 48 h, and (C) 72 h in ES2, MDA-MB-231, T47D, U937, and MM.1S cancer cell lines.

To extend this further, the percentage of change in CT (relative to controls) induced by 0.01 and 0.1 μM Dox (seen in section 4.6) were also analysed against the matched percentage of apoptotic cells (previously determined by flow cytometry in section 4.3) for all cell lines examined shown in Figure 4.11. The CT was shown to decrease, thus increasing the coagulability, with increasing numbers of Dox-induced apoptotic cells (with the exception of MM.1S at 0.01 μM Dox), suggesting that tumour cells become procoagulant through the enhanced exposure of PS as a result of chemotherapy-induced apoptosis. However, there was no statistical correlation (Pearson's correlation coefficient r = 0.50, P = 0.055) found between PCA and the degree of apoptosis for all cell lines combined (Figure 4.11).

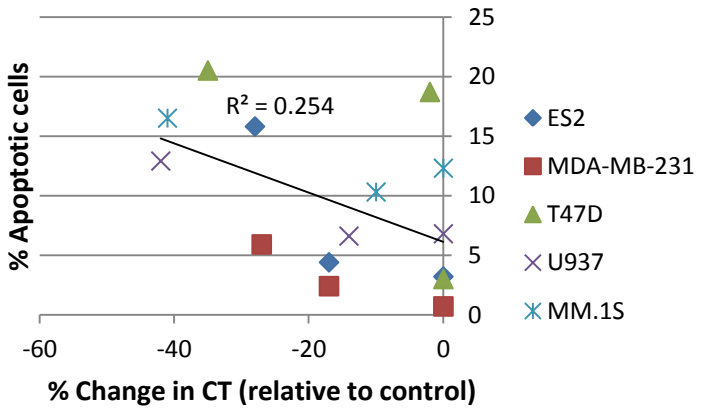
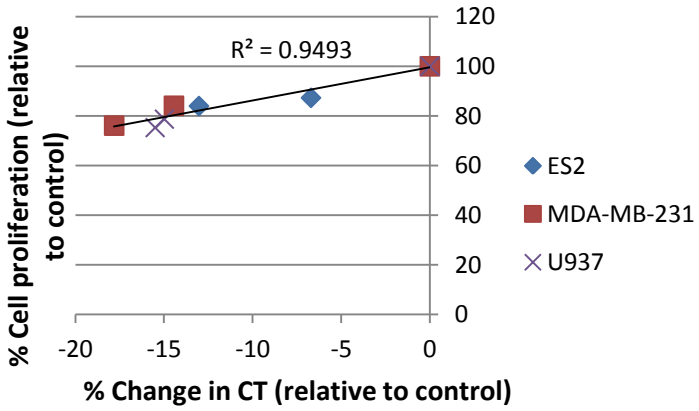


Figure 4.11: Relationship between Dox-enhanced cancer cell surface-mediated PCA and degree of apoptosis. The percentage of apoptotic cells plotted against the percentage change in CT induced by Dox treatment (0.01 and 0.1 μM) after 48 h in ES2, MDA-MB-231, T47D, U937, and MM.1S cancer cell lines.

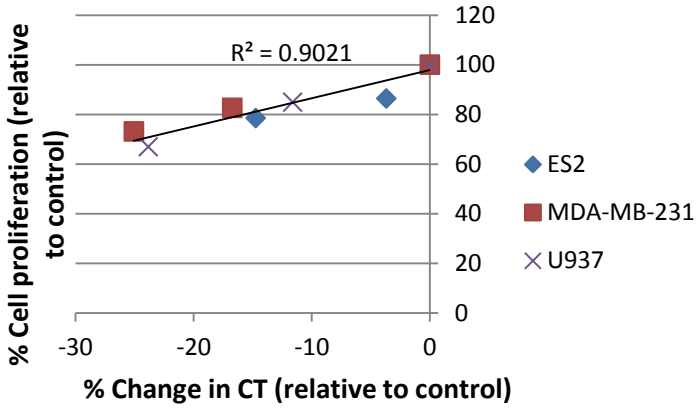
Furthermore, to establish whether or not a similar relationship exists between the drug–induced increase in cell-free supernatant-mediated PCA with the tumour cell viability, the cell lines with procoagulant potential associated with MP, specifically ES2, MDA-MB-231, and U937, were selected for further study at 0.01 and 0.1 μM Dox concentrations (similar to therapeutic concentrations). The percentage of change in cell-free supernatant CT (relative to controls) induced by 0.01 and 0.1 μM Dox (seen in section 4.7) were plotted against the matched cell viability (previously determined by an MTS assay in section 4.2) for ES2, MDA-MB-231, and U937 at 24, 48, and 72 h (Figure 4.12 A to C, respectively). Statistical correlations (Pearson's correlation coefficient *r* = 0.95 to 0.97, P < 0.01) were found to exist between enhanced PCA and reduced cell viability for ES2, MDA-MB-231,

and U937 at 24, 48, and 72 h, respectively, in terms of observed decreases in cell-free supernatant-mediated CT with decreasing cell proliferation.

(A) 24 h



(B) 48 h



(C) 72 h

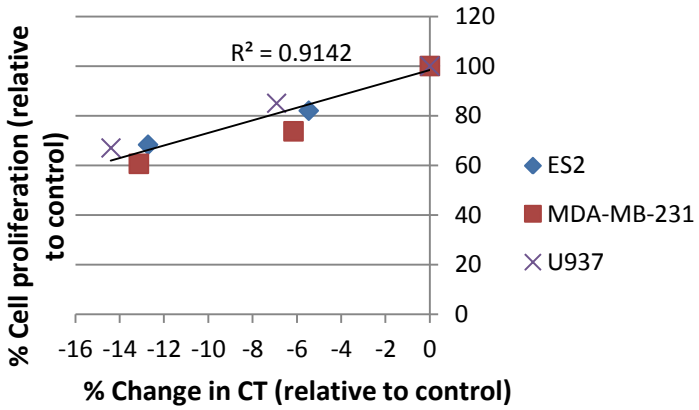
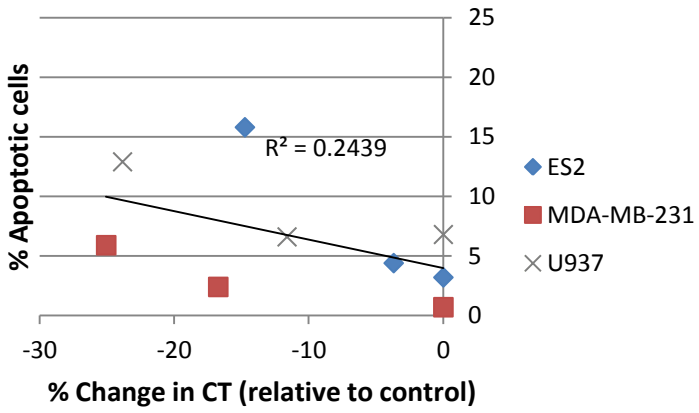


Figure 4.12: Dox-enhanced cancer cell supernatant-mediated PCA is related to cell viability.
The percentage of cell proliferation (relative to control) plotted against the percentage change in CT induced by Dox treatment (0.01 and 0.1 μM) after (A) 24 h (B) 48 h, and (C) 72 h in the conditioned cell supernatants of ES2, MDA-MB-231, and U937 cancer cell lines.

Furthermore, the percentage decrease in cell-free supernatant CT seen in section 4.7 paralleled the increase in apoptotic cells for ES2, MDA-MB-231, and U937 cancer cell lines following 48 h Dox treatment (0.01 and 0.1 μM) previously determined in section 4.2, shown in Figure 4.13, suggesting that apoptosis induction by Dox enhances PCA via tumour cell-derived MP. Although, this relationship was found not to be statistically significant for all 3 cell lines combined (Pearson's correlation coefficient r = -0.50, P < 0.174; Figure 4.13).



4.13: Relationship between Dox-enhanced cancer cell supernatant-mediated PCA and degree of apoptosis.
The percentage of apoptotic cells plotted against the percentage change in CT induced by Dox treatment (0.01 and 0.1 μM) after 48 h in conditioned cell supernatants of ES2, MDA-MB-231, and U937 cancer cell lines.

4.9 Discussion

An association between the occurrence of thrombosis and anticancer treatment with a range of cytotoxic drugs, hormonal therapies, antiangiogenic agents, and IMiD has been established, although the molecular mechanisms by which these agents trigger a prothrombotic state are poorly understood (Furie and Furie, 2006, Falanga and Russo, 2012). In particular, combination chemotherapy with IMiD are associated with a high risk of VTE in MM, up to 35% in newly diagnosed patients (Zangari et al., 2004, Carrier et al., 2011). Thus the initial aim of this study was to examine the effects of the newer IMiD, specifically Len and Pom, on the PCA of various haematological cancer cells, including H929, JJN3, and MM.1S MM cell lines and the lymphoma cell line U937. The various mechanisms of action of IMiD in myeloma involve direct cytotoxicity of malignant plasma cells through caspase-8-mediated apoptosis (Mitsiades et al., 2002) as well as through indirect effects on tumour immunity (Anderson, 2005, Kotla et al., 2009). However, both

Len and Pom failed to elicit cytotoxicity at concentrations of 10 µM and below following 24, 48, and 72 h incubations in any of the 4 haematological cancer cell lines tested in this study, determined by an MTS cell proliferation assay. Previously MM cell lines have been shown to exhibit variable degrees of sensitivity to Len and Pom with typically high concentrations and prolonged exposure of these drugs required to sufficiently reduce cell viability (Zhu et al., 2011). Therefore, subsequent experiments focused on the well-known cytotoxic drug Dox, which was used as an alternative to investigate the hypothesis that chemotherapeutic treatment induces tumour cell apoptosis-associated PCA.

Thrombosis has frequently been encountered with Dox in combination with other chemotherapeutic agents, especially IMiD in MM patients (Zangari et al., 2002b, Zangari et al., 2004, Barlogie et al., 2006). Dox is used in the treatment of a broad range of cancers, including non-Hodgkin's and Hodgkin's lymphoma, MM, acute leukaemias, lung, ovarian, gastric, thyroid, breast, bladder, and sarcoma, among others. The *in vitro* cytotoxic effect of Dox with ovarian (ES2), breast (MDA-MB-231, T47D), and haematological (MM.1S, U937) cancer cells was determined via an MTS cell proliferation assay. In the current study, Dox was shown to reduce the cell viability in a time- and dose-dependent manner in ovarian (ES2), breast (MDA-MB-231, T47D), and haematological (MM.1S, U937) cancer cells, with all cell lines exhibiting relatively similar sensitivity to Dox. The two major mechanisms responsible for the antiproliferative and cytotoxic effects of the anthracycline antibiotic Dox include the intercalation into DNA leading to inhibition of the DNA synthesis or disruption of topoisomerase-II-mediated DNA repair, and the generation of reactive oxygen species (ROS) leading to DNA and cell membrane damage (Gewirtz, 1999).

Moreover, several studies have demonstrated that Dox can induce apoptosis *in vivo* and *in vitro* in various types of cancer cells (Wang et al., 2004, Lerma-Diaz et al., 2006, Du et al., 2012, Harati et al., 2012), and has been shown to be mediated through a number of pathways, such as accumulation of p53, followed by caspase-3 and caspase-9 activation, and DNA fragmentation (Wang et al., 2004). Thus the next aim of this study was to determine the degree of Dox-induced apoptosis in these solid and haematological cancer cells via flow cytometry, using annexin V to stain for exposed PS on the surface of apoptotic and necrotic cells, and propidum iodide a fluorescent dye that ascertains membrane integrity to distinguish apoptotic populations from necrotic. In agreement with

the cell proliferation results, the cell viability was shown to typically decrease in a dose-dependent manner by flow cytometry after 48 h incubation of ovarian (ES2), breast (MDA-MB-231, T47D), and haematological (MM.1S, U937) cancer cells with concentrations of Dox that are similar to therapeutic doses. Moreover, Dox was shown to dose-dependently increase both the number of apoptotic (annexin V+/propidium iodide-) and necrotic (annexin V+/propidium iodide+) cancer cells. The fold-increase in the degree of apoptosis alone and the total PS exposure (apoptosis and necrosis) in cancer cells incubated with various concentrations of Dox relative to the untreated controls was found to be higher in the solid malignant TF-bearing cells (i.e. MDA-MB-231, ES2, and T47D) in comparison with the haematological cancer cells that express no detectable cell surface TF by flow cytometry (i.e. MM.1S and U937). Interestingly, a previous study found that the tumours that stained negative for TF were more frequently resistant to Dox than the tumours bearing TF in 140 patient samples of NSCLC (Koomagi and Volm, 1998).

In the current study, Dox did not induce an upregulation of TF cell surface expression on any of the cancer cell lines examined, as determined by flow cytometry. The expression of TF was actually dose-dependently reduced in Dox-treated breast MDA-MB-231 and ovarian ES2 cancer cells with high and moderate TF expression, respectively, and no TF was subsequently detected on T47D breast cancer cells with low TF expression after Dox treatment. Although the exact mechanism of the reduction of tumour cell surface TF was not investigated in the current study, it is presumably a result of Dox-mediated downregulation of the TF gene *(F3)* and/or the impact of Dox-induced apoptosis on the membrane physiology in which TF may be shed from the tumour cells on the membrane surfaces of MP. Indeed, Swystun et al. observed a downregulation of TF mRNA levels in endothelial cells treated with Dox and another anthracycline chemotherapy drug called epirubicin, although the total cellular amount of TF was not altered (Swystun et al., 2009a).

Subsequently the procoagulant potential of these Dox-treated apoptotic tumour cells and their isolated cell-free supernatant (containing suspensions of MP) was assessed by a one-stage clotting assay. Importantly, Dox was shown to enhance the PCA of ovarian (ES2), breast (MDA-MB-231, T47D), and haematological (MM.1S, U937) cancer cells relative to untreated controls, over various culture incubations and in a dose-dependent manner at

the most therapeutically relevant concentrations (i.e. 0.01 and 0.1 μM). These results are in agreement with other studies investigating the procoagulant effects of Dox and other anticancer agents on tumour cells. For example, thrombin generation was previously shown to be increased in Dox-treated human bladder carcinoma cells (Paredes et al., 2003). Furthermore, in Dox-treated human acute monocytic leukaemia THP-1 cells the PCA was shown to be enhanced via a one-stage clotting assay (Hoshi et al., 2011), and in another study there was a reported dose-dependent increase in the TF activity of THP-1 cells demonstrated with both Dox and another anthracycline chemotherapy drug called daunorubicin (Boles et al., 2012). In an earlier study, enhanced thrombin generation was demonstrated in NB4 and HL-60 human promyelocytic leukaemia cells with the chemotherapeutic agent camptothecin, and a statistical correlation was observed between thrombin generation and camptothecin-induced apoptosis ($P < 0.0005$) in both cancer cell lines (Wang et al., 2001). Similarly in this study, Dox-induced PCA was shown to parallel the observed reduction in cell proliferation, induction of apoptosis, and exposure of PS in all 5 tumour cell lines, although statistically significant correlations ($P < 0.01$) were only found to exist between the cell proliferation and cell-mediated PCA induced by Dox, using all cell line combined data for each incubation time point. However, if more drug concentrations between 0.01 and 0.1 μM Dox had been examined in this study, thereby allowing each cell line to be analysed individually, significant correlations may have been identified between the degree of apoptosis and the cell-mediated PCA, as the range of spontaneous apoptosis under baseline conditions varied for all cell lines examined.

PS exposure occurs in the early stages of apoptosis, preceding DNA fragmentation, membrane blebbing, and loss of membrane integrity (Martin et al., 1995). Furthermore, enhanced PS levels on the outer membrane surface of cells potentiates blood coagulation by extending the surface area available for the assembly of coagulation complexes (Zwaal et al., 1998). In this study, it is postulated that Dox exerts its procoagulant effects on tumour cells of different histological types by increasing cell surface PS exposure. However, it is also possible that Dox may induce TF activation through decryption without upregulating TF antigen levels, as the TF activity of these Dox-treated cancer cells was not determined. Given more time, the precise mechanism of Dox-enhanced PCA could have been explored further by measuring TF activity and performing coagulation inhibition assays using annexin V and an anti-TF antibody to determine the definitive contribution of

PS and TF to the PCA of Dox-treated cancer cells, respectively. In particular, the Dox-induced PCA on human acute monocytic leukaemia THP-1 cells has been shown to correlate with cell surface PS exposure as annexin V suppressed PCA in all Dox treated and Dox in combination with Thal samples, relative to baseline levels (Hoshi et al., 2011). Furthermore, the increased TF activity in daunorubicin-treated THP-1 cells ceased after the addition of either annexin V or lacterherin (a PS-binding milk protein) (Boles et al., 2012).

Moreover, at a higher concentration of Dox (1 μM), the PCA of the 2 cancer cell lines bearing the most TF (MDA-MB-231 and ES2) was found to be decreased relative to controls, despite the exposure of procoagulant PS. This may be explained by the observed reduction in both the level of cell surface TF antigen and the number of viable cells (due to enhanced apoptotic and necrotic cell death) in MDA-MB-231 and ES2 cells, which have presumably fallen below the critical thresholds necessary to sustain baseline coagulation, as cell-mediated PCA was shown to be dependent on the cell count and cell surface concentration of TF in the previous chapter. Additionally, previous work in this laboratory has shown that the induction of necrosis decreases the PCA of human pancreatic cancer cell lines relative to control levels (Yates et al., 2011).

In addition to cancer cells, many studies have examined the procoagulant effect of Dox with endothelial cells due to the high association of this chemotherapeutic drug with cardiomyopathy and the *in vitro* and *in vivo* findings that associate Dox chemotherapy with vascular injury, via the induction of endothelial cell apoptosis, leading to endothelial dysfunction (Murata et al., 2001, Wu et al., 2002, Kaushal et al., 2004, Chow et al., 2006, Yamac et al., 2006, Bar-Joseph et al., 2011). Furthermore, Dox has been shown to disrupt the endothelium-based protein C anticoagulant pathway by free radical metabolites, which facilitate decreases in cell surface levels of the endothelial cell protein C receptor that is responsible for the conversion of protein C to the functional anticoagulant activated protein C (Woodley-Cook et al., 2006). The generation of ROS is a common mechanism of action for many chemotherapeutic agents and it has been reported by Swystun et al. that Dox enhanced the thrombin generation of endothelial cells *in vitro* by ROS-induced PS exposure, which facilitated the activation of TF (Swystun et al., 2009a). In support of the concept that anthracyclines induce PCA of cultured endothelial cells through PS exposure, Fu et al. demonstrate that blocking PS by lactadherin dramatically suppresses the PCA of

daunorubicin-treated endothelial cells *in vitro*. Furthermore, Dox has been shown to significantly enhance PCA in platelets *in vitro* and in an *in vivo* rat thrombosis model through increased PS exposure mediated by ROS generation, Ca^{2+} increase, ATP depletion, and caspase-3 activation that induces platelet cytotoxicity and may contribute to the development of thrombocytopenia following treatment (Kim et al., 2009, Kim et al., 2011). In addition, Swystun et al. suggest that the TF activity levels on both endothelial cells and smooth muscle cells *in vitro* are also increased via ROS-mediated mechanism involving PS exposure using the cyclophosphamide chemotherapeutic metabolite acrolein (Swystun et al., 2011a). In another study, the balance between procoagulant and anticoagulant factors in relation to the properties of endothelial cells *in vitro* has been shown to be impaired by a combination of antiangiogenic and chemotherapy drugs, via enhancing TF activity and reducing TFPI, the primary inhibitor of the TF-FVIIa complex (Ma et al., 2005).

