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Novel Endothelial-Protective and Anti-Thrombotic Effects of Therapeutic Agents in Malignant and Inflammatory Diseases: Molecular Mechanisms and Translational Relevance

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Student Number: 13212583

This thesis is submitted to University College Dublin in fulfilment of the requirements for the Degree of Doctor of Philosophy in the School of Medicine and Medical Science

Head of School: Professor Patrick Murray

Principal Supervisor: Associate Professor Fionnuala Ní Áinle

May 2017
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ABSTRACT

While the primary physiological function of the blood coagulation system is to prevent bleeding following vascular injury, a complex interplay is known to exist between components of the blood coagulation cascade and the activity of various other biological processes and systemic diseases including inflammation, tumour metastasis and pulmonary vascular disease. Moreover, the potential of this interplay to provide novel therapeutic opportunities is increasingly representing a source of scientific and translational interest, particularly in the setting of clinical scenarios where therapeutic options are limited, such as in the treatment and prevention of cancer metastasis and in the management of thrombotic risk among individuals at high risk of bleeding.

The work described within this thesis was conducted with the aim of exploring the nature of this interplay in the context of two disease states, cancer metastasis and Eisenmenger syndrome, and to explore potential novel therapeutic strategies which target the interaction between coagulation activation and the underlying systemic disease in both cases. LMWH, an anticoagulant drug, appears to mediate an anti-metastatic effect in vivo but the underlying mechanisms remain to be elucidated and the associated risk of bleeding precludes its use in cancer solely for the prevention of tumour dissemination. Eisenmenger syndrome is associated with a significant risk of both haemorrhage and thrombosis, the underlying mechanisms remain poorly understood and the nature of the competing thrombotic and haemorrhagic risks presents significant clinical dilemmas.

LMWH was found to support endothelial barrier function in vitro (a key barrier to tumour metastasis) and to inhibit tumour cell trans-endothelial migration. This activity does not appear to be linked to its anticoagulant function and both a non-anticoagulant LMWH fraction and a combination of low dose LMWH with a statin were found to represent a novel means of delivering the cytoprotective effects of LMWH in vitro in the absence of a significant anticoagulant effect.

Abnormal platelet procoagulant activity was found to represent a key determinant of the prothrombotic phenotype in Eisenmenger syndrome but, interestingly, platelets were also found to modulate the activity of endogenous anticoagulant pathways in a manner which would be predicted to increase the bleeding risk (reflecting the clinical phenotype). Remarkably, following a course of treatment with macitentan, a dual endothelin-1 receptor antagonist which does not directly affect haemostasis, the
derangements in both procoagulant and anticoagulant pathways were found to be attenuated.

In summary, the cross-talk between coagulation and systemic disease represents a potential source of novel therapeutic opportunities. The in vitro results described in this thesis may, if replicated in vivo, represent a means of addressing significant clinical dilemmas in two high risk patient groups.
STATEMENT OF ORIGINAL AUTHORSHIP

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree of Doctor of Philosophy, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

Signed: _______________________

Dr. Barry Kevane BMedSc, MB, BCh, BAO, MRCPI

Date: ________________________
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**Published Editorials**


**Book chapters**


**Oral Presentations**


**International Poster Presentations**


National posters


5. Crowley MP, Kevane B, O'Shea SI, Egan S, Quinn S, Gilligan O, Ní Áinle F. Evolving patterns of thrombin generation and sensitivity to activated protein C in
patients with myeloma at diagnosis and in response to therapy. **Haematology Association of Ireland Meeting**, Sligo, October 2014.

Awards

   **Science Foundation Ireland (SFI)/ Enterprise Ireland, December 2015**

2. **Best Oral Poster Presentation** – (Low Molecular weight heparin inhibits thrombin and VEGF induced endothelial Permeability).  
   **Haematology Association of Ireland Annual Meeting, Sligo, October 2014**

3. **Best Oral Presentation** – (Low molecular weight heparin inhibits thrombin and VEGF induced endothelial permeability).  
   **Contempory Issues in Hospital Practice, Dublin, November 2014**

Invention disclosures registered with UCD technology transfer office (UCD Nova):

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Patent applications:

2. OUTPUTS ARISING FROM TECHNIQUES AND CLINICAL COLLABORATIONS FORMED AS A RESULT OF THE WORK PUBLISHED IN THIS THESIS

Published papers


Oral Presentations


2. Egan K, Dillon A, Galvin Z, Kevane B, Neary E, Stewart S, Ní Áinle F. Thrombin generation varies with fibrosis score in patients with well-

**International Posters**


6. Egan K, Dunne E, Dillon A, **Kevane B**, Galvin Z, Kenny D, Stewart S, Ní Áinle F. Increased soluble GPVI levels in cirrhosis: evidence for collagen induced...
platelet activation in vivo. 57th Annual Meeting and Exposition of the American Society of Haematology, Orlando, December 5th-8th 2015.

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<td>APC</td>
<td>Activated protein C</td>
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<td>APTT</td>
<td>Activated partial thromboplastin time</td>
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<td>AT</td>
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<td>ATAP2</td>
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<td>CHD-PAH</td>
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<td>CTI</td>
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<td>LMS</td>
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<td>Micro-particle</td>
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<td>NET</td>
<td>Neutrophil extracellular trap</td>
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<td>PAI-2</td>
<td>Plasminogen activator inhibitor-2</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease activated receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>PK</td>
<td>Pre-kallikrein</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
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<tr>
<td>PolyP</td>
<td>Polyphosphate</td>
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<tr>
<td>PPP</td>
<td>Platelet-poor plasma</td>
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<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>Protein S</td>
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<tr>
<td>P-Selectin</td>
<td>Platelet-selectin</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>Platelet-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinyl difluoride</td>
</tr>
<tr>
<td>RCL</td>
<td>Reactive centre loop</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERPIN</td>
<td>Serine protease inhibitor</td>
</tr>
<tr>
<td>SFLLRN</td>
<td>Ser-Phe-Leu-Leu-Arg-Asn-amide</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and rich in cysteine</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with tween</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UFH</td>
<td>Unfractionated heparin</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VKA</td>
<td>Vitamin K antagonist</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous thromboembolism</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zona occludens-1</td>
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CHAPTER 1: INTRODUCTION

1.1 Blood coagulation

1.1.1 Overview

The normal haemostatic response to vascular injury consists of the activation of blood coagulation, a pathway which is characterised by a series of sequential reactions which culminate in the generation of thrombin and the formation of a stable fibrin clot at the site of injury (1, 2). The initial events in the process of fibrin clot formation (primary haemostasis) are characterised by the deposition of von Willebrand factor (VWF) at the site of injury with the subsequent interaction between VWF, collagen and circulating platelets leading to the formation of the initial platelet plug, which, following activation of coagulation (secondary haemostasis; a process promoted by procoagulant platelet activity), leads to the generation of a stable, insoluble thrombus composed primarily of cross-linked fibrin (3). The activation of this complex pathway rapidly leads to the sealing of vascular defects but in order to ensure that the extent of fibrin deposition is restricted to the site of vessel injury (in order to prevent vessel occlusion or thromboembolism) the activity of this procoagulant pathway is tightly regulated by endogenous anticoagulant and fibrinolytic pathways (2, 4-6).

While normal physiological blood coagulation activation is only initiated following exposure of extra-vascular tissue factor (TF, a procoagulant glycoprotein considered to represent the sole activator of \textit{in vivo} coagulation) to the circulating blood, pathological activation of coagulation may arise in the absence of vessel injury where circulating elements, such as tumour cells with aberrant surface TF expression, can promote inappropriate and potentially harmful intra-vascular coagulation activation and fibrin deposition (7-9).
1.1.2 Primary haemostasis

Following vessel wall injury, pro-coagulant sub-endothelial elements such as collagen become exposed to the circulating blood. VWF (a multimeric glycoprotein present in plasma but also stored in platelet α-granules and endothelial Weibel-Palade bodies) binds to exposed collagen upon which it becomes unwound from its globular structure to expose platelet binding sites. The initial interactions between VWF, sub-endothelial collagen and circulating platelets (platelet adhesion) are mediated through the platelet glycoprotein complexes GP 1b-V-IX and GP VI(3, 10). These initial weak interactions facilitate platelet rolling and initial tethering on the surface of VWF, with signalling events initiated by these initial interactions between VWF and the platelet glycoproteins leading to the activation of platelet integrins such as integrin αIIbβ3 (platelet glycoprotein IIb/IIIa; mediates interactions with VWF and fibrinogen) and integrin α2β1 (platelet glycoprotein 1a; mediates interactions with collagen) which leads to secure adhesion of platelets to VWF and collagen. Integrin αIIbβ3, through the formation of fibrinogen bridges, also mediates platelet-platelet interactions (platelet aggregation)(3, 10).

As well as leading to stable adhesion interactions between VWF and platelet integrins, the initial ligand-receptor interactions also initiate intra-cellular events which promote release of platelet storage granules (including α-granules containing fibrinogen, coagulation factor V, platelet factor-4, VWF etc.; dense granules containing serotonin, adenosine diphosphate, adenosine triphosphate etc.), with the release of these contents promoting further platelet recruitment, activation and aggregation culminating in the formation of a haemostatic platelet plug at the site of injury(3, 10) (Figure 1.1).

In order to ensure adequate haemostasis, the initial platelet aggregate must be replaced by a stable cross-linked fibrin thrombus, the generation of which arises during the process of ‘secondary haemostasis’, comprising the generation of a series of procoagulant serine proteases which act in a cascade of sequential reactions culminating in the generation of thrombin which cleaves soluble fibrinogen to form an insoluble fibrin clot(1, 11).
Figure 1.1 Primary haemostasis

Initial binding of platelets to VWF and sub-endothelial collagen is mediated through GP 1b-V-IX and GP VI (A). The activation of the integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ leads to more stable interactions between the platelet and the sub-endothelial tissues as well as the formation of ‘fibrinogen bridges’ between activated platelets (B), a process which is also promoted by the release of contents of platelet granules from activated platelets at the site of injury (which is promoted by initial platelet binding interactions) and leads to the recruitment of more activated platelets to the site of the developing platelet plug.
1.1.3 Secondary haemostasis

*In vivo*, secondary haemostasis occurs in parallel with primary haemostasis, with these processes being dependent on each other for achieving effective cessation of bleeding. This inter-dependence is illustrated by the role of activated platelet membranes in providing the anionic phospholipid surface which is crucial to the progression of the coagulation cascade and the role of the products of the coagulation cascade (such as thrombin) in acting as a potent agonists for further platelet activation\(^{(10-12)}\).

Following vessel injury, exposure of the procoagulant glycoprotein TF leads to the activation of physiological blood coagulation. TF is a 47KDa transmembrane glycoprotein, the expression of which is normally limited to extra-vascular tissues. Its primary site of constitutive expression is in the sub-endothelial adventitia of blood vessels where it forms a ‘haemostatic envelope’ to prevent loss of blood from the vasculature, by activating blood coagulation when it is exposed to its circulating ligand activated coagulation factor VII (FVIIa). The interaction between TF and FVIIa forms the extrinsic tenase complex, a procoagulant complex which, in the presence of calcium ions and following effective assembly on an anionic phospholipid surface, leads to the activation of the zymogens coagulation factors IX and X to form the serine proteases activated factors IX and X (FIXa and FXa)\(^{(13, 14)}\).

The activity of the extrinsic tenase in generating FXa is limited by the activity of the tissue factor pathway inhibitor pathway (TFPI), and the picomolar concentrations of thrombin generated by FXa formed by the extrinsic tenase prior to inactivation (and in the absence of its co-factor) is insufficient to lead to the formation of a fibrin clot. However, the thrombin generated in this initiation phase of coagulation activation primes the clotting cascade for enhanced thrombin generation by activating the co-factors, coagulation factors VIII and V (FVIII and FV), and the serine protease, activated factor XI (FXIa). Activated factor VIII (FVIIia) acts as co-factor for the proteolytic activity of FIXa (generated by the extrinsic tenase and through the activity of FXIa) to form the intrinsic tenase complex (assembled in the presence of an anionic phospholipid membrane and calcium ions) which proceeds to generate micromolar concentrations of FXa from zymogen FX. FXa then forms the prothrombinase complex with its co-factor, FVa (in the presence of anionic and calcium ions) which proceeds to generate micromolar concentrations of thrombin from its zymogen precursor, prothrombin\(^{(2, 11, 12)}\).
Fibrinogen is a plasma glycoprotein consisting of pairs of disulphide bond-linked Aα, Bβ and γ polypeptide chains.(4, 15) Thrombin cleaves fibrinopeptide A from the Aα chain and fibrinopeptide B from the Bβ chain, revealing novel n-terminal domains which interact leading to polymerisation and the formation of insoluble fibrin. Coagulation factor XIII then catalyses the formation of covalent bonds between residues on the α and γ fibrin polypeptide chains (fibrin crosslinking) in order to enhance the strength of the formed fibrin clot.(4, 16) (Figure 1.2).
Figure 1.2 Secondary haemostasis

Vascular injury leads to the exposure of TF, a procoagulant glycoprotein, which initiates coagulation by interacting with circulating FVIIa, leading to the formation of the extrinsic tenase complex. The subsequent calcium and phospholipid-dependent proteolytic reactions mediated by the extrinsic & intrinsic tenase complexes and the prothrombinase complex culminates in the generation of vast amounts of thrombin, the key effector protease of the blood coagulation pathway. The activity of this procoagulant pathway is regulated by the TFPI, antithrombin and activated protein C pathways which inhibit components of the extrinsic tenase, intrinsic tenase and prothrombinase complexes as well as mediating a direct inhibitory effect on the procoagulant activity of thrombin.
1.1.4 Contact pathway of blood coagulation activation

While extra-vascular TF-mediated activation of blood coagulation is the primary physiological pathway involved in initiating thrombus formation in response to injury, an alternative pathway of coagulation activation exists comprised entirely of elements present within the circulating blood and which is not dependent on exposure to extra-vascular molecules in order to initiate its activation. This ‘contact pathway’ appears to have little, if any, contribution to physiological haemostasis but increasing evidence suggests that it may be implicated in pathological coagulation activation and, as such, may represent a potential target for novel therapeutic agents (17-19).

The contact pathway consists of the zymogen coagulation factors XI, XII (FXI & FXII) & prekallikrein (PK) and the non-enzymatic co-factor high molecular weight kininogen (HMWK). FXII becomes auto-activated (FXIIa) upon contact with specific negatively-charged surfaces/substances. Previously, FXII activation was thought to arise primarily following its exposure to artificial surfaces in vitro, such as those provided by glass, silica, kaolin etc. However, an increasing number of in vivo activators of the contact pathway have been described (primarily in the setting of pathological coagulation activation) and include negatively-charged components of the bacterial cell wall, platelet polyphosphates (released from platelet dense-granules following activation), bacterial polyphosphates, circulating free-DNA/RNA and misfolded protein aggregates (such as amyloid plaque) etc. The activation of FXII is enhanced in the presence of the co-factor HMWK which promotes the assembly of the components of the contact pathway on the anionic surface. Binding of FXII to the anionic surface enhances its auto-activation and the small amounts of FXIIa generated at this point lead to further FXII activation and, in addition, also activate PK which cleaves and activates further amounts of zymogen FXII. FXIIa can then promote blood coagulation activation by its role in cleaving and activating FXI, which supports thrombin generation by activating FIX, a key component of the intrinsic tenase complex. In addition to the procoagulant activity of the contact pathway, a pro-inflammatory effect has also been described following its activation which appears to be primarily mediated by PK-induced degradation of HMWK, which leads to the generation of bradykinin, a key inflammatory mediator, which promotes vasodilation, vascular permeability and neutrophil chemotaxis (17, 18, 20).
1.1.5 Endogenous inhibitors of coagulation

Tissue factor pathway inhibitor (TFPI), the activated protein C (APC) pathway and antithrombin are the primary endogenous anticoagulants which act to prevent excessive thrombin generation following tissue injury.

TFPI is a kunitz-type serine protease inhibitor (serpin) which inhibits the initial stages of coagulation activation by suppressing the activity of the extrinsic tenase complex. Stores of TFPI are found in endothelial cells, platelets and in circulation. It inhibits extrinsic tenase activity in a step-wise manner by binding initially to the TF-VIIa complex and then to FXa to form a quaternary complex which is subsequently cleared from the circulation (21, 22).

The APC pathway consist of zymogen protein C (PC), its co-factor protein S (PS) and the endothelial receptors thrombomodulin (TM) and endothelial protein C receptor (EPCR). It mediates its anticoagulant activity by the specific cleavage and inactivation of the co-factors FVIIIa and FVa, leading to the inhibition of the activity of the intrinsic tenase and prothrombinase complexes respectively. Protein C becomes activated by thrombin following the formation of a complex between TM and thrombin on the endothelial cell surface. This interaction with TM re-directs the activity of thrombin towards the activation of protein C and inhibits its procoagulant function. Binding of protein C to EPCR facilitates thrombin-induced PC activation by localising PC in proximity to the thrombin-TM complex. APC then dissociates from EPCR and binds its co-factor PS which enhances APC-mediated FVa and FVIIIa proteolysis by orientating APC on an anionic phospholipid membrane in a manner which optimises its interaction with its target substrates (5).

Antithrombin is a plasma glycoprotein and serpin which acts as the primary inhibitor of thrombin and FXa. In its native conformation it exhibits limited efficacy in inhibiting the procoagulant activity of these proteases but following an interaction with glycosaminoglycan molecules (GAGs) such as heparin or endogenous heparan sulphates, this activity is greatly enhanced (discussed in 1.3.2.2).
1.1.6 Fibrinolysis

In addition to the endogenous anticoagulant pathways, the extent of fibrin deposition is also regulated by the fibrinolytic pathway which acts by degrading the cross-linked fibrin formed following thrombin-induced cleavage and polymerisation of fibrinogen. This ensures that the deposition of fibrin is limited to the site of injury only. The fibrinolytic system consists of plasminogen (the glycoprotein zymogen of the serine protease plasmin), tissue plasminogen activator (tPA), plasminogen activator inhibitor type 1 and 2 (PAI-1 & -2), α2-antiplasmin and thrombin-activated fibrinolytic inhibitor (TAFI)(4).

tPA is secreted by endothelial cells in response to various stimuli including thrombin, vessel occlusion, strenuous exercise and adrenaline. Following release and binding to fibrin its activity in cleaving and activating plasminogen is enhanced, with the plasmin generated by cleavage of plasminogen then acting by degrading its primary substrates, fibrin and fibrinogen (although other targets for plasmin-induced proteolytic degradation include FV and FVIII)(4, 23).

Inhibition of plasmin activity is mediated by PAI-1, PAI-2, α2-antiplasmin and TAFI. PAI-1 is present in endothelial cells and platelet α-granules. It binds and inhibits both fibrin-bound and free tPA (with greatest inhibitory activity observed in its interaction with free tPA). PAI-2 is produced by the placenta and appears to have a role in inhibition of fibrinolysis during pregnancy. α2-antiplasmin is synthesised in the liver and is the primary direct inhibitor of plasmin. TAFI is activated by TM-bound thrombin and acts by cleaving plasminogen and tPA binding sites from fibrin, thereby inhibiting the effective activation of plasmin(23).
1.2 The interplay between blood coagulation and systemic disease

1.2.1 Overview

While the primary physiological function of the blood coagulation system is the formation of a fibrin clot at the site of vascular injury in order to prevent bleeding, a complex interplay also exists between components of the blood coagulation cascade and the activity of various other biological processes and systemic diseases including inflammation, tumour metastasis and pulmonary vascular disease(7, 9, 24-26).

Inflammatory cytokines and other molecules generated as a consequence of acute inflammation have been shown to modulate the activity of blood coagulation in a manner which promotes the activity of procoagulant pathways and downregulates the activity of endogenous anticoagulant and fibrinolytic pathways leading to an overall hypercoagulable state. Similarly, in the setting of malignancy, particularly in the context of metastatic disease, the activity of the blood coagulation system is modulated in a manner which promotes the increased generation of procoagulant proteases such as thrombin and FXa. Pulmonary vascular diseases, such as idiopathic pulmonary hypertension and Eisenmenger syndrome are also associated with abnormal blood coagulation activation through mechanisms which remain poorly understood but which may be linked (directly or indirectly) to the activity of pro-inflammatory mediators implicated in the pathogenesis of the underlying vascular pathology (such as endothelin-1)(27-30).