The PCA of the cell-free supernatants isolated from ovarian ES2, breast MDA-MB-231, and lymphoma U937 cancer cell lines was enhanced with Dox treatment, specifically at 0.01 and 0.1 µM concentrations that are similar to therapeutic plasma levels *in vivo*, relative to untreated controls, across 24, 48, and 72 h incubation. It is important to note that this PCA was associated with MP as demonstrated via the removal of MP from suspension by 0.1 µm filtration leading to a dramatic suppression of PCA in Dox-treated cancer cells, and also the PCA was only modified by Dox in those cancer cell lines that demonstrate procoagulant potential associated with MP at baseline. This implies that the inherent ability of tumour cells to spontaneously release MP into the circulation may predict the thrombotic risk following chemotherapeutic treatment. Recently this premise was explored in relation to the number of circulating TF+ MP, whereby the risk of VTE was shown to be increased following treatment in patients that had a higher level of circulating TF+ MP prior to chemotherapy in comparison with patients expressing low levels of TF+ MP (Zwicker et al., 2013). Furthermore, the Dox-induced PCA in ES2, MDA-MB-231, and U937 was shown to correspond with the observed reduction in cell proliferation, induction of apoptosis, and PS exposure. However, as similar to findings of the Dox-treated cancer cells, statistically significant correlations ($P < 0.01$) were only found to exist between the cell proliferation and ES2, MDA-MB-231, and U937 cell-free supernatant PCA induced by Dox over 24, 48, and 72 h incubations.

The results from the current study are consistent with other studies that have demonstrated chemotherapy-induced PCA via the induction of apoptosis and the subsequent generation of procoagulant MP. In particular, daunorubicin treatment of human acute monocytic leukaemia THP-1 cells increased the number of TF+ MP with PCA (Boles et al., 2012), while various chemotherapeutic agents, such as cisplatin, daunorubicin, and arsenic trioxide have been shown to increase the number of PS+ EMP with PCA independent of TF (Lechner et al., 2007, Fu et al., 2010, Zhou et al., 2011). In addition, Dox was found to increase the generation of PS+ PMP with PCA (Kim et al., 2011). In contrast to monocyte-derived MP, PMP were shown not to possess TF activity and propagated coagulation by exposing PS and triggering thrombin generation independent of TF in a contact-dependent manner via FXIIa (Aleman et al., 2011, Van Der Meijden et al., 2012). In cases where chemotherapeutic drug treatment does not upregulate cell surface levels of TF, the number of TF+ MP generated through chemotherapy-induced apoptosis will most likely be dependent upon the baseline cellular TF expression.

In summary, this *in vitro* study demonstrates that Dox induces an increase in PS-rich solid and haematological cancer cell membranes, therefore enhancing the cell-mediated PCA and the PCA of tumour cell-derived MP in suspension. These results taken together with some of the above-mentioned findings, suggest that chemotherapy induced apoptosis enhances the PCA of tumour cells, endothelial cells, and platelets by cell surface exposure of PS, and also releases procoagulant MP into the circulation that may contribute to hypercoagulability observed in cancer patients. Thus, it may be necessary to measure cellular biomarkers of tumour and host cell activation/injury (including cell-specific MP), in addition to TF+ MP, in order to elucidate the underlying prothrombotic mechanism in clinical studies.

Chapter 5: Modulation of plasma PCA and release of circulating MP in MM patients after administration of Thal- or Len-based therapies

5.1 Introduction

Although the underlying mechanism of cancer-associated thrombosis is incompletely understood, recent findings suggest that the tumour mass may be partly responsible for the elevated PCA of circulating MP, particularly in pancreatic cancer (one of the most prothrombotic solid malignancies). For example, highly elevated TF activity associated with MP in metastatic pancreatic cancer patients have been demonstrated in a number of studies (Tesselaar et al., 2007, Thaler et al., 2012, Thaler et al., 2013a, Thaler et al., 2014). Furthermore, Thaler et al. correlated TF activity with CA 19-9 levels, a specific tumour marker for pancreatic cancer (Thaler et al., 2013a). Previously, levels of MP expressing both TF and the tumour antigen MUC-1 were shown to decrease after surgical tumour resection in three pancreatic cancer patients (Zwicker et al., 2009), while more recently, the PCA associated with MP was found to be significantly reduced after pancreatectomy (n=18) and the levels of TF+ MP declined, although this difference was not shown to be statistically significant (Echrish et al., 2014). This hypothesis is further supported by Davila et al. demonstrating in an orthotopic mouse model of pancreatic cancer that TF+ MP were directly released from pancreatic cancer cells into the circulation, and in another study Wang et al. show in a xenograft mouse model of pancreatic cancer, that mice with elevated levels of tumour-derived TF+ MP exhibited increased thrombosis in a ferric chloride-induced saphenous vein model (Wang et al., 2012).

The number of TF+ MP and TF activity associated with MP in malignancy have been shown to correlate with markers of haemostatic activation such as D-dimers in a number of studies (Hron et al., 2007, Haubold et al., 2009, Sartori et al., 2011, Bharthuar et al., 2013, Sartori et al., 2013, Thaler et al., 2013a), as previously discussed in section 1.6.4, suggesting that TF+ MP play a key role in the pathogenesis of the hypercoagulable state in cancer. Initially, Khorana et al. prospectively evaluated the prognostic potential of TF activity associated with MP to predict subsequent thrombosis in a small cohort of pancreatic cancer patients (Khorana et al., 2008a), which was recently confirmed in a larger prospective study of pancreaticobiliary cancer patients (n=117), whereby elevated

levels of TF activity associated with MP at the time of diagnosis correlated with future development of thrombosis (Bharthuar et al., 2013). Furthermore, elevated TF activity associated with MP in pancreatic cancer patients leads to significantly shortened overall survival (Tesselaar et al., 2009, Thaler et al., 2012, Bharthuar et al., 2013, Thaler et al., 2013a). Numerous lines of evidence indicate that TF plays an important role in cancer growth and progression via clotting dependent (see section 1.4.1) and clotting independent (see section 1.5.1) pathways (Rickles et al., 2003, Yu et al., 2005). In immunohistochemical studies, high intratumoural TF expression has been associated with increased angiogenesis, advanced stage, and shortened survival in a variety of solid malignancies (Nakasaki et al., 2002, Khorana et al., 2007a, Chen et al., 2010, Abu Saadeh et al., 2013, Echrish et al., 2014).

Haematological malignancies are also associated with a high frequency of thrombosis particularly individuals with MM receiving treatment with IMiD, such as Thal or Len, concurrently with chemotherapeutic drugs or Dex (previously described in more detail in section 1.8.3), in whom, up to 35% may develop VTE in the absence of thromboprophylaxis (Zangari et al., 2004). However, the mechanisms underlying the development of thrombosis in solid malignancies, which appear to be driven by tumour-derived TF, may differ from the mechanisms driving the process in haematological malignancies, as recent evidence demonstrates TF is not expressed by tumour cells in MM (Cesarman-Maus et al., 2012) or lymphoid malignancies of both T- and B-cell origins (Cesarman-Maus et al., 2014). Furthermore, these studies do not exclude a role for TF from non-tumour cell sources such as the activated endothelium, platelets, and monocytes, as a consequence of the host response to cancer or its treatment, in the development of thrombosis or in tumour biology. Indeed, Auwerda et al. recently found that the circulating levels of TF activity associated with MP in untreated MM patients were significantly elevated in comparison with healthy controls, and that these levels remain high in patients who develop VTE, but decline in those that do not (Auwerda et al., 2011). Moreover, the absence of TF on myeloma cells may explain why the development of thrombosis in most of these patients is not predictive of poor overall survival (Zangari et al., 2007, Zangari et al., 2010).

Furthermore, several lines of evidence suggest a contribution of platelets to the pathogenesis of chemotherapy-associated thrombosis in MM as aspirin significantly

reduces the risk of thromboembolic events in low-risk MM receiving chemotherapy with IMiD and was equally efficient to LMWH (Palumbo et al., 2011, Larocca et al., 2012). In particular, increased platelet surface P-selectin expression and platelet-monocyte complexes were detected as a result of Thal treatment in 5 MM patients, suggesting platelet activation (Dunkley and Gaudry, 2007). Following aspirin prophylaxis both P-selectin levels and platelet-monocyte complexes returned to levels of platelet activation seen at baseline in these patients prior to Thal treatment (Dunkley and Gaudry, 2007). It is not currently known whether platelet activation is directly induced by Thal or indirectly via Thal-induced endothelial cell activation. Endothelial dysfunction has been demonstrated in MM patients receiving chemotherapy with IMiD, that may propagate coagulation. In particular, significantly lower circulating levels of the endothelial cell-derived anticoagulant thrombomodulin were seen in 13 relapsed MM patients after the first cycle of low-dose Thal and Dex in comparison with baseline (Corso et al., 2004), while the plasma levels of the endothelial cell injury marker vWF were increased during chemotherapy with either vincristine, Thal, or Bor in combination with Dox and Dex (van Marion et al., 2008).

The number of circulating MP from various cellular origins were evaluated in this study as TF expression reflects only one aspect of MP function, and it is likely that MP influence thrombotic risk through multiple other pathways, including endothelial dysfunction, platelet activation, and PS exposure. The main aims of this chapter were to examine the effects of chemotherapy containing IMiD, such as Thal or Len, on the generation of EMP, PMP, monocyte-derived MP, plasma cell-derived MP, TF+ MP, and PS+ MP, secretion of soluble endothelial dysfunction markers (sCD106 and sCD54), and also the PCA of PFP in MM patients to develop a better understanding of their potential contribution to thrombotic complications.

5.2 Clinical trials

5.2.1 Design and patient groups

The project document was developed for the main clinical trial upon which this thesis is based, entitled ‘study of apoptosis related changes and endothelial responses of MM patients treated with chemotherapy’ that included information regarding the background

and rationale of the study, research aims and objectives, study design and methodology, safety reporting, quality control, data handling, details of those involved in the study and sponsor, along with patient information sheets and patient consent forms (see Appendices A and B for examples, respectively), which was submitted to the relevant bodies via the Integrated Research Approval System. This study was classified as a clinical trial of an investigational medicinal product and granted clinical trial authorisation from the Medicines and Healthcare products Regulatory Agency in 2012. Subsequently, this study was given a favourable opinion from a local Research Ethics Committee (REC; ref 12/YH/0328), and was approved by the Hull and East Yorkshire (HEY) Research and Development (R&D) department. The primary aim was to study the disruption of the endothelium, platelets, and the coagulation cascade caused by novel agents and chemotherapeutic combinations for the treatment of MM.

The highest risk of VTE in MM patients is observed during the first few months of Thal- or Len-based therapies in most studies, but still occurs after several months of therapy (Kristinsson, 2010). Therefore, MM patient blood samples were scheduled prior to chemotherapy administration (T1), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4). Sample collection occurred between October 2012 and October 2013 in this prospective study, which included newly diagnosed or relapsed MM patients treated with standard chemotherapy at the Queen's Centre for Oncology and Haematology, Castle Hill Hospital (CHH; HEY Hospitals NHS Trust). Eligibility criteria included age $\geq$ 18 years; the ability to provide written informed consent; and patients with a confirmed diagnosis of symptomatic MM (based on the presence of a paraprotein in serum and/or urine, bone marrow clonal plasma cells or plasmacytoma, and the presence or absence of related organ or tissue impairment including hypercalcaemia, anaemia, renal insufficiency, or bone lesions) requiring treatment for their myeloma either at presentation or at the time of relapse. While, the exclusion criteria for this study included patients with conditions known or suspected to independently increase levels of MP specifically, patients with 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); patients treated with long term anti-coagulants (e.g. warfarin for DVT or atrial fibrillation); and patients with recent thrombosis still on secondary prophylaxis. In addition, a small number of patients with a confirmed diagnosis of a MPD, specifically either polycythemia vera or myelofibrosis, served as a 'non-myeloma' control group that have a known propensity to develop thromboses irrespective of treatment.

Furthermore, this thesis includes blood samples obtained from pancreatic cancer patients as part of an ongoing clinical trial, entitled 'Pancreatectomy and Tissue Factor', within the Queen's Centre for Oncology and Haematology, CHH (HEY Hospitals NHS Trust) that had previously been approved by a local REC (ref 08/H1305/59) and HEY R&D department. Written informed consent was obtained from all patients prior to study entry. Plasma samples were isolated from whole blood samples from pancreatic cancer patients prior to surgical resection of the tumour between April 2009 and October 2012 (processed by Dr Hussein Echrish prior to April 2011) and analysed for circulating TF+ MP (as described in section 2.5.3). This group served as a 'TF-dependent cancer' control since pancreatic cancer patients have a known propensity to develop thromboses, which is believed to be mediated by high tumour TF expression and elevated circulating tumour cell-derived TF+ MP (Echrish et al., 2014). Clinical and demographic data was collected for all patients recruited to both clinical trials by trial unit data managers (see example Case Report Form [CRF] in Appendix C). Furthermore, all clinical samples were anonymised and all laboratory assays were carried out in a 'blinded' manner. 'Unblinding' was only undertaken at the stage of statistical analyses.

5.2.2 Blood sample processing

All venous blood samples were drawn by a standard venipuncture technique from the antecubital vein into two 3.2% tri-sodium citrate BD Vacutainer tubes (9 parts blood: 1 part citrate anticoagulant; BD Biosciences) and one serum-separating BD Vacutainer tube (BD Biosciences). PFP samples were identically prepared for MM, MPD, and pancreatic cancer patients using serial centrifugation steps; firstly sodium citrate-anticoagulated whole blood tubes were spun at 180 x *g* for 10 min (at room temperature to avoid cold-induced platelet activation) and the platelet-rich plasma obtained was subsequently spun

at 12,000 x *g* for 10 min to remove platelets. For MM and MPD patients, the PCA of freshly isolated PFP was immediately assessed by a one-stage coagulation assay as described in section 2.6.2. In addition, fresh PFP from MM, MPD, and pancreatic cancer patients was immediately studied for the detection of MP as described in section 2.5.3. All PFP samples were kept at room temperature and processed within 3 h. Serum-separating blood tubes were processed within 3 h and upon collection were immediately stood in an upright position and allowed to clot for 30 min at 4°C. The serum samples were subsequently isolated by centrifugation at 700 x *g* for 10 min, and aliquots were stored in a -80°C freezer. Serum samples for all MM patients were evaluated for sCD106 and sCD54 by quantitative ELISA (method detailed in section 2.7.2).

5.3 Statistical analysis

Data are expressed as mean ± SD or median and interquartile range (IQR) according to the normality of distribution. The number of all MP were logarithmically transformed to the natural base e ($\log_e$) as they varied over one order of magnitude between patients, and to obtain a normal distribution before statistical comparison. Pearson's correlation coefficient (*r*) was used to describe correlations and the associated two-tailed test was used for statistical significance. The analysis of variance (ANOVA) with Tukey's post-hoc analysis was used to evaluate the level of differences in the mean age at enrolment, gender, and the $\log_e$ TF+ MP between three independent patient groups (MM, MPD, and pancreatic cancer), while the unpaired Student's *t* test was used to evaluate the level of difference in $\log_e$ EMP, PMP, monocyte-derived MP, plasma cell-derived MP, and PS+ MP between two independent patient groups (MM and MPD).

The statistical comparison of each of the quantitative variables measured in this study (i.e. haematological parameters, subtypes of MP, and CT) over time (i.e. before, during, and after combination therapy with IMiD) was performed by Dr Eric Gardiner (Statistical advisor, Department of Psychological Health and Wellbeing, University of Hull, Hull, UK). Specifically, a marginal model (with no random effects) was fitted to the $\log_e$ transformed haematological parameters, subtypes of MP, and CT values collected for each variable using the SPSS MIXED procedure that accommodates for missing data points. An unstructured correlation matrix with no pattern at all assumed for the variances and

covariances of the variable values within a patient at each time point was used. The significant differences between model-estimated marginal means for time points T1 (before chemotherapy), T2 (after cycle 1), T3 (after cycle 2) and the reference time point T4 (6-8 weeks after chemotherapy) for each variable was calculated using paired Student's *t* tests. P values < 0.05 were considered to be statistically significant. All statistical analyses were performed with SPSS computer software, version 20.0 (IBM Corp.).

5.4 Patient characteristics

Overall a total of 17 newly diagnosed and relapsed MM patients were enrolled in this study. However, the analysis included data from only 15 of these patients as one patient was initially excluded due to blood sample haemolysis prior to chemotherapy administration, and another because they were the only patient recruited receiving Bor/Dex (therapy without IMiD; see consort diagram in Figure 5.1). Furthermore, not all patient samples could be collected at subsequent time points during and after chemotherapy (T2 to T4) for various reasons, including death, adverse events, and loss due to patients not finishing the chemotherapy course during the thesis study period (summarized in Figure 5.1). Ultimately, analysed blood samples were obtained prior to the administration of chemotherapy at T1 for 15 MM patients, and subsequent samples were collected from; 66.6% (10 out of 15) of patients after the completion of the first cycle and before the initiation of the second cycle, on cycle 2 day 1 at T2; 66.6% (10 out of 15) of patients after completion of the second cycle and before initiation of the third cycle, on cycle 3 day 1 at T3; and 33.3% (5 out of 15) of patients following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy at T4. In addition, a single blood sample was obtained for 6 MPD patients during a regular follow-up visit in a haematology outpatient clinic. Furthermore, consented and eligible patients with suspected pancreatic cancer who were scheduled to undergo surgical resection of the tumour were enrolled in the 'Pancreatectomy & TF' clinical trial, however after 'unblinding' of the clinical data, only those patients with confirmed pancreatic adenocarcinoma (according to histopathology reports) were included in the analysis. A total of 35 pre-operative pancreatic cancer plasma samples were collected over the full clinical trial study period (processed by Dr Hussein Echrish prior to April 2011) and statistically analysed in this thesis.

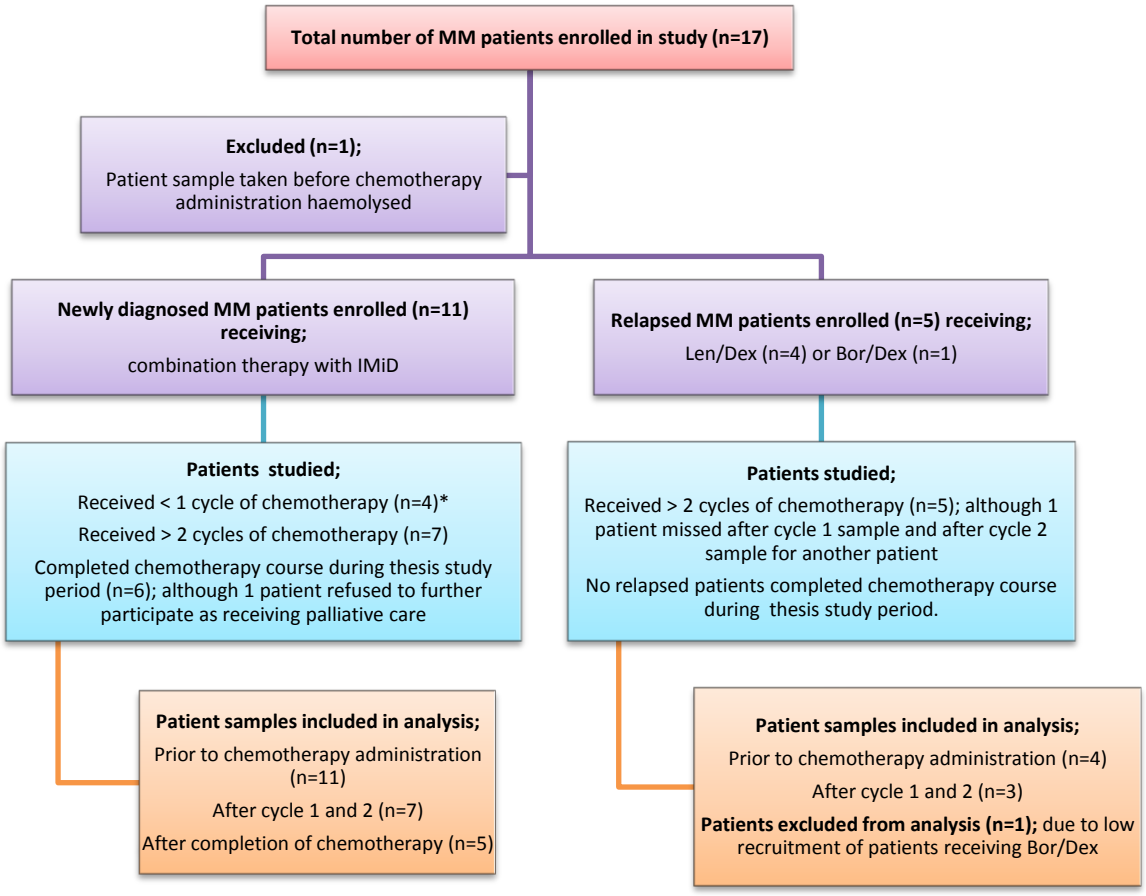


Figure 5.1: CONSORT flow diagram of enrolled myeloma study patients.
* Due to death by stroke (n=2) or adverse event (n=2), including one patient admitted to hospital with suspected respiratory tract infection or pulmonary oedema, and another with chest pain but no evidence of pulmonary embolism or infection. As a result of these adverse events the decision was made by the treating clinician to stop the prescribed combination chemotherapy in these two patients and continue treatment with alternative chemotherapeutic drug regimens.