While the enhanced procoagulant activity associated with inflammation, malignancy and pulmonary vascular disease clearly promotes the formation of occlusive thrombosis and the risk of thromboembolism, the morbidity associated with the interplay between systemic disease and blood coagulation is not limited to the risk of thrombosis. Products of blood coagulation activation (such as thrombin, FXa, activated platelets and fibrin) have themselves been shown to promote the activity of inflammatory and metastatic pathways and appear to modulate the natural history of the associated disease process. Conversely, components of endogenous anticoagulant systems (such as antithrombin, activated Protein C and endogenous heparan sulphates) have been shown to exhibit anti-inflammatory and anti-metastatic properties(31-33).
Consequently, therapies directed at influencing the activity of blood coagulation may have reciprocal effects in modulating the natural history of associated systemic diseases and similarly, therapies which target the activity of systemic diseases associated with coagulation activation may alter the haemostatic balance in a manner which attenuates the thrombotic risk (24, 27, 34).
1.2.2 The role of inflammation in promoting blood coagulation activation

1.2.2.1 Enhanced procoagulant pathway activity

As outlined in 1.1.3, tissue factor acts as the primary activator of blood coagulation in the setting of vascular injury(31). However, in addition to its role in physiological coagulation activation, TF appears to play a key role in the initiation of pathological coagulation activation in the context of inflammation(2, 7, 31).

While TF is not normally expressed in the intra-vascular compartment, abnormal expression of TF on circulating blood cells has been described in the context of acute inflammation, leading to abnormal intravascular coagulation activation. The primary site of abnormal TF expression in the setting of acute inflammation is on the surface of leucocytes such as monocytes and macrophages, which arises following their exposure to pro-inflammatory mediators such as TNF-α, IL-1 (cytokines secreted by cellular components of the immune system in response to specific pathogen- and damage-associated molecular patterns) and endotoxin (cell wall associated bacterial lipopolysaccharides)(35-37). In addition, both circulating soluble TF and TF-bearing platelet and leucocyte derived microparticles have also been described in the setting of acute inflammation and may contribute to the thrombotic risk(38). Endothelial cell TF expression has also been described in vitro in response to inflammatory mediators although its significance in vivo remains to be determined(14, 35).

In addition to TF-mediated pathological coagulation activation, the contact pathway (which plays no role in physiological haemostasis) also appears to be implicated in pathological coagulation activation in the setting of inflammation(17). As outlined in 1.1.4, the contact pathway becomes activated following assembly on an anionic surface, leading to the generation of both procoagulant factors (factor XIa which promotes thrombin generation through the intrinsic pathway) and pro-inflammatory mediators (bradykinin; generated following kallikrein-mediated proteolytic cleavage of HMWK). Pathological sources of contact pathway activation have been reported to include components of bacterial cell membranes, bacterial polyphosphates and neutrophil extra-cellular traps (NETs; complexes of histones and DNA released by neutrophils in response to specific pathogens)(18, 39).
1.2.2.2 Diminished endogenous anticoagulant activity in the setting of inflammation

The activity of TF in promoting the initiation of thrombin generation is mediated through the extrinsic tenase procoagulant enzyme complex, which results in the generation of activated factors IX and Xa (FIXa and FXa). The procoagulant activity of the extrinsic tenase is countered by the activity of TFPI (21, 22). In the setting of inflammation, the pathological procoagulant activity of TF (as outlined above) can be further augmented through the reduction in TFPI activity mediated through its cleavage and inactivation by various pro-inflammatory enzymes such as neutrophil elastases and micro-organism associated proteases (40-42).

Downstream of the extrinsic tenase complex activity, the co-factors FVIIIa and FVa act to amplify thrombin generation through their function as components of the intrinsic tenase and prothrombinase complexes respectively and play vital roles in the generation of the vast amounts of FXa and thrombin which are required for fibrin clot formation in the setting of vascular injury (7). In order to prevent excessive intrinsic tenase and prothrombinase activity, the activity of FVa and FVIIIa is limited by the protein C pathway which cleaves and inactivates these factors following its own activation by the thrombin-thrombomodulin complex (5). The effective function of the protein C pathway is dependent on endothelial thrombomodulin expression in order to direct thrombin activity towards the generation of activated protein C (43). In addition, the expression of the endothelial protein C receptor augments the interaction between the thrombin-thrombomodulin complex and zymogen protein C (5, 43). This crucial anticoagulant pathway is downregulated by inflammatory mediators such as TNF-α and IL-1 which impair endothelial expression of TM and EPCR and leucocyte-derived proteases which actively cleave TM from the endothelial cell surface (44). Moreover, reduced hepatic synthesis and increased consumption and clearance of protein C and its co-factor protein S, further contribute to deficient protein C pathway activity in the setting of sepsis and acute inflammation (7).

In addition to the loss of endothelial TM and EPCR, which can arise in the setting of acute inflammation, diminished endothelial cell heparan sulphate proteoglycan expression (as a consequence of increased proteolytic cleavage and cytokine-meditated inhibition of its expression), leads to an attenuation in the activity of antithrombin, a serpin which acts as an endogenous inhibitor of thrombin and FXa (45). The activity of antithrombin is dependent on an interaction with heparin-like compounds (primarily the endothelial HSPG), which induce a conformational change in its structure which is crucial to its function (46). The increased rate of consumption and diminished
hepatic synthesis of antithrombin which can arise in acute inflammatory states also contributes to state of relative antithrombin deficiency(7).

The activity of the endogenous fibrinolytic pathway is also impaired in the setting of acute inflammation, with sustained increases in plasma levels of the fibrinolysis inhibitor PAI-1 reported, arising secondary to the signalling activity of IL-1, endotoxin and TNF-α on endothelial cells, leading to an overall hypofibrinolytic state(2) (Figure 1.3).
Figure 1.3 Inflammation is associated with a hypercoagulable, hypofibrinolytic state

Pro-inflammatory responses (including the release of inflammatory mediators such as TNF-α and IL-1, the release of leucocyte specific proteases and the presence of specific pathogen associated molecules such as endotoxin) promote enhanced activation of blood coagulation, characterised by aberrant leucocyte and endothelial cell expression of TF and diminished endogenous anticoagulant and fibrinolytic pathway activity (including loss of the endogenous endothelial anticoagulant phenotype with diminished TM & EPCR expression and cleavage of HSPG) leading to an overall hypercoagulable state.
1.2.3 The role of blood coagulation pathway activity in modulating inflammation

1.2.3.1 Procoagulant activity promoting inflammation

The primary components of procoagulant pathway activity which exhibit pro-inflammatory properties are TF, the serine proteases thrombin and FXa, fibrinogen/fibrin and activated platelets(2, 7, 31, 32).

TF, in complex with FVIIa, exhibits proteolytic activity in activating protease activated receptors (PARs) on monocytes and macrophages leading to the initiation of intracellular signalling events which promote the expression of molecules involved in immune responses, such as the major histocompatibility complex receptors, and in the increased generation of reactive oxygen species(13, 47). Thrombin also elicits pro-inflammatory responses through its activation of PARs on the surface of leucocytes, epithelial cells, fibroblasts, platelets and endothelial cells leading to enhanced cytokine and chemokine production, such as increased IL-8, IL-6 and monocyte chemotactic-1 (MCP-1) production as well as eliciting other inflammatory responses (such as increased vascular permeability, mediated through PAR-1 induced endothelial cell actin cytoskeleton re-arrangement)(31, 48-50). Fibrinogen and fibrin signalling has also been shown to directly stimulate the expression of TNF-α and IL-1β in mononuclear cells and have also been shown to induce IL-8 and MCP-1 production in endothelial cells and fibroblasts(16, 32, 51).

Platelets, in addition to their central role in primary and secondary haemostasis, exhibit pro-inflammatory activity following activation characterised by the release of inflammatory mediators, cytokines and adhesion molecules (such as CD40 ligand, Platelet-factor 4, IL-1β, IL-1, P-selectin) which act to regulate leucocyte chemotaxis; promote neutrophil granular release; stimulate endothelial IL-6 and IL-8 release; promote monocyte to macrophage differentiation and stimulate endothelial expression of adhesion molecules such as E-selectin, VCAM-1 and ICAM-1(52, 53).
1.2.3.2 The effect of endogenous anticoagulant pathways in modulating inflammation

In contrast to the pro-inflammatory activity exhibited by the procoagulant elements of the blood coagulation system, the anticoagulant APC pathway, antithrombin and TFPI have been shown to mediate anti-inflammatory properties(2, 7, 31, 32).

Antithrombin has been reported to attenuate leucocyte adhesion interactions by down-regulating the expression of the CD11b/CD18 adhesion molecule on the leucocyte cell surface and by inhibiting TF and IL-6 expression in endothelium and monocytes and has also been shown to impair chemokine-induced neutrophil migration through an interaction with a specific heparan sulphate-like receptor on the neutrophil cell surface(54-56). Moreover, in animal models of sepsis, infusion of recombinant antithrombin has previously been shown to reduce inflammatory cytokine expression and also to reduce mortality(57, 58).

With regards to the protein C pathway, TM, protein C and activated protein C (both EPCR-bound and unbound) have been shown to suppress inflammatory pathway activity(59). Thrombin mediated pro-inflammatory activity is indirectly attenuated by TM, which when bound to thrombin directs its proteolytic activity towards the activation of protein C, thereby preventing it from activating pro-inflammatory cell signalling events initiated by proteolysis of PARs. APC when bound to EPCR has been shown to inhibit endothelial cell apoptosis, a cytoprotective effect which appears to be mediated through PAR-1 and APC has also been shown to suppress monocyte pro-inflammatory cell signalling activity, as well as also mediating an inhibitory effect on leucocyte chemotaxis & adhesion to endothelium and in inhibiting thrombin-induced endothelial permeability(60, 61).

TFPI has also been reported to elicit anti-inflammatory effects, including the down-regulation of IL-6 expression. Its administration has also previously been shown to improve outcomes in animal models of sepsis(62, 63).
1.2.4 Blood coagulation and cancer

The role of malignant tumours in promoting abnormal blood coagulation activation is well established and is reflected in the substantially increased risk of thrombosis which has been consistently reported among patients with cancer (64). Several investigators have attempted to estimate the magnitude of thrombotic risk associated with malignancy and have demonstrated evidence to suggest that the risk of thrombosis among affected individuals is likely to be at least four-fold greater than that observed among the general population, with even higher risks reported in the context of certain specific tumour types and among individuals with advanced disease (64-66). Similarly, the risk of recurrent thrombosis is much higher among cancer patients relative to the general population (even despite the use of anticoagulant therapy) (67-69). Moreover, thrombosis represents a leading cause of mortality among patients with cancer and its occurrence is associated with shortened overall survival and diminished quality of life (70, 71).

In addition to the clinical data which support the hypothesis that malignant tumours drive abnormal blood coagulation activation, laboratory evidence of hypercoagulability among patients with cancer has been widely reported. Several investigators have demonstrated evidence of increases in plasma levels of circulating procoagulant factors (such as FVIII and fibrinogen) as well as reductions in the anticoagulant activity of endogenous anticoagulant pathways (such as the APC pathway) (72-77). Reflecting this shift in haemostatic balance towards a more hypercoagulable phenotype, a number of studies have also reported overall enhanced plasma thrombin generation and increased platelet activation within this cohort (77-80).

The molecular mechanisms underlying cancer hypercoagulability have been extensively studied (24, 33). Direct tumour cell-mediated procoagulant activity appears to represent a key pathway through which cancers induce a prothrombotic state. The aberrant, constitutive expression of procoagulant TF and phospholipid (crucial components of coagulation activation) on the surface of tumour cells has been well described and is likely to represent one of the primary molecular mechanisms underlying the cancer-associated thrombotic risk (81-83). The release of TF and phospholipid bearing microparticles of tumour cell origin into the circulation has also been reported (84, 85). Similarly, the aberrant expression and release of cancer procoagulant, a cysteine protease expressed exclusively in malignant tumour cells (and in some placental tissues), occurs in certain tumour cell types including acute
leukaemia and colon cancer and mediates a procoagulant effect through its ability to
directly activate FX(86-90). Tumour cells have also been reported to express other
proteases which modulate the activity of the fibrinolytic pathway in a manner which
gives rise to a hypofibrinolytic state(91). The secretion of heparanase, an enzyme
which acts by cleaving glycosaminoglycan-based molecules, is upregulated in cancer
and is implicated in tumour growth and dissemination(92-94). Its expression also
mediates a procoagulant effect, primarily through its activity in cleaving the
endothelial cell surface and by cleaving endothelial TFPI which promotes the procoagulant activity of the
TF/VIIa complex(95).

Several investigators have also reported evidence of malignant tumours eliciting pro-
flammatory responses which may also contribute to the overall prothrombotic state.
In particular, tumour cells and tumour-specific antigens have been shown to induce
enhanced expression of inflammatory cytokines such as TNF-α and IL-1 which promote
coaulation through their ability to induce leucocyte and endothelial cell TF expression,
down-regulate the activity of endogenous anticoagulant pathways and which
promote the expression of endogenous inhibitors of fibrinolysis(33).

Interestingly, the interplay between cancer and blood coagulation does not appear to
represent a one-way process, with strong evidence from in vitro and in vivo studies
suggesting that coagulation activation and tumour cell procoagulant activity supports
tumour growth and metastasis(24, 96). Expression of TF on the tumour cell surface
has been shown to confer a more aggressive phenotype and is characterised by
enhanced tumour growth and angiogenesis (possibly related to increased TF-induced
eexpression of VEGF)(81, 83, 97). Localised generation of insoluble fibrin (the end
product of coagulation activation) has been postulated to confer protection upon
circulating cancer cells from host defences and may also facilitate cancer cell adhesion
interactions with other cells (such as platelets and leucocytes) and to the vascular
endothelium, a process which is required for cancers to metastasize(24, 98-100).
Moreover, the direct signalling activity of procoagulant proteases (such as thrombin
and FXa) on endothelial and tumour cells, independent of their actions in promoting
clot formation, has been implicated in the process of cancer growth and dissemination.
In particular, thrombin-mediated signalling via the PAR family of receptors has been
shown to promote the transcription of specific pro-metastatic genes in incipient
metastatic tumour cells as well as also inducing expression and release of various
pro-angiogenic and pro-metastatic substances from endothelial cells and platelets,
such as VEGF, P-selectin and P-selectin glycoprotein ligand 1 (PSGL-1) (50, 101-106).
The TF/VIIa complex and its chief product, FXa, formed during the initiation of blood coagulation also exhibit signalling activities on malignant tumour cells and has been shown to illicit pro-metastatic responses by signalling via PAR-2, which appears to be primarily characterised by enhanced tumour angiogenesis(107).
1.2.5 Derangements of haemostasis in Eisenmenger syndrome

Eisenmenger syndrome (ES) is a very rare but devastating complication of congenital heart disease with pulmonary artery hypertension (CHD-PAH), which is estimated to affect 2-6 per million adults in the developed world (108, 109).

ES arises among individuals who are born with specific cardiac defects (such as atrial or ventricular septal defects) which allow the abnormal flow of blood from the high-pressure environment of the left ventricle into the low pressure environment of the right side of the heart. The increased flow of blood under high pressure into the right atrium and ventricle causes increased pressure and congestion in the pulmonary vasculature (a low pressure environment which receives deoxygenated blood from the right ventricle and which returns oxygenated blood to the left atrium), leading to progressive pulmonary vascular disease characterised by vascular re-modelling & hypertrophy and a progressive increase in pulmonary vascular resistance. The pressure within the pulmonary vasculature eventually rises to a point where the pressures within the left ventricle are either matched or exceeded at which point the direction of blood flow through the cardiac defect is reversed or becomes bidirectional. As a consequence of this reversal in the direction of flow, deoxygenated blood becomes shunted away from the pulmonary vasculature (where under physiological circumstances it would become oxygenated prior to returning to the systemic circulation) and enters the systemic circulation. ES arises at the point of reversal of blood flow through the cardiac defect, with the impairment of normal oxygenation of blood leading to chronic hypoxaemia and progressive cardio-respiratory failure(108, 110, 111).

Patients with ES and other forms of pulmonary hypertension have an increased risk of thromboembolism, including potentially lethal complications such as stroke (108, 112). Paradoxically, in ES the bleeding risk is also increased(108, 112). The mechanisms underlying these co-existing thrombotic and bleeding tendencies remain poorly understood (113-118).

The onset of ES is characterised by a marked increase in morbidity and mortality (26, 30, 119). While therapeutic options remain limited, a number of targeted therapies have emerged which have been shown to improve outcomes(120-122). Although the aetiology of the coagulation abnormalities in ES is thought to be related to the underlying pulmonary vascular disease, the effects (if any) of these targeted therapies in restoring normal haemostasis is unknown (discussed in 1.3.3).
Pulmonary artery thrombosis represents the most frequently described manifestation of the ES-associated procoagulant phenotype, reported in up to 20-30% of affected adults (113, 123, 124). As well as representing an important cause of morbidity and mortality, thrombosis within the pulmonary vasculature contributes to the progression of pulmonary vascular disease, an observation which is reflected in current clinical practice where anticoagulant therapy is routinely offered to individuals with idiopathic pulmonary hypertension (111, 125). However, the role of anticoagulant therapy in ES remains controversial in view of the co-existing bleeding diathesis (34). Haemoptysis is the most common and clinically significant manifestation of the ES-associated bleeding tendency and has been reported as representing the eventual cause of death in a significant proportion of ES patients (26, 115).

A number of potential mechanisms by which a prothrombotic phenotype might emerge in ES have been postulated. The precise aetiology remains unclear although the abnormal shear forces generated by the abnormal vascular intra-vascular pressures and the associated hyperviscosity are thought to be implicated in the abnormal platelet and coagulation activation which is thought to arise in this disorder. Deranged blood flow is a well-recognised predisposing factor for coagulation activation. Hyperviscosity, biventricular failure and the structurally abnormal vessels which arise in ES would be predicted to precipitate turbulent blood flow through the pulmonary vasculature and contribute to the risk of thrombosis. Similarly, the abnormal shear forces generated within the vasculature of the lungs in ES have been reported to impair the endogenous anticoagulant activity of healthy endothelium in this condition, with reduced plasma levels of thrombomodulin (an endothelium-associated anticoagulant protein) and increased plasma levels of VWF reported in this context (25, 29, 114, 116, 126-128).

Derangements in platelet activity have been investigated as potential mechanisms underlying ES-associated hypercoagulability. Platelets normally circulate in an inactive state and only mediate haemostatic activity at the site of vessel injury. Platelets support haemostasis not only through the formation of physical barriers to bleeding (platelet aggregates) but also by promoting thrombin generation through the release of procoagulant substances and through the expression of procoagulant surface phospholipids (129). Several studies have reported evidence of enhanced platelet activation in ES (118, 130, 131). In particular, evidence of increased plasma levels of circulating platelet-derived microparticles (MP) have been reported in ES and are thought to represent markers of increased platelet-activation as well as possibly exhibiting direct procoagulant properties through the expression of various coagulation factors (such as factor Va) and anionic procoagulant phospholipids. The generation of
platelet-derived MPs (and abnormal platelet activation) is thought to also be linked to the exposure of platelets to the abnormal shear forces which arise within the pulmonary vasculature in ES(118).

Paradoxically, derangements of platelet activity have also been implicated in the aetiology of the ES-associated haemorrhagic risk. Thrombocytopenia is frequently observed and diminished platelet aggregation has been reported (114, 128). The aetiology of the haemorrhagic platelet dysfunction is unknown but the abnormal platelet activation which has been reported to arise in ES (and which potentially predisposes to intravascular coagulation activation) may result in a significant proportion of circulating platelets having exhausted their haemostatic potential (‘spent’ platelets), a phenomenon which could account for the bleeding risk which arises in this prothrombotic disorder(132).

While a definitive explanation for the various haemostatic and prothrombotic abnormalities which arise in ES is currently lacking, the evidence to date suggests that the derangements in pulmonary blood flow which arise directly as a consequence of the underlying vascular pathology in ES may be implicated. In particular haemodynamic stress in the form of abnormal shear forces appears to impair the normal anticoagulant properties of vascular endothelium as well as influencing platelet activity in a manner which may induce opposing haemorrhagic tendencies (in the form of diminished platelet aggregation in response to injury) and prothrombotic tendencies (as a result of increased platelet activation and generation of prothrombotic platelet MPs).
1.3 The crosstalk between blood coagulation pathway activity and systemic disease as a potential therapeutic target

1.3.1 Overview

For decades, the heparins (comprising unfractionated heparin (UFH) and its derivatives the low molecular weight heparins (LMWH)) have been widely used for the treatment and prevention of arterial and venous thrombosis(133, 134). However, increasing evidence suggests that these naturally-occurring agents mediate additional biological effects when administered to humans which do not appear to be linked directly to their anticoagulant activity and which may be of significant clinical benefit. In particular, the role of the heparins in the modulation of inflammatory responses in various disease states has represented a significant source of both scientific and clinical interest in recent years and, in addition, a growing body of evidence from both pre-clinical and clinical studies suggests that the use of LMWH confers a significant survival benefit upon sub-groups of patients with cancer(135-137).

The precise mechanism underlying the anti-cancer effects of the LMWHs remains to be fully elucidated, however data from pre-clinical studies and results from clinical trials which indicate a survival benefit primarily among patients with early stage disease, suggests that this additional beneficial effect of LMWH is mediated through the inhibition of metastasis(138-140).

Cancer metastasis, as opposed to primary tumour growth, accounts for the vast majority of all cancer deaths. The process of metastasis is complex and involves the migration of a tumour cell with metastatic potential from the primary tumour bulk, through the systemic circulation to a secondary site where it must breach the vascular endothelium and proliferate in the new microenvironment to form a secondary tumour deposit. Clearly, therapeutic agents which arrest this process of metastasis would be predicted to greatly improve patient outcomes(141, 142).

Unfortunately, the risk of haemorrhage associated with the administration of LMWH for its potential anti-metastatic effect limits its application in cancer and it is unlikely that this agent could be used for its non-anticoagulant cytoprotective activity in this high-risk patient group until such time as the risk of haemorrhage can be addressed(143).
Interestingly, the interplay between blood coagulation and systemic disease may also present potential therapeutic opportunities in non-malignant disease states. Eisenmenger syndrome is a rare form of pulmonary hypertension characterised by a significant burden of multi-system morbidity(30). While the primary source of morbidity in this disorder is the underlying progressive cardio-pulmonary failure, it has long been recognised that this disease is associated with a significant risk of both thrombosis and haemorrhage, both of which contribute significantly to the risk of early mortality(26). The molecular mechanisms underlying these haemostatic derangements remain poorly understood (as outlined in 1.2.5) and therapeutic options are limited, given that current conventional anticoagulant agents would be predicted to exacerbate the competing haemorrhagic risk and vice versa. Potential therapeutic strategies which are directed against the molecular mechanisms driving the ES-associated haemostatic derangements (as opposed to agents which directly modulate haemostasis) may represent a means of reducing the morbidity and mortality associated with the blood coagulation anomalies associated with this disorder, without impairing normal haemostasis.
1.3.2 Low molecular weight heparin: current role and potential novel therapeutic application in the prevention of cancer metastasis

1.3.2.1 Sources and structure of commercially utilised heparin formulations

Heparins are naturally occurring glycosaminoglycans (GAG) which are synthesised and stored in mast cells of most mammalian species. GAG compounds (including both heparin and the related heparan sulphate) are comprised of repeating disaccharide units consisting of uronic acid (either D-glucoronic or L-iduronic acid) linked covalently to an amino sugar (D-galactosamine or D-glucosamine) (6, 144). Significant structural heterogeneity is generated in the biosynthesis of heparin/heparan GAGs, an occurrence which is primarily mediated by the highly variable pattern and degree of sulphation which arises on individual GAG chains. This structural variation in conjunction with the significant variability in GAG chain length which is observed in commercial formulations appears to account for the significant functional diversity which has been reported, both in terms of their anticoagulant activity and in terms of their other potential biological effects (137, 144).

The heparin compounds which are currently in commercial use are largely derived from GAGs extracted from porcine intestinal mucosa. The manufacture of these compounds involves initially the separation of the mucosa from the porcine intestines with the subsequent solubilisation, precipitation and purification of crude heparin products leading ultimately to the generation of UFH, a polymer of heparin polysaccharide chains of varying length, with a molecular weight range of between 5 and 30KDa and a mean molecular weight generally in the range of 12-15 KDa (134, 144).

LMWHs are fractions of UFH (mean molecular weight in the region of 4.5-6KDa) which are derived by various methods of chemical and enzymatic depolymerisation, with the various commercial LMWH formulations being derived by different depolymerisation methods (e.g. the LMWH tinzaparin is derived by heparinase-mediated enzymatic depolymerisation while LMWH enoxaparin is derived by nitrous acid-induced chemical depolymerisation) (134). These varying methods of manufacture employed in the generation of these LMWH fractions leads to a degree of structural diversity also being present among these formulations, which may account for the heterogeneity observed with regard to their anticoagulant function (specifically with regard to the ratio of
inhibitory activity directed against FXa versus thrombin) and their other potential non-
anticoagulant biological functions(134, 144).
1.3.2.2 The anticoagulant mechanism of action of UFH and LMWH and their role in clinical practice

The anticoagulant activity of both UFH and LMWH is primarily mediated through antithrombin (AT), a plasma glycoprotein and serpin which acts as one of the chief physiological regulators of blood coagulation through its activity in inhibiting the procoagulant activity of the serine proteases thrombin and FXa (as briefly outlined in 1.1.5).

The AT gene, SERPINC1, is located on the long arm of chromosome 1, spanning 13.5 kbp and consisting of 7 exons and 6 introns. Transcription of SERPINC1 and synthesis of the AT glycoprotein occurs in hepatocytes, where a 464 amino acid polypeptide is initially synthesised and, following post-translational modification and cleavage of a 32 residue signalling peptide, is ultimately released into the circulation as a 58KDa, 432 amino acid polypeptide(145). Over 300 point mutations as well as partial and whole gene deletion mutations affecting the SERPINC1 gene have been described to date. AT qualitative and quantitative defective states of varying severity may occur as a result, with the associated increased rates of venous thrombosis (and the lethal phenotype observed in the SERPINC1 knock-out state) reflecting the importance of AT as the primary physiological inhibitor of blood coagulation(46).

The protein structure of AT comprises 3 beta-sheets (sheets A-C) and 9 alpha-helices (helices A-I) arranged in an upper barrel domain and lower helical domain with the central beta-sheet A acting as a bridge between the two domains(46, 146). A 22 residue, highly flexible loop, known as the reactive centre loop (RCL) projects above the main structure of the protein(147). At the centre of this loop lies a sessile bond between the arg393 and ser394 which acts as the site of protease mediated cleavage (and subsequent protease tethering)(46). In the native state, AT appears to exist in a state of equilibrium between at least two conformations where the RCL is either accessible to attacking proteases or in a conformation wherein the N-terminal end (hinge region) of the RCL is inserted into the central A-sheet and the P1 arginine orientated away from the direction of potential protease interaction(146, 148). This greatly limits the inhibitory function of AT, however, following binding of AT with a glycosaminoglycan (GAG) such as heparin or the physiological heparan sulphate proteoglycans, conformational change occurs within the AT structure making it more amenable to protease interaction(146, 147).
While the initial heparin-AT interaction is sufficient to cause physiologically significant FXa inhibition, acceleration of the rate of thrombin inhibition is dependent on the formation of a ternary structure between thrombin, the heparin polysaccharide chain and AT. This requires a minimum heparin chain length of 18 monosaccharide units (generally only present in significant concentrations in UFH formulations) which facilitates simultaneous binding of helix D of the AT structure and to exosite II of thrombin (the heparin-binding site). The formation of the ternary structure brings thrombin in closer proximity to the RCL, facilitating an enhanced rate of inhibition.
1.3.2.3 Current clinical applications of UFH and LMWH

UFH is administered parenterally, through either the subcutaneous or through the intravenous routes (149). UFH exhibits limited bioavailability and a delayed onset of action when administered subcutaneously but following intravenous administration the onset of anticoagulant effect is immediate, a characteristic which is particularly useful in clinical scenarios when immediate therapeutic anticoagulation may be necessary, such as in the setting of haemodynamic compromise secondary to pulmonary embolism. Similarly, the anticoagulant effect is rapidly lost following either discontinuation of the UFH infusion or following the administration of protamine sulphate and so UFH may also represent a potential therapeutic option in scenarios where rapid reversal of anticoagulation may be indicated(150). UFH is primarily cleared through the reticuloendothelial system with very limited renal clearance and so this agent may also be an attractive therapeutic option in the setting of significant renal impairment.

UFH exhibits unpredictable pharmacokinetics and consequently regular dose-response monitoring using the activated partial thromboplastin time (APTT) is necessary. Moreover, exposure to UFH is associated with a risk of developing the severe prothrombotic disorder heparin-induced thrombocytopenia (HIT), which affects up to 2-3% of certain high-risk subgroups of patients treated with this agent (a disorder which arises following an interaction between platelet factor-4 and heparin polysaccharides, leading to a prothrombotic immunological response directed against the platelet factor-4/heparin complex). In addition, prolonged UFH therapy is associated with a significant risk of osteopenia and symptomatic vertebral osteoporotic fracture (estimated at 2-3% of patients exposed to at least 4 weeks of UFH therapy). These adverse effects appear to be mediated through interactions between specific plasma proteins and the longer heparin polysaccharide chains which appear to be present primarily in UFH and so consequently these adverse effects occur much less frequently among patients treated with LMWH(133).

The LMWHs were developed to address the disadvantages associated with the use of UFH as outlined above. LMWH is administered through the subcutaneous route, with peak plasma levels normally achieved within 3 to 4 hours following administration. LMWH is primarily cleared through the renal route and in an individual with normal renal function loss of anticoagulant effect following subcutaneous administration of a therapeutic dose would be anticipated to occur within 24 hours. No specific reversal agents exist currently, however partial reversal may be achieved through administration
of protamine sulphate (which binds with greatest affinity to the longer heparin polysaccharides) (150).

Despite limited activity against thrombin, the LMWH's have been shown to be at least as effective as UFH in the treatment and prevention of VTE and appear to confer a lower risk of bleeding. Moreover, LMWH exhibits a very stable pharmacokinetic profile which facilitates once-daily subcutaneous dosing without the requirement for routine monitoring (133, 151). Consequently, the LMWHs have superseded UFH as the heparin-based anticoagulant of choice for the vast majority of clinical indications.

In current clinical practice, the use of UFH/LMWH therapy in the setting of uncomplicated venous thromboembolism is generally restricted to the initial short period of 'lead-in' treatment during which time the patient is becoming established on oral therapy with either a vitamin K antagonist (VKA; such as warfarin) or a novel direct oral anticoagulant (such as dabigatran or edoxaban; LMWH lead-in therapy is not required during initial treatment with apixaban and rivaroxaban).

While the oral agents represent the mainstay of VTE treatment, LMWH continues to be the agent of choice for certain specific patient sub-groups, including pregnant patients (where the oral agents are contra-indicated on account of the associated risk of teratogenicity and fetal haemorrhage) and in the setting of cancer-associated thrombosis.
1.3.2.4  The role of LMWH in the treatment of cancer-associated thrombosis

Venous thromboembolism is a leading cause of cancer-associated mortality (second only to cancer progression itself)(152). The mechanisms through which abnormal blood coagulation activation arises in the setting of active cancer are varied but include direct cancer cell mediated processes, including the aberrant expression of TF and procoagulant proteases coupled with a direct inhibitory effect on the activity of endogenous anticoagulant and fibrinolytic pathways(33).

In addition to the observation that the risk of VTE is higher among patients with cancer relative to the general population (with unprovoked VTE occasionally representing the first manifestation of an occult malignancy), it is also well established that the risk of VTE recurrence among patients with cancer who suffer a first VTE event is also higher than what is observed in the general population despite conventional anticoagulant therapy(153).

The 'comparison of LMWH versus oral anticoagulant therapy for long term anticoagulation in patients with cancer trial (CLOT trial)' was the first large, randomised, controlled trial which aimed to establish the optimal anticoagulant therapy strategy for patients with cancer who develop VTE. In this international, multi-centre study, over 600 patients with cancer who developed either a proximal lower limb deep vein thrombosis or pulmonary embolism (or both) were randomised to receive either conventional therapy (VKA arm) consisting of LMWH dalteparin 200IU/Kg during a 5-7 day lead in period (with concurrent initiation of a VKA) followed by VKA alone for 6 months, or the alternative treatment arm consisting of dalteparin alone for the entire duration of the 6 month study period. The results of this study demonstrated the superiority of LMWH over VKA for the treatment of cancer-associated thrombosis with a significantly lower rate of VTE recurrence observed in the dalteparin arm following completion of the period of follow-up (17% recurrence in the VKA arm versus 9% in the LMWH arm)(154).

Similarly, in the subsequent comparisons of acute treatments in cancer haemostasis trial (CATCH trial) the investigators assessed the efficacy of the LMWH tinzaparin given as a daily dose of 175IU/Kg as an alternative to oral anticoagulant therapy with a VKA (preceded by 5-7 days of a tinzaparin lead-in period) for the treatment of cancer-associated VTE. At the completion of the 6 month period of follow-up, among the 900 subjects enrolled and randomised to either the VKA or LMWH therapy arms, no
significant difference in overall VTE recurrence rates were observed although a significantly lower rate of symptomatic lower limb DVT and clinically relevant non-major bleeding was observed among the subjects who were randomised to receive 6 months of tinzaparin. The investigators postulated that the failure to detect a significant difference in overall rate of VTE recurrence between treatment arms in this study may be attributed to the lower than anticipated rate of VTE recurrence observed in the cohort as a whole, with the subjects recruited to the CATCH study representing a more low-risk group of cancer patients than had been recruited to the CLOT trial (where a higher proportion of patients had metastatic disease and were actively receiving anti-cancer therapy)(155).

The results of the CLOT and CATCH trials, in conjunction with data from several smaller studies, suggest that LMWH is a more effective agent for the prevention of cancer-associated thrombosis than conventional management with a VKA(156, 157). These findings are reflected in current clinical practice guidelines which recommend the use of LMWH in this setting(143, 149). The precise molecular mechanism underlying the superiority of LMWH relative to other anticoagulant agents for the prevention of thrombosis in the setting of cancer-provoked blood coagulation activation remains to be elucidated.
1.3.2.5 *LMWH administration confers a survival benefit upon sub-groups of cancer patients which is independent of its anticoagulant activity*

Given the extensive data in support of the hypothesis that a complex relationship exists between blood coagulation pathways and cancer biology and in particular given the association between the activity of procoagulant proteases and the progression of tumour growth and metastasis, it is not surprising that anticoagulant therapy has represented a significant focus of interest for decades as a potential therapeutic adjunct for the treatment of malignant tumours. Warfarin, UFH and LMWH have all been subjected to clinical studies exploring their efficacy as adjuncts to conventional anti-cancer therapies. The most convincing data to date has been generated by clinical trials exploring the role of LMWH in cancer and consequently this agent currently represents the primary focus of pre-clinical and clinical studies exploring the role of anticoagulation in cancer(24, 158, 159).

Several early studies, including small randomised controlled trials, demonstrated modest improvements in patient outcomes in the setting of specific tumour types (particularly small cell and non-small cell lung carcinoma) when patients also received warfarin therapy, however these benefits were somewhat negated by the incidence of haemorrhagic complications (including cases of fatal haemorrhage) and subsequent studies have failed to demonstrate a definite survival advantage associated with the use of warfarin in cancer(160-162). The administration of UFH to patients with small cell lung cancer in a small randomised controlled trial was also associated with a survival benefit but the anti-cancer effect of UFH has not been confirmed in larger studies and definitive evidence in support of its use in this context is therefore lacking(24, 163).

In a study exploring the efficacy of LMWH as an alternative to UFH in the treatment of proximal lower limb deep vein thrombosis, the investigators reported evidence suggesting a survival advantage associated with LMWH in the small sub-group of patients with cancer who were included in the trial(164). The LMWH-associated survival advantage in cancer has been subsequently studied in several large clinical trials designed specifically for the purpose of exploring the anti-cancer effects of LMWH, as well as also in the context of post-hoc analyses of large trials initially conducted for the purposes of investigating the anti-thrombotic efficacy of these agents.
In response to the growing body of *in vitro* and *in vivo* data supporting the hypothesis that LMWH mediates an anti-cancer effect, the investigators of the CLOT trial conducted a post-hoc analysis of their cohort and demonstrated a significant benefit in terms of survival at 12 months among subjects without metastatic disease who received LMWH as opposed to a VKA (hazard ratio 0.5 in favour of LMWH group; 95% CI 0.27-0.95, p=0.03)(138). In the FAMOUS study (the first randomised controlled trial designed to assess the effect of LMWH on survival in cancer patients without VTE), 385 subjects with cancer were randomised to receive either dalteparin 5000IU or placebo once daily for 1 year and the investigators reported that within this cohort of patients (comprised primarily of patients with advanced disease and with heterogenous tumour subtypes) that the use of LMWH was not associated with a survival benefit for the cohort as a whole. However, among the cohort who survived in excess of 17 months (having completed 12 month of LMWH therapy) that statistically significant improvements in survival were observed in the LMWH group both at 2 years and 3 years following randomisation (albeit in a smaller sub-cohort with 55 subjects remaining in the LMWH arm and 47 subjects in the placebo arm)(139). In a subsequent randomised, placebo-controlled trial where 302 subjects with advanced malignancy were recruited to receive either placebo or a brief course (six weeks) of nadroparin a significant difference in survival was observed for the cohort as a whole among the subjects randomised to the LMWH arm (mortality at 12 and 24 months reduced by 10% and 12% respectively with median survival in the LMWH arm observed to be at 8 months versus 6.6 months in the placebo arm)(165). Interestingly, and reflecting that which had been observed in the CLOT and FAMOUS studies, the survival benefit associated with LMWH was more marked within the sub-group of patients who were identified at recruitment as having a predicted survival in excess of 6 months. In this sub-cohort, the use of LMWH was associated with an increase in median survival from 9.4 to 15.4 months(165).

A meta-analysis of randomised controlled trials exploring the role of LMWH as an anti-cancer agent was published in 2007, incorporating the results of the above studies as well as additional smaller trial conducted in 2004 which demonstrated significant improvements in progression-free survival and overall survival among subjects with small cell lung cancer who also received dalteparin in conjunction with standard anti-cancer therapy. The authors of this meta-analysis concluded that the addition of LMWH appeared to confer a survival advantage upon sub-groups of patients with cancer but highlighted the need for more targeted studies aimed at determining the optimum dosing regimen, treatment duration and LMWH formulation in this context as
well as also determining the patient sub-group (in terms of tumour type and disease stage) which was most likely to benefit from this intervention(166).

Reflecting the need for a more targeted approach to clinical studies in this area, the ABEL trial was conducted with the aim to explore the efficacy of the addition of prophylactic dose bemiparin to standard therapy in improving survival among a cohort of patients with limited-stage small cell lung carcinoma. This study was limited by its small cohort size (just 38 patients were recruited) however it reported marked improvements in progression-free survival (272 vs 410 days, hazard ratio 2.58 (CI 95% 2.15-5.8; p=0.02) and overall survival (345 vs 1133 days; p=0.02) in favour of the LMWH arm(140).

A subsequent meta-analysis published in 2014 and incorporating data from the trials included in the initial 2007 meta-analysis in conjunction with data from 5 additional studies published in the interim, including the ABEL trial, failed to detect a definite survival benefit associated with LMWH use in cancer however the authors highlighted the marked heterogeneity in terms of tumour type, tumour stage, presence of metastatic disease and LMWH dosing strategy utilised in these studies and concluded that while the meta-analysis did not identify a LMWH associated survival benefit across all patients subgroups, that a benefit could not be excluded in specific sub-groups, such as in the context of early-stage, non-metastatic disease in specific tumour subtypes(167).

The bulk of the evidence supporting the use of LMWH for the purposes of its potential anti-cancer effect in a single specific tumour sub-type has been generated in the setting of lung tumours. The FRAGMATIC study, published in 2015, was the first large randomised controlled trial which aimed specifically to investigate the role of dalteparin as an adjunct to standard therapy in small cell or non-small cell lung carcinoma. This study failed to detect any significant difference in either metastasis-free survival or in overall survival as a result of dalteparin use(39). However the majority of patients (61%) had metastatic disease at the time of recruitment, a factor which may have impacted on the ability of the study to detect a LMWH-associated anti-cancer effect, particularly if this effect is generated through an effect on the process of cancer metastasis(39).

The results of the TILT study, a randomised controlled trial investigating the effects of tinzaparin on patient outcomes specifically in subjects with completely resected, non-metastatic non-small cell lung carcinoma, are awaited. This trial will be the first large trial to explore the role of LMWH in early stage lung tumours exclusively(168).
In conclusion, the results generated from clinical trials conducted to date clearly suggest that LMWH mediates an anti-cancer effect (a hypothesis which is supported by *in vitro* data and results from pre-clinical cancer models; discussed in 1.3.2.6), however this survival benefit appears unlikely to be observed across all sub-groups of cancer patients. The precise mechanism underlying this effect and the sub-group of patients that is most likely to derive benefit from the addition of LMWH to their standard therapy remains to be determined. The data to date suggest that LMWH is likely to be most effective in early-stage disease, possibly as a result of the inhibition of cancer metastasis specifically (a hypothesis which is also supported by pre-clinical data) and not as a result of a reduction in thrombotic risk, as evidenced by the low rates of VTE overall in the placebo arms of these studies and the persistence of the survival benefit following completion of the LMWH treatment course. Moreover, if the anti-cancer effect of the LMWH is found to be entirely independent of its anticoagulant activity, it is likely that this agent could be more widely studied and utilised in clinical practice for this purpose, given that the significant risk of bleeding associated with anticoagulant use in cancer represents a significant obstacle to its application in cancer, even in the event of more definitive evidence in support its anti-metastatic activity becoming available (68, 69, 169).
Cancer metastasis is the multi-step, highly complex process by which malignant cells detach from a primary tumour and migrate through the systemic circulation to a distant tissue or organ where they invade and proliferate leading to the development of new, secondary tumour deposits (141). Complications arising as a result of the proliferation of metastatic tumours account for the vast majority of cancer associated deaths and consequently therapeutic strategies which interrupt this process would be predicted to improve patient survival (141).