Table 5.1 summarizes the patient characteristics of all three study groups (MM, MPD, and pancreatic cancer patients). A significant variation was found between the mean age at enrolment in the MM, MPD, and pancreatic cancer patient groups (69.2 ± 10.6, 68.2 ± 6.0, and 61.2 ± 11.1 years, respectively) by ANOVA (Table 5.1). Post-hoc Tukey's analysis showed that this difference was significant between the MM and pancreatic cancer patient groups (P = 0.015), but not MM and MPD (P = 0.96) or MPD and pancreatic cancer (P = 0.93) patient groups. In addition, no significant variation in gender was found between the three groups (P = 0.72), in particular women comprised 33.3% (5 out of 15) of the MM patient group, 16.6% (1 out of 6) of the MPD patient group, and 37% (13 out of 35) of the pancreatic cancer patient group (Table 5.1). Furthermore, the mean paraprotein

concentration was 29.0 ± 18.3 g/l amongst the MM patients and myeloma IgG was found to be the most frequent subtype (53.3%; 8 out of 15 patients) as shown in Table 5.1.

A pre-chemotherapy haemoglobin level of < 100 g/l, platelet count of ≥ 350 x10^9/l, and leukocyte count of > 11.0 g/l have all individually been associated with an increased risk of VTE in cancer patients (Khorana et al., 2008b), and were found in 26.7% (4 out of 15), 6.7% (1 out of 15), and 13.3% (2 out of 15) of MM patients prior to combination therapy with IMiD, respectively, in this study. The mean haemoglobin level for MM patients prior to chemotherapy (108.0 ± 14.7 g/l) was below the normal references ranges for men (135 to 175 g/l) and women (120 to 160 g/l) provided by the CHH laboratory performing the tests, while other haematological parameters analysed, specifically platelet, leukocyte, neutrophil, and monocyte count, were within normal ranges. Although the mean haemoglobin, platelet, leukocyte, and neutrophil count were all within normal ranges in MPD patients, they were shown to be significantly elevated in comparison with the relative mean levels in MM patients (P = 0.01, P = 0.04, P = 0.023, and P = 0.031, respectively) using the unpaired Student's *t*-test (Table 5.1).

Table 5.1: Patient characteristics.

Patient characteristics	MM (n=15)	MPD (n=6)	Pancreatic cancer (n=35)	P value
Age (mean ± SD years)	69.2 ± 10.6	68.2 ± 6.0	61.2 ± 11.1	**P = 0.015***
Male/female	10/5	5/1	22/13	P = 0.72*
Paraprotein concentration (mean ± SD; g/l)	29.0 ± 18.3	N/A	N/A	
Myeloma type				
IgG	8	N/A	N/A	
IgA	3	N/A	N/A	
Other	4	N/A	N/A	
Haematological parameter (mean ± SD)				
Haemoglobin (g/l)	108.0 ± 14.7	136.5 ± 17.6	N/A	**P = 0.01****
Platelet count (x10^9/l)	207.9 ± 91.0	308.7 ± 104.7	N/A	**P = 0.04****
Leukocyte count (x10^9/l)	6.6 ± 3.0	10.7 ± 4.4	N/A	**P = 0.023****
Neutrophil count (x10^9/l)	3.9 ± 2.3	6.4 ± 1.8	N/A	**P = 0.031****
Monocyte count (x10^9/l)	0.6 ± 0.4	0.6 ± 0.4	N/A	P = 0.68**

Abbreviations: N/A, not applicable. *Denotes P value for the comparison of the three groups by ANOVA, while ** denotes the P value for the comparison of two groups by the unpaired Student's *t*-test.

Treatment schedules for all MM patients are shown in Table 5.2. In particular, newly diagnosed MM patients received CTD (21 day cycle; n=4), CTDa (28 day cycle; n=6), or an attenuated dose of Len, cyclophosphamide, and Dex (RCDa; 28 day cycle; n=1), whereas relapsed patients received Len/Dex (28 day cycle; n=4). Since all MM patients received

combination therapy with IMiD, they were consequently prescribed concurrent LMWH anticoagulation (at the discretion of the treating clinician), specifically a prophylactic dose of dalteparin (typically 5000 U/day) to reduce the incidence of thrombotic complications during the course of chemotherapy. Thus, no symptomatic DVT or pulmonary embolism events were observed in any of the MM patients in this study. However, 2 out of the 15 MM patients (13%) did develop cardiovascular events during chemotherapy; these were both newly diagnosed patients receiving either CTD or CTDa and endured fatal stokes (or cerebrovascular accidents) within the first cycle of chemotherapy (associated with progressive disease). Additionally, antibiotics and antiemetic prophylaxis were given to patients during treatment according to local protocols. Furthermore, it is important to note that after 'unblinding' it was discovered that 2 out of the 6 (33.3%) MPD patients were receiving chemotherapeutic agents at the time of blood sampling, specifically hydroxycarbamide (500 mg).

Table 5.2: Treatment schedules with CTD (21 day cycle), CTDa (28 day cycle), and RCDa (28 day cycle) in newly diagnosed MM patients and Len/Dex (28 day cycle) in relapsed MM patients.

Regimen	Daily oral dose	Days administered	n =
CTD (21 day cycle)*			4
Cyclophosphamide	500 mg	1, 8, and 15	
Thal	Initially 50 mg and increasing up to 200 mg in some cases	1 – 21	
Dex	40 mg	1 – 4 and 12 – 15	
CTDa (28 day cycle)*			6
Cyclophosphamide	500 mg	1, 8, 15, and 22	
Thal	Initially 50 mg and increasing up 100 mg in some cases	1 – 28	
Dex	20 mg	1 – 4 and 15 – 18	
RCDa (28 day cycle)*			1
Len	25 mg	1 – 21	
Cyclophosphamide	500 mg	1 and 8	
Dex	20 mg	1 – 4 and 15 – 18	
Len/Dex (28 day cycle)			4
Len	25 mg**	1 – 21	
Dex	40 mg**	1, 8, 15, and 22	

* Denotes in the absence of disease progression patients should receive a minimum of 4 cycles of induction chemotherapy in the intensive pathway (CTD) or 6 cycles in the non-intensive pathway (CTDa or RCDa) as long as they are responding and should continue to maximum response or intolerance. ** Denotes dose reductions were required in some cases due to toxicity.

5.5 Haematological parameters in MM patients before, during, and after chemotherapy

The mean ± SD haemoglobin level for MM patients prior to chemotherapy (108.0 ± 14.7 g/l; T1) was similar to the mean ± SD haemoglobin level after cycle 1 (105.5 ± 12.5 g/l; T2)

and cycle 2 (107.1 ± 13.1 g/l; T3) of chemotherapy, and slightly increased after chemotherapy (119.4 ± 24.2 g/l; T4) as shown in Figure 5.2, but remained below normal references ranges for men (135 to 175 g/l) and women (120 to 160 g/l). Statistical analysis following $\log_e$ transformation of the data and using a marginal model that accommodated for missing data showed that the estimated marginal mean haemoglobin levels were not significantly elevated after treatment (T4) in comparison with before (T1) and during (T2 and T3) chemotherapy (P = 0.84, P = 0.92, and P = 0.88, respectively; Table 5.3).

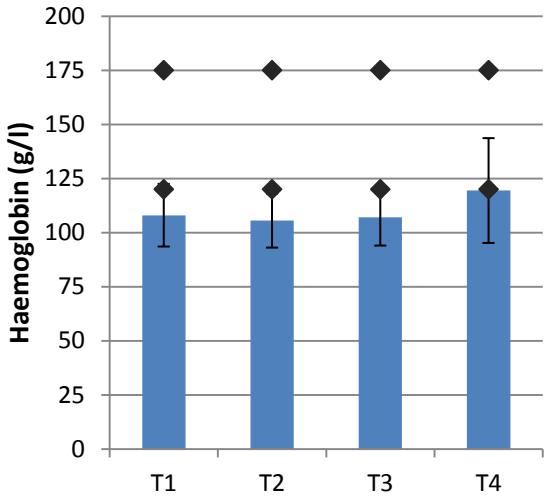


Figure 5.2: Haemoglobin levels (g/l) in patients with MM before, during, and after chemotherapy.
Haemoglobin concentration (g/l) of newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5). Data are the mean ± SD of all collected blood samples at each time point (T1 to T4). ♦ Indicates upper and lower limit of normal reference range.

Table 5.3: Haemoglobin levels (g/l) in patients with MM before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Mean ± SD (g/l)	P value vs. T4*
T1	15	108.0 ± 14.5	P = 0.84
T2	10	105.5 ± 12.5	P = 0.92
T3	10	107.1 ± 13.1	P = 0.88
T4	5	119.4 ± 24.2	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.
*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

Although the mean ± SD platelet count remained within the normal reference range (150.0 to 400.0 x10^9/l) before, during, and after chemotherapy, it was shown to increase after cycle 1 of chemotherapy (247.1 ± 82.5 x10^9/l; T2) as compared with before chemotherapy (207.9 ± 91.0 x10^9/l; T1) and remained elevated after cycle 2 of chemotherapy (236.9 ± 48.0 x10^9/l; T3) and after the completion of the prescribed chemotherapy course (214.2 ± 41.1 x10^9/l; T4), shown in Figure 5.3. Statistical analysis following $\log_e$ transformation of the data and using a marginal model that accommodated for missing data showed that the estimated marginal mean platelet counts (x10^9/l) were significantly higher during cycle 1 (P = 0.048; T2) and cycle 2 of chemotherapy (P = 0.013; T3) in comparison with after chemotherapy (T4), and did not significantly differ (P = 0.77) between before (T1) and after chemotherapy (T4) as shown in Table 5.4.

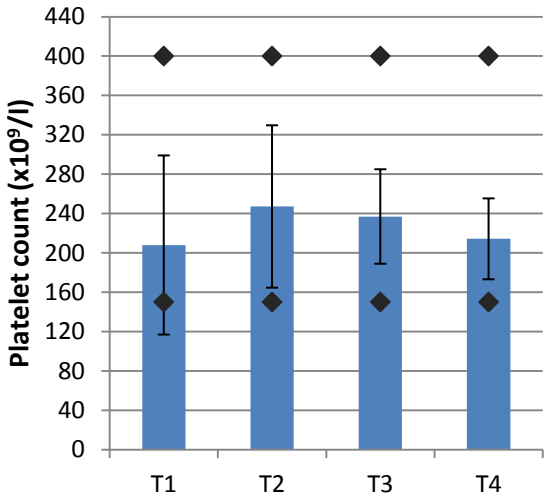


Figure 5.3: Platelet count (x10^9/l) in patients with MM before, during, and after chemotherapy.
Platelet count (x10^9/l) of newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5). Data are the mean ± SD of all collected blood samples at each time point (T1 to T4). ♦ Indicates upper and lower limit of normal reference range.

Table 5.4: Platelet count (x10^9/l) in patients with MM before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Mean ± SD (x10^9/l)	P value vs. T4*
T1	15	207.9 ± 91.0	P = 0.77
T2	10	247.1 ± 82.5	**P = 0.048**
T3	10	236.9 ± 48.0	**P = 0.013**
T4	5	214.2 ± 41.1	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.
*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

The mean ± SD leukocyte count (x10^9/l) was shown to decrease after cycle 1 (4.9 ± 2.5 x10^9/l; T2) and cycle 2 of chemotherapy (4.9 ± 1.4 x10^9/l; T3), and also after the completion of the prescribed course of chemotherapy (4.6 ± 1.0 x10^9/l; T4) in MM patients as compared with the mean ± SD leukocyte count prior to chemotherapy (6.6 ± 3.0 x10^9/l; T1) shown in Figure 5.4, but remained within the normal reference range (4.0 to 11.0 x10^9/l). Statistical analysis following $\log_e$ transformation of the data and using a marginal model that accommodated for missing data showed that there was no significant differences among the before (T1) and during (T2 and T3) chemotherapy estimated marginal means of leukocyte counts (x10^9/l) in comparison with after chemotherapy (T4) as shown in Table 5.5.

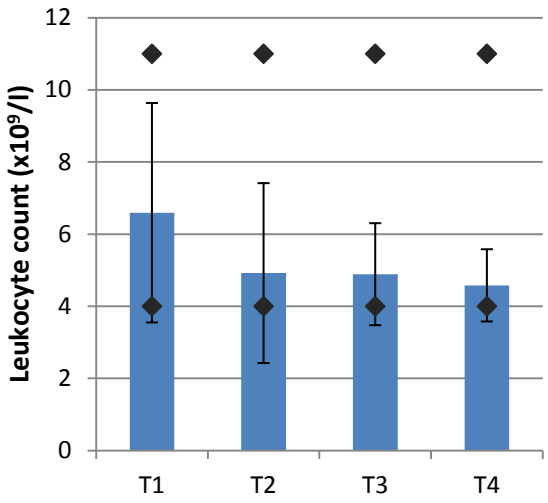


Figure 5.4: Leukocyte count (x10^9/l) in patients with MM before, during, and after chemotherapy.
Leukocyte count (x10^9/l) of newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5). Data are the mean ± SD of all collected blood samples at each time point (T1 to T4). ♦ Indicates upper and lower limit of normal reference range.

Table 5.5: Leukocyte count (x10^9/l) in patients with MM before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Leukocyte count	P value vs. T4*
T1	15	6.6 ± 3.0	P = 0.067
T2	10	4.9 ± 2.5	P = 0.63
T3	10	4.9 ± 1.4	P = 0.98
T4	5	4.6 ± 1.0	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.
*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

The mean ± SD neutrophil count (x10^9/l) was shown to decrease after cycle 1 (2.8 ± 1.5 x10^9/l; T2) and cycle 2 of chemotherapy (2.8 ± 0.9 x10^9/l; T3), and also after the completion of the prescribed course of chemotherapy (3.0 ± 0.6 x10^9/l; T4) in MM patients as compared with the mean ± SD neutrophil counts prior to chemotherapy (3.9 ± 2.3 x10^9/l; T1) shown in Figure 5.5, but remained within normal reference ranges (2.0 to 7.7 x10^9/l). Statistical analysis following $\log_e$ transformation of the data and using a marginal model that accommodated for missing data showed that there was no significant differences among the before (T1) and during (T2 and T3) chemotherapy estimated

marginal means of neutrophil counts (x10^9/l) in comparison with after chemotherapy (T4) as shown in Table 5.6.

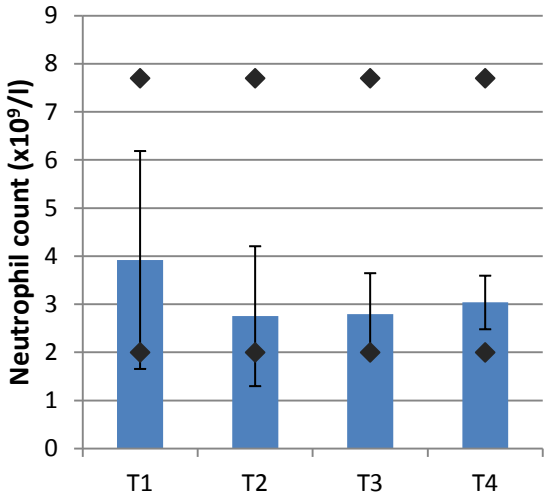


Figure 5.5: Neutrophil count (x10^9/l) in patients with MM before, during, and after chemotherapy.
Neutrophil count (x10^9/l) of newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5). Data are the mean ± SD of all collected blood samples at each time point (T1 to T4). ♦ Indicates upper and lower limit of normal reference range.

Table 5.6: Neutrophil count (x10^9/l) in patients with MM before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Neutrophil count	P value vs. T4*
T1	15	3.9 ± 2.3	P = 0.61
T2	10	2.8 ± 1.5	P = 0.13
T3	10	2.8 ± 0.9	P = 0.24
T4	5	3.0 ± 0.6	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.
*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

In addition, the mean ± SD monocyte count (x10^9/l) was similar prior to chemotherapy administration (0.64 ± 0.39 x10^9/l; T1) to after cycle 1 (0.68 ± 0.43 x10^9/l; T2) and cycle 2 of chemotherapy (0.66 ± 0.30 x10^9/l; T3), and decreased after the completion of the prescribed chemotherapy course (0.43 ± 0.16 x10^9/l; T4) shown in Figure 5.6, but remained within the normal reference range (0.2 to 0.8 x10^9/l). The estimated marginal

mean monocyte count (x10^9/l) was significantly elevated after the second cycle of chemotherapy (T3) in comparison with after chemotherapy (P = 0.025; T4), but there was no significant difference after treatment (T4) in comparison with before (T1) and after cycle 1 (T2) of chemotherapy (P = 0.15 and P = 0.073, respectively; T4) as shown in Table 5.7.

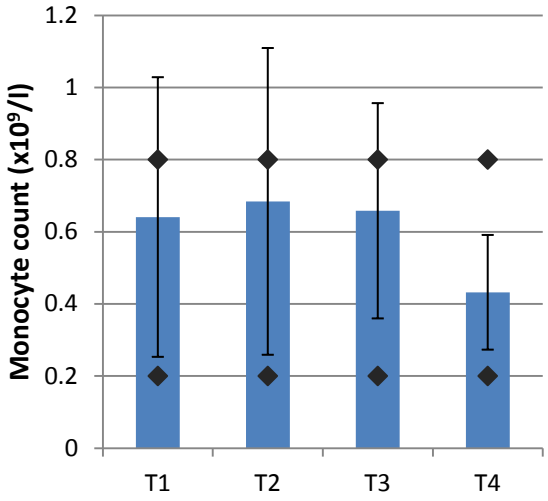


Figure 5.6: Monocyte count (x10^9/l) in patients with MM before, during, and after chemotherapy.
Monocyte count (x10^9/l) of newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5). Data are the mean ± SD of all collected blood samples at each time point (T1 to T4). ♦ Indicates upper and lower limit of normal reference range.

Table 5.7: Monocyte count (x10^9/l) in patients with MM before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Monocyte count	P value vs. T4*
T1	15	0.64 ± 0.39	P = 0.15
T2	10	0.68 ± 0.43	P = 0.073
T3	10	0.66 ± 0.30	**P = 0.025**
T4	5	0.43 ± 0.16	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.
*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

5.6 Enumeration of MP by flow cytometry

MP were identified by their flow cytometric scatter properties and the presence of a cell-specific antigen that allows identification of their cellular origin or the presence of procoagulant markers (exposed PS or TF on MP). To detect MP by flow cytometry, an initial size gate for MP was set with a defined upper and lower limit (seen in Figure 5.7 and represented by R1) using a mixture of fluorescent latex beads (Megamix) with diameters of 0.5, 0.9, and 3.0 μm to distinguish the cell-derived populations of MP from platelets, apoptotic bodies, and background noise in PFP. This strategy is recommended by the ISTH Scientific and Standardization Subcommittee in Vascular Biology following a collaborative workshop that this laboratory was involved in along with another 39 laboratories; specifically, the primary objective of this workshop was to standardize the enumeration of PMP ≥ 0.5 μm by flow cytometry (Lacroix et al., 2010). Moreover, this size-standardized gate (R1; Figure 5.7) allows reproducible quantification of MP and was subsequently applied as a stable template to all data analyses of MP.

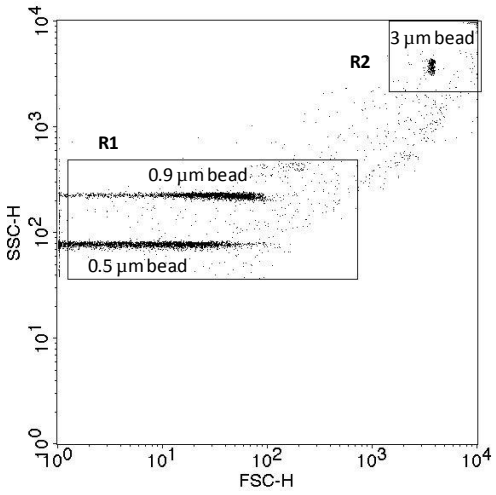


Figure 5.7: Flow cytometric size-standardized gate for MP.
The size gate for the detection of cell-derived MP in PFP shown in region 1 (R1) was defined using calibration beads by flow cytometrty with FSC and SSC set at a logarithmic gain to cover a wide size range following a consensus guideline on the measurement of MP. The 0.9 μm latex beads facilitate the placement of the upper limit of the region providing an approximation of a maximal size of 1 μm, while the 0.5 μm beads aid the set up of the FSC resolution threshold and lower detection limit for MP. The 3 μm beads are shown in region 2 (R2).

PFP was processed from blood samples taken from MM, MPD, and pancreatic cancer patients as described in section 5.2.2 and was analysed for subpopulations of MP (methodology detailed section 2.5.3) according to their positivity for various specific

antigens expressed on their membrane surfaces, which are listed in Table 5.8 (only TF+ MP were studied in pancreatic cancer patients). Since Thal and Len are antiangiogenic agents, it is hypothesised that they will elicit endothelial dysfunction in this study, therefore a panel of four different monoclonal antibodies (anti-CD105, anti-CD106, anti-CD54, and anti-CD144) that are all directed against antigens expressed on endothelial cells was used to detect EMP and distinguish the specific phenotypes (i.e. derived from resting, activated, or injured endothelial cells). In particular, CD144 is believed to be constitutively exposed on most types of endothelial cells (Breier et al., 1996), while CD105 is expressed at low levels on the surface of resting endothelial cells, but is abundantly expressed on angiogenic endothelial cells (Duff et al., 2003). In addition, CD106 and CD54 are cell adhesion molecules upregulated by cytokine stimulation on the surface of activated endothelial cells (Lechleitner et al., 1998, Zadeh et al., 2000). For the detection of PMP and monocyte-derived MP, the CD42b (or GPIbα) platelet activation marker (Krueger et al., 2002) and CD14 cell surface antigen (strongly expressed on monocytes and macrophages (Ziegler-Heitbrock and Ulevitch, 1993)) were studied, respectively. Furthermore, CD138 expression was determined by flow cytometry for the detection of plasma cell-derived MP or more specifically, tumour cell-derived MP in MM patients as CD138 is a cell surface antigen known to be expressed on plasma cells and myeloma cells (Wijdenes et al., 1996). The cell surface antigen CD142 (or TF), expressed by various cell types, was studied to specifically detect populations of procoagulant TF+ MP. Lastly, annexin V is a common marker used with flow cytometry to measure the expression of the procoagulant phospholipid PS. In particular, PS is widely considered a general marker of MP, although not all MP expose PS. Fluorescently labelled annexin V was used accordingly in this study to detect MP exposing PS.

Table 5.8: Antibodies used to identify cell-specific subtypes of MP.

Subtype	Monoclonal antibody details (all mouse anti human)	Marker details
EMP	1. Anti-CD144 FITC (vascular endothelial cadherin) BD biosciences, clone 55-7H1	1. CD144, a member of the cadherin family of calcium-dependent adhesion molecules, is a cell surface GP expressed by endothelial cells. It may play a role in the organization of lateral endothelial junctions and in the control of permeability of vascular endothelium.
	2. Anti-CD105 FITC (endoglin) AbD Serotec, clone SN6	2. CD105 is a GP homodimer of 95-kDa subunits and part of the transforming growth factor-β receptor complex involved in the control of vascular endothelial cell proliferation, adhesion and migration. It is expressed at low levels on the surface of resting endothelial cells and is increased on active endothelium in tumours undergoing angiogenesis.
	3. Anti-CD106 FITC (VCAM-1) AbD Serotec, clone 1.G11B1	3. CD106 is a cell surface GP expressed at high levels on the surface of cytokine-stimulated endothelium, thereby facilitating the adhesion of various leukocytes to the vascular endothelium. In contrast, it is expressed at minimal levels on unstimulated endothelium.
	4. Anti-CD54 FITC (ICAM-1) AbD Serotec, clone 15.2	4. CD54 is a cell surface GP member of the Ig superfamily expressed by endothelial cells, lymphocytes, epithelial cells, macrophages and dendritic cells following activation by inflammatory mediators, such as cytokines. CD54 binds to various integrins and may participate in inflammatory reactions and antigen-specific immune responses.
PMP	Anti-CD42b FITC (GPIbα) AbD Serotec, clone AK2	CD42b is expressed by platelets and megakaryoctes, which serves as the vWF surface receptor involved in the adhesion of platelets to the subendothelium of damaged vascular walls.
Monocyte-derived MP	Anti-CD14 FITC AbD Serotec, clone MEM-18	CD14, a glycosylphosphatidylinositol-anchored single chain GP, is strongly expressed on the surface of monocytes and macrophages. It functions as a pattern recognition receptor in innate immunity for a variety of ligands.
Plasma cell-derived MP	Anti-CD138 FITC (Syndecan-1) AbD Serotec, clone B-A38	CD138 is a member of the transmembrane heparan sulphate proteoglycan family and is expressed on the surface of plasma cells and myeloma cells. It acts as an extracelluar matrix receptor, involved in many cellular functions, including cell binding, cell signalling and cytoskeletal organization through cell adhesion and cell matrix adhesion. In MM, CD138 controls tumour cell survival, growth, adhesion and bone cell differentiation.
TF+ MP	Anti-CD142 FITC (TF) AbD Serotec, clone CLB/TF-5	CD142 is a cell surface GP known as TF and expression can be induced on monocytes, macrophages, endothelial cells, and cancer cells by various stimuli. CD142 initiates coagulation by binding to FVIIa. CD142 also plays an important role in inflammation, angiogenesis, and the pathophysiology of thrombosis and cancer.
PS+ MP	Annexin V PE BD biosciences	PS is a membrane phospholipid and in activated or apoptotic cells PS is translocated from the inner to the outer plasma membrane leaflet, thus MP generated during these processes expose PS on their external membrane surface. Annexin V, a calcium dependent phospholipid-binding protein, has a high affinity for PS and binds to membranes with exposed PS.

Positive events of the various subpopulations of MP studied (including, EMP, PMP, monocyte-derived MP, plasma cell-derived MP, and TF+ MP) were counted as the difference in labelling between cell-specific or TF-specific FITC-labelled monoclonal antibodies and their respective isotype-matched control antibodies (all IgG1). Furthermore, PS exposure on MP was determined by PE-labelled annexin V binding in the presence of $CaCl_2$, and unspecific PE-labelled annexin V-binding was evaluated in the

absence of $CaCl_2$. All dilution buffers were sterile-filtered using 0.1 μm filters to limit background noise from dust and crystals that have the same size range as MP, which could therefore potentially influence or disturb the analysis. Furthermore, the absolute counts of MP present in the samples were based on the addition of 25 μl of known quantities of standardized fluorescent beads A and B (AccuCheck counting beads) to the same volume of sample prior to flow cytometry acquisition. These two fluorescent beads (A and B) were used as a double internal standard that provide reproducible and accurate measurements of the number of MP in PFP, and were provided at a known concentration (around 1000 beads/μl) by the supplier. Subsequently, the absolute counts of MP were calculated using the following equation;

$$\text{Absolute count} = \frac{\text{Number of MP counted}}{\text{Total number of beads counted (A + B)}} \times \text{known concentration of beads/μl} \qquad \text{(Equation 5.1)}$$

The representative example of an analysis for CD105+ MP in Figure 5.8 shows all events detected within the size range of MP (seen in R1), all CD105+ MP detected in relation to the negative control (7835), and the number of counting beads A (3474) and B (3317) detected in a PFP sample from a newly diagnosed MM patient after chemotherapy. Thus, the absolute count of MP in this example is calculated following substitution of the appropriate values (see Figure 5.8) into equation (5.1);

$$7835/(3474 + 3317) \times 1037 = \textbf{1196 CD105+ MP/μl PFP} \qquad \text{(Equation 5.2)}$$

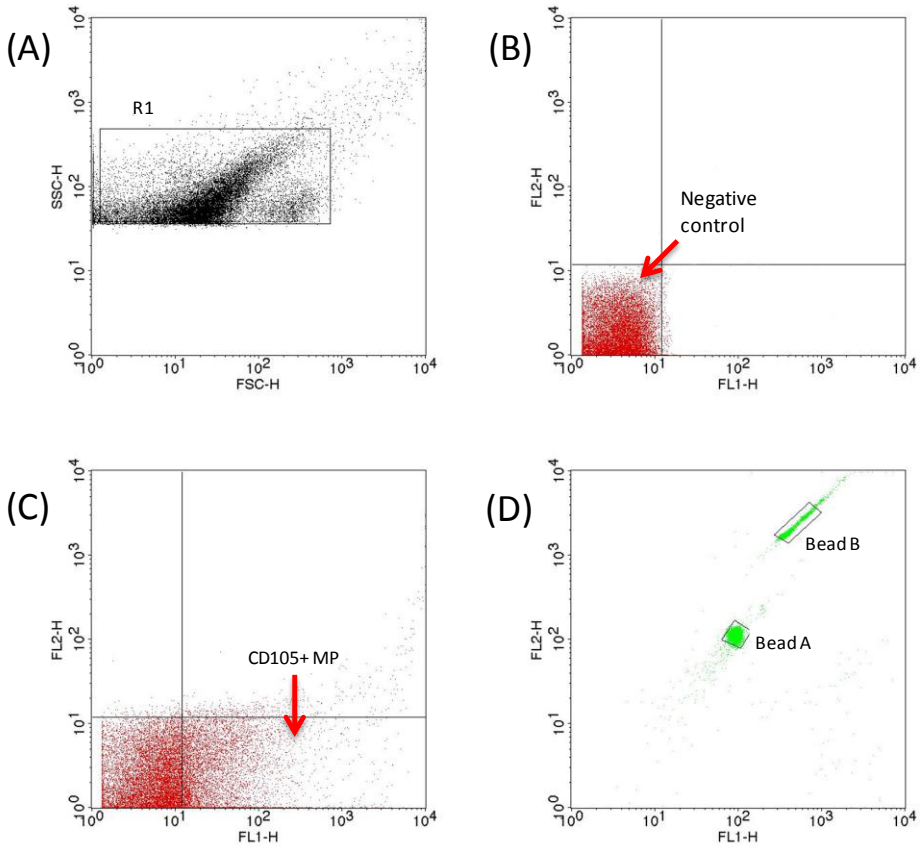


Figure 5.8: Representative flow cytometric analysis of CD105+ MP in PFP.
(A) All events detected within the size-standarized gate (R1) for MP (B) MP stained with 5 µl of FITC-labelled IgG1 isotype negative control were analysed by flow cytometry and a representative fluorescent threshold is shown with all non-specific binding contained within the lower left quadrant. (C) MP stained with 5 µl of FITC-labelled IgG1 anti-CD105 antibody were analysed by flow cytometry and positive events are shown in the lower right quadrant (D) detection of two fluorescent counting beads that present different fluorescence intensities allowing their differentiation; beads A shown in R3 and beads B shown in R4.

5.6.1 Comparison of circulating MP subsets in MM patients at baseline with MPD patients

The median number of different subtypes of MP, including EMP (CD105+, CD106+, CD54+, CD144+), PMP (CD42b+), monocyte-derived MP (CD14+), plasma cell-derived MP (CD138+), and PS+ MP, for 15 MM patients at baseline (prior to chemotherapy) and 6 MPD patients are shown in Figures 5.9 and 5.10, respectively, as determined by flow cytometry (described in section 2.5.3). The median number of CD105+, CD106+, CD54+, and CD144+ EMP were approximately 3- to 4-fold higher in MPD patients as compared with MM patients, and the differences between the mean levels of the various subtypes of EMP in the two study populations were all shown to be significantly different ($P < 0.0005$; Table

5.9) following $\log_e$ transformation of the data to obtain a normal distribution and using the unpaired Student's *t*-test for two independent samples. Furthermore, the median (IQR) number of CD42b+ PMP was slightly higher in MPD patients as compared with MM patients (9438.0 [3773.5–18860.3] versus 6074.0 [4156.0–7479.0]/µl PFP), although no significant difference was found between the mean of $\log_e$ transformed levels of CD42b+ PMP in MPD and MM patients (P = 0.41; Table 5.9). In addition, the median number of CD14+ monocyte-derived MP, CD138+ plasma cell-derived MP, and PS+ MP were elevated by 3.8-fold, 3.4-fold, and 2.4-fold, respectively, in MPD patients as compared with MM patients, and the differences between the mean levels of these various subtypes of MP were all shown to be significantly different (P < 0.0005, P < 0.0005, and P < 0.001, respectively) following $\log_e$ transformation of the data and using the unpaired Student's *t*-test for two independent samples (Table 5.9).

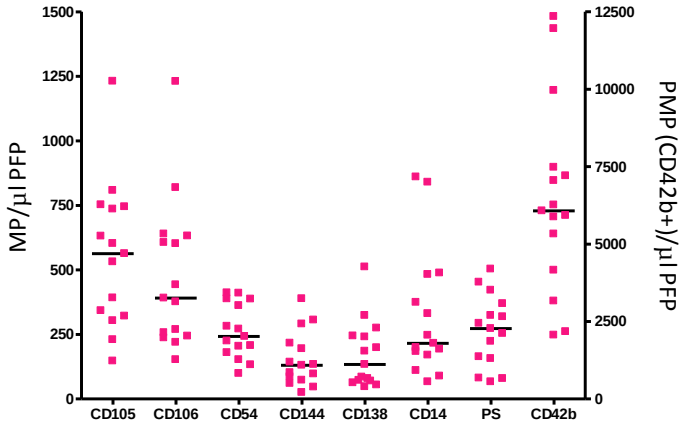


Figure 5.9: Number of circulating MP (per µl in PFP) measured in MM patients according to their specific cell of origin and PS positivity.

Number of CD105+, CD106+, CD54+, and CD144+ EMP, CD138+ plasma cell-derived MP, CD14+ monocyte-derived MP, PS+ MP, and CD42b+ PMP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (n=15), determined by flow cytometry. The horizontal line indicates the median level.

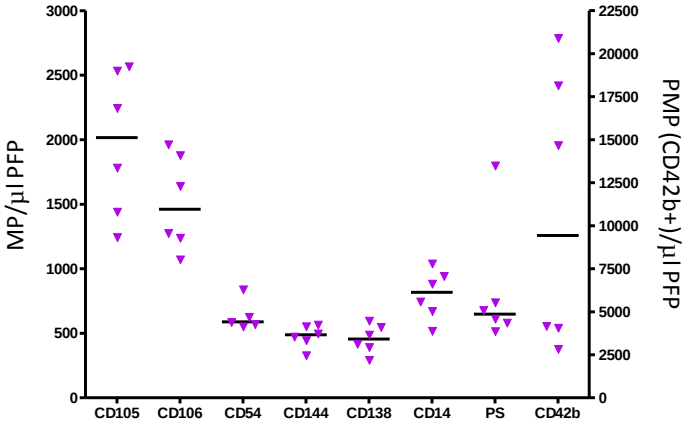


Figure 5.10: Number of circulating MP (per µl in PFP) measured in MPD patients according to their specific cell of origin and PS positivity.

Number of CD105+, CD106 +, CD54+, and CD144+ EMP, CD138+ plasma cell-derived MP, CD14+ monocyte-derived MP, PS+ MP, and CD42b+ PMP (per µl in PFP) in MPD patients (n=6), determined by flow cytometry. The horizontal line indicates the median level.

Table 5.9: Number of circulating MP (per µl of PFP) in MM and MPD patients according to their specific cell of origin and PS positivity and P value for two-tailed significance.

MP subtype	MM (n = 15) median (IQR)	MPD (n = 6) median (IQR)	P value*
EMP:			
CD105	563.0 (321.0 - 744.0)	2016.0 (1394.8 - 2545.3)	**P < 0.0005**
CD106	391.0 (243.0 - 631.0)	1461.0 (1199.3 - 1902.8)	**P < 0.0005**
CD54	242.0 (179.0 - 387.0)	608.5 (567.8 - 693.6)	**P < 0.0005**
CD144	130.0 (73.0 - 216.0)	488.0 (418.8 - 559.0)	**P < 0.0005**
PMP (CD42b)	6074.0 (4156.0 - 7479.0)	9438.0 (3773.5 - 18860.3)	P = 0.41
Monocyte-derived MP (CD14)	215.0 (170.0 - 483.0)	817.0 (635.5 - 970.0)	**P < 0.0005**
Tumour-derived MP (CD138)	133.0 (70.0 - 244.0)	455.5 (370.0 - 563.0)	**P < 0.0005**
PS+ MP	273.0 (156.0 - 369.0)	648.0 (567.3 - 1006.3)	**P = 0.001**

* P value for two-tailed significance was calculated following natural $\log_e$ transformation of the data to obtain a normal distribution and using the unpaired Student's *t*-test for two independent samples.

5.6.2 Comparison of the number of TF+ MP in MM, MPD, and pancreatic cancer patients

The median number of TF+ MP for 15 MM patients at baseline (prior to chemotherapy), 6 MPD patients, and 35 pancreatic cancer patients prior to surgical resection of the tumour are shown in Figure 5.11, as determined by flow cytometry (described in section 2.5.3). Subsequent to $\log_e$ transformation of the data to obtain a normal distribution, an ANOVA test yielded significant variation among the mean levels of TF+ MP in these three populations ($P < 0.0005$). The median (IQR) number of TF+ MP was 17.5-fold higher in pancreatic cancer patients (3421.0 [1297.0–5623.0]/µl PFP) as compared with MM

patients (195.2 [129.1–367.0]/µl PFP) shown in Figure 5.11, and a post-hoc Tukey's test of $\log_e$ transformed data showed that the difference between the mean levels of TF+ MP were highly statistically significant (P < 0.0005) as shown in Table 5.10. In addition, the median (IQR) number of TF+ MP was 3.3-fold higher in pancreatic cancer patients (3421.0 [1297.0–5623.0]/µl PFP) as compared with MPD patients (1051.5 [695.3–1359.3]/µl PFP) shown in Figure 5.11, although there was no significant difference (P = 0.094) found between the mean levels of TF+ MP in these two populations using $\log_e$ transformed data and a post-hoc Tukey's test, as shown in Table 5.10. Furthermore, the median (IQR) number of TF+ MP was 5.4-fold higher in MPD patients (1051.5 [695.3–1359.3]/µl PFP) as compared with MM patients (195.2 [129.1–367.0]/µl PFP) shown in Figure 5.11, but there was no significant difference (P = 0.065) found between the mean levels of TF+ MP in these two populations using $\log_e$ transformed data and a post-hoc Tukey's test, as shown in Table 5.10. Moreover, levels of TF+ MP above the 75th percentiles (367.0/µl PFP) of MM patients were seen in all 6 MPD patients and nearly all (94.3%) pancreatic cancer patients, indicating that MM patients have very low levels of circulating TF+ MP prior to treatment in comparison with pancreatic cancer patients prior to surgical resection of the tumour and MPD patients.