Tumour cells which enter the circulation and succeed in surviving the shear forces of the circulating blood for a sufficient amount of time to allow them to reach their target organ must attach to the vascular wall and extravasate through the relatively impermeable barrier presented by normal endothelium in order for metastasis to proceed. Once extravasation has been completed, the hostile nature of the healthy micro-environment of the target tissue presents the final obstacles to the invading cells, which if surmountable, permits the proliferation of the tumour cells leading to the growth of secondary tumour deposits (141, 170).

The metastatic process is therefore inefficient, and important barriers to successful completion of metastasis exist at the latter stages of the process (in particular, barriers to tumour cell extravasation from the circulation into the target tissue and forces which inhibit tumour cell proliferation and angiogenesis in the foreign micro-environment). These are reflected in the observation that, while the vast majority of cancer patients (even patients with early stage disease who never develop metastases) demonstrate evidence of circulating tumour cells, only a tiny minority of these circulating cells (<0.01%) successfully establish secondary tumours in a distant site (141, 170-173). Therefore, therapeutic strategies which directly enhance the activity of these physiological barriers to tumour cell extravasation and proliferation would be predicted to limit the successful completion of cancer metastasis (170, 172, 174).

Reflecting the data from clinical studies which have suggested that LMWH administration confers a survival benefit upon cancer patients with limited-stage disease, results from numerous pre-clinical studies incorporating animal models of experimental metastasis have demonstrated that exposure to LMWH inhibits the formation of secondary tumour deposits (with minimal inhibitory effects reported for LMWH in the context of primary tumour growth in vitro), suggesting that the anti-cancer
effects of LMWH are mediated through an inhibition of metastasis rather than through an effect on the primary tumour bulk. The precise mechanism underlying this activity remains a significant focus of scientific interest with the majority of studies to date focusing on investigating the effects of LMWH in modulating the key 'rate-limiting' events which arise in the latter stages of the metastatic cascade as outlined above. In particular, the role of LMWH in modulating adhesion interactions between circulating tumour cells and the vessel wall and the effect of LMWH in inhibiting tumour angiogenesis following the extravasation of tumour cells into the target site have represented key areas of interest(24, 135).

Angiogenesis is the process through which proliferating tumours induce the formation of blood vessels within the growing tumour bulk in order to facilitate further growth and, eventually, to provide a means of further dissemination of metastatic cells(175). The proliferation of tumour cells following extravasation into the target organ is dependent on this process and its inhibition would be predicted to severely limit the growth of these secondary tumour deposits. LMWH has been shown to inhibit tumour-induced angiogenesis both in vitro and in vivo with several investigators reporting evidence to suggest that the anti-angiogenic activity of heparin polysaccharides is closely linked to their molecular weight with maximal inhibitory activity exhibited by shorter heparin polysaccharides (such as those which predominate in LMWH fractions)(176-179). The underlying mechanisms remain to be fully elucidated however the binding of LMWH polysaccharides to pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) and the subsequent interruption of the interaction between these growth factors and their receptors on the endothelial cell surface has been postulated as a potential mechanism through which LMWH might inhibit this process(180, 181). LMWH-mediated inhibition of pro-angiogenic signalling induced by procoagulant proteases including the TF-VIIa protease complex may also be implicated in this process which appears to be achieved through LMWH-induced enhanced endothelial cell release of TFPI, a key inhibitor of TF and TF/VIIa activity(182-184). LMWH-mediated inhibition of tumour-associated heparanase (a key enzyme involved in the proteolytic degradation of the extracellular matrix of the tumour microenvironment in order to facilitate further blood vessel growth) has also been proposed as a potential mechanism through which LMWH might attenuate tumour neoangiogenesis(185).

In order for tumour cells to invade from the circulation and into the parenchyma of the target organ, they must survive the shear forces of the circulating blood and adhere to
the vessel wall at the site of extravasation (141, 186). These adhesion interactions between the tumour cell and the vascular endothelium are facilitated by the expression on the tumour cell surface of ligands with affinity specific for adhesion molecules expressed on the endothelial cell surface (as well as on the surface of leucocytes and platelets which mediate important functions in promoting the arrest of tumour cells in the vasculature and their tethering to the endothelium). In particular, the expression of mucin ligands bearing highly sialylated fucosylated glycan motifs (such sialyl lewis $^x$), which act as ligands for the selectins (a family of adhesion molecules expressed on the surface of activated platelets, endothelium and leucocytes), promotes the tethering of the tumour cell to the vessel wall and is associated with the progression of metastasis (187). Loss of P-selectin expression has been shown to reduce tumour cell-platelet aggregation in vivo and is associated with a reduction in the formation of metastatic tumour deposits (187). Interestingly, LMWH polysaccharide chains have been shown to inhibit P-selectin and L-selectin adhesion interactions in vitro and heparin has been reported to inhibit metastasis formation in vivo in a P-selectin dependent manner (188).

While the inhibition of tumour neoangiogenesis and selectin-mediated vascular adhesion interactions have represented two key areas of research exploring the anti-metastatic properties of LMWH, the precise mechanism (or mechanisms) underlying this activity remain to be fully characterised. A number of other components of the metastatic cascade have also been explored as potential targets for the anti-cancer effects of LMWH (albeit to a lesser extent), such as LMWH mediated-inhibition of chemokine-induced organ-specific metastatic dissemination (189). Conversely, several other components of the metastatic process have yet to be explored as potential targets for LMWH therapy. In particular, the role of the endothelial barrier to tumour cell extravasation (and the potential effect which LMWH might exert on endothelial barrier function) has yet to be extensively investigated in this context.
The migration of circulating tumour cells through the normally impermeable barrier presented by vascular endothelium is a key step in the progression of cancer metastasis\(^{(174, 190)}\). Malignant tumours have been shown to induce abnormal endothelial permeability in the vascular beds of their metastatic targets in order to promote tumour cell invasion and recently a number of investigators have demonstrated that the inhibition of tumour-induced vascular permeability attenuates metastasis \textit{in vivo}, a finding which highlights this phenomenon as a potential novel therapeutic target\(^{(191, 192)}\).

Disassembly of inter-endothelial junctions leading to transient increases in endothelial barrier permeability most commonly arises following the interaction of various inflammatory mediators with their receptor on the endothelial cell surface, as occurs during the process of inflammation to facilitate the migration of immune cells through the inter-endothelial junctions and into the surrounding tissues. Inflammatory mediators such as thrombin and VEGF (both of which are also upregulated in cancer and both of which are associated with progression of metastasis) are frequently implicated in the disruption of IEJ and are among the most potent known endogenous inducers of endothelial barrier permeability\(^{(49, 174)}\).

Thrombin is a procoagulant serine protease generated during the process of blood coagulation activation but which is also upregulated in cancer through various pathological mechanisms but primarily as the result of the expression of procoagulant glycoproteins and proteases on the circulating tumour cell surface. Previous investigators have demonstrated that thrombin exerts its effects on endothelial barrier permeability through its interaction with protease activated receptor-1 (PAR-1), a G-protein coupled receptor expressed on the surface of endothelial cells. Thrombin-mediated signalling via PAR-1 initiates various downstream signalling events which culminate in the enhanced phosphorylation and activation of myosin light chain-2 (MLC-2), the regulatory subunit of the endothelial cell actinomyosin contractile machinery. MLC-2 diphosphorylation, which is also implicated in endothelial barrier dysfunction following exposure to other permeability-inducing agonists such as VEGF, bradykinin and histamine, results in contraction of the actin cytoskeleton and the disassembly of IEJ\(^{(49, 193, 194)}\).
A number of investigators have described several distinct mechanisms through which malignant tumours induce vascular permeability, leading to enhanced metastasis. The expression of specific sets of genes or metastatic gene ‘signatures’ in cells of a primary tumour such as the expression of the lung metastasis signature (LMS) in breast carcinoma cells confers a predilection for pulmonary metastasis with *in vivo* and *in vitro* data demonstrating that the activity of these upregulated genes appears to mediate endothelial barrier dysfunction in the pulmonary vasculature prior to the arrival of the metastatic cells, priming the lung vessels to facilitate cancer cell extravasation(170, 171, 195). Similarly, the release of primary tumour-derived exosomes (small membrane bound vesicles of endosomal origin) expressing various oncoproteins or specific pro-metastatic microRNA have been shown to prime the pre-metastatic niche for the arrival of tumour cells, promoting organ-specific tropism of metastatic cells and enhanced permeability in the vasculature of the target organ(192, 196, 197). In addition to factors released from the primary tumour itself, the increased activity of pro-metastatic factors in the primary tumour microenvironment, such as TGFβ, have also been reported to prime incipient metastatic cells for disruption of endothelial barrier function in the target vasculature(171, 198). Moreover, at the interface between the tumour cell and the vascular endothelium, the secretion of factors such as VEGF and tumour-derived SPARC protein as well as the enhanced generation of thrombin also serve to induce loss of barrier integrity(191). Crucially, inhibition of these various mechanisms in animal models of metastasis has been shown to attenuate tumour-mediated vascular permeability and metastasis formation(191, 192, 195, 198).

Heparins have been shown to oppose abnormal vascular permeability in experimental models of sepsis, preeclampsia and acute lung injury but the underlying molecular mechanisms remain poorly understood(199-202). While LMWH exposure has been shown to exhibit anti-metastatic properties *in vivo*, the precise underlying mechanism also remains to be conclusively determined and the effects of LMWH in supporting endothelial barrier activity in the context of tumour metastasis have not been previously investigated.
1.3.3 The role of dual-endothelin-1 receptor antagonism in the management of Eisenmenger syndrome and its potential to restore normal haemostasis in this disorder

Having initially considered the role of potential novel therapeutic strategies in optimizing the balance of benefit and risk (in terms of bleeding side effects) in cancer patients, a number of other disease states may also present potential therapeutic opportunities to maximize the balance of competing risks relevant to blood coagulation. One such disease area is Eisenmenger syndrome (discussed in section 1.2.5), which is characterized by challenging competing bleeding and thrombotic risks due to coagulation and platelet activation.

Endothelin-1 (ET-1) is a 21 amino acid peptide which is synthesised primarily in endothelial and smooth muscle cells and which exhibits very potent vasoconstrictive and smooth muscle mitogenic properties\(^{203, 204}\). ET-1 has also been shown to exhibit pro-inflammatory properties in addition to its effects in promoting increased vascular tone and vascular re-modelling (hallmarks of pulmonary vascular disease in pulmonary hypertension), with several investigators demonstrating evidence of increased pro-inflammatory cytokine expression, increased endothelial cell adhesion molecule expression and enhanced leucocyte recruitment to the vascular endothelium mediated by ET-1\(^{205-208}\). Factors implicated in the induction of ET-1 synthesis include hypoxia, pulsatile stretch and shear stress and its expression, particularly within the pulmonary vasculature, has been reported to be significantly enhanced in pulmonary hypertension\(^{203, 204, 209}\). In the pulmonary vasculature, ET-1 mediates its effects through the endothelin-A (ET\(_A\)) and endothelin-B (ET\(_B\)) receptors. ET\(_A\) is expressed solely on smooth muscle cells while ET\(_B\) is expressed both on smooth muscle and endothelial cells. Binding of ET-1 to either ET\(_A\) or ET\(_B\) receptors on smooth muscle cells induces vasoconstriction and smooth muscle cell proliferation while the interaction between ET-1 and ET\(_B\) receptors specifically on endothelial cells promotes vasodilation. endothelin-1 receptor antagonism (both dual receptor and ET\(_A\) specific) has been shown to improve clinical outcomes among individuals with pulmonary hypertension (including patients with Eisenmenger syndrome)\(^{121, 210, 211}\).

Recently, in a large, randomised placebo-controlled trial, the novel dual endothelin-1 receptor antagonist macitentan has been shown to improve clinical outcomes and, crucially, to reduce mortality among individuals with pulmonary hypertension\(^{120}\). The effects of macitentan specifically within the high-risk sub-group of Eisenmenger syndrome remains to be determined.
The precise mechanism underlying the pro-thrombotic tendency observed among individuals with Eisenmenger syndrome remains to be determined (as outlined in 1.2.5). Interestingly, there is some limited evidence to suggest that elevated plasma ET-1 concentrations may be associated with systemic coagulation activation and intravascular thrombosis \textit{in vivo} although the underlying mechanisms are unclear (28). Platelets have been shown to express ET-1 receptors but the implications of ET-1 signalling for platelet function are unknown (27, 28, 212). The competing risk of major haemorrhage which arises in ES represents a major barrier to the use of anti-coagulant and anti-platelet drugs in this disorder despite the significant risk of thrombosis. Given that the use of the ET-1 receptor antagonists does not appear to be associated with a risk of haemorrhage (120), these agents could represent an attractive therapeutic option for the reduction of this thrombotic risk associated with ES should ET-1 receptor antagonism be found to attenuate abnormal blood coagulation in this disorder (either directly through an inhibitory effect on platelet pro-coagulant activity or indirectly, by opposing the pro-inflammatory activity of ET-1 or by ameliorating the vascular and endothelial dysfunction which can promote abnormal coagulation activation in this disorder) (213-215).
1.4 Objectives

1.4.1 To characterise LMWH-mediated endothelial barrier protection and to investigate the molecular mechanisms underlying this phenomenon

As outlined in 1.3.2.5 and 1.3.2.6, LMWH has been shown to mediate an anti-cancer effect through mechanisms which remain to be fully elucidated but which appear to be mediated through a suppression of cancer metastasis. Data from several recent studies have illustrated the key role of endothelial barrier function in the prevention of cancer metastasis and have highlighted this phenomenon as a potential novel therapeutic target(191, 192). The potential effects of LMWH in augmenting endothelial barrier function in the context of metastasis have not been investigated as a possible mechanism underlying the LMWH anti-cancer effect. The first objective of this thesis was to characterise LMWH-mediated endothelial barrier protection and LMWH-mediated inhibition of tumour cell trans-endothelial migration in vitro.

While the anticoagulant effect of LMWH is mediated through the indirect inhibition of the proteolytic function of thrombin and FXa, it remains to be determined if the cytoprotective properties of LMWH are directly related to its anticoagulant activity(46). This is of particular clinical relevance as the risk of haemorrhage associated with the use of LMWH limits its use among patients with cancer, among whom the risk of major haemorrhage would be predicted to negate the potential survival benefit which might otherwise be associated with LMWH use(143). Therefore, the second objective of this thesis was to investigate the mechanism underlying LMWH-mediated endothelial barrier protection and specifically to determine if this cytoprotective effect is dependent on anticoagulant function.
1.4.2 To optimise the relative anticoagulant and cytoprotective properties of LMWH in the context of cancer metastasis

Given that the risk of haemorrhage associated with LMWH represents a significant obstacle to its use as an adjunct to conventional anti-cancer therapies, the third objective of this thesis was to explore potential strategies that might permit clinicians to limit the exposure of cancer patients to the anticoagulant properties of LMWH while maximising the endothelial barrier protective effect.

Specifically, the relative anticoagulant and cytoprotective properties of fractions of LMWH tinzaparin were investigated, aiming to identify a fraction which exhibited limited anticoagulant activity but which retained endothelial barrier protective properties.

In addition, the potential additive effect on endothelial barrier function exhibited by combinations of sub-anticoagulant concentrations of tinzaparin in conjunction with clinically relevant concentrations of simvastatin (a lipid lowering drug which has been shown to mediate endothelial barrier protective properties in vitro when utilised in supra-physiological concentrations) was explored(216, 217), aiming to identify a drug combination which would maximise the LMWH-mediated endothelial barrier protective effect at the lowest possible LMWH concentration.
1.4.3 To characterise the mechanisms underlying the haemostatic derangements associated with Eisenmenger syndrome

As outlined in 1.2.5, derangements of haemostasis are common in ES and represent a significant source of morbidity and mortality. The mechanisms underlying ES-driven hypercoagulability are unknown but are thought to be related to the underlying vascular pathology (25, 117, 123, 128). Similarly, the aetiology of the haemorrhagic tendency in this disorder remains to be determined. Currently the management of the competing thrombotic and haemorrhagic risks represents a significant clinical challenge and in particular the role of anticoagulant therapy remains controversial in view of the bleeding tendency despite the significant risk of thromboembolic complications (34, 112). The fourth objective of this thesis was to investigate the mechanisms underlying these thrombotic and haemorrhagic tendencies in ES.
1.4.4 To explore the effects of dual endothelin-1 receptor antagonist therapy in ameliorating derangements of haemostasis in Eisenmenger syndrome

Dual ET-1 receptor antagonism has been shown to improve survival in patients with pulmonary hypertension by inhibiting the signalling activity of ET-1, a key mediator of vasoconstriction and vascular re-modelling within the pulmonary vasculature in this and related disorders (including ES)\(^2\text{10}\).

These agents are not known to mediate any direct effect on haemostasis (although platelets express ET-1 receptors and ET-1 has been postulated to induce abnormal coagulation activation)\(^2\text{7, 28}\). However, given that the underlying inflammatory, vascular pathophysiological changes in ES are presumed to drive the abnormal activation of coagulation and the impairment of normal haemostasis, the fifth objective of this thesis was to determine if dual endothelin-1 receptor antagonism would ameliorate the haemostatic derangements which are typical of this disorder.
CHAPTER 2: MATERIALS AND METHODS

2.1. Endothelial permeability assay: optimisation of endothelial monolayer confluence and barrier function

An *in vitro* assay of endothelial barrier function was optimised as previously described (61, 218, 219). EA.hy926 (an immortalised, PAR-1 expressing human umbilical vein endothelial cell line; kind gift from Dr C. Edgell, University of North Carolina Chapel Hill, North Carolina, USA) was cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen™, California, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen™, California USA), 5% penicillin-Streptomycin (pen-strep 10,000U/mL; Invitrogen™, California, USA) and 5% sodium hypoxanthine-aminopterin-thymidine (HAT supplement; Invitrogen™, California, USA) at 37°C in a humidified atmosphere of 5% carbon dioxide in air.

The EA.hy926 cells were then seeded at a concentration range of 10-30x10^4 on polyethylene terephthalate (PET) membranes in the inner chamber of trans-well culture plate inserts (Millicell® Hanging Cell Culture Inserts, 3.0µm pore size, membrane surface area 4.5cm^2; Merck Millipore Corporation, Massachusetts, USA) and cultured for 72 hours in 6-well plates containing supplemented-DMEM (outer chamber, 4 mls; inner chamber, 2mLs).

At 72 hours, the EA.hy926 cell layers were washed twice in warmed, sterile phosphate-buffered saline (PBS; Invitrogen™, California, USA) and returned to a 6-well plate containing serum-free DMEM (4mLs DMEM in the outer chamber, no culture media added to the inner chamber). An Evans Blue (0.67mg/mL; Sigma-Aldrich® Inc., Missouri, USA)-conjugated bovine serum albumin (BSA, 4%; ThermoFisher Scientific Inc., Massachusetts, USA) solution was added to the inner chamber of each trans-well insert (2mLs per insert) and 200uL aliquots of cell culture media from the outer chamber of the trans-well plate were then sampled at 2 minute intervals (0-10 minutes) and added to a 96-well plate. Using a plate reader (SpectraMax®M2 Microplate Reader; Molecular Devices LLC, California, USA) in conjunction with SoftMax Pro® software (Version5.4.1; Molecular Devices LLC, California, USA) the permeability of the endothelial cell layers was determined by measuring the increase in absorbance at 650nm in the sampled media as a result of transmigration of the Evans Blue-BSA solution through the endothelial cell layer and into the outer chamber of the trans-well plate over time.
2.2. Assessment of thrombin-induced endothelial permeability

Assessment of thrombin-induced endothelial barrier permeability using an Evans blue-albumin trans-endothelial migration assay was conducted as previously described (61, 218, 219). EA.hy926 cells were seeded at a concentration of 20x10⁴/mL on PET trans-well inserts and grown to confluence in a 6-well plate as described in 2.1 in FBS-supplemented DMEM. At 72 hours, the FBS-supplemented DMEM was aspirated and the EA.hy926 monolayers were washed in sterile PBS and returned to a 6-well cell culture plate containing 2mls serum-free DMEM per well. 500µl serum-free DMEM was then added to the inner chamber of the trans-well insert following which thrombin (human alpha-thrombin, 1-10nM; Haematologic Technologies Inc., Vermont, USA) was added to the inner chamber and incubated with the endothelial cell monolayers for 10 minutes at 37°C.

The 500µL of culture media in the inner chamber (which had been incubated with thrombin) was then aspirated and the monolayers washed twice in warmed, sterile PBS prior to incubation with the Evans Blue-BSA solution. Thrombin-mediated disruption of endothelial barrier function was assessed by measurement of the change in absorbance in the outer chamber of the trans-well plate due to transmigration of the Evans Blue-BSA solution through the thrombin-treated EA.hy926 cell monolayers as described in 2.1.
2.3 Characterisation of endothelial cell actin cytoskeleton activation in response to thrombin

In order to characterise the normal pattern of distribution of filamentous actin (F-actin) in confluent monolayers of EA.hy926 cells, glass coverslips were seeded with cells (20x10⁴) and cultured in FBS-supplemented DMEM until confluent cell layers were formed. The cell culture media was then aspirated and the coverslips were washed twice in warmed, sterile PBS following which the coverslips were fixed in 3.7% paraformaldehyde (ThermoFisher Scientific Inc., Massachusetts, USA) for 10 minutes at room temperature. The paraformaldehyde solution was then aspirated and discarded and the coverslips washed three times in sterile PBS. The EA.hy926 cells were then permeabilised by incubating the coverslips in triton-X-100 (0.1% in PBS; ThermoFisher Scientific Inc., Massachusetts, USA) for 5 minutes at room temperature and then washed three times in PBS. The coverslips were then blocked in a 10% BSA solution for 1 hour at room temperature following which they were washed with PBS and incubated with a phalloidin probe (directed against F-actin) (AlexaFlour488®phalloidin; Invitrogen™, California, USA) for 30 minutes in darkness at room temperature. The coverslips were then mounted on glass slides and the EA.hy926 cells visualised by immunofluorescence microscopy (Axioplan 2 imaging® fluorescence microscope; Carl Zeiss AG, Gottingen, Germany) in conjunction with Axiovision software (Version 4.2.8; Carl Zeiss AG, Gottingen, Germany). The presented images were obtained using the 100x objective oil immersion lens with fluorescence excitation filter BP 436/10nm, emission filter LP 470nm.

In order to characterise the effect of thrombin on actin cytoskeleton dynamics and F-actin localisation, confluent monolayers of EA.hy926 were incubated with thrombin (1nM) in serum-free DMEM for 10 minutes prior to fixation with paraformaldehyde. Treated coverslips were then permeabilised, incubated with the phallloidin probe and mounted on glass slides as described above.
2.4 Characterisation of the cytoplasmic localisation of the endothelial tight junction-associated protein zona occludens-1 in response to thrombin.

EA.hy926 monolayers were grown on glass coverslips and fixed in 3.7% paraformaldehyde and permeabilised as described in 2.3. In order to characterise the normal pattern of localisation of ZO-1, the fixed EA.hy926 monolayers were blocked in a 10% solution of goat serum (Invitrogen™; California, USA) in PBS for 1 hour following which they were washed in PBS and incubated with a rabbit anti-ZO-1 primary antibody with reactivity against human tissues (1:200 dilution in 10% goat serum-PBS; Invitrogen™, California, USA) for 2 hours at room temperature as previously described(220). Coverslips were washed again in sterile PBS and then incubated with a fluorescent goat-anti-rabbit IgG secondary antibody (Alexa Flour 488 Goat-anti-rabbit; Invitrogen™, California, USA) for 30 minutes at room temperature. The coverslips were then mounted on glass slides and the pattern of localisation of ZO-1 was examined by immunofluorescence microscopy.

In order to characterise the effect of thrombin on the localisation of ZO-1 in endothelial cells, the monolayers of EA.hy926 were incubated in serum-free DMEM with thrombin (1nM) prior to fixation with paraformaldehyde. Incubation with the primary and secondary antibodies was then carried out as described above and the pattern of ZO-1 localisation visualised by immunofluorescence microscopy.
2.5 Assessment of vascular endothelial growth factor (VEGF)-induced endothelial permeability

EA.hy926 cells were seeded at a concentration of 20x10⁴/mL on PET trans-well inserts and grown to confluence in a 6-well plate as described in 2.1. Confluent monolayers were then incubated with VEGF (recombinant human VEGF₁₆₅, 1-10nM; Sigma-Aldrich™, Missouri, USA) in serum-free DMEM for 30 minutes at 37°C. VEGF-induced endothelial permeability was assessed by measurement of transmigration of Evans Blue-BSA through the EA.hy926 cell layer as outlined in 2.1.
2.6 LMWH-mediated support of baseline endothelial barrier function

EA.hy926 monolayers were cultured on PET membrane inserts in FBS-supplemented DMEM as described in 2.1. At 72 hours, the culture media was aspirated and the inserts washed twice with warmed, sterile PBS. The inserts were returned to a 6 well plate containing 2mLs serum-free DMEM per well with an additional 500µL serum-free media added to the inner chamber of the trans-well insert. The EA.hy926 monolayers were then incubated for 3 hours at 37°C in the presence or absence of either the LMWH tinzaparin (Innohep®, 2IU/mL; Leo Pharma®, Ballerup, Denmark) or equivalent concentrations of a tinzaparin-derived LMWH fraction (2.8KDa LMWH fraction, 20µg/mL; Leo Pharma®, Ballerup, Denmark) by directly adding the LMWH to the cell culture media in the inner chamber of the trans-well insert.

After 3 hours, the cell culture media was aspirated and the EA.hy926 monolayers washed twice in warmed, sterile PBS. The inserts were then returned to a 6-well tissue culture plate containing 4mLs serum-free DMEM per well. 2mLs of the Evans Blue-BSA solution was added to each insert and 200µL of cell culture media were aspirated from the outer chamber of each trans-well at 10 minute intervals for at least 40 minutes and this media was then transferred to a 96-well plate. The absorbance of the aspirated media at 650nm at each of the time points was then assessed using a plate reader as described in 2.1.
2.7 Characterisation of LMWH-mediated attenuation of thrombin-induced endothelial permeability

Confluent monolayers of EA.hy926 cells were cultured on PET membrane trans-well inserts as described in 2.1. The EA.hy926 monolayers were then washed in sterile PBS and incubated in serum-free DMEM (outer chamber, 2mLs; inner chamber 500µL) in the presence or absence of LMWH (tinzaparin, 0-2IU/mL; or the 2.8KDa LMWH fraction, 0-20µg/mL) for three hours at 37°C as described in 2.7.

At 3 hours, the trans-well inserts were washed in sterile PBS and returned to a cell culture 6-well plate containing fresh serum-free DMEM (outer chamber, 2mLs; inner chamber 500µL) and the EA.hy926 monolayers were then incubated with thrombin in the inner chamber of the trans-well plate (1nM) for 10 minutes at 37°C.

At ten minutes, the cell culture media in the inner chamber was aspirated and the inserts were washed in sterile PBS. The inserts were then returned to a 6-well plate containing 4mLs of serum-free DMEM per well. 2mLs of the Evans Blue-BSA solution was then directly added to the inner chamber of each trans-well insert. At ten minutes, 200µL of cell culture media was sampled from the outer chamber and the absorbance of this sampled media at 650nm was calculated as described in 2.1.

In order to compare results generated from different assays, permeability of endothelial cell layers was calculated as a percentage using the following formula:

\[
P (%) = \frac{(X-C)}{(F-C)} \times 100
\]

Where \(P\) is permeability (%), \(X\) is the LMWH treated OD 650, \(C\) is the untreated OD650 and \(F\) is the thrombin treated OD650.
2.8 Assessment of LMWH-mediated attenuation of VEGF-induced endothelial permeability

EA.hy926 monolayers were grown on PET trans-well inserts and incubated in the presence or absence of LMWH as described in 2.7.

At three hours, the inserts were washed in sterile PBS and returned to 6-well culture plates containing 2mLs serum-free DMEM per well with an additional 500µL serum-free DMEM directly added to the inner chamber of the trans-well insert. The EA.hy926 monolayers were then incubated for 30 minutes at 37°C in the presence or absence of VEGF in the inner chamber of the trans-well (final VEGF concentration, 1nM).

At 30 minutes, the cell culture media was aspirated and the permeability assay completed as described in 2.1.
2.9 Characterisation of endothelial cell myosin light chain-2 phosphorylation status

EA.hy926 cell monolayers were incubated with a sample buffer (125mM TRIS pH 6.8, 4% SDS w/v, 0.1% Bromophenol blue w/v, 20% Glycerol v/v, 100mM dithiothreitol (DTT), 1% v/v protease and phosphatase inhibitor cocktail) and cell lysates were generated by scraping. Samples were denatured by heating to 95°C for 3 minutes in the presence of sodium dodecyl sulphate. Cell lysate protein samples were then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using pre-cast 10% polyacrylamide Tris-HCL gels exposed to a current of 75 volts for 15 minutes followed by 100 volts for a further 60 minutes.

Following SDS-PAGE, the separated proteins were transferred to a polyvinyl difluoride (PVDF) membrane, blocked for 1 hour in blocking buffer (3% BSA in 1X TBS-T (20mM 54 TRIS, 150mM NaCl, 0.1% Tween, pH 7.4), washed and then probed with the targeting antibodies (rabbit anti-phospho-MLC-2 Thr/Ser19 antibody, 1:1000; rabbit anti-zona occludens-1 antibody, 1:1000) overnight at 4°C.

Membranes were then washed three times in Tris-buffered saline with tween (TBS-T) for 5 minutes per wash. HRP-conjugated specific secondary (2°) antibodies were prepared (1:25,000 dilution in 1X TBS-T in 3% BSA) and incubated with the membranes for 1 hour at room temperature. Membranes were then washed 3 times for 5 minutes and once for 15 minutes in TBS-T and then washed 3 times for 5 minutes and once for 15 minutes in dH2O. Supersignal West Pico Chemiluminescent reagent (prepared according to manufacturer’s instructions) was then added to each membrane for 1 minute. HRP - conjugated antibodies were detected with a BioSpectrum Imaging System (UVP Ltd; UK).

The degree of MLC-2 diphosphorylation observed in endothelial lysates was quantified by particle densitometry (ImageJ® software) with the numerical arbitrary units generated for each band in control and LMWH-treated cells expressed as a percentage of the mean maximum particle density observed in bands representing thrombin-induced MLC-2 diphosphorylation.
2.10 Confirmation of expression of PAR-1 on the surface of EA.hy926

EA.hy926 endothelial cells were incubated with murine anti-PAR-1 receptor (ATAP2; Sigma-Aldrich®, Missouri, USA) antibody or a murine IgG control (2µg/mL) for 60 minutes on ice. The EA.hy926 cells were then incubated with a fluorescent secondary antibody (AlexaFluor488® goat anti-mouse IgG, 1:1000; Invitrogen™, California, USA) for 30 minutes on ice and the expression of PAR-1 determined by the measurement of the cellular fluorescence intensity by flow cytometry (BD accuri® C6 Flow Cytometer).
2.11 Characterisation of PAR-1 dependent thrombin-induced endothelial permeability

EA.hy926 monolayers were cultured on PET trans-well cell culture inserts as described in 2.1. At 72 hours, the monolayers were washed in sterile PBS and returned to a trans-well plate containing serum-free DMEM (outer chamber, 2mLs; inner chamber, 500µL). In order to investigate the role of PAR-1 in mediating thrombin-induced permeability, the media in the inner chamber of the trans-well inserts was incubated for 3 hours at 37°C with either an anti-PAR-1 receptor antibody which inhibits thrombin-mediated PAR-1 cleavage and activation (ATAP2, 20µg/mL; Sigma-Aldrich®, Missouri, USA), RWJ56110, a selective PAR-1 antagonist which inhibits PAR-1 signalling activity independent of PAR-1 cleavage (20µM/mL; R&D Systems Inc., Minnesota, USA) or a mouse IgG control (IgG from mouse serum, 20µg/mL; Sigma-Aldrich®, Missouri, USA).

At 3 hours, thrombin (1nM) was added to the cell culture media in the inner chamber of the trans-well insert and the assessment of endothelial barrier permeability completed as described in 2.2.
2.12 Characterisation of the role of LMWH in modulating PAR-1 dependent thrombin-mediated signalling

EA.hy926 cell monolayers were cultured in trans-well inserts and incubated with tinzaparin (2IU/mL) in serum-free DMEM for three hours as described in 2.8. The endothelial cell monolayers were then incubated with either thrombin (1nM) or a selective PAR activating peptide (Ser-Phe-Leu-Leu-Arg-Asn-amide) which activates PAR-1 signalling independent of PAR-1 cleavage (SFLLRN, 50µM; Sigma-Aldrich®, Missouri, USA) for 10 minutes at 37°C. The effect of tinzaparin in attenuating endothelial permeability was then assessed as described in 2.7.
2.13 Assessment of the effect of HSPG cleavage from the endothelial cell surface on LMWH-mediated endothelial barrier protection

EA.hy926 monolayers were grown on PET trans-well inserts as described in 2.1. At 72 hours, the cell culture media was aspirated and the inserts were washed in sterile PBS and returned to the 6-well plate containing fresh supplemented DMEM (outer chamber, 2mLs; inner chamber 500µL). The EA.hy926 monolayers in the inner chambers of the trans-well inserts were then incubated either in the presence or absence of heparinase (Heparinase III from Flavobacterium Heparinum, 1 unit/mL; Sigma Aldrich®, Missouri, USA) for 2 hours at 37°C.

At 2 hours, the cell culture media from the inner chambers was aspirated and the inserts were washed twice in sterile PBS and returned to the 6-well plate containing fresh serum-free DMEM (outer chamber, 2mLs; inner chamber 500µL). The EA.hy926 monolayers were then incubated either in the presence or absence of LMWH (tinzaparin, 2IU/mL) for 3 hours at 37°C.

At 3 hours, the culture media was aspirated and the inner chambers of the trans-well inserts were again washed with sterile PBS prior to treatment with thrombin (1nM). Assessment of endothelial barrier permeability was then completed as described in 2.1.
2.14 Tumour cell trans-endothelial migration assay

EA.hy926 cells were seeded at a concentration of $5 \times 10^4$ on PET membranes in the inner chamber of trans-well culture plate inserts (Millicell® Hanging Cell Culture Inserts, 8.0µm pore size, 0.3cm² surface area; Merck Millipore Corporation, Massachusetts, USA) and cultured for 72 hours in 24-well plates containing DMEM supplemented with FBS (10%) (outer chamber, 900µL; inner chamber, 200µL).

At 72 hours, each insert was washed in warmed, sterile PBS and returned to a well containing 900µL of serum-free DMEM. 200µL of serum-free DMEM was then added to each inner chamber. The cell culture media in these inner chambers was then supplemented with LMWH tinzaparin (0-2IU/mL) or a 2.8KDa LMWH tinzaparin fraction (0-20µg/mL) or equivalent volume of PBS (control) and incubated for 3 hours at 37°C.

DU145 metastatic prostate carcinoma cells (ATCC®; Virginia, USA) were cultured in DMEM-FBS, then detached from the cell culture flask using Accutase® (2mL; Sigma aldrich®, Missouri, USA) and re-suspended in serum-free and phenol red-free DMEM at a concentration of $100 \times 10^4$/mL. The DU145 carcinoma cell line is known to exhibit a marked metastatic potential and, in particular, is known to exhibit an aggressive tendency towards trans-endothelial migration (although the precise underlying mechanisms are unclear)(221, 222). 3mL of this DU145 cell suspension was aspirated and incubated with calcein blue-AM (10µg/mL; Sigma aldrich®, Missouri, USA) for 30 minutes at 37°C.

The cell suspension was then centrifuged with the cell pellet formed re-suspended in fresh serum-free, phenol-red free DMEM media (repeated twice).

The EA.hy926 cell monolayers cultured on the PET membrane trans-well inserts (inner chamber of trans-well plate) were again washed in warmed, sterile PBS and returned to wells containing phenol red-free, FBS (10%)-supplemented DMEM. 200µL of the calcein blue-AM labelled DU145 cell suspension was then added to the inner chamber of each trans-well. The plates were incubated for 3 hours at 37°C.

At 3 hours, the trans-well inserts were removed and the fluorescence intensity (350/460nm) of the cell culture media in the outer chamber of the trans-well was determined using a plate reader (SpectraMax®M2 Microplate Reader; Molecular Devices LLC, California, USA) in conjunction with SoftMax Pro® software (Version5.4.1; Molecular Devices LLC, California, USA).
2.15 Assessment of simvastatin-mediated attenuation of thrombin-induced endothelial permeability

Monolayers of EA.hy926 were seeded onto PET membranes and cultured in supplemented DMEM as described in 2.1. After 48 hours, the cell culture media in the inner and outer chambers of the trans-well inserts were incubated with simvastatin (1-40nM; Sigma Aldrich®, Missouri, USA) for a further 24 hours at 37°C until confluent monolayers were formed.

The cell culture media was then aspirated and the cell culture inserts washed twice in warmed, sterile PBS prior to incubation with thrombin in serum-free media, with the permeability assay completed as described in 2.1.
2.16 Assessment of the effect of co-incubation of simvastatin with LMWH in modulating endothelial barrier function

EA.hy926 monolayers were cultured in the presence of simvastatin (1-20nM) as described in 2.15. At 72 hours, the cell culture media was aspirated and inserts washed in sterile PBS following which they were incubated in serum-free DMEM either in the presence or absence of LMWH at a concentration which would be predicted to be lower than concentrations that would exhibit anticoagulant activity if observed in plasma (0.1IU/mL). Simvastatin-treated EA.hy926 monolayers were also separately co-incubated with the 2.8KDa tinzaparin-derived LMWH fraction (5µg/mL).

At 3 hours, the monolayers were washed in PBS prior to incubation with thrombin in serum free-DMEM. The permeability assay was then completed as described in 2.1.

This experiment was repeated with EA.hy926 cell monolayers which were incubated at 48 hours with a low concentration of simvastatin (5nM) and, after a further 24 hours, the simvastatin-treated monolayers were incubated with a range of concentrations of tinzaparin (0-0.5IU/mL) with permeability of the endothelial cell layers following incubation with thrombin then assessed as described in 2.1.
2.17: Recruitment of subjects for assessment of derangements of anticoagulant and procoagulant pathways in Eisenmenger syndrome and for investigating the influence of dual endothelin-1 antagonism on the prothrombotic phenotype in Eisenmenger syndrome.