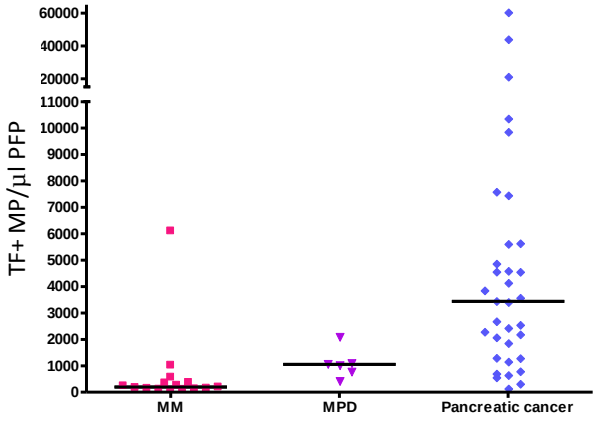


Figure 5.11: Number of circulating TF+ MP (per µl of PFP) measured in MM, MPD, and pancreatic cancer.

Number of TF+ MP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (n=15), MPD patients (n=6), and pancreatic cancer patients prior to surgical resection of the tumour (n=35), determined by flow cytometry. The horizontal line indicates the median level.

Table 5.10: Number of circulating TF+ MP (per µl of PFP) measured in MM, MPD, and pancreatic cancer patients and P value for two-tailed significance.

Group 1: MM (n = 15) median (IQR)	Group 2: MPD (n = 6) median (IQR)	Group 3: pancreatic cancer (n = 35) median (IQR)	P value (between groups)*
195.2 (129.1 - 367.0)	1051.5 (695.3 - 1359.3)	3421.0 (1297.0 - 5623.0)	1 and 2 P = 0.065 1 and 3 **P < 0.0005** 2 and 3 P = 0.094

* P value for two-tiled significance was calculated using the post-hoc Tukey's test following $\log_e$ transformation of the data (for normal distribution) and a significant ANOVA.

5.6.3 Number of EMP before, during, and after chemotherapy in MM patients

In all analysed samples, the median (IQR) level of circulating CD105+ EMP before chemotherapy (563.0 [321.0–744.0]/µl PFP; T1) was similar to the median level of CD105+ EMP after the first cycle of chemotherapy (605.0 [339.5-1766.3]/µl PFP; T2), but was shown to considerably increase after the second cycle of chemotherapy (T3) by 2.4-fold (1375.0 [361.3–1794.0]/µl PFP), and remained elevated after patients had finished the full chemotherapy course (T4) by 2.1-fold (1190.0 [763.5–2184.5]/µl PFP) in comparison with the median level of CD105+ MP before chemotherapy, as shown in Figure 5.12. Statistical analysis following $\log_e$ transformation of the data to obtain a normal distribution and using a marginal model that accommodated for missing data revealed that the estimated marginal mean of circulating CD105+ EMP was significantly elevated after chemotherapy (T4) in comparison with before (T1) and during (T2 and T3) chemotherapy ($P < 0.0005$, $P = 0.001$, and $P = 0.005$, respectively; Table 5.11).

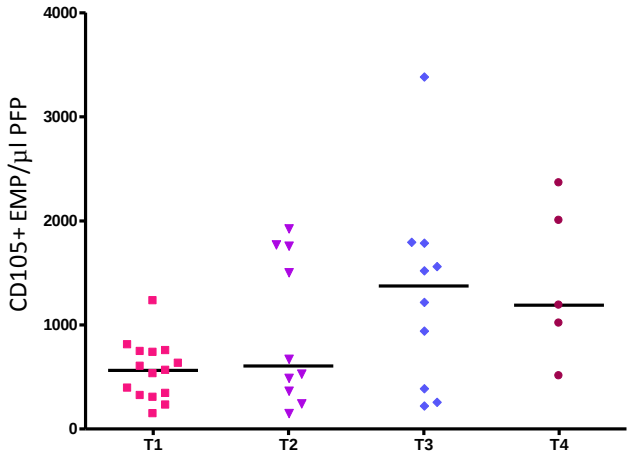


Figure 5.12: Number of circulating CD105+ EMP (per µl in PFP) measured in MM patients before, during, and after chemotherapy.

Number of CD105+ EMP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5), determined by flow cytometry. The horizontal line indicates the median level.

Table 5.11: Number of circulating CD105+ EMP (per µl of PFP) measured in MM patients before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Median (IQR)	P value vs. T4*
T1	15	563.0 (321.0-744.0)	**P <0.0005**
T2	10	605.0 (339.5-1766.3)	**P = 0.001**
T3	10	1375.0 (361.3-1794.0)	**P = 0.005**
T4	5	1190.0 (763.5-2184.5)	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.

*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

The median (IQR) level of circulating CD106+ EMP (391.0 [243.0–631.0]/µl PFP) measured before chemotherapy (T1) was shown to sequentially increase after each time point measured, specifically after cycle 1 (T2) by 1.5-fold (599.5 [314.8–1413.0]/µl PFP) and cycle 2 (T3) by 2.7-fold (1058.5 [298.8–1514.0]/µl PFP) of chemotherapy, and after patients had completed the prescribed course of chemotherapy (T4) by 3.8-fold (1491.0 [994.5–1588.0]/µl PFP) in all analysed samples (Figure 5.13). Statistical analysis following $\log_e$ transformation of the data to obtain a normal distribution and using a marginal model that accommodated for missing data showed that the estimated marginal mean of

circulating CD106+ EMP was significantly elevated after chemotherapy (T4) in comparison with before (T1) and during (T2 and T3) chemotherapy (P = 0.001, P = 0.004, P = 0.031, respectively; Table 5.12).

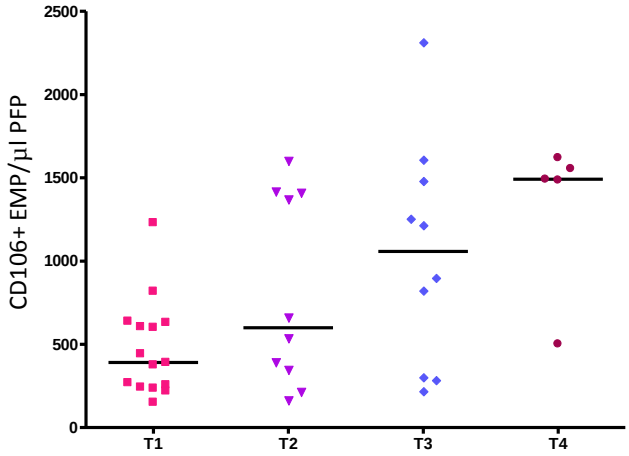


Figure 5.13: Number of circulating CD106+ EMP (per µl in PFP) measured in MM patients before, during, and after chemotherapy.

Number of CD106+ EMP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5), determined by flow cytometry. The horizontal line indicates the median level.

Table 5.12: Number of circulating CD106+ EMP (per µl of PFP) measured in MM patients before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Median (IQR)	P value vs. T4*
T1	15	391.0 (243.0-631.0)	**P = 0.001**
T2	10	599.5 (314.8-1413.0)	**P = 0.004**
T3	10	1058.5 (298.8-1514.0)	**P = 0.031**
T4	5	1491.0 (994.5-1588.0)	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.

*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

Similarly, in Figure 5.14 there is a clear trend of increasing median (IQR) levels of circulating CD54+ EMP from before chemotherapy (242.0 [179.0–387.0]/µl PFP; T1) to after cycle 1 (T2) by 1.7-fold (400.5 [240.0–601.8]/µl PFP) and cycle 2 (T3) by 1.9-fold (465.5 [283.8–960.8]/µl PFP) of chemotherapy, and after patients had completed the

prescribed course of chemotherapy (T4) by 2.8-fold (680.0 [560.5–764.0]/µl PFP) in all analysed samples. Statistical analysis following $\log_e$ transformation of the data to obtain a normal distribution and using a marginal model that accommodated for missing data revealed that the estimated marginal mean of circulating CD54+ EMP was significantly elevated after chemotherapy (T4) in comparison with before (T1) and after cycle 1 (T2) of chemotherapy (P 0.0005 and P = 0.011, respectively; Table 5.13). However, there was no significant difference found between after cycle 2 of chemotherapy (T3) and after the full course of chemotherapy (T4; P = 0.16; Table 5.13).

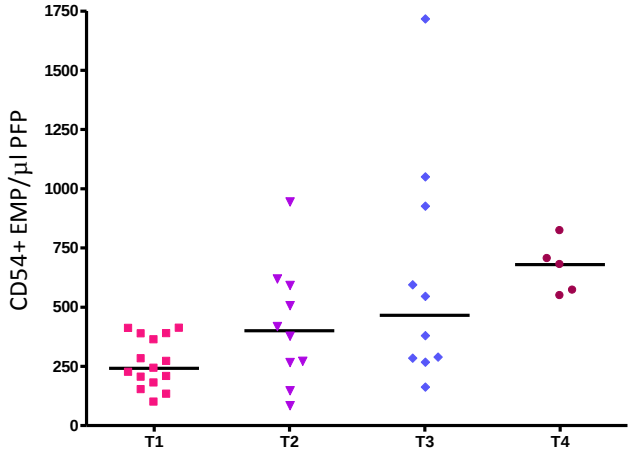


Figure 5.14: Number of circulating CD54+ EMP (per µl in PFP) measured in MM patients before, during, and after chemotherapy.

Number of CD54+ EMP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5), determined by flow cytometry. The horizontal line indicates the median level.

Table 5.13: Number of circulating CD54+ EMP (per µl of PFP) measured in MM patients before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Median (IQR)	P value vs. T4*
T1	15	242.0 (179.0-387.0)	**P < 0.0005**
T2	10	400.5 (240.0-601.8)	**P = 0.011**
T3	10	465.5 (283.8-960.8)	P = 0.16
T4	5	680.0 (560.5-764.0)	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.

*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

The median (IQR) level of circulating CD144+ EMP measured before chemotherapy (130.0 [73.0–216.0]/µl PFP; T1) was similar to the median levels of CD144+ EMP after cycle 1 (145.0 (50.5–422.5]/µl PFP; T2) and cycle 2 (128.5 [76.3–287.0]/µl PFP; T3) of chemotherapy, but increased after patients had completed the prescribed chemotherapy course (T4) by 2.4-fold (305.0 [162.0–400.5]/µl PFP) in all analysed samples (Figure 5.15). As shown in Table 5.14, statistical analysis following $\log_e$ transformation of the data to obtain a normal distribution and using a marginal model that accommodated for missing data showed that the estimated marginal mean of circulating CD144+ EMP was significantly elevated after chemotherapy (T4) in comparison with CD144+ EMP before chemotherapy (T1; P = 0.017), but not in comparison with after cycles 1 and 2 of chemotherapy (T2 and T3; P = 0.12 and P = 0.15, respectively).

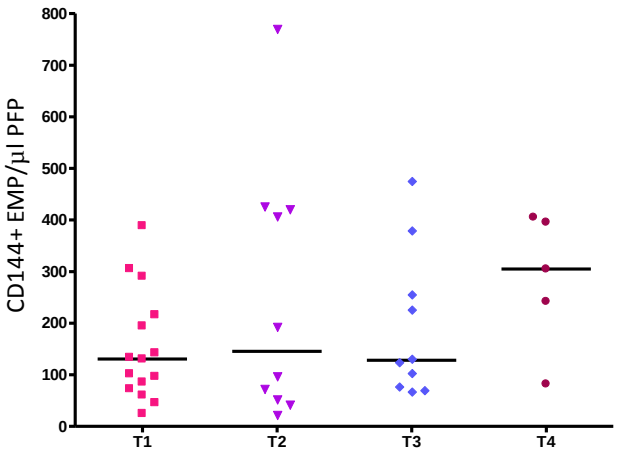


Figure 5.15: Number of circulating CD144+ EMP (per µl in PFP) measured in MM patients before, during, and after chemotherapy.

Number of CD144+ EMP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5), determined by flow cytometry. The horizontal line indicates the median level.

Table 5.14: Number of circulating CD144+ EMP (per µl of PFP) measured in MM patients before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Median (IQR)	P value vs. T4*
T1	15	130.0 (73.0-216.0)	**P = 0.017**
T2	10	145.0 (50.5-422.5)	P = 0.12
T3	10	128.5 (76.3-287.0)	P = 0.15
T4	5	305.0 (162.0-400.5)	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.
*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

5.6.4 Number of PMP before, during, and after chemotherapy in MM patients

For all analysed samples shown in Figure 5.16, the median (IQR) level of circulating CD42b+ PMP measured before chemotherapy (6074.0 [4156.0–7479.0]/µl PFP; T1) was similar to the median levels of CD42b+ PMP after cycle 1 (6117.0 [3281.8–8660.5]/µl PFP; T2) and cycle 2 (6144.0 [4206.0–9385.5]/µl PFP; T3) of chemotherapy, but increased after patients had completed the prescribed chemotherapy course (T4) by 2.1-fold (12530.0 [9305.5–13678.5]/µl PFP) in comparison with the before chemotherapy (T1) median level of CD42b+ MP. Statistical analysis following $\log_e$ transformation of the data to obtain a normal distribution and using a marginal model that accommodated for missing data showed that the estimated marginal mean of circulating CD42b+ PMP was significantly elevated after chemotherapy (T4) in comparison with before (T1) and during (T2 and T3) chemotherapy ($P < 0.0005$, $P = 0.007$, $P = 0.008$, respectively; Table 5.15). However, there was no statistical correlation found between the platelet count and the level of CD42b+ PMP in MM patients before, during, and after chemotherapy (Pearson's correlation coefficient $r = 0.25$, $P = 0.13$).

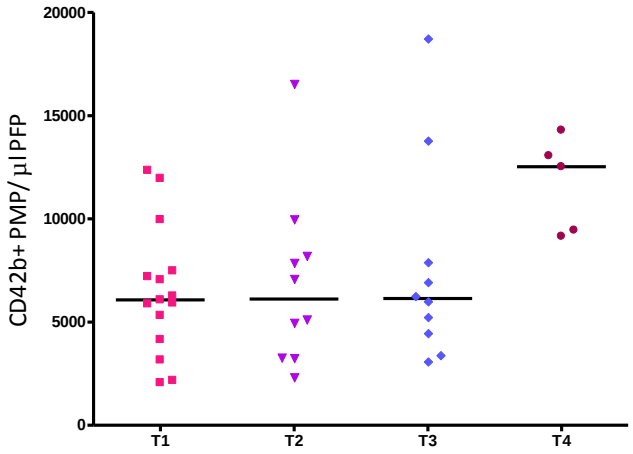


Figure 5.16: Number of circulating CD42b+ PMP (per µl in PFP) measured in MM patients before, during, and after chemotherapy.

Number of CD42b+ PMP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5), determined by flow cytometry. The horizontal line indicates the median level.

Table 5.15: Number of circulating CD42b+ PMP (per µl of PFP) measured in MM patients before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Median (IQR)	P value vs. T4*
T1	15	6074.0 (4156.0-7479.0)	**P < 0.0005**
T2	10	6117.0 (3281.8-8660.5)	**P = 0.007**
T3	10	6144.0 (4206.0-9385.5)	**P = 0.008**
T4	5	12530.0 (9305.5-13678.5)	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.

*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

5.6.5 Number of monocyte-derived MP before, during, and after chemotherapy in MM patients

For all analysed samples shown in Figure 5.17, the median (IQR) levels of circulating CD14+ monocyte-derived MP were shown to be increased after cycle 1 (T2) by 1.6-fold (333.5 [244.8–902.5]/µl PFP) and cycle 2 (T3) by 3.2-fold (684.5 [313.0–928.0]/µl PFP) of chemotherapy, and also after patients had completed the prescribed chemotherapy course (T4) by 2.6-fold (551.0 [357.5–840.5]/µl PFP) in comparison with the before chemotherapy (T1) median level of CD14+ monocyte-derived MP (215.0 [170.0–483.0]/µl

PFP; T1). However, statistical analysis following $\log_e$ transformation of the data to obtain a normal distribution and using a marginal model that accommodated for missing data showed that the estimated marginal mean of circulating CD14+ monocyte-derived MP was not significantly elevated after chemotherapy (T4) in comparison with before (T1) and during (T2 and T3) chemotherapy (P = 0.33, P = 0.70, and P = 0.68, respectively; Table 5.16). Furthermore, there was no statistical correlation found between the monocyte count and the level of CD14+ monocyte-derived MP in MM patients before, during, and after chemotherapy (Pearson's correlation coefficient *r* = -0.13, P = 0.44).

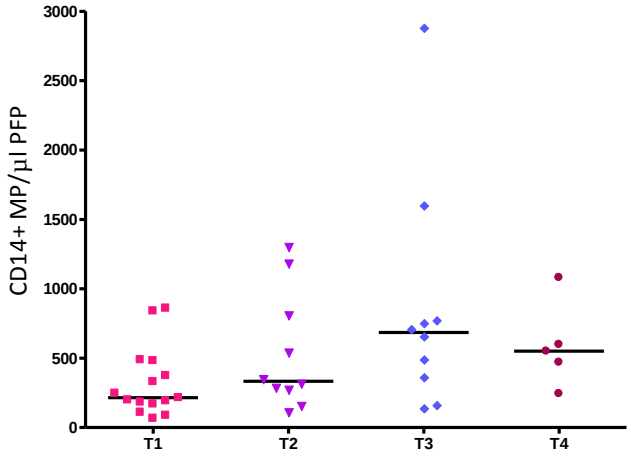


Figure 5.17: Number of circulating CD14+ monocyte-derived MP (per µl in PFP) measured in MM patients before, during, and after chemotherapy.

Number of CD14+ monocyte-derived MP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5), determined by flow cytometry. The horizontal line indicates the median level.

Table 5.16: Number of circulating CD14+ monocyte-derived MP (per µl of PFP) measured in MM patients before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Median (IQR)	P value vs. T4*
T1	15	215.0 (170.0-483.0)	P = 0.33
T2	10	333.5 (244.8-902.5)	P = 0.70
T3	10	684.5 (313.0-982.0)	P = 0.68
T4	5	551.0 (357.5-840.5)	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.

*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

5.6.6 Number of plasma cell-derived MP before, during, and after chemotherapy in MM patients

In Figure 5.18, there is a clear trend of increasing median (IQR) levels of circulating CD138+ plasma cell-derived MP from before chemotherapy (133.0 [70.0–244.0]/µl PFP; T1) to after cycle 1 (T2) by 2.2-fold (292.5 [223.5–677.8]/µl PFP) and cycle 2 of chemotherapy (T3) by 2.5-fold (331.5 [209.5–813.52]/µl PFP) and after patients had finished the full chemotherapy course (T4) by 3.1-fold (414.0 [179.0–558.0]/µl PFP) in all analysed samples. Statistical analysis following $\log_e$ transformation of the data to obtain a normal distribution and using a marginal model that accommodated for missing data revealed that the estimated marginal mean of circulating CD138+ plasma cell-derived MP was significantly elevated (P = 0.004; Table 5.17) after chemotherapy (T4) in comparison with before chemotherapy (T1). However, there was no significant difference found between the estimated marginal mean of circulating CD138+ plasma cell-derived MP after cycle 1 or 2 of chemotherapy (T2 and T3, respectively), in comparison with after treatment (T4; P = 0.95 and P = 0.98; respectively; Table 5.17).

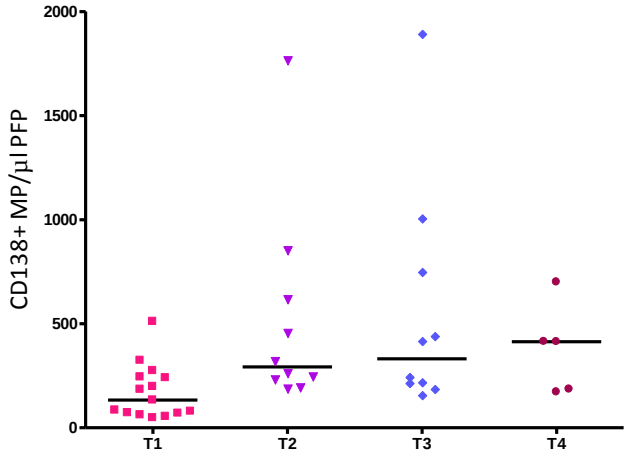


Figure 5.18: Number of circulating CD138+ plasma cell-derived MP (per µl in PFP) measured in MM patients before, during, and after chemotherapy.