Ethical approval to proceed with the recruitment of subjects for participation in a prospective cohort study aimed at investigating derangements of anticoagulant and procoagulant pathways in Eisenmenger syndrome was granted by the Mater Misericordiae University Hospital & Mater Private Hospital research ethics committee. In collaboration with the national adult congenital cardiac disease service based at the Mater Misericordiae University Hospital, treatment-naive subjects with Eisenmenger syndrome who were about to commence dual endothelin-1 antagonist therapy (Macitentan, 10mg daily; Actelion Pharmaceuticals Ltd, Allschwill, Switzerland) for the treatment of complications related to ES, were identified. Subjects underwent a clinical assessment to screen for the presence of existing haemorrhagic symptoms or a history of arterial or venous thromboembolism. Inclusion criteria for participation in the study consisted of age greater than 18 years and the presence of a diagnosis of cyanotic congenital cardiac disease complicated by Eisenmenger syndrome requiring medical therapy. Exclusion criteria consisted of the presence of a known hereditary thrombophilia or bleeding disorder; a history of malignancy, liver disease or surgery within the 6 months preceding recruitment; current or previous treatment with a dual endothelin-1 antagonist or other targeted pharmacological agent for the treatment of ES (e.g. sildenafil); exposure to an anticoagulant drug in the month prior to recruitment. Informed consent was obtained from participants (or from the legal guardian in situations where participants lacked capacity to provide informed consent due to intellectual disability, in which case patient assent was obtained in addition to guardian consent); 30-40 mLs of whole blood was then obtained by direct venepuncture at the antecubital fossa using minimal tourniquet. Blood was collected directly into collection tubes (S-monovette® 10 mL aspiration tube, Citrate 3.2% (1:10), final concentration 0.109M; Sarstedt AG & Co., Numbrecht, Germany) and into collection tubes which had been additionally supplemented with corn trypsin inhibitor (CTI, final concentration 50µg/mL; Haematologic Technologies Inc. Vermont, USA). Whole blood samples were also collected into tubes containing potassium-EDTA (S-monovette® 1.6mL aspiration tube, potassium-EDTA; Sarstedt AG & Co., Numbrecht, Germany) for analysis of whole blood cell counts. Among the subjects with Eisenmenger syndrome with a haematocrit in excess of 0.55, the volume of trisodium citrate was adjusted in the collection tubes prior to blood collection as per standard protocols.
2.18 Preparation of platelet-rich and platelet-poor plasma

Citrated whole blood samples were hand-delivered to the laboratory for immediate preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP). In order to extract PRP, whole blood was centrifuged at 170g for 10 minutes at room temperature. Samples of PRP extracted from whole blood obtained from patients were immediately analysed in parallel with samples obtained from the matched healthy volunteers. PPP was prepared by centrifuging whole blood at 2000g for 10 minutes at room temperature. The resulting plasma fraction was directly aspirated from the blood collection tube and frozen immediately in 1mL aliquots at -80°C.
2.19 Measurement of whole blood and platelet-rich plasma cell counts

Full blood count analysis (including measurement of cell counts, haematocrit and mean platelet volume) was performed using the ADVIA® 2120i haematology analyser (Siemens Healthcare GmbH, Erlangen, Germany) on whole blood obtained from study participants and collected into tubes containing EDTA. Similarly, platelet counts in PRP prepared from whole blood collected into trisodium citrate were also determined using this instrument.
2.20 Assessment of clotting times, fibrinogen concentration, D-Dimer level and anti-Factor Xa activity level in citrated platelet poor plasma

Measurements of prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen concentration, D-dimer level and plasma anti-Factor Xa activity level was undertaken using the ACL TOP® 500 haematology analyser (Instrumentation Laboratory, Bedford, MA 01730-2443, USA) in conjunction with the HemosIL® RecombiPlasTin2G PT reagent, the HemosIL® SynthASil APTT reagent, the HemosIL® Fibrinogen-C XL reagent, the HemosIL® D-Dimer HS 500 reagent and the HemosIL® Liquid Anti-Xa reagent (Instrumentation Laboratory, Bedford, MA 01730-2443, USA).
2.21 Measurement of plasma thrombin generation

Plasma thrombin generation was assessed by calibrated automated thrombography using a Fluoroskan Ascent® Plate Reader (ThermoLab Systems®, Helisinki, Finland) in conjunction with Thrombinscope™ software (Thrombinscope BV, Maastricht, The Netherlands).

For the assessment of tissue factor (TF)-stimulated thrombin generation in platelet-poor plasma, 80μl aliquots of citrated platelet poor plasma were incubated with 20 μL of platelet-poor-plasma reagent (PPP-Low reagent; Thrombinscope BV, Maastricht, The Netherlands) containing 1 pM TF and 4 μM phospholipids (composed of 60% phosphatidylcholine, 20% phosphatidylserine, and 20% phosphatidylethanolamine) in a 96-well round bottom polystyrene plate (Nunc™ microwell plates; ThermoScientific™, Massachusetts, USA). Thrombin generation was initiated by automatic dispensation of a fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC.HCl; Thrombinscope BV, Maastricht, The Netherlands) and 100 mM CaCl2 into each well (final concentrations, Z-Gly-Gly-Arg-AMC.HCl, 0.42 mM and CaCl2, 16.67 mM) and assessment of thrombin generation parameters was completed using a thrombin generation standard (Thrombin Calibrator; Thrombinscope BV, Maastricht, The Netherlands). The lagtime to initiation of thrombin generation, peak thrombin generation, time to peak thrombin generation and the area under the thrombin generation curve (endogenous thrombin potential; ETP) was determined for each plasma sample. Experiments were performed in duplicate and data were reported as mean ± SEM. Statistical analysis was performed using the software package, Prism™ (Version 5.0; GraphPad Software Inc., California, USA).

For the assessment of TF-stimulated thrombin generation in platelet-rich plasma, 80μl aliquots of plasma were incubated with 20μl of platelet-rich plasma reagent (PRP-reagent; Thrombinscope BV, Maastricht, The Netherlands), containing 1pm TF but no exogenous phospholipid (as the subject’s platelets provide the source of phospholipid which is required for blood coagulation enzyme activity). Initiation and measurement of thrombin generation was then completed as outlined above.

In order to characterise contact pathway activation-independent thrombin generation, the experiment was also conducted in plasma collected into collection tubes which had been supplemented with corn trypsin inhibitor (CTI, an inhibitor of coagulation factor XII activation, final concentration 50μg/mL; Haematologic Technologies Inc., Vermont, USA).
In order to characterise TF-independent plasma thrombin generation in platelet-rich or platelet-poor plasma, the PPP-low or PRP-reagents were substituted with PBS, with the remainder of the experiment completed as outlined above.

The role of the activated protein C pathway in attenuating thrombin generation was assessed by incubating plasma with APC (human activated protein C, 0-10nM; Haematologic Technologies Inc., Vermont, USA) prior to initiating TF-stimulated thrombin generation as outlined above.
CHAPTER 3: THE ENDOTHELIAL BARRIER-PROTECTIVE PROPERTIES OF LOW MOLECULAR WEIGHT HEPARIN

3.1 Introduction

Cancer metastasis is the highly complex process through which cancer cells that are shed from a primary tumour migrate via the systemic circulation to distant organs where they invade and proliferate leading to the growth of new tumour deposits. Complications relating to the proliferation of these metastatic tumours account for the vast majority of cancer-associated deaths and consequently therapeutic strategies which interrupt this process would be predicted to improve patient survival (142, 186).

The low molecular weight heparins (LMWH) are a class of anticoagulant drug which are widely used in current clinical practice for the treatment and prevention of thrombosis (134). Remarkably, data from the clinical trials which have explored the use of LMWH specifically in the setting of cancer (which is a well-recognised prothrombotic state) have suggested that an additional survival benefit is conferred upon sub-groups of cancer patients following LMWH exposure which is independent of the reduction in thrombotic risk (138-140, 166). This survival benefit appears to be limited to patients with early-stage disease (prior to the occurrence of cancer dissemination), suggesting that LMWH may exert an inhibitory effect on the process of cancer metastasis. This hypothesis is supported by reports from animal models of human metastasis which suggest that while LMWH does not influence primary tumour growth, it does appear to suppress the development of secondary tumour deposits (187, 223). However, the precise mechanisms underlying these potential anti-metastatic effects of LMWH have yet to be fully elucidated.

In order for cancer metastasis to proceed successfully, malignant cells must migrate through the vascular endothelium at the site of the primary tumour (where the cancer cells intravasate into the systemic circulation) and again at the site of the target tissue (where the cancer cells attach to the endothelium and migrate into the tissues of the target organ) (141, 171, 190). However, under normal physiological conditions the intact vascular endothelium forms an impermeable barrier to the cellular components of the circulating blood. Agonists such as thrombin, VEGF and other various factors which are secreted by malignant cells induce vascular permeability, permitting cellular trans-endothelial migration. Recent data suggests that this tumour-mediated impairment of endothelial barrier function is critical to metastasis and moreover that
inhibition of tumour-induced endothelial permeability suppresses cancer dissemination (191, 192, 224).

The following experiments were undertaken with the aim of addressing the hypothesis that LMWH mediates an inhibitory effect on the process of cancer metastasis by supporting endothelial barrier function. Specifically, the effects of LMWH in attenuating agonist-induced endothelial permeability (focusing on the activity of cancer-associated agonists such as thrombin and VEGF) and tumour cell trans-endothelial migration were investigated in addition to an exploration of the potential signalling mechanisms underlying these observations.
3.2 Optimisation of an \textit{in vitro} assay of endothelial barrier permeability

In order to establish an assay of endothelial barrier function comprising of confluent monolayers of endothelial cells, a range of concentrations of EA.hy926 cells (10-30x10^4) were seeded on PET trans-well membranes and cultured for 72 hours as described in 2.1, with the barrier function of the endothelial cell layers assessed by measuring the change in absorbance in the outer chamber of the trans-wells as a result of flux of an Evans blue-conjugated albumin solution through the EA.hy926 monolayers over time.

EA.hy926, a permanent endothelial cell line generated through the fusion of human umbilical vein endothelial cells (HUVECs) and the A549 bronchial carcinoma cell line, has been well characterised and widely utilised as a source of endothelial cells in \textit{in vitro} experiments of vascular function. The stability and extended life-span of the EA.hy926 cell line permits reproducibility in repeated experiments over time and negates the requirement for repeated isolation of primary HUVECs and the influence of potential variability in such serial isolates from different donors(225).

Albumin, a plasma protein, migrates through vascular endothelium under certain physiological and pathological conditions. Paracellular diffusion of the albumin molecule through gaps formed between contiguous endothelial cells appears to represent a key mechanism underlying the trans-endothelial flux of this protein, a phenomenon which is enhanced through the effects of various agonists such as thrombin, bradykinin and histamine in activating endothelial actin-myosin skeletal contraction leading to disassembly of inter-endothelial cell junctions(194, 226). Transcellular migration of albumin through caveolae-dependent and independent mechanisms have also been described(194, 227).

The mean OD650nm of the cell culture media in the outer chamber of the plates prior to the addition of the Evans blue-albumin solution to the inner chamber of the plate was measured at 0.04±0.005. The OD650nm of undiluted Evans blue-albumin solution was measured at 3.02±0.005. Following the addition of the Evans blue solution to the inner chamber of the trans-wells, confluence and barrier function of the cell layers was assessed by measuring the change in optical density of the cell culture media in the outer chamber (as a result of transmigration of the Evans blue-BSA solution through the endothelial monolayers) over time (with measurement of cell culture media absorbance undertaken at ten minutes following the addition of the Evans-blue solution to the trans-well as previously described(219)).
Marked transmigration of the Evans Blue-BSA solution was observed through the PET trans-well membranes which had been seeded with EA.hy926 cells at a concentration of 10x10^4 and 15x 10^4 with the optical density of the cell culture media in the basolateral chamber of these wells measured at 0.87±0.13 and 0.59±0.18 at ten minutes respectively, suggesting that confluent monolayers with intact barrier function had not been formed at these cell concentrations (Figure 3.1).

At a cell concentration of 20x10^4 only very limited Evans Blue-BSA transmigration was observed, with the optical density of the cell culture media in the basolateral chamber at ten minutes measured at 0.17±0.04 which was significantly lower than that observed with either the 10x10^4 (p<0.001) or 15x10^4 (p<0.05) cell concentration. Seeding of trans-well PET membranes with a EA.hy926 cell concentration of 30x10^4 led to a similar response as that observed with the 20x10^4 cell concentration (optical density of cell culture media 0.14±0.04) which was significantly lower than that observed with either the 10x10^4 (p<0.001) or the 15x10^4 (p<0.01) EA.hy926 cell concentration but which was not significantly different to that observed with the 20x10^4 cell concentration, suggesting that confluent EA.hy926 monolayers with intact barrier function are formed on PET trans-well membranes when seeded at a concentration of 20-30x10^4 and cultured in supplemented DMEM for 72 hours, as described in 2.1.
Figure 3.1 Seeding of PET membranes with EA.hy926 endothelial cells at a concentration range of 20-30x10⁴ leads to the formation of endothelial monolayers with intact barrier function

A range of concentrations of EA.hy926 cells were seeded on PET membrane trans-well inserts and cultured in supplemented-DMEM for 72 hours. The membranes were then washed with sterile PBS and incubated with an Evans blue-conjugated albumin solution and the degree of permeability of the endothelial cell layers was determined by the measurement of the flux of the albumin solution through the cell layer and into the outer chamber. Intact barrier function was exhibited by endothelial layers which had formed on PET membranes seeded at a concentration of 20-30x10⁴ where limited transmigration of Evans Blue-BSA through the intact monolayer was observed as determined by measurement of the optical density at 650nm in the cell culture media of the basolateral chamber of these trans-wells. Experiments were performed in duplicate and results are expressed as mean±SEM of 4 independent experiments (One-Way ANOVA with Bonferroni’s multiple comparison test; ns= non-significant, *=p<0.05, **=p<0.01, ***=p<0.001).
3.3 Characterisation of thrombin-induced endothelial monolayer permeability

In order to determine the optimum concentration of thrombin required to induce measurable endothelial barrier permeability, confluent monolayers of EA.hy926 cells (seeded at a concentration of 20x10⁴ on PET membranes and grown to confluence as described in 2.1) were incubated with thrombin at a concentration range of 0-10nM and endothelial permeability was assessed at 10 minutes as described in 2.2.

Increased transmigration of Evans Blue-BSA through the endothelial monolayers was observed with increasing concentrations of thrombin (as determined by measurement of optical density of the culture media in the basolateral chamber of the trans-well) (Figure 3.2). At a thrombin concentration of 1nM, the optical density in the basolateral chamber as a result of Evans Blue-BSA transmigration through the endothelial monolayer was significantly increased relative to that observed through endothelial monolayers which had not been incubated with thrombin (0nm thrombin, OD650nm: 0.14±0.4; 1nM thrombin, OD650nm: 0.82±0.1; p<0.001). Evans Blue-BSA transmigration was also significantly enhanced through monolayers incubated with 10nM thrombin relative to the untreated monolayer (OD650nm: 0.6±1.4; p<0.01) but no significant difference in monolayer permeability was observed using this higher concentration relative to the 1nM thrombin concentration, suggesting that measurable endothelial barrier permeability can be achieved by incubating confluent endothelial monolayers with 1nM thrombin.
Figure 3.2 Incubation of endothelial monolayers with thrombin leads to endothelial barrier permeability

EA.hy926 monolayers were grown to confluence and incubated with thrombin (0-10nM). Progressive loss of endothelial barrier function was observed with increasing thrombin concentrations as determined by assessing the degree of Evans Blue-BSA transmigration through treated endothelial monolayers. Experiments were performed in duplicate and results are expressed as mean±SEM of 3 independent experiments (One-Way ANOVA with Bonferroni’s multiple comparison test; **=p<0.01, ***=p<0.001).
3.4 Characterisation of thrombin-mediated endothelial cell actin cytoskeleton activation and inter-endothelial junction disruption

Monolayers of EA.hy926 cell were cultured on glass coverslips and incubated with thrombin or PBS prior to fixation in paraformaldehyde. The coverslips were then incubated with either a fluorescent phalloidin probe (directed against F-actin) or a monoclonal antibody directed against Zona Occludens-1 (ZO-1, an integral component of inter-endothelial adherens junctions) as described in 2.3 and 2.4.

In endothelial cell monolayers which were not exposed to thrombin, intact inter-endothelial junctions were observed with the normal cortical F-actin ring, cortical ZO-1 localisation and minimal cytoplasmic actin stress fibres were demonstrated (Figure 3.3 A-B).

In the presence of thrombin, marked cellular contraction was observed with loss of the cortical actin ring, loss of the normal peripheral ZO-1 localisation, marked cytoplasmic actin stress fibre formation and inter-cellular gap formation (Figure 3.3 C-D).
Figure 3.3 Thrombin-mediated endothelial barrier disruption is characterised by loss of inter-endothelial cell junctions and actin cytoskeleton activation

EA.hy926 monolayers which had been cultured on glass coverslips, fixed in 3.7% paraformaldehyde and permeabilised in triton-X-100 were incubated with a fluorescent phalloidin probe directed against F-actin (A-B) or a monoclonal antibody directed against ZO-1. The normal pattern of F-actin localisation (A, white arrow) was lost following incubation with thrombin, with marked actin stress fibre formation (B, red arrow) and inter-cellular gap formation (B, yellow arrow). Similarly, the normal cortical localisation of ZO-1 (C, blue arrow) was lost following incubation with thrombin (D, grey arrow). Experiments were performed in duplicate and the presented images are representative of three independent experiments.
3.5 LMWH Tinzaparin supports endothelial barrier function

In order to determine whether LMWH enhances baseline endothelial barrier function, confluent monolayers of EA.hy926 endothelial cells were incubated in the presence or absence of LMWH tinzaparin (2IU/mL) for three hours in serum-free DMEM prior to assessment of endothelial barrier permeability as described in 2.7.

In the presence of tinzaparin, endothelial barrier function was enhanced with significantly reduced Evans Blue-BSA transmigration observed at 40 minutes in endothelial monolayers exposed to tinzaparin in comparison to that observed in the untreated endothelial monolayers (0IU/mL Tinzaparin, OD650nm: 0.63±0.1 vs. 2IU/mL, OD650nm: 0.34±0.1; p<0.01), suggesting that tinzaparin enhances baseline endothelial barrier activity (Figure 3.5).
Figure 3.4 LMWH Tinzaparin enhances endothelial barrier activity

Incubation of confluent monolayers of EA.hy926 endothelial cells with tinzaparin (2IU/mL) for three hours leads to a significant reduction in transmigration of an Evans Blue-BSA solution through the treated monolayers at 40 minutes suggesting that LMWH enhances endothelial barrier function. All experiments were performed in duplicate and results are expressed as the mean±SEM of three independent experiments (n=6; Wilcoxon matched pairs test; **=p<0.01).
3.6 LMWH attenuates thrombin-induced endothelial permeability

Confluent EA.hy926 monolayers were incubated with LMWH tinzaparin at a concentration range of 0-2IU/mL for three hours prior to incubation with thrombin as described in 2.8.

Within this clinically relevant concentration range, a marked suppression of thrombin-induced endothelial permeability was observed with increasing concentrations of LMWH (Figure 3.6). At 0.25IU/mL, 0.5IU/mL, 1IU/mL and 2IU/mL, thrombin induced endothelial permeability was suppressed to 38.2±14.4% (p<0.01), 21.9±14.2% (p<0.01), 14.3±4.4% (p<0.001) and 5.7±4.3% (p<0.001) respectively. At a concentration of 0.1IU/mL which is below the range that would be considered to mediate a clinically relevant anticoagulant effect in plasma, no significant attenuation of thrombin-induced permeability was observed (98.43±19.6% of baseline).
Figure 3.5 Tinzaparin attenuates thrombin-induced endothelial permeability

EA.hy926 monolayers were incubated with tinzaparin (0-2IU/mL) for three hours prior to incubation with thrombin (1nM). A concentration-dependent attenuation of thrombin-induced endothelial permeability was observed. Experiments were performed in duplicate and results are expressed as the mean±SEM of three independent experiments (n=6 for each tinzaparin concentration; One-Way ANOVA with Bonferroni’s multiple comparison test; **=p<0.01, ***=p<0.001).
3.7 LMWH-mediated endothelial barrier protection is observed across a range of commercial LMWH formulations

In order to characterise the endothelial barrier protective properties of a range of LMWH preparations and in order to compare the magnitude of protective activity exhibited by the various LMWH preparations, a selection of LMWH standards (enoxaparin, dalteparin and tinzaparin; European Pharmacopoeia Reference Standards) were obtained and their barrier protective properties were assessed as described previously. In these experiments, commercial formulations of the various LMWH preparations were not used as the units of LMWH concentration used differed between formulations (e.g. commercial tinzaparin is only available in IU/mL of anti-Xa activity which cannot be precisely converted to micrograms of tinzaparin per mL, while commercial enoxaparin and dalteparin are available in micrograms per mL of LMWH). The reference compounds were reconstituted in micrograms per mL in order to allow direct comparisons between these agents at the same concentrations.

All three LMWH preparations significantly attenuated thrombin-induced endothelial permeability. At a concentration of 20µg/mL, the tinzaparin standard attenuated permeability to 55.1±10.6% (P<0.05), the enoxaparin standard attenuated permeability to 40.7±7% (p<0.01) and the dalteparin standard attenuated permeability to 29.8±8.7% (p<0.001). The differences in magnitude of effect between the three LMWH standard preparations did not reach significance (Figure 3.6).
Figure 3.6 The LMWH preparations tinzaparin, dalteparin and enoxaparin exhibit similar endothelial-barrier protective activity

Incubation of endothelial cell monolayers with tinzaparin (20µg/mL), enoxaparin (20µg/mL) or dalteparin (20µg/mL) leads to an attenuation of thrombin-induced endothelial permeability. Experiments were performed in duplicate and results are expressed as the mean±SEM of 3 independent experiments (One-Way ANOVA with Bonferroni’s multiple comparison test; *=p<0.05, **=p<0.01, ***=p<0.001).
3.8 LMWH tinzaparin attenuates thrombin-induced myosin light chain-2 diphosphorylation

Myosin light chain-2 (MLC-2) is one of the key regulators of actin cytoskeleton dynamics and in its phosphorylated state, promotes cytoskeletal contraction resulting in disassembly of inter-endothelial cell junctions and the loss of endothelial barrier integrity. Thrombin, a potent inducer of endothelial permeability, stimulates MLC-2 diphosphorylation leading to the generation of actin stress fibres and actin cytoskeletal contraction within endothelial cells.

The effect of tinzaparin in attenuating thrombin-induced MLC-2 diphosphorylation was assessed by SDS-PAGE and western blotting using endothelial cell lysates which had been treated with LMWH tinzaparin prior to incubation with thrombin as described in 2.9. The degree of MLC-2 diphosphorylation was quantified by particle densitometry and the numerical arbitrary units generated for each band in control and LMWH-treated cell lysates was expressed as a percentage of the mean maximum particle density observed in bands representing thrombin-induced MLC-2 diphosphorylation. Levels of ZO-1 protein (a tight junction-associated membrane protein with a molecular weight in excess of MLC-2 and which is present in endothelial cells) were used as a control.

A significant increase in MLC-2 diphosphorylation was observed in EA.hy926 cell lysates following incubation with thrombin (13.1±4.8 to 100±19.5%, p=0.003). However, the degree of MLC-2 diphosphorylation was significantly attenuated in lysates prepared from endothelial cells which had been incubated with LMWH prior to exposure to thrombin (mean maximum thrombin-induced MLC-diphosphorylation reduced to 51.4±9.9%, p=0.03).
(A) 240KDa

ZO-1 Junctional Protein

18KDa

Diphosphorylated MLC-2

Thrombin (1nM)  
-  +  +

Tinzaparin (10IU/)  
-  -  +

(B)

Thrombin (1nM)  
-  +  +

Tinzaparin (10IU/mL)  
-  -  +
Figure 3.7 LMWH tinzaparin attenuates thrombin-induced endothelial cell MLC-2 diphosphorylation

EA.hy926 endothelial cell MLC-2 diphosphorylation status was determined by SDS-PAGE and western blotting. Incubation of endothelial cells with thrombin (1nM) led to a significant increase in MLC-2 diphosphorylation as predicted, however pre-treatment with LMWH tinzaparin (10IU/mL) significantly attenuated thrombin-induced diphosphorylation. Experiments were performed in duplicate and results represent the mean±SEM of 4 independent experiments (*=p<0.05; **=p<0.01).
3.9 Thrombin-induced endothelial barrier permeability is PAR-1 dependent

The role of the PAR-1 signalling pathway in mediating thrombin-induced endothelial permeability was investigated as described in 2.14.

Confluent EA.hy926 monolayers were incubated in the presence or absence of thrombin and ATAP2 (a murine monoclonal antibody directed against the PAR-1 thrombin cleavage site). Permeability of the endothelial cell monolayers was significantly increased in the presence of thrombin as previously observed but where EA.hy926 monolayers had been pre-incubated with ATAP2, the thrombin-induced permeability was completely abolished (Figure 3.8). Similarly, when EA.hy926 monolayers were incubated with RWJ56110, a PAR-1 inhibitory peptide which suppresses PAR-1 signalling independent of thrombin-induced PAR-1 cleavage, thrombin-mediated permeability was again abolished (Figure 3.8), suggesting that thrombin-mediated endothelial barrier permeability is PAR-1 dependent.
Figure 3.8 Thrombin-induced endothelial permeability is PAR-1 dependent

Endothelial cell monolayers were incubated in the presence of thrombin (1nM); a mouse monoclonal antibody directed against the PAR-1 cleavage site (ATAP2, 20µg/mL); a mouse immunoglobulin control (20µg/mL); and RWJ56110, a PAR-1 signalling inhibitory peptide (20µg/mL). Thrombin-induced endothelial permeability was abolished in the presence of ATAP2 and RWJ56110 suggesting that thrombin-induced permeability of EA.hy926 monolayers is PAR-1 dependent. Experiments were performed in duplicate and results are expressed as the mean±SEM of three independent experiments (One-Way ANOVA with Bonferroni’s multiple comparison test; ***=p<0.001).
3.10 LMWH tinzaparin does not modulate endothelial PAR-1 expression and does not inhibit thrombin-induced PAR-1 cleavage and activation

Given that thrombin-induced cell signalling activity is PAR-1 dependent, LMWH-mediated inhibition of thrombin-induced PAR-1 cleavage was explored as a potential mechanism by which LMWH attenuates thrombin-induced endothelial barrier dysfunction.

EA.hy926 endothelial cells were washed and incubated with either the ATAP2 anti-PAR-1 antibody (an antibody which binds epitopes which are retained following receptor cleavage and so binds to both cleaved and intact PAR-1(228, 229)), or a mouse IgG control, following incubation with tinzaparin and prior to incubation with thrombin as described in 2.15. Thrombin-induced cleavage and activation of PAR-1 (or specific peptide-induced receptor activation, such as SFLLRN-mediated receptor activation) leads to internalisation of the cleaved receptor and therefore prevents binding of ATAP2 to PAR-1 on the cell surface(230). Consequently, the incubation of endothelial cells with thrombin would be predicted to result in diminished mean fluorescence intensity as measured by flow cytometry as a result of receptor internalisation (following incubation with a fluorescent secondary antibody directed against ATAP2).

As expected, fluorescence intensity was significantly lower following incubation of EA.hy926 with thrombin. Incubation of EA.hy926 cells with tinzaparin (2IU/mL) did not significantly alter the measured fluorescence intensity, suggesting that LMWH does not alter PAR-1 expression. Moreover, the fluorescence intensity measured in cells incubated with thrombin following pre-incubation with tinzaparin was similar to that measured in cells treated with thrombin alone, suggesting that LMWH does not impair thrombin-mediated PAR-1 activation and internalisation (Figure 3.9).
Figure 3.9 LMWH does not alter endothelial PAR-1 expression and does not inhibit thrombin-mediated PAR-1 cleavage

EA.hy926 endothelial cells were incubated with ATAP2 (20µg/mL), a PAR-1 binding antibody and PAR-1 expression determined by the measurement of fluorescence intensity with flow cytometry following incubation with a fluorescent secondary antibody directed against ATAP2. Fluorescence was diminished following incubation of EA.hy926 cells with thrombin as a result of thrombin-mediated PAR-1 internalisation. Tinzaparin did not alter PAR-1 expression and did not inhibit the thrombin-mediated PAR-1 internalisation. Experiments were performed in duplicate and results are expressed as the mean±SEM of 3 independent experiments (One-Way ANOVA with Bonferroni’s multiple comparison test; **=p<0.01, ***=p<0.001).
3.11 LMWH inhibits PAR-1 mediated endothelial barrier permeability

In order to assess the ability of LMWH to oppose PAR-1 mediated endothelial barrier permeability, confluent monolayers of EA.hy926 were incubated with a selective PAR activating peptide (Ser-Phe-Leu-Leu-Arg-Asn-amide; SFLLRN) which activates PAR signalling independent of PAR-1 cleavage following pre-incubation with LMWH tinzaparin as described in 2.16.

As expected, endothelial barrier permeability was significantly increased in the presence of the PAR-1 activating peptide (Figure 3.10) but this effect was attenuated in endothelial monolayers which had been incubated with tinzaparin prior to incubation with the peptide, where permeability was suppressed to 50.5±6.2% of that observed in the absence of pre-incubation with tinzaparin (p<0.01). This finding suggests that while LMWH does not appear to impair agonist-induced PAR-1 activation as demonstrated in 3.10, the signalling activity of the PAR-1 pathway leading to endothelial permeability is suppressed following treatment of endothelial cells with this agent.
Figure 3.10 LMWH attenuates PAR mediated endothelial permeability

EA.hy926 monolayers were incubated in the presence or absence of tinzaparin (2IU/mL) prior to incubation with the PAR activating peptide (50µM) which activated PAR-1 signalling independent of PAR-1 cleavage. LMWH significantly attenuated the endothelial barrier permeability induced by the PAR activating peptide. Experiments were performed in duplicate and results are expressed as the mean±SEM of three independent experiments (Unpaired t-test; **=p<0.01).
3.12 Cleavage of endothelial cell surface heparan sulphate proteoglycans does not impair LMWH-mediated endothelial barrier protection

Endothelial cell surface heparan sulphate proteoglycans (HSPG) have been shown to exhibit co-factor activity in supporting thrombin-mediated cell signalling activity. In order to characterise the role of cell surface HSPG in modulating thrombin-induced endothelial permeability and to investigate whether LMWH-mediated attenuation of thrombin-induced endothelial barrier permeability arises through the inhibition of the HSPG-thrombin interaction, EA.hy926 monolayers were treated with heparinase III (derived from Flavobacterium Heparinum and which specifically cleaves heparan sulphates) prior to assessing monolayer permeability as described in 2.10.

As expected, thrombin-induced endothelial barrier permeability was diminished as demonstrated by the reduction in Evans Blue-BSA transmigration which was observed through endothelial monolayers which had been treated with heparinase III prior to incubation with thrombin, suggesting that cell surface HSPG supports thrombin-induced endothelial cell signalling activity leading to endothelial barrier dysfunction (Figure 3.11A).

However, the relative attenuation of thrombin-induced endothelial barrier dysfunction by LMWH was similar in both the heparinase III-treated endothelial monolayers and in the untreated monolayers as evidenced by the ratios of reduction in outer chamber absorbance in the heparinase III-treated and untreated trans-wells respectively (Figure 3.11B OD ratio of 0.46±0.1 and 0.3±0.1; p=0.14).
A

![Bar chart showing optical density at 650nm for different treatments: Thrombin (1nM), Tinzaparin (2IU/mL), and Heparinase III (1U/mL).](chart.png)

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![Bar chart showing Thrombin:Thrombin + Tinzaparin OD650nm for different treatments: Heparinase III (1U/mL).](chart.png)

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**Statistical Significance**

- ****: p < 0.01
- ***: p < 0.001
Figure 3.11 Cleavage of endothelial heparan sulphate proteoglycans diminishes thrombin-induced endothelial permeability but does not impair LMWH-mediated endothelial barrier protection.

EA.hy926 cell monolayers were incubated with heparinase III (1U/mL) in order to cleave cell surface HSPG prior to incubation with thrombin (1nM) and tinzaparin (2IU/mL). Thrombin-induced endothelial permeability was reduced following HSPG cleavage (A). The degree of LMWH-mediated attenuation of thrombin-induced endothelial permeability was similar in heparinase III-treated and untreated endothelial monolayers (B). Experiments were performed in duplicate and results are expressed as the mean±SEM of 4 independent experiments (One-Way ANOVA with Bonferroni’s multiple comparison test; **=p<0.01, ***=p<0.001).
3.13 Characterisation of VEGF-induced endothelial barrier permeability

In order to determine the optimum concentration of VEGF required to induce measurable endothelial barrier permeability, confluent monolayers of EA.hy926 cells (seeded at a concentration of 20x10⁴ on PET membranes and grown to confluence as described in 2.1) were incubated with recombinant human VEGF at a concentration range of 0-5nM and endothelial permeability was assessed as described in 2.6.

No significant increase in Evans Blue-BSA transmigration was observed following incubation of EA.hy926 monolayers with VEGF in the concentration range of 0-0.5nM however in the presence of 1nM VEGF, endothelial permeability was significantly increased relative to that observed in the absence of incubation with VEGF (0nM VEGF, OD650nm: 0.1±0.01; 1nM VEGF, OD650nm: 0.3±0.05; p<0.05). With VEGF concentrations in excess of 1nM, no significant loss of endothelial barrier function was observed suggesting that VEGF induces maximal endothelial barrier permeability at the concentration of 1nM when incubated with monolayers of EA.hy926 endothelial cells (Figure 3.12).
Figure 3.12 Incubation of endothelial monolayers with VEGF leads to a loss of endothelial barrier function

EA.hy926 monolayers were grown to confluence and incubated with VEGF (0-5nM). Loss of endothelial barrier function was observed in the presence of VEGF at a concentration of 1nM. Higher concentrations of VEGF (5nM) did not appear to disrupt barrier function in vitro. Experiments were performed in duplicate and results are expressed as mean±SEM of at 5 independent experiments (One-Way ANOVA with Bonferroni’s multiple comparison test; *=p<0.05).
3.14 LMWH attenuates VEGF-induced endothelial permeability

In order to determine whether the effect of LMWH in attenuating agonist-induced endothelial permeability would also be observed in the presence of agonists other than thrombin, the ability of LMWH (2IU/mL) in attenuating VEGF-induced permeability of endothelial monolayers was also assessed as described in 2.9.

Pre-incubation of endothelial monolayers with tinzaparin led to a significant reduction in transmigration of Evans Blue-BSA through treated monolayers in comparison to that which was observed in monolayers which were not incubated with tinzaparin prior to exposure to VEGF, suggesting that the effect of LMWH in suppressing agonist-induced endothelial permeability is not specific to a single agonist (Figure 3.13).
Incubation of endothelial monolayers with tinzaparin (2IU/mL) prior to incubation with VEGF (1nM) leads to a significant attenuation of VEGF-induced endothelial barrier permeability. Experiments were performed in duplicate and results are expressed as the mean±SEM of 3 independent experiments (One-Way ANOVA with Bonferroni’s multiple comparison test; **=p<0.01, ***=p<0.001).
3.15 LMWH tinzaparin inhibits tumour cell trans-endothelial migration

In order to determine if the endothelial barrier protective activity of LMWH would be reflected in diminished tumour cell trans-endothelial migration, a modified endothelial barrier permeability assay was utilised where confluent EA.hy926 endothelial cell monolayers were incubated with a suspension of calcein blue AM-labelled DU145 metastatic prostate carcinoma cells and the degree of transmigration through the endothelial cell layer was then determined by measuring the change in fluorescence in the outer chamber of the trans-well plate (as outlined in 2.14 and as previously described(231)).

In the initial optimisation experiments, the relative contributions of the confluent endothelial monolayer and the trans-well insert (comprised of a PET membrane; 0.3cm² surface area, 8.0µM pore size) to the prevention of tumour cell migration were assessed by measuring the mean fluorescence intensity in cell culture media in wells where either the DU145 cell suspension was added directly into wells in the absence of an trans-well insert (i.e. 200µL of DU145 suspension added directly to 2mLs of cell culture media) or where the DU145 cell suspension was added to a trans-well insert containing a membrane which had not been seeded with EA.hy926 (i.e. no endothelial monolayer present and so the insert membrane represented the only obstacle to cell migration) (Figure 3.15).

In the control wells (endothelial monolayers grown on trans-well inserts cultured in phenol red-free DMEM) in the absence of any calcein blue AM-labelled DU145 cells, the mean baseline fluorescence intensity of the cell culture media was measured at 2447±258.2 arbitrary units. When this cell culture media was directly incubated with 200µL of the DU145 cell suspension, the mean fluorescence intensity was significantly increased at 7707±1800 arbitrary units (measured at 3 hours; p=<0.001). In the presence of the trans-well inserts containing the porous PET membrane (but lacking a confluent endothelial monolayer), the addition of 200µL of cell suspension to the inner chamber resulted in an increase in outer chamber fluorescence to 5946±27 arbitrary units from baseline at 3 hours, which was significantly greater than control fluorescence intensity (p=<0.05) but which appeared to be less than that observed when labelled tumour cells were directly incubated with outer chamber media (suggesting that the insert membrane itself represents a barrier to tumour cell migration, although this did not reach significance).

When the experiment was repeated using a trans-well insert containing a membrane on which an endothelial monolayer had been cultured, the fluorescence intensity in the
outer chamber at 3 hours was measured at 4494±91.7 arbitrary units, which was lower than that observed in the trans-wells containing an insert which lacked the endothelial monolayer, suggesting that the presence of the monolayer further attenuates tumour cell migration into the outer chamber.

Following a 3 hour incubation period with the DU145 carcinoma cell suspension, a significant increase in outer chamber fluorescence was observed as a consequence of migration of the carcinoma cells through the endothelial cell layer in comparison to that which was observed in trans-well plates incubated in the absence of the carcinoma cell suspension (fluorescence intensity 4753±139.9 vs 2732±45 arbitrary units, p=0.006) (Figure 3.15).

Confluent endothelial cell layers were then incubated with LMWH tinzaparin 0-2IU/mL for three hours prior to incubation with the carcinoma cell suspension and the degree of cellular transmigration was again determined. In order to compare results generated from independent experiments, the values obtained for fluorescence intensity in each trans-well were normalised using the following formula,(61), in order to express the fluorescence intensity measured in the LMWH treated trans-wells as a percentage of the fluorescence generated by tumour-cell transmigration at baseline (100%):

\[ F (\%) = \frac{(X-C)}{(Y-C)}*100 \]

Using this formula, F is fluorescence intensity (%), X is the LMWH treated fluorescence intensity, C is the baseline fluorescence intensity measured in trans-wells in the absence of the fluorescence-labelled carcinoma cell suspension (control) and Y is the fluorescence intensity measured in the trans-wells in the presence of the carcinoma cell suspension.

A significant reduction in carcinoma cell trans-endothelial migration was observed through endothelial monolayers which had been treated with tinzaparin. In the presence of tinzaparin concentrations of 0.1IU/mL, 0.5IU/mL and 2IU/mL, tumour cell trans-endothelial migration was attenuated to 67.5±5.9% (p<0.01), 65.1±8% (p<0.001) and 73.9±5.7% (p<0.05) of baseline respectively (Figure 3.16).
Figure 3.14 Schematic representation of the tumour cell trans-endothelial migration assay

Monolayers of confluent endothelial cells grown on trans-well insert membranes and incubated with a calcein blue-AM labelled DU145 carcinoma cell suspension and the degree of migration of these cells was assessed by measuring the change in fluorescence intensity in the outer chamber of the trans-well as a result of migration of the DU145 cells through the endothelial monolayer and the underlying membrane.
Figure 3.15 The endothelial cell monolayer attenuates tumour cell migration

Tumour cell trans-endothelial migration was assessed using a dual-chamber trans-well model where the fluorescence intensity of cell culture media was measured in the presence or absence of calcein blue AM-labelled DU145 metastatic carcinoma cells which had either been directly incubated with the cell culture media in the trans-well outer chamber or added to cell culture media of a trans-well insert, the base of which comprised of a porous membrane which was either bare or upon which an EA.hy926 monolayer had been grown. At three hours following incubation, the cell culture media in the outer chamber was sampled and the fluorescence intensity of the sampled media was determined. The presence of DU145 cells was associated with a significant increase in fluorescence, however this was less marked in the wells where the DU145 cells were incubated in the inner chamber of the trans-well (both in the presence and absence of the endothelial monolayer) suggesting that both the insert membrane and the monolayer present obstacles to tumour cell migration into the outer chamber of the plate in this experimental model of tumour cell migration. Experiments were performed in duplicate. (n=3; *=p<0.05, **=p<0.001; One-way ANOVA with Bonferroni multiple comparisons test).
Figure 3.16 LMWH tinzaparin attenuates tumour cell trans-endothelial migration

Endothelial cell monolayers were incubated with LMWH tinzaparin (0-2IU/mL) prior to incubation with a suspension of calcein blue AM-labelled DU145 metastatic prostate carcinoma cells (100x10^4 cells/mL). Mean fluorescence intensity in the outer chamber of the trans-well plates was significantly lower where endothelial cell layers had been incubated with LMWH prior to incubation with the cell suspension as a consequence of reduced migration of the labelled carcinoma cells through the endothelial monolayers. Experiments were performed in duplicate and results are expressed as the mean±SEM of 4 independent experiments (One-Way ANOVA with Bonferroni's multiple comparison test; *=p<0.05; **=p<0.01; ***=p<0.001).
3.16 Summary of key findings

Tumour-cell induced vascular permeability is a hallmark of cancer metastasis and may represent a potential therapeutic target(191, 192).

The findings described in this chapter support the hypothesis that LMWH enhances endothelial barrier function. Moreover, these results suggest that LMWH attenuates the abnormal endothelial permeability which is induced by cancer-associated agonists and inhibits tumour cell trans-endothelial migration.

Thrombin, a procoagulant serine protease which is generated during the process of blood coagulation (and which is upregulated in cancer), is a potent physiological inducer of vascular permeability(104, 106, 194). It mediates its effect on endothelial barrier function by proteolytic cleavage and activation of the endothelial cell PAR-1 receptor(50). LMWH was found to oppose thrombin-induced, PAR-1 mediated endothelial permeability, however the effects of LMWH in attenuating thrombin-induced PAR-1 signalling did not appear to be dependent on inhibition of thrombin-induced PAR-1 activation as evidenced by the similar pattern of thrombin-induced attenuation of ATAP2 fluorescence (as determined by flow cytometry) which was seen in the presence and absence of LMWH (with ATAP2 binding being dependent on a specific motif on the extracellular PAR-1 domain which is internalised following thrombin-mediated receptor activation). This finding suggests that the cytoprotective activity of LMWH is not linked to its anticoagulant activity (which is mediated, indirectly, through the inhibition of thrombin proteolytic activity. Moreover, the endothelial-barrier protective effects of LMWH were also observed in the setting of endothelial permeability induced by VEGF, another potent agonist associated with vascular permeability and cancer progression. Interestingly, the effect of VEGF in inducing endothelial permeability appeared to be reduced at higher concentrations in our experiments. The precise mechanisms underlying VEGF-induced endothelial permeability are not fully understood and other investigators have also previously demonstrated the opposing cell signalling effects of VEGF on endothelial function when applied at different concentrations (232). The mechanism underlying our observations are unknown, although VEGF is thought to influence the activity of various independent signalling pathways which are implicated in endothelial barrier function regulation and perhaps at certain concentrations signalling events are initiated which promote barrier stability in vitro and oppose the permeability-inducing signalling which is mediated through the better characterised VEGF-mediated endothelial signalling pathways(194), however the precise mechanism remains to be determined.
Collectively, these *in vitro* findings suggest that LMWH-mediated suppression of metastasis may arise as a result of its inhibitory effect on agonist-induced endothelial barrier dysfunction and tumour cell trans-endothelial migration. Crucially, the effect of LMWH on endothelial barrier function does not appear to be dependent on the same mechanism of action underlying its anticoagulant activity.