Number of CD138+ plasma cell-derived MP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5), determined by flow cytometry. The horizontal line indicates the median level.

Table 5.17: Number of circulating CD138+ plasma cell-derived MP (per µl of PFP) measured in MM patients before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Median (IQR)	P value vs. T4*
T1	15	133.0 (70.0-244.0)	**P = 0.004**
T2	10	292.5 (223.5-677.8)	P = 0.95
T3	10	331.5 (209.5-813.5)	P = 0.98
T4	5	414.0 (179.0-558.0)	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.
*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

5.6.7 Number of PS+ MP before, during, and after chemotherapy in MM patients

The median (IQR) level of circulating PS+ MP measured before chemotherapy (273.0 [156.0–369.0]/µl PFP; T1) was similar to the median levels of PS+ MP measured after cycle 1 (274.0 (187.0–430.8]/µl PFP; T2) and cycle 2 (241.0 [125.8–369.8.0]/µl PFP; T3) of chemotherapy, and also after patients had completed the prescribed chemotherapy course (301.0 [226.5–774.5]/µl PFP; T4) in all analysed samples shown in Figure 5.19. Statistical analysis following $\log_e$ transformation of the data to obtain a normal distribution and using a marginal model that accommodated for missing data showed that the estimated marginal mean of circulating PS+ MP was significantly elevated after chemotherapy (T4) in comparison with before chemotherapy (T1; P = 0.012; Table 5.18), but PS+ MP were not significantly different after treatment (T4) in comparison with after cycles 1 and 2 of chemotherapy (T2 and T3; P = 0.20 and P = 0.066, respectively; Table 5.18).

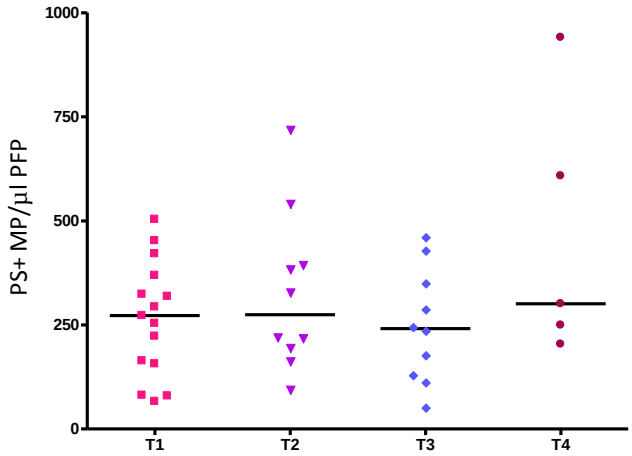


Figure 5.19: Number of circulating PS+ MP (per µl in PFP) measured in MM patients before, during, and after chemotherapy.

Number of PS+ MP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5), determined by flow cytometry and annexin V binding. The horizontal line indicates the median level.

Table 5.18: Number of circulating PS+ MP (per µl of PFP) measured in MM patients before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Median (IQR)	P value vs. T4*
T1	15	273.0 (156.0-369.0)	**P = 0.012**
T2	10	274.0 (187.0-430.8)	P = 0.20
T3	10	241.0 (125.8-369.8)	P = 0.066
T4	5	301.0 (226.5-774.5)	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.

*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

5.6.8 Number of TF+ MP before, during, and after chemotherapy in MM patients

For all analysed samples shown in Figure 5.20, the median (IQR) levels of circulating TF+ MP were shown to be increased after cycle 1 (T2) by 2.7-fold (532.9 [303.8–2236.7]/µl PFP) and cycle 2 (T3) by 1.6-fold (304.1 [118.2–666.6]/µl PFP) of chemotherapy, and also after patients had completed the prescribed chemotherapy course (T4) by 1.5-fold (285.7 [148.7–431.8]/ µl PFP) in comparison with the before chemotherapy (T1) TF+ MP median level (195.2 [129.1–367.0]/µl PFP). However, statistical analysis following $\log_e$

transformation of the data to obtain a normal distribution and using a marginal model that accommodated for missing data showed that the estimated marginal mean of circulating TF+ MP was not significantly elevated after treatment (T4) in comparison with before (T1) and during (T2 and T3) chemotherapy (P = 0.41, P = 0.20, and P = 0.95, respectively; Table 5.19).

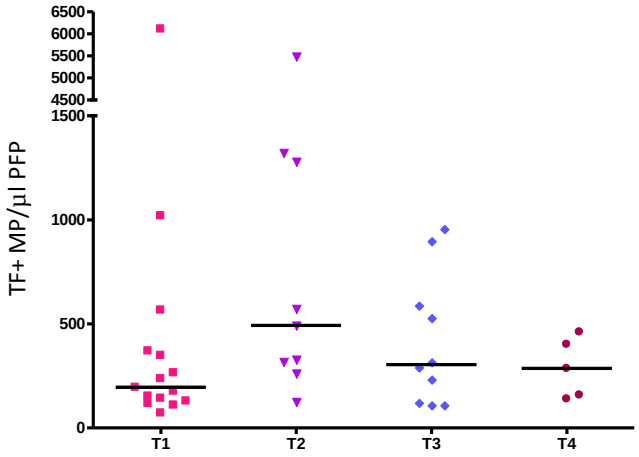


Figure 5.20: Number of circulating TF+ MP (per µl in PFP) measured in MM patients before, during, and after chemotherapy.

Number of TF+ MP (per µl in PFP) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5), determined by flow cytometry. The horizontal line indicates the median level.

Table 5.19: Number of circulating TF+ MP (per µl of PFP) measured in MM patients before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Median (IQR)	P value vs. T4*
T1	15	195.2 (129.1-367.0)	P = 0.41
T2	10	532.9 (303.8-2236.7)	P = 0.20
T3	10	304.1 (118.2-666.6)	P = 0.95
T4	5	285.7 (148.7-431.8)	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.

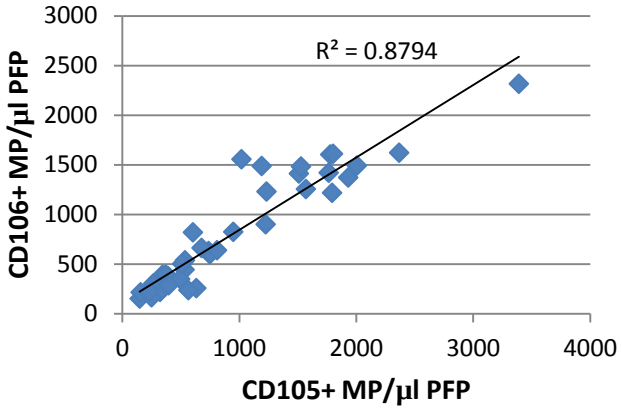
*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. Under a specified assumption, this model facilitated the estimation of the marginal means for each time point, which was analysed by Student's *t* tests to determine significant differences. P values < 0.05 were considered to be statistically significant.

5.6.9 Relationship between different marker subtypes of MP in MM patients before, during, and after chemotherapy

All four markers of EMP used in this study, specifically CD105, CD106, CD54, and CD144, were plotted against each other following $\log_e$ transformation of the data to obtain a normal distribution. Strong statistical correlations were found to exist between all markers of EMP (Pearson's correlation coefficient r = 0.53 to 0.94, P < 0.0005) as shown in Table 5.20 and a representative graph depicting the relationship between CD105+ and CD106+ EMP is shown in Figure 5.21, indicating that the detected MP were of homogenous/endothelial origin. Thus, one of these markers (i.e. CD105) was chosen to represent the population of EMP in further correlation tests among the different subsets of MP including, PMP, monocyte-derived MP, tumour-derived MP, PS+ MP, and TF+ MP. Subsequently, it was found that statistical correlations exist between EMP and PMP (r = 0.39, P = 0.01); monocyte-derived MP (r = 0.85, P < 0.0005); and plasma cell-derived MP (r = 0.54, P < 0.0005) as shown in Table 5.21.

Furthermore, there are significant positive correlations between the level of PS+ MP and EMP (r = 0.53, P < 0.0005; Table 5.21); PMP (r = 0.63, P < 0.0005; Table 5.22); monocyte-derived MP (r = 0.54, P < 0.0005; Table 5.22); and plasma cell-derived MP (r = 0.51, P = 0.001; Table 5.22), suggesting that these MP of different origins are potentially released simultaneously with PS+ MP or that a proportion of these subsets co-express PS. In addition, a significant correlation was found to exist between monocyte-derived MP and plasma cell-derived MP (r = 0.63, P < 0.0005) as shown in Table 5.22, in MM patients before, during, and after chemotherapy. As shown in Table 5.22, PMP were not found to significantly correlate with monocyte-derived MP (r = 0.31, P = 0.053) or plasma cell-derived MP (r = 0.26, P = 0.11). Lastly, as shown in Table 5.22 there was no significant difference found between the level of TF+ MP and PS+ MP (r = -0.05, P = 0.75) EMP (r = 0.11, P = 0.49); PMP (r = -0.25, P = 0.11); and monocyte-derived MP (r = 0.03, P = 0.85), indicating it is unlikely that TF+ MP were released simultaneously with any of these subtypes of MP or that they significantly co-express TF. However, there was a weak statistically significant correlation between TF+ MP and plasma cell-derived MP (r = 0.34, P = 0.03) as shown in Table 5.22.

(A)



(B)

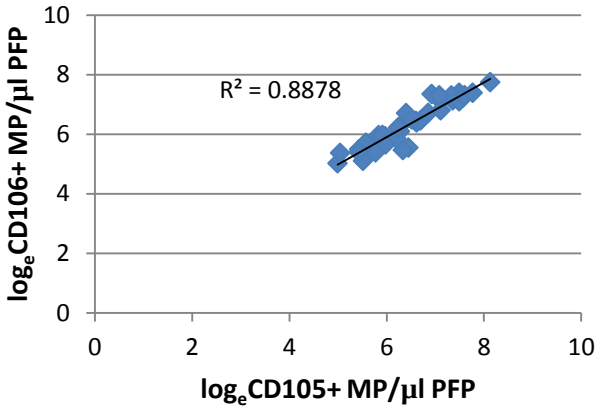


Figure 5.21: Relationship between CD105+ and CD106+ markers of EMP in MM patients before, during, and after chemotherapy.

(A) The number of CD105+ circulating EMP (per µl PFP) determined by flow cytometry in newly diagnosed and relapsed MM patients prior to chemotherapy administration (n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (n=5) was plotted against the relative number of CD106+ circulating EMP (per µl PFP). (B) The variation in CD105+ circulating EMP (per µl PFP) with CD106+ circulating EMP (per µl PFP) on $\log_e$ transformed data.

Table 5.20: Correlation between the $\log_e$ transformed number of CD105+, CD106+, CD54+, and CD144+ circulating EMP in patients with MM before, during, and after chemotherapy.

Correlation between markers of EMP	Pearson's correlation coefficient (*r*)	P value (two-tailed)
CD105 and CD106	0.94	**P < 0.0005**
CD105 and CD54	0.80	**P < 0.0005**
CD105 and CD144	0.66	**P < 0.0005**
CD106 and CD54	0.84	**P < 0.0005**
CD106 and CD144	0.72	**P < 0.0005**
CD54 and CD144	0.53	**P < 0.0005**

Table 5.21: Correlation between the log_e transformed number of circulating EMP and other subsets of MP in patients with MM before, during, and after IMiD-based therapy.

Correlation between EMP and other subtypes of MP	Pearson's correlation coefficient (*r*)	P value (two-tailed)
EMP and PMP CD105 and CD42b	0.39	**P = 0.01**
EMP and monocyte-derived MP CD105 and CD14	0.85	**P < 0.0005**
EMP and plasma cell-derived MP CD105 and CD138	0.54	**P < 0.0005**
EMP and PS+ MP CD105 and annexin V	0.53	**P < 0.0005**
EMP and TF+ MP CD105 and CD142	0.11	P = 0.49

Table 5.22: Correlation between the log_e transformed number of circulating PMP, monocyte-derived MP, plasma cell-derived MP, PS+ MP, and TF+ MP in patients with MM before, during, and after chemotherapy.

Correlations between other subtypes of MP	Pearson's correlation coefficient (*r*)	P value (two-tailed)
PMP and monocyte-derived MP CD42b and CD14	0.31	P = 0.053
PMP and plasma cell-derived MP CD42b and CD138	0.26	P = 0.11
PMP and PS+ MP CD42b and annexin V	0.63	**P < 0.0005**
PMP and TF+ MP CD42b and CD142	-0.25	P = 0.11
Monocyte-derived MP and plasma cell-derived MP CD14 and CD138	0.63	**P < 0.0005**
Monocyte-derived MP and PS+ MP CD14 and annexin V	0.54	**P < 0.0005**
Monocyte-derived MP and TF+ MP CD14 and CD142	0.03	P = 0.85
Plasma cell-derived MP and PS+ MP CD138 and annexin V	0.51	**P = 0.001**
Plasma cell-derived MP and TF+ MP CD138 and CD142	0.34	**P = 0.03**
PS+ MP and TF+ MP Annexin V and CD142	-0.05	P = 0.75

5.7 The PCA of patient plasma samples

The effect of PFP samples containing suspensions of MP on fibrin clot formation were determined in MM patients before, during and after chemotherapy and MPD patients (at a single time point) by a one-stage clotting assay, as described in section 2.6.2. Briefly, the time taken for PFP to clot following recalification (25 mM $CaCl_2$) was recorded in triplicate (if less than 999.9 s).

5.7.1 Comparison of the PCA of MM patient PFP at baseline to the PFP of MPD patients

The median CT of MPD patients (430.7 s) was similar to the median CT of MM patients (424.5 s) measured at baseline prior to chemotherapy administration (T1) for all analysed samples (Figure 5.22), and was shown not to significantly differ (P = 0.72) following $\log_e$ transformation of the data to obtain a normal distribution and using the unpaired Student's t-test for two independent samples (Table 5.23).

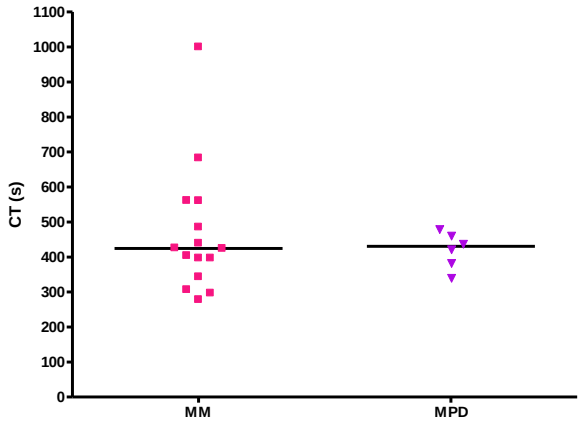


Figure 5.22: The PCA of MM and MPD patient PFP.
CT (s) of newly diagnosed and relapsed MM patients prior to chemotherapy administration (n=15) and MPD patients (n=6), determined by a one-stage coagulation assay. Any PFP sample that did not clot was given a value of 999.9 s. The horizontal line indicates the median level.

Table 5.23: The PCA of MM and MPD patient PFP and P value for two-tailed significance.

MM (n = 15) median (IQR) CT (s)	MPD (n = 6) median (IQR) CT (s)	P value
424.5 (343.5-560.9)	430.7 (373.0-466.5)	P = 0.72

5.7.2 The PCA of MM patient PFP before, during, and after chemotherapy

The median (IQR) CT was found to slightly increase and the coagulability decrease during chemotherapy, specifically after cycle 1 (T2; 512.7 [414.1–658.3] s) and 2 (T3; 478.7 [332.3–579.8] s) of chemotherapy as compared with before chemotherapy (T1; 424.5 [343.5–560.9] s) as shown in Figure 5.23. In contrast, the CT was shown to decrease and coagulability increase after chemotherapy (T4) by 1.3-fold (318.9 [267.2–472.0] s) as compared with before chemotherapy (424.5 [343.5–560.9] s; T1), and shown in Figure 5.23. Statistical analysis following $\log_e$ transformation of the data to obtain a normal

distribution and using a marginal model that accommodated for missing data showed that the estimated marginal mean of the CT was significantly decreased and the coagulability significantly elevated after chemotherapy (T4) in comparison with before chemotherapy (T1; P = 0.007; Table 5.24), and after the first cycle of chemotherapy (T2; P = 0.03; Table 5.24). Furthermore, a trend towards significance was demonstrated in relation to a reduction in the estimated marginal mean of CT after chemotherapy (T4) in comparison with after the second cycle of chemotherapy (T3; P = 0.05; Table 5.24).

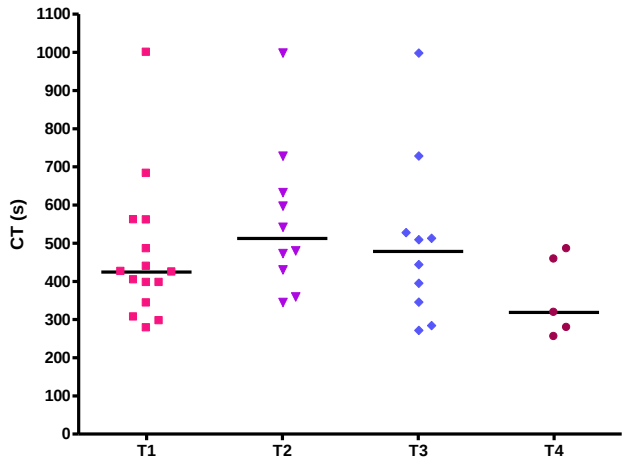


Figure 5.23: The PCA of MM patient PFP before, during, and after IMiD-based therapy.

CT (s) of newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1; n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2; n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3; n=10), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4; n=5), determined by a one-stage clotting assay. Any PFP sample that did not clot was given a value of 999.9 s. The horizontal line indicates the median level.

Table 5.24: The PCA of MM patient PFP before, during, and after chemotherapy and P value for two-tailed significance.

Time point	Number of patients	Median (IQR)	P value vs. T4*
T1	15	424.5 (343.5 – 560.9)	**P = 0.007**
T2	10	512.7 (414.1 – 658.3)	**P = 0.03**
T3	10	478.7 (332.3 – 579.8)	P = 0.05
T4	5	318.9 (267.2 – 472.0)	

Abbreviations: T1, before chemotherapy; T2, after cycle 1; T3, after cycle 2; T4, after chemotherapy.

*Prior to statistical analysis, all collected values for each patient were $\log_e$ transformed and a marginal model was fitted to this data that estimated missing data patterns. 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 differences. P values < 0.05 were considered to be statistically significant.

5.8 The sCD106 and sCD54 serum levels before, during and after chemotherapy in MM patients

To estimate the effects of Thal- and Len-based therapies on endothelial cells *in vivo*, circulating sCD106 and sCD54 were measured, serum levels of which increase in states of endothelial dysfunction (Burger and Touyz, 2012). Specifically, the serum concentration of sCD106 and sCD54 were quantified by ELISA in all MM patient samples collected before, during, and after chemotherapy as described in section 2.7.2 (with the exception of one after cycle 2 sample as a serum separating blood tube was not obtained). Subsequently, the percentage change was calculated for each patient at the following time points T2, T3, and T4 relative to the paired T1 measurements, shown in Figure 5.24. The mean ± SD percentage change in the serum concentration of sCD106 increased by 25.8% after the first cycle of chemotherapy (T2; n=10) relative to the paired samples analysed at T1 prior to chemotherapy administration (given the arbitrary value of 100%). These levels subsequently decreased after the second cycle of chemotherapy (T3; n=9) and after the completion of the prescribed course of chemotherapy (T4; n=5), but were still higher than baseline levels (15.5% and 15.3% increase in comparison to baseline, respectively). In contrast, the mean ± SD percentage change in the serum concentration of sCD54 relative to the paired samples analysed at T1 prior to chemotherapy administration (given the arbitrary value of 100%) were similar after the first cycle (T2; n=10) and the second cycle of chemotherapy (T3; n=9), but increased by 15.0% after the completion of the prescribed course of chemotherapy (T4; n=5).

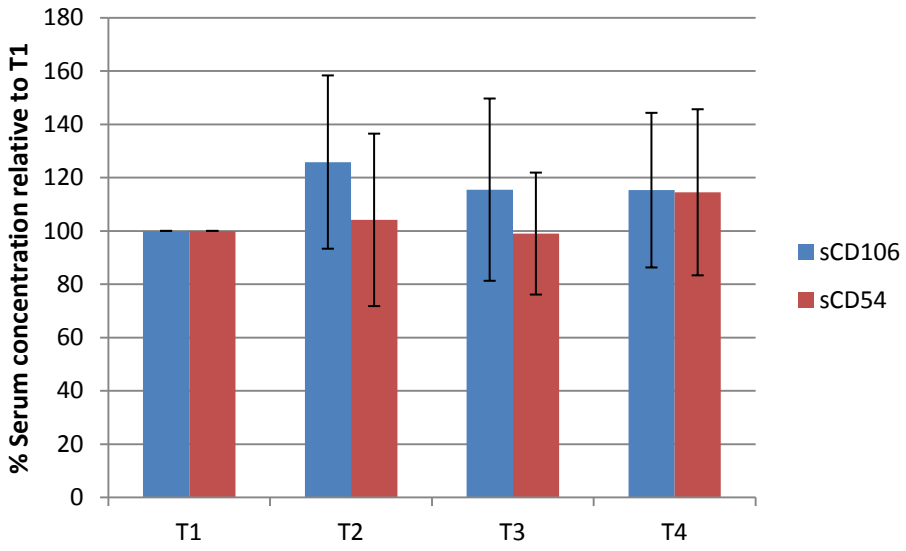


Figure 5.24: The percentage of sCD106 and sCD54 serum levels during and after chemotherapy, relative to before chemotherapy administration in MM patients.

The serum concentration of sCD106 and sCD54 were quantified by ELISA in newly diagnosed and relapsed MM patients prior to chemotherapy administration (T1), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (T2), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (T3), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (T4). The data represent the mean ± SD percentage of both sCD106 and sCD54 serum levels at T2 (n=10), T3 (n=9), and T4 (n=5) calculated relative to T1, which was adjuseted to 100%.

Additionally, a statistical correlation was found to exist between the serum concentration of sCD106 and sCD54 (Pearson's correlation coefficient $r = 0.84$, $P < 0.0005$) determined by ELISA for all analysed serum samples (n=39) as shown in Figure 5.25. However, there was no statistical correlations found between the serum concentration of sCD106 and the level of CD106+ EMP in MM patients before, during, and after chemotherapy (Pearson's correlation coefficient $r = -0.028$, $P = 0.87$), or between the serum concentration of sCD54 and the level of CD54+ EMP (Pearson's correlation coefficient $r = 0.094$, $P = 0.57$).

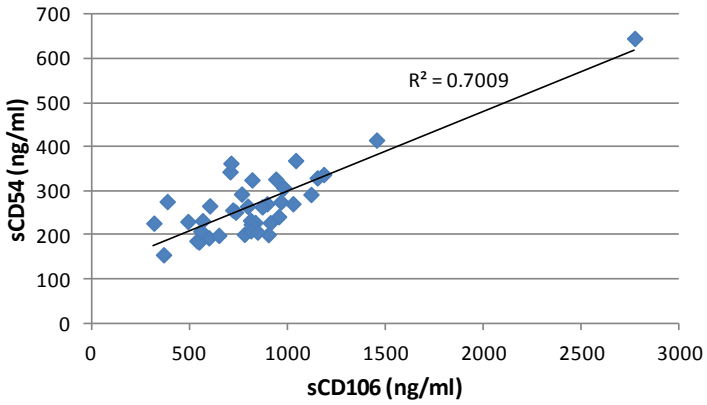


Figure 5.25: Relationship between sCD106 and sCD54 serum levels (ng/ml) before, during, and after chemotherapy in MM patients.

The serum concentration of sCD106 (ng/ml) in newly diagnosed and relapsed MM patients prior to chemotherapy administration (n=15), after completion of the first cycle and before the initiation of the second cycle on cycle 2 day 1 (n=10), after completion of the second cycle and before the initiation of the third cycle on cycle 3 day 1 (n=9), and following the completion of the prescribed chemotherapy course (minimum of 4 cycles) approximately 6-8 weeks after therapy (n=5) was plotted against the relative serum concentration of sCD54 (ng/ml), quantified by ELISA. Data are the average of duplicate measurements.

5.9 Discussion

In cancer, tumour- and host-derived MP have been demonstrated to contribute to angiogenesis, metastatic progression, immune escape, and thrombogenicity (as previously described in section 1.6.1) (van Doormaal et al., 2009, Castellana et al., 2010, Martinez and Andriantsitohaina, 2011). The absolute number of MP reported in the literature varies considerably due to the study population, multiple pre-analytical variables, and the wide range of methods used for MP quantification. In cancer, the tumour type, tumour stage, and/or treatment received may all potentially influence the number of MP detected. Yet, it is likely that different techniques for processing plasma for MP measurement contribute significantly to the variability (Yuana et al., 2011). For example, different centrifugation protocols can result in the loss of MP or residual contaminating platelets, which interfere with the detection of MP (Yuana et al., 2011). Furthermore, some researchers quantify MP using frozen-thawed plasma, yet a consensus decision on the effect of a freeze/thaw cycle on MP has currently not been agreed upon. In one study, the number of PMP determined by flow cytometry were found to be 10-fold higher using frozen-thawed PPP prepared by a single centrifugation at 2,000 x *g* for 30 min (Mobarrez et al., 2010). It is presumed that platelets present in PPP fragment during a freeze/thaw cycle, resulting in an increase in the level of inferred PMP and PS+ MP. In the current study, PFP samples were analysed for

MP detection within 3 h of blood collection, thus eliminating any potential MP artefacts that might be generated by freezing and thawing samples.

The number of different subtypes of circulating MP (including EMP, PMP, monocyte-derived MP, plasma cell-derived MP, TF+ MP, and PS+ MP) and the PCA of PFP were initially evaluated in 15 MM patients prior to chemotherapy administration and 6 MPD patients. To date, few studies have investigated MP in MM, and to the best of the authors' knowledge this is the first study specifically assessing the number and cellular origins of MP in MM patients. The risk of thrombosis in MM is approximately 3% (Zangari et al., 2002b, Klein et al., 2009), which dramatically increases in patients receiving treatment with IMiD in combination with chemotherapeutic drugs or Dex (Carrier et al., 2011). In addition, thrombosis is a leading cause of morbidity and mortality in MPD patients irrespective of treatment (Falanga and Marchetti, 2012b). Previously, PMP were found to be statistically higher in MPD patients in comparison with healthy controls (Villmow et al., 2002). Also, elevated plasma circulating PCA of MP, determined by a widely used chromogenic FXa generation assay (Zymuphen MP-activity), was observed in 44 MPD patients as compared with controls (Duchemin et al., 2010).

In this study, PMP made up the majority of MP evaluated in both MM and MPD patients, a finding that is consistent with other studies showing PMP to be the predominant circulating subtype of MP (Horstman and Ahn, 1999, Berckmans et al., 2001). However, only EMP, PMP, monocyte-derived MP, plasma-cell-derived MP, TF+ MP, and PS+ MP, were evaluated in this study, therefore the presence of MP from other cellular origins cannot be excluded, such as neutrophils that have recently been shown to play a key role in the development of venous thrombosis (von Bruhl et al., 2012, Martinod et al., 2013). Furthermore, the numbers of all MP subtypes measured were higher in MPD patients in comparison with MM patients at baseline, although the levels of PMP or TF+ MP were not found to be significantly different. These results may be explained by the numerous lines of evidence that suggest that the endothelium, platelets, and leukocytes are activated in MPD patients (Falanga and Marchetti, 2012b). For example, increased plasma levels of soluble P-selectin, E-selectin, and leukocyte selectin (Cella et al., 2010), circulating platelet-leukocyte aggregates (Falanga et al., 2005, Marchetti and Falanga, 2008), and the number of circulating endothelial cells have been reported in MPD patients (Alonci et al., 2008,

Trelinski et al., 2010, Belotti et al., 2012). In addition, there was no significant difference between the fibrin clot formation time in MM patients at baseline and MPD patients, which may be explained in part by the fact that there was no significant difference found between the levels of TF+ MP in these patients. However, these results should be interpreted with caution due to very small patient numbers, and the fact that 2 out of the 6 MPD patients were receiving chemotherapy at the time of blood sampling, which may have confounded these results.

The number of TF+ MP were also evaluated in 35 untreated pancreatic cancer patients, which were found to be significantly higher in comparison with MM patients. Pancreatic cancer has a high association with VTE, which has been linked to TF expression (Khorana et al., 2007a). Furthermore, elevated TF activity associated with MP has been demonstrated in pancreatic cancer patients in comparison with healthy controls (Tesselaar et al., 2007, Thaler et al., 2013a, Thaler et al., 2014). Indeed it is believed that tumour cell-derived TF+ MP may contribute to the pathogenesis of thrombosis (Geddings and Mackman, 2013). Thus, the difference in the level of circulating TF+ MP found between pancreatic cancer and MM patients in this study was not surprising as pancreatic cancer cells have been shown to highly express surface TF (Kakkar et al., 1995b, Khorana et al., 2007a, Thaler et al., 2013a), whereas TF is absent on myeloma cells (Cesarman-Maus et al., 2012). However, host cells may also be stimulated to express TF and various studies have identified TF+ MP that also possess endothelial cell-, platelet-, and monocyte-derived surface antigens in various conditions including meningococcal sepsis (Nieuwland et al., 2000), type 2 diabetes mellitus (Diamant et al., 2002), sickle cell disease (Shet et al., 2003), and cancer (Del Conde et al., 2007, Hron et al., 2007). Bone marrow biopsies in MM show positive TF staining within non-myeloma cells such as megakaryocytes and endothelial cells (Cesarman-Maus et al., 2012), which may be the source of at least part of the plasma TF+ MP detected in this study. In particular, Hron et al. show that elevated levels of TF+ MP in 20 advanced colorectal cancer patients are mainly derived from platelets (Hron et al., 2007). However, activated platelets free of monocytes have recently been shown not to express active TF (Osterud and Olsen, 2013), and the acquisition of the TF receptor by platelets is believed to occur via membrane transfer from monocyte-derived MP (Rauch et al., 2000, Del Conde et al., 2005). In the current study, the cellular source(s) of circulating TF+ MP in MM, MPD, and pancreatic cancer patients are unknown, although this study

was not designed to address this issue. The differences in the level of circulating TF+ MP found between pancreatic cancer and MM patients in this study are presumably due to differences in tumour behaviour and may explain at least in part the reported differences between the occurrence of thrombosis in pancreatic cancer and MM patients prior to treatment and the markedly different effect of some of the conventional thromboprophylaxis schedules in these two disease entities.

The main aims of this chapter were to prospectively evaluate the level of various subtypes of circulating MP (including EMP, PMP, monocyte-derived MP, plasma cell-derived MP, TF+ MP, and PS+ MP) and the PCA of PFP in MM patients before, during, and after treatment with Thal- or Len-based therapies. Newly diagnosed MM patients were treated with CTD (n=4), CTDa (n=6), or RCDa (n=1), while relapsed patients received Len/Dex (n=4). Specifically, EMP, PMP, plasma cell-derived MP, and PS+ MP were elevated in MM patients after treatment relative to baseline levels. In addition, the PCA of PFP containing suspensions of MP was also higher in these patients after treatment, although the faster CT did not correlate with any of the subtypes of MP evaluated in this study.

As previously mentioned, the normal endothelium functions to protect against thrombosis by preventing the attachment of platelets and leukocytes, expressing anticoagulants (TFPI, thrombomodulin, endothelial protein C receptor, and heparan sulphate proteoglycans) and releasing platelet inhibitors (nitric oxide and prostacyclin) (Watson, 2009, Esmon and Esmon, 2011). However under pathological conditions, endothelial cell injury may lead to loss of vascular integrity, platelet activation, thrombus formation, increased permeability, and leukocyte adhesion mediated by mechanisms including increased expression of adhesion molecules, cytokine production, changes in the phenotype from antithrombotic to prothrombotic, increased ROS bioavailability, and decreased nitric oxide bioavailability. Elevated plasma levels of EMP reflect endothelial cell injury and are now considered a biomarker of vascular dysfunction (Burger and Touyz, 2012). Furthermore, previous studies have demonstrated that MP from various cellular origins, including EMP and leukocyte-derived MP, may also induce endothelial dysfunction, particularly by shifting the balance between nitric oxide and ROS production and release from endothelial cells (VanWijk et al., 2002, Brodsky et al., 2004, Mostefai et al., 2008). In particular, high levels of EMP have been observed in cardiovascular diseases, including acute coronary

syndromes, stroke, metabolic syndrome, and hypertension (Mallat et al., 2000, Gonzalez-Quintero et al., 2004, Koga et al., 2005, Bernard et al., 2009, Morel et al., 2009, Lee et al., 2012). Furthermore, in VTE, marked activation of the endothelium, platelets, and leukocytes has been reported and shown to involve the generation of EMP and formation of EMP-monocyte conjugates and platelet-leukocyte conjugates (Chirinos et al., 2005). In addition, endothelial cell damage has been described as a mechanism for the increased incidence of VTE observed when antiangiogenic drugs were combined with chemotherapy agents such as cisplatin or gemcitabine (Kuenen et al., 2002b). In this study, the findings of elevated plasma EMP and PCA after chemotherapy in combination with IMiD, either Thal or Len that have antiangiogenic effects, lend further support to the idea that endothelial cell injury is a mechanism involved in the thrombogenicity associated with anticancer agents *in vivo*, and in the context of this study, particularly in MM patients.

Although the exact mechanism of the generation of MP has not been evaluated in this study, an *in vitro* study found that the levels of EMP expressing CD105, a constitutive endothelial marker, were markedly increased in apoptosis, while those EMP expressing the inducible cell adhesion marker CD54 were only increased following cell activation (Jimenez et al., 2003). Therefore, it is hypothesised that EMP shed into the peripheral circulation of MM patients that received chemotherapy with IMiD, either Thal or Len, might be a consequence of endothelial cell apoptosis and activation as both CD105+ and CD54+ EMP are elevated in the current study following treatment. In addition, the serum levels of both sCD106 and sCD54 were elevated by 15% after chemotherapy with IMiD, either Thal or Len, relative to before treatment levels in the 5 MM patients that completed the prescribed course, which further implies an effect of chemotherapy regimens with IMiD on the vascular endothelium. In agreement with these results, serum levels of sCD106 were previously shown to be significantly elevated in 30 patients with relapsed chronic lymphocytic leukaemia after 1 week on Len indicating Len-induced endothelial dysfunction, which were associated with the subsequent development of DVT (Aue et al., 2011). Furthermore, increased levels of sCD106 were recently associated with inferior survival in newly diagnosed MM patients treated with Thal- or Len-based therapies (Terpos et al., 2013).

In this study, 4 newly diagnosed MM patients received pulsed high-dose Dex (40 mg; days 1-4 and 12-15 of a 21 day cycle of CTD) and 4 relapsed MM patients received weekly high-dose Dex (40 mg; days 1, 8, 15, and 22 of a 28 day cycle of Len/Dex). Previously, Kerachian et al. demonstrated that treating endothelial cells *in vitro* with high-dose Dex significantly elevates CD106 and CD54 mRNA expression levels determined by real-time reverse transcriptase polymerase chain reaction (RT-PCR) assays (Kerachian et al., 2009). In addition, several studies have reported that Dex modulates the expression of endothelial haemostatic elements; specifically inducing vWF (Jilma et al., 2005, Kerachian et al., 2009) and TF (Kerachian et al., 2009, Hoshi et al., 2011, Isozumi et al., 2013), while down-regulating thrombomodulin (Kerachian et al., 2009). Similarly, IMiD, either Thal or Len, may also elevate vWF (Baz et al., 2005, van Marion et al., 2008) and TF (Hoshi et al., 2011, Valsami et al., 2011, Isozumi et al., 2013). Furthermore, EMP derived from TNF-α-stimulated endothelial cells have been shown to bind to monocytes *in vitro*, involving the interaction between CD54 on EMP and β2 integrin on monocytes, which induces TF-dependent PCA in monocytes (Sabatier et al., 2002). Interestingly, TF mRNA was not detected by RT-PCR in these EMP excluding the possibility of a TF mRNA transfer to monocytes during co-incubation (Sabatier et al., 2002). Moreover, the PCA of LPS-stimulated peripheral blood mononuclear cells and the TF antigen levels of purified monocytes were increased in 10 MM patients after 3-4 cycles of either Thal- or Len-based treatment, relative to baseline levels in these patients and also in comparison with 12 MM patients who received Bor-based treatment (Kornberg et al., 2013).

Similarly, monocyte-derived MP can adhere to endothelial cell membranes and have been found to increase endothelial cell TF activity, antigen level, and mRNA expression (Aharon et al., 2008). They may also induce endothelial cell apoptosis and capillary tube formation, which may indicate an angiogenic effect (Aharon et al., 2008). Furthermore, monocyte-derived MP from LPS-treated monocytes have been shown to bind to endothelial cells *in vitro* resulting in the activation of intracellular signalling pathways, such as ERK1/2 and NF-κB, which in turn induce the expression of the cellular adhesion molecules CD106, CD54, and E-selectin on endothelial cells (Wang et al., 2011). Monocyte-derived MP appear to be the primary cellular source of circulating TF in healthy subjects and can transfer the TF receptor to other cellular types, including platelets and neutrophils, amplifying PCA (Rauch et al., 2000, Del Conde et al., 2005, Egorina et al., 2005). Previous studies have

demonstrated that MP derived from polymorphonuclear leukocytes, most likely neutrophil-derived MP, can activate endothelial cells and induce their expression of TF (Mesri and Altieri, 1998, Mesri and Altieri, 1999). In addition, leukocyte-derived MP have been associated with venous thrombus formation in animal models (Myers et al., 2003, Ramacciotti et al., 2009), and elevated levels of monocyte-derived MP bearing TF were found in a septic patient with disseminated intravascular coagulation (Nieuwland et al., 2000). In the current study, both monocyte-derived MP and TF+ MP were not elevated after chemotherapy with IMiD, either Thal or Len, relative to before or after the first and second cycles of treatment. However, an initial transient elevation of monocyte-derived MP in MM patients during treatment cannot be excluded since these MP may rapidly be internalized by various cell types *in vivo*.

Activated platelets release inflammatory proteins that promote leukocyte chemoattraction, vascular inflammation, and further modify the endothelial phenotype (Gawaz et al., 2005). In addition, activated platelets also generate PMP that are elevated in arterial thrombosis (platelet-rich so called 'white clots') (Lee et al., 1993, Katopodis et al., 1997, Mallat et al., 2000, van der Zee et al., 2006, Skeppholm et al., 2012), but PMP are less clearly defined in venous thrombosis (fibrin-rich so called 'red clots' because they also contain erythrocytes). Nonetheless, experimental and morphological studies imply that inflammation and platelet activation are also involved in venous thrombogenesis (as previously described in section 1.3) (Sobieszczyk et al., 2002, Myers et al., 2003, von Bruhl et al., 2012). P-selectin on isolated platelets and PMP were higher in 6 patients with pulmonary embolism in comparison with healthy controls, indicating platelet activation (Inami et al., 2003), and thrombus weight was shown to positively correlate with PMP in an experimental model of thrombosis induced by IVC ligation (Ramacciotti et al., 2009). However, Chirinos et al. reported that the levels of PMP were not significantly elevated in 25 patients with VTE (Chirinos et al., 2005). It is currently speculated that elevated levels of PMP may be considered as a marker of ongoing thrombosis rather than a crucial component in venous thrombogenesis (Myers et al., 2003, Ramacciotti et al., 2009). However, the role of PMP in venous thrombosis requires further evaluation *in vivo*.

Platelets bearing CD42b (GPIbα) are known to bind vWF in the subendothelial matrix or plasma, P-selectin on activated platelets or endothelial cells, and leukocyte Mac-1 integrin.

A recent experimental model of thrombosis induced by IVC stenosis demonstrated the importance of the platelet CD42b receptor in the development of DVT, which facilitated both platelet and leukocyte recruitment, and stimulated the formation of NET by neutrophils (von Bruhl et al., 2012). In the current study, the finding of increased levels of CD42b+ PMP after chemotherapy in combination with IMiD, either Thal or Len, may suggest treatment-induced platelet activation. In support of this finding, other studies have previously demonstrated *ex vivo* platelet activation mediated by some of the anticancer agents that patients received in the current study. For example, a significant increase of the platelet activation marker P-selectin was found on isolated platelets in 31 MM patients after 4 weeks of treatment with Thal and Dex (Robak et al., 2012). Furthermore, Jilma et al. observed elevated plasma levels of soluble P-selectin in 9 healthy men 48h after receiving high-dose Dex (Jilma et al., 2005). Furthermore, in castration-resistant prostate cancer patients treated with docetaxel-based chemotherapy, high PMP levels were associated with a short survival (Helley et al., 2009).

In this study, the number of circulating plasma cell-derived MP or more specifically, tumour cell-derived MP in MM patients were elevated after chemotherapy with IMiD, either Thal or Len, relative to baseline, suggesting that the tumour cells have undergone apoptosis in the presence of cytotoxic agents as the number of tumour cell-derived MP correlated with the number of PS+ MP. In addition, previous studies have shown that human acute monocytic leukaemia THP-1 cells exposed to daunorubicin *in vitro* release higher numbers of MP than untreated tumour cells (Boles et al., 2012), and also breast cancer patients exhibit increased numbers of circulating tumour cell-derived MP (MUC-1+) 24 h after the first cycle of Dox and cyclophosphamide (n = 11) or paclitaxel (n = 9) as compared to the numbers of tumour cell-derived MP at baseline (Fremder et al., 2014).

Elevated levels of PS+ MP were found after chemotherapy in combination with IMiD, either Thal or Len, which may at least in part contribute to the greater PCA observed in MM patients following treatment in this study by providing a catalytic surface for the assembly of components of the coagulation cascade. However, no correlation was observed between the number of PS+ MP and the CT. Exposed PS on MP was determined by annexin V binding, although the extent of annexin V binding to the membrane is related to many variables, including the free annexin V concentration, membrane PS content,

phosphatidylethanolamine content, and ambient Ca^{2+} concentration. A growing number of studies are showing that lactadherin has an increased affinity for anionic phospholipids in comparison with annexin V and is a more sensitive marker of small changes in PS expression, particularly when examining PMP and stored platelets (Albanyan et al., 2009, Hou et al., 2011), adherent platelets at the site of vascular damage in animal models of thrombosis (Shi et al., 2008), and apoptotic leukaemia cell lines (Shi et al., 2006, Zhou et al., 2010). Thus, the use of annexin V in this study may have underestimated the number of PS+ MP detected. Furthermore, the expression of PS is widely regarded as a critical marker for MP, but subpopulations of MP without exposed PS are increasingly being reported in the literature (Connor et al., 2010). Interestingly, studies have shown that the PS composition of circulating procoagulant MP varies depending on the type of stimulation (Jimenez et al., 2003, Abid Hussein et al., 2008).

Furthermore, several cytokines and transcription factors that are elevated in MM patients, such as IL-6, TNF-α, VEGF, and NF-κB, have the ability to upregulate TF expression in both monocytes and endothelial cells (Bode and Mackman, 2014), although no significant alteration of the levels of TF+ MP was observed in this study after chemotherapy with IMiD, either Thal or Len, relative to before or after the first and second cycles of treatment. However, several studies have shown that the number of TF+ MP does not correlate with TF activity associated with MP in cancer patients (Tesselaar et al., 2007, Haubold et al., 2009, Tesselaar et al., 2009, van Doormaal et al., 2012). In this study we did not specifically evaluate TF activity, therefore an increase in the TF activity associated with MP may have potentially contributed to the higher PCA observed after treatment. Yet, Auwerda et al. previously showed that the circulating levels of TF activity associated with MP declined after chemotherapy in 122 untreated newly diagnosed MM patients (except in those patients who developed VTE) in comparison with before treatment levels, although not all patients in this study were receiving novel agent-based therapies (Auwerda et al., 2011). Thus, the TF activity of MM patients receiving chemotherapy in combination with IMiD requires further exploration as functional assays may better reflect the thrombogenicity of MP.

All patients received thromboprophylaxis with LMWH alongside therapy, which do not significantly interfere with conventional prothrombin time assays at therapeutic

concentrations (Funk, 2012). However, recent data suggests the PCA of MP is not limited to stimulation of the extrinsic pathway or the supply of a negatively charged phospholipid surface for the initiating and prothrombinase complexes, which are measured by the clotting assay used in this study. In particular, evidence is emerging that demonstrate cell-derived MP, specifically erythrocytes and platelets, from patients' plasma can also increase contact-dependent thrombin generation (van Beers et al., 2009, Aleman et al., 2011, Van Der Meijden et al., 2012, Rubin et al., 2013). Therefore, thrombin generation may be a more appropriate test to use to assess the hypercoagulable state in patients' plasma with diseases known to have elevated MP, as this method assesses all phases of thrombin generation including, initiation, amplification, and inhibition of thrombin generation as well as the integral amount of generated thrombin (van Veen et al., 2008). However, standardization of this method is required before wide clinical use of this assay and even when a single well standardized protocol is available it is likely that it would have to be modified for different clinical situations.

It is important to note that the results presented here are a preliminary report of a clinical study, which requires confirmation upon trial completion. The approved protocol for this clinical study specified that a maximum of 50 newly diagnosed MM patients could be recruited prior to the administration of various chemotherapy regimens (with and without the inclusion of IMiD), 25 relapsed MM patients receiving Len/Dex, and 25 relapsed MM patients receiving Bor/Dex. However, due to regulatory delays encountered during the approval stage, only a small cohort of patients were recruited and subsequently suitable for analysis during the thesis study period (n=15; see Figure 5.1). Therefore, the limitations of this study are the small sample size and heterogeneity of the patient group i.e. newly diagnosed and relapsed MM patients receiving a variety of treatment regimens including CTD, CTDa, RCDa, and Len/Dex. In contrast, the patient group is representative of the overall population of MM patients, which might be regarded as a strength of the study. Furthermore, all patients analysed in this pilot study received thromboprophylaxis with LMWH, which was successful as no symptomatic VTE events were observed in the MM patients before, during and for up to 8 weeks after chemotherapy. Although a larger patient cohort without any thromboprophylaxis might better elucidate the role of procoagulant MP in VTE associated with Thal- or Len-based therapies in MM patients, it would be unethical to design such studies. Furthermore, important limitations regarding

light scatter flow cytometry for MP sizing and detection need to be addressed. The number of MP determined using current standard flow cytometers is an underestimation of the true count, due to the intrinsic resolution limit for the size-related parameter (500 nm), and more recently the described swarm effect, whereby multiple vesicles smaller than 220 nm are aggregated and counted as a single event at sufficiently high concentrations (van der Pol et al., 2012b). Thus, the concentration of MP detected in this study must be interpreted with caution due to the potential presence of aggregated microvesicle artefacts. Furthermore, the accuracy of determining the cellular origin of MP can be affected by the ability of MP or the parental cells to obtain other cell-specific receptors through membrane fusion.

Despite the relatively small size of the study population, the data presented in this pilot study suggests that the host response to treatment through endothelial cell dysfunction may contribute to the relatively increased thrombogenicity observed in MM patients who received chemotherapy in combination with IMiD, either Thal or Len. Furthermore, the procoagulant profile of MM in this study does not appear to be mediated by the level of TF+ MP, which is markedly different to the one that has been demonstrated in solid malignancies, particularly pancreatic cancer. This study also lends support to further clinical evaluation of new strategies aimed at reducing the risk of VTE in these patients through more effective thromboprophylaxis, including defibrotide and aspirin, which specifically target endothelial stress and platelet functions, respectively, and have both shown promise in this setting (Mitsiades et al., 2009, Palumbo et al., 2010c, Palumbo et al., 2011, Larocca et al., 2012). Furthermore, a reduced risk for thrombosis has been suggested to be one of the unintended benefits of lipid-lowering therapy with statins via a number of proposed mechanisms including the decreased expression of TF, release of procoagulant MP, and alteration of coagulation factors, such as FV, thrombin, and TFPI (Takemoto and Liao, 2001, Camera et al., 2002, Undas et al., 2005, Camino-Lopez et al., 2007, Owens et al., 2012). However, the clinical significance of the antithrombotic activity of statins in cancer patients has yet to be established. On the basis of these results, further studies are justified to evaluate the impact of endothelial dysfunction and the resulting increase in circulating procoagulant MP in chemotherapy-associated VTE in cancer patients. However, this mechanism may be different in other tumour types, particularly those with high TF expression, with the administration of different anticancer agents.

Chapter 6: General discussion

It is of great importance to delineate the mechanism(s) through which cancer and anticancer agents can alter the thrombotic risk in order to achieve better screening for at-risk patients and safer thromboprophylactic strategies. The current study has established a number of findings that contribute to the understanding of how coagulation can be initiated (specifically through the intrinsic and common pathways) in cancer cells and be potentiated by the cytotoxic treatment of malignant disease, with respects to the specific expression of TF and PS, and generation of MP.

Numerous studies have shown that the majority of cancer cells, particularly those of epithelial origin, express TF and generate TF+ MP (Geddings and Mackman, 2013). The majority of clinical studies (previously described in section 1.6.5) suggest an association between the level of tumour TF and number/activity of circulating TF+ MP with cancer-associated VTE. However, this premise has mainly been demonstrated in patients with pancreatic cancer, which is one of the most prothrombotic malignancies. The results presented in Chapter 3 strengthen these existing observations by demonstrating TF expression on various cancer cells *in vitro*, and provide an explicit link between the level of tumour cell TF expression and PCA. Furthermore, those tumour cells that possess procoagulant potential, particularly those that express TF, demonstrate PCA associated with MP.

In addition, the risk of VTE is further increased in cancer patients receiving single-agent or multi-agent chemotherapeutic regimens by mechanisms that have not yet been fully elucidated. The results from Chapter 4 of this study show that the cytotoxic chemotherapeutic agent Dox increased PCA in cancer cells, which was not related to an upregulation of surface TF antigen. Furthermore, increased PCA associated with the fraction of MP in the cell-free supernatant was found to increase in response to chemotherapy (exclusively in those cancer cell lines that demonstrated baseline MP-associated PCA), which was inversely correlated with the cell viability. Thus, suggesting that chemosensitive tumours are likely to be associated with VTE, as they are more susceptible to apoptosis and consequently the generation of procoagulant MP induced by chemotherapy. Indeed this theory is supported by studies performed in MM that report

higher VTE incidences in newly diagnosed MM patients receiving IMiD in combination with Dex (approximately 12-26%), in comparison with relapsed or refractory patients (apporoximately 2-15%) (Rajkumar et al., 2002, Cavo et al., 2005, Rajkumar et al., 2006, Weber et al., 2007, Rajkumar et al., 2008, Klein et al., 2009). Moreover, increased levels of tumour cell-derived MP were found in Chapter 5 in MM patients treated with Thal- or Len-based therapies indicating tumour cell apoptosis, and these also correlated with the number of procoagulant PS+ MP.

A major finding of this study was elevated levels of EMP, sCD106, and sCD54 that reflect endothelial damage in MM patients after combination therapy with Thal or Len. These results are consistent with previous clinical studies that have also observed markers of endothelial injury related to Thal or Len treatment (Aue et al., 2011, van Marion et al., 2008, Corso et al., 2004). Furthermore, the levels of MP expressing the platelet activation marker CD42b were increased in this study following treatment with Thal- or Len-therapies in MM patients, potentially indicating platelet activation. Previously, platelet activation induced by Thal therapy was indicated in 5 MM patients by elevated levels of platelet P-selectin expression and platelet-monocyte complexes (Dunkley and Gaudry, 2007). Unfortunately due to time, resources, and logistical issues these effects were not confirmed in this preliminary study by endothelial and platelet function experiments, which are included in the approved study protocol for this clinical trial and should be performed in the future continuation of this work.

Another novel finding of this study is that although the PCA was increased in MM patients following treatment, this was not a consequence of increased levels of TF+ MP, which remained unchanged throughout the study. However, this preliminary result needs to be validated upon completion of this clinical study. Furthermore, as previously mentioned an increase in TF activity cannot be excluded as a potential explanation for the treatment-induced hypercoagulable state as this was not specifically measured in this study. However in a similar study, Auwerda et al. found that TF activity was higher in MM patients before chemotherapy compared to controls and remained elevated after chemotherapy only in patients with VTE during follow-up, although this association between increased levels of TF activity associated MP and future VTE in MM patients was not found to be statistically significant (Auwerda et al., 2011). Furthermore, the initial procoagulant response to

chemotherapy administered mainly in the adjuvant clinical setting in breast cancer patients demonstrated by circulating thrombin-antithrombin complex levels was not associated with either TF activity on MP or plasma levels of TF antigen (Mukherjee et al., 2010, Kirwan et al., 2008).

In contrast to the aforementioned clinical settings, a highly prothombotic tumour-derived TF phenotype already exists in the majority of pancreatic cancer patients prior to treatment. Indeed untreated pancreatic cancer patients in this study were found to have a markedly higher baseline level of TF+ MP in comparison to MM patients. It is possible that the endothelial dysfunction observed in MM patients in this study that was induced by chemotherapy in combination with IMiD (either Thal or Len) may be an indiscriminate effect of the cytotoxic treatment in cancer. Thus, direct damage exerted by anticancer agents on the vascular endothelium leading to endothelial dysfunction may be further augumented by a high baseline level of tumour-derived TF+ MP that would likely bind to the damaged endothelium or activated platelets and elicit coagulation, changing the vascular phenotype from anticoagulant to prothrombotic. However, this hypothesis would need to be verified in other cancer groups as different chemotherapy regimens are used in other malignancies, and different rates of VTE are observed with the type of chemotherapy drug administered. For example, the risk of thromboembolism was higher in gastroesophageal cancer patients treated with cisplatin based-therapy in comparison with oxaliplatin-based therapy (Al-Batran et al., 2008, Starling et al., 2009). In addition, Thal and Len possess known antiangiogenic activity and are the main agents used in MM therapy, which may be the reason why endothelial dysfunction was so evident in this study and why VTE is such a frequent complication in MM patients receiving treatment. Recently, the risk of VTE was shown to be increased following chemotherapy in cancer patients that had a higher baseline level of circulating TF+ MP in comparison with patients expressing low levels of TF+ MP (Zwicker et al., 2013). However, it is essential to standardise methods for measuring the number or activity of TF+ MP prior to clinical application, and the most appropriate assay for clinical implementation will be the one that is found to consistently correlate with a clinically relevant outcome, such as thrombotic risk and/or cancer progression.

Finally, as this work was reaching its conclusion the potential impact of neutrophils on venous thrombosis in these patients has received new impetus through the description of NET (previously described in section 1.3). During infection and sepsis, neutrophils have been demonstrated to provide intravascular immunity by releasing NET (a network of DNA fibres containing histones and antimicrobial proteins) that capture and kill circulating pathogens, thereby preventing their dissemination throughout the body (McDonald et al., 2012). In addition, NET have been implicated in venous thrombogenesis by promoting platelet adhesion, activation, and aggregation, recruiting red blood cells, triggering fibrin deposition, and inducing thrombus formation (Fuchs et al., 2010, Massberg et al., 2010, Brill et al., 2012, von Bruhl et al., 2012). It is therefore proposed that they may also capture circulating procoagulant MP. In this study, no significant difference was detected between the number of neutrophils before, during, and after chemotherapy; however, a marker to detect neutrophil-derived MP was not used. In particular, it has been suggested that chemotherapy administration in breast cancer patients promotes the release of cell-free DNA from injured cells, predominantly of neutrophil origin, that possess the ability to trigger PCA through activation of the intrinsic pathway (Swystun et al., 2011b). Thus, the role of NET in cancer-associated thrombosis and during chemotherapy requires further investigation.

In conclusion, the data presented here has strengthened the notion that endothelial dysfunction is a mechanism of chemotherapy-induced thrombosis in MM patients receiving Thal- or Len-based therapies. Therefore, strategies for VTE prevention that are focused on protecting the endothelium and reducing the prothrombotic/proinflammatory response of the perturbed endothelium may be more beneficial for MM patients treated with Thal- or Len-based therapies. Furthermore, it is proposed that in other malignancies chemotherapy may indiscriminately elicit insult to endothelial cells by altering their normal antithrombotic and antiadhesive phenotype or by directly damaging the endothelial monolayer, thereby exposing the highly procoagulant subendothelial cell matrix to the blood and increasing the risk of thrombosis. However, this hypothesis would need to be prospectively evaluated in further clinical studies on a tumour type basis with regards to the specific chemotherapeutic agent(s) administered.

Future work

Recommendations for future work in this project include the continuation of this clinical study to reach maximum recruitment limits of all study arms and inclusion of endothelial and platelet function tests, to support and extend the findings of this preliminary report. Endothelial function can be assessed by a new non-invasive technique known as peripheral arterial tonometry (PAT) that uses fingertip plethysmography to measure pulse volume amplitude (PVA). The PVA changes to reactive hyperemia (induced following upper arm occlusion of systolic blood pressure) compared to baseline have been shown to be nitric oxide-dependent, implying that PAT is a measure of endothelial function (Nohria et al., 2006). Furthermore, platelet function can be determined by whole blood flow cytometry, specifically measuring platelet surface activation markers (i.e. CD42b, P-selectin, and CD154) and platelet-leukocyte aggregates. In addition, the chemotherapy-induced hypercoagulable state in this study could be further investigated by measuring markers of blood coagulation activation, such as D-dimer, prothrombin fragments 1+2, thrombin-antithrombin complexes, and fibrin monomer complexes. Furthermore, the work in this clinical study suggests that chemotherapy-induced initiation of coagulation may be enhanced by endothelial activation/damage. Thus, the anticoagulant properties of the endothelium could be investigated in MM patients before, during, and after chemotherapy by measuring plasma levels of protein C, activated protein C, protein S, TFPI, and/or antithrombin III. In particular, Mukherjee et al. have reported that chemotherapy impairs endothelium-based protein C anticoagulant pathway in 26 breast cancer patients (Mukherjee et al., 2010), and protein C was also significantly decreased in 32 patients with relapsed chronic lymphocytic leukaemia after 1 week on Len (Aue et al., 2011). Lastly, after recent demonstration of the importance of neutrophils in venous thrombogenesis and their formation of NET (von Bruhl et al., 2012), a neutrophil marker could be included in this study to detect neutrophil-derived MP, and additionally plasma biomarkers of NET (e.g. cell-free DNA and citrullinated histone H3) may be a further avenue worth investigating.

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Appendix A: Patient information sheet for newly diagnosed MM patients undergoing chemotherapy

Hull and East Yorkshire Hospitals NHS
NHS Trust

Queen's Centre for Oncology & Haematology
Castle Hill Hospital
Castle Road
Cottingham
HU16 5JQ

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, reduced

production 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?
<u>Blood samples</u>:

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.

<u>Part 2</u>

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?
<u>Complaints</u>:
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.

<u>Harm</u>:
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.

Date:

Version number:

Protocol reference:

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 B: Consent form for newly diagnosed MM patients undergoing chemotherapy

Hull and East Yorkshire Hospitals NHS
NHS Trust

Queen's Centre for Oncology & Haematology
Castle Hill Hospital
Castle Road
Cottingham
HU16 5JQ

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

When completed: 1 copy for patient, 1 copy to be kept in medical notes, 1 original for researcher site file.

Appendix C: The CRF for newly diagnosed MM patients undergoing chemotherapy

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 <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 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 <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**

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**