The risk of haemorrhage associated with the anticoagulant properties of LMWH currently precludes its use in the setting of cancer except for the treatment or prevention of thrombosis. Therapeutic strategies which would permit the use of LMWH while minimising the associated haemorrhage risk would likely be of enormous clinical relevance.

These findings must be interpreted in the context of several limiting factors. Firstly, these experiments were carried out using an immortalised endothelial cell line and while this cell line is widely used in the investigation of vascular function in vitro, the replication of these results in experiments using primary endothelial cells would have enhanced the translational relevance of the results. Secondly, these observations were not confirmed using other methods for the assessment of endothelial barrier function, such as trans-endothelial electrical resistance models, which if utilised would have also allowed us to monitor the effects of various compounds in modulating barrier function over more prolonged periods. Thirdly, while the LMWH-mediated effect in inhibiting tumour cell trans-endothelial migration would likely be of significant clinical relevance in replicated in vivo, our in vitro assay (although also utilised by other investigators(231)) is also somewhat limited by its failure to reflect certain specific factors which would be predicted to influence cell migration in vivo (such as the presence of a basement membrane) and, in addition, in our model it was not possible to account for the loss of migrating cells which may have become adhered to the artificial membranes underlying the endothelial monolayers (although this limitation would be predicted to have had a similar effect in influencing cell migration in both the control and LMWH-treated monolayers).
CHAPTER 4: OPTIMISATION OF THE RELATIVE ANTICOAGULANT AND CYTOPROTECTIVE PROPERTIES OF LOW MOLECULAR WEIGHT HEPARIN

4.1 Introduction

The anticoagulant activity of LMWH is mediated through its ability to interact with antithrombin, a plasma glycoprotein which acts as one of the primary physiological inhibitors of blood coagulation(46). In its native conformation, antithrombin has limited capacity to bind to its primary targets, the procoagulant proteases thrombin and activated coagulation factor X (FXa). The binding of LMWH to antithrombin induces a conformational change in the antithrombin protein structure which exposes its reactive centre loop, facilitating a more efficient interaction with its targets. This leads to a marked acceleration in its anticoagulant activity. This interaction between heparin compounds and antithrombin is mediated through a specific pentasaccharide sequence which is present on a proportion of the longer heparin polysaccharide chains found in commercial heparin compounds (in unfractionated heparin only approximately one-third of the heparin polysaccharides contain the antithrombin-binding sequence)(134). Heparin chains which lack the pentasaccharide would not be predicted to exhibit anticoagulant activity.

The normal haemostatic response to tissue injury consists of the activation of procoagulant pathways leading ultimately to the generation of FXa, thrombin and the formation of a fibrin clot(11, 12). Given that all pharmacological agents which are currently used for the prevention and treatment of thrombosis ultimately suppress the generation of thrombin and fibrin formation, their use leads to an impairment of normal haemostasis and confers a risk of haemorrhage. The annual risk of major haemorrhage associated with the use of therapeutic anticoagulant therapy with agents such as LMWH in the general population has been estimated at 1-2%. However, the risk of haemorrhage specifically among patients with cancer receiving this therapy is likely to be in excess of the predicted risk in the general population in view of the multiple risk factors for bleeding which frequently arise in association with active cancer(68). Consequently, despite evidence to suggest that LMWH may confer a survival benefit in cancer, current clinical practice guidelines recommend against its use for this indication alone in view of the potential morbidity and mortality which could arise in the setting of major haemorrhage, which may be in excess of any potential survival benefit which could arise as a result of the LMWH-mediated anti-cancer effects(133, 143).
As outlined in chapter 3, the endothelial cytoprotective properties of LMWH do not appear to be mediated through its anticoagulant activity. LMWH compounds with diminished anticoagulant activity would therefore be predicted to retain cytoprotective function.

The statins are a widely-prescribed class of lipid-lowering drugs. *In vitro* data suggests that they may also mediate a protective effect on endothelial barrier function by inhibiting the production of a lipid metabolite which is crucial to the activity of the RhoGTPase signalling pathway, one of the key signalling pathways regulating actin cytoskeleton dynamics. The evidence to date suggests that this effect is observed at statin concentrations which are in excess of the predicted plasma concentrations which would be encountered in clinical practice (216, 217).

The following experiments were undertaken with the aim of addressing the hypothesis that the anticoagulant activity of LMWH can be decoupled from its cytoprotective activity in a manner which would permit exposing patients with cancer to the potential anti-metastatic properties of LMWH without conferring a haemorrhage risk. Specifically, these experiments aimed to investigate the anticoagulant and cytoprotective properties of a LMWH fraction comprising of primarily shorter heparin polysaccharide chains (which would be predicted to exhibit diminished anticoagulant activity) and to characterise the potential additive effects on endothelial barrier function when standard LMWH at non-anticoagulant concentrations is combined with a statin at a clinically relevant concentration.
4.2 Plasma thrombin generation in pooled normal plasma

Thrombin generation was assessed in pooled normal plasma (80µL; PNP) by calibrated automated thrombography (CAT), firstly in the absence of either exogenous phospholipid (PL) or tissue factor (TF) and then in the presence of either PL alone (4µM) or PL and TF (1-5pM) in combination, as described in 2.24. Lagtime to initiation of thrombin generation, time to reaching peak thrombin generation, peak thrombin, thrombin generation velocity index and endogenous thrombin potential were determined. Previous investigators have demonstrated evidence of correlations between various thrombin generation parameters and thrombotic or haemorrhagic events, with the bulk of this evidence to date demonstrating clinical correlations with the parameters of peak thrombin and endogenous thrombin potential(79, 233, 234).

As expected, in PNP lacking in endogenous PL (as a result of removal of platelets by centrifugation) and in the absence of an exogenous TF stimulus, no measurable thrombin generation was detected (Figure 4.1). Following supplementation of plasma with exogenous PL (MP reagent, 4µM PL), plasma thrombin generation was initiated with peak thrombin generation reaching 26.4±7.7nM (Figure 4.2 A) and with the area under the thrombin generation curve (endogenous thrombin potential; ETP) measured at 393.4±112nM*min (Figure 4.2 B). Lag time to initiation of thrombin generation and time to achieving peak thrombin generation in the presence of PL were measured at 25.3±2.7 mins (Figure 4.2 C) and 35.6±2.6 mins (Figure 4.2 D) respectively.

TF is the primary trigger of plasma thrombin generation in vivo. When PNP was incubated with exogenous TF (PPP reagent, 1-5pM TF) in addition to PL, thrombin generation was significantly enhanced. At 1pM TF, peak thrombin generation rose to 93.2±1.9nM (p<0.05) (Figure 4.2 A), ETP was measured at 1279±65.4nM*min (Figure 4.2 B) and both lag time to thrombin generation and time to achieving peak thrombin generation were shortened relative to that observed in the presence of PL alone (8.3±0.3mins, p<0.05; and 15±0.3mins, p<0.001; respectively) (Figure 4.2 C-D). In the presence of a 5pM TF trigger plasma thrombin generation was further enhanced, with peak thrombin generation reaching 276±18.2nM (p<0.01), ETP was measured at 2453±102.4nM*min (p<0.01) and lag time to initiation of thrombin generation and time to peak thrombin generation were further shortened to 3.2±0.2 mins and 7.1±0.7 mins respectively (p<0.01) (Figure 4.2 A-D).
Figure 4.1: Thrombin generation in pooled normal plasma.

Thrombin generation in pooled normal plasma was determined by CAT in recalcified pooled normal platelet poor plasma (80μL) in the absence of phospholipid and tissue factor (yellow), in the presence of phospholipid vesicles (4μM; green) and in the presence of tissue factor (1pM; blue, 5pm; red) in addition to phospholipid vesicles. Thrombin generation was assessed by comparing the rate of hydrolysis of a thrombin-specific fluorogenic substrate to a thrombin standard. Experiments were performed in triplicate.
Figure 4.2: Parameters of thrombin generation in pooled normal platelet-poor plasma following incubation with phospholipid vesicles and tissue factor. Plasma thrombin generation was assessed by CAT. The peak thrombin generation (A), ETP (B), lagtime to initiation of thrombin generation (C) and time to peak thrombin generation (D) in plasma were determined using Thrombinscope® software. Thrombin generation was enhanced in the presence of PL (4µM) and increasing concentrations of TF (1-5pM). Experiments were performed in triplicate and results are expressed as mean±SEM of 3 independent experiments (One-Way ANOVA with Bonferroni’s Multiple comparison test;*=p<0.05, **=p<0.01).
4.3 LMWH tinzaparin suppresses thrombin generation in pooled normal plasma

LMWH mediates its anticoagulant effect by enhancing the anticoagulant activity of antithrombin (a plasma glycoprotein which binds to and inhibits the proteolytic activity of thrombin and FXa) and would be predicted to suppress plasma thrombin generation in vitro.

In order to characterise LMWH tinzaparin-mediated suppression of thrombin generation, pooled normal plasma was supplemented with tinzaparin at a range of clinically relevant concentrations (0-0.5IU/mL; in vivo peak plasma tinzaparin concentrations between approximately 0.2-1IU/mL would be predicted to be achieved in the setting of standard LMWH dosing strategies in humans).

In the presence of a 5pM TF stimulus, a concentration-dependent suppression of plasma thrombin generation was observed in plasma incubated with tinzaparin. At a concentration of 0.5IU/mL (within the target plasma range for thrombosis therapy in humans) a profound attenuation of thrombin generation was observed with peak thrombin generation just reaching 4.2±0.7nM from a baseline of 308.2±20nM (p<0.001) (Figure 4.4 A), representing a tinzaparin-mediated attenuation of peak plasma thrombin generation of 98.6%. Similarly, 0.5IU/mL tinzaparin suppressed ETP to 110.5±17.1nM*min from a baseline of 2229±136nM*min (p<0.001) (Figure 4.4 B), representing an attenuation of ETP of approximately 95%. Baseline lagtime to thrombin generation (2.9±0.2 mins) and time to peak thrombin generation (6.2±0.6 mins) were prolonged to 7.1±1.1 mins (p<0.05) and 23.7±1.7 mins (p<0.01) respectively (Figure 4.4 C-D).

Suppression of peak plasma thrombin generation and ETP was also observed (although to a lesser degree) with plasma tinzaparin concentrations which would be considered to be below the target range for effective anticoagulant therapy in vivo and which would not be predicted to confer a significant bleeding risk. At 0.1IU/mL of tinzaparin, peak thrombin generation was suppressed to 125.9±16.2nM from a baseline of 308.2±20nM (p<0.001) and ETP was suppressed to 1290±73.5nM*min from the baseline ETP level of 2229±136nM*min (p<0.001) (Figure 4.4 A-B).
Figure 4.3: LMWH Tinzaparin-mediated suppression of thrombin generation in pooled normal plasma.

Pooled normal plasma was incubated with TF (5pM) & PL vesicles (4µM) and incubated with a range of concentrations of tinzaparin (0IU/mL, red; 0.1IU/mL, green; 0.25IU/mL, blue; 0.5IU/mL, yellow). Plasma thrombin generation was assessed by CAT. A progressive suppression of thrombin generation was observed with increasing plasma concentrations of tinzaparin. Experiments were performed in triplicate.
Figure 4.4: Parameters of plasma thrombin generation in the presence of LMWH tinzaparin.

Parameters of plasma thrombin generation were determined by CAT using Thrombinscope software® (5pM TF stimulus). Peak thrombin generation (A), ETP (B), lagtime to initiation of thrombin generation (C) and time to peak thrombin generation (D) were determined in plasma incubated with tinzaparin (0-0.5IU/mL). A progressive suppression of ETP & peak thrombin generation and a progressive prolongation of lagtime & time to peak thrombin generation were observed with increasing tinzaparin concentrations. Experiments were performed in triplicate and results expressed as mean±SEM of 3 independent experiments (One-way ANOVA with Bonferroni’s multiple comparison test; *=p<0.05, **=p<0.01, ***=p<0.001).
4.4 A 2.8KDa tinzaparin-derived LMWH fraction suppresses plasma thrombin generation to a lesser extent than that observed with equivalent plasma concentrations of standard tinzaparin

The interaction between LMWH and antithrombin which accelerates the anticoagulant activity of antithrombin is dependent on a specific antithrombin-binding pentasaccharide sequence which is found in a proportion of heparin polysaccharide chains (less than one-third of heparin polysaccharides in standard commercial LMWH preparations contain the antithrombin-binding pentasaccharide)\(^{(46, 134)}\). Given that this pentasaccharide sequence is generally restricted to the longer heparin chains, LMWH preparations comprising primarily of shorter polysaccharides would be predicted to exhibit diminished anticoagulant activity.

In order to characterise the anticoagulant activity of a LMWH preparation comprising primarily of shorter heparin polysaccharides, thrombin generation was measured in pooled normal plasma incubated with a range of concentrations of a LMWH fraction derived from standard tinzaparin but consisting of heparin polysaccharide chains with a mean molecular weight of 2.8KDa (standard commercial tinzaparin consists of a heterogenous mixture of heparin polysaccharide chains with a mean molecular weight of 6.5KDa). This fraction was provided to us by LEO Pharma, the manufacturers of tinzaparin, and was extracted from commercial grade tinzaparin during the manufacture process. The concentration range of the 2.8KDa fraction used in this experiment (0-5µg/mL) is approximately equivalent to plasma tinzaparin concentrations in the range of 0-0.5IU/mL (commercial tinzaparin, dosed in IU of anti-Xa activity, exhibits anti-Xa activity of approximately 100IU per milligram, therefore a 20 micrograms/mL solution of the 2.8KDa fraction would be predicted to contain approximately the same concentration of LMWH as a 2IU/mL tinzaparin solution; commercial grade tinzaparin is not manufactured in units of measurement other than IU/mL of anti-Xa activity).

The 2.8KDa LMWH fraction attenuated plasma thrombin generation (Figure 4.5) but to a lesser extent than that observed when plasma was incubated with the equivalent concentrations of tinzaparin (Figure 4.3). At a 2.8KDa LMWH plasma concentration of 5µg/mL (equivalent to 0.5IU/mL tinzaparin), mean ETP was only attenuated from 2184±65.4nM*min to 1532±123nM*min (p<0.01) (Figure 4.6 A) and mean peak plasma thrombin generation was only suppressed from 273.8±43.7nM to 211±27.4nM (Figure 4.6 B). Lagtime to initiation of thrombin generation and time to peak thrombin generation were similar across the range of LMWH concentrations (Figure 4.6 C-D).
Figure 4.5: A 2.8KDa LMWH Tinzaparin fraction attenuates thrombin generation in pooled normal plasma.

Pooled normal plasma was incubated with a 2.8KDa LMWH fraction (0µg/mL, red; 1µg/mL, green; 2.5µg/mL, blue; 5µg/mL, yellow) and plasma thrombin generation was determined by CAT as previously described following incubation with TF (5pM). Increasing plasma concentrations of the LMWH fraction were associated with a progressive suppression of thrombin generation but the effect was less marked than that observed with equivalent concentrations of standard tinzaparin. All experiments were performed in triplicate.
A

B

C

D

2.8KDa LMWH (μg/mL)

2.8KDa LMWH (μg/mL)

2.8KDa LMWH (μg/mL)

2.8KDa LMWH (μg/mL)
Figure 4.6: Parameters of plasma thrombin generation in pooled normal plasma spiked with a 2.8KDa fraction of LMWH tinzaparin.

Parameters of plasma thrombin generation in pooled normal plasma were determined by CAT (5pM TF stimulus). ETP (A) & peak thrombin generation (B) were suppressed in the presence of increasing concentrations of the LMWH fraction (0-20µg/mL) but not to the extent observed with equivalent concentrations of standard tinzaparin (0-2IU/mL). Lagtime to initiation of thrombin generation (C) & time to peak thrombin generation (D) were largely unaffected. Experiments were performed in triplicate and results are expressed as mean±SEM of 3 independent experiments (One-Way ANOVA with Bonferroni’s multiple comparison test; *=p<0.05).
4.5 A 2.8KDa Tinzaparin-derived LMWH fraction exhibits diminished plasma anti-factor Xa activity

Chromogenic assays of plasma anti-factor Xa activity are widely used in clinical practice for the assessment of the intensity of anticoagulation achieved following administration of LMWH. Moreover, target plasma ranges for both preventative and therapeutic dosing of LMWH have been established using this assay(133, 235).

Tinzaparin exhibits anti-factor Xa activity of approximately 100IU of anti-Xa per milligram. In order to determine the relative anti-factor Xa activity exhibited by concentrations of the 2.8KDa LMWH fraction in comparison to equivalent tinzaparin concentration, PNP was incubated with the 2.8KDa fraction at a concentration range of 0-20µg/mL (approximately equivalent to 0-2IU/mL of tinzaparin) and the resulting plasma anti-FXa activity was determined as described in 2.23.

Increasing plasma anti-FXa activity levels were observed with increasing plasma concentrations of the 2.8KDa fraction, however the levels achieved were substantially lower than the anti-Xa activity levels achieved with equivalent plasma concentrations of standard tinzaparin (at a plasma concentration of 5µg/mL, the fraction exhibited 0.18±0.01 IU/mL of anti-FXa activity, a value which would not be expected to confer a significant bleeding risk) (Figure 4.7).
Figure 4.7: A 2.8KDa LMWH fraction derived from tinzaparin exhibits diminished plasma anti-factor Xa activity relative to that observed with standard tinzaparin.

PNP was incubated with standard tinzaparin (0-2IU/mL; A) or a 2.8KDa tinzaparin-derived fraction (0-20µg/mL; B) and plasma anti-factor Xa activity was determined using the ACL TOP® 500 haematology analyser and the HemosIL® liquid anti-Xa reagent. Experiments were performed in triplicate. Results are expressed as mean±SEM of 3 independent experiments.
4.6 Incubation of endothelial cell monolayers with the 2.8KDa LMWH fraction does not enhance baseline endothelial barrier function

Incubation of endothelial cell monolayers with standard LMWH tinzaparin improves baseline endothelial barrier function (Figure 3.5) in addition to attenuating thrombin and VEGF induced endothelial permeability (Figures 3.6; 3.14). In order to determine if similar endothelial barrier protective properties were exhibited by the 2.8KDa LMWH fraction, EA.hy926 monolayers were cultured on trans-well inserts as described in 2.1 and at 72 hours the endothelial cell monolayers were incubated with the 2.8KDa LMWH fraction (20µg/mL) in serum-free DMEM at 37°C for a further three hours. Assessment of endothelial barrier function was then completed as described in 2.7.

At 40 minutes no significant improvement in endothelial barrier function was observed. The mean optical density at 650nm as a result of transmigration of Evans Blue-BSA through the LMWH-treated monolayers was measured at 0.29±0.4 in comparison to the untreated monolayers where the optical density was measured at 0.27±0.5 (p=0.63), suggesting that the 2.8KDa fraction does not support baseline endothelial barrier function.
Figure 4.8: The 2.8KDa LMWH tinzaparin-derived fraction does not enhance baseline endothelial barrier function.

EA.hy926 cell monolayers were incubated with the 2.8KDa LMWH fraction (20µg/mL) and endothelial barrier function was assessed at 40 minutes by assessing the degree of trans-endothelial migration of an Evans blue-BSA solution through the treated monolayers over time. No significant difference in Evans Blue-BSA migration was observed in LMWH-treated cell monolayers in comparison to untreated monolayers. Experiments were performed in triplicate. Results are expressed as mean±SEM of 3 independent experiments.
4.7 A 2.8KDa LMWH fraction attenuates thrombin-induced endothelial permeability

Confluent EA.hy926 cell layers were cultured as described in 2.8 and incubated in serum-free DMEM with the 2.8KDa LMWH fraction for three hours at 37°C. The endothelial cell layers were then incubated with thrombin (1nM) and endothelial permeability was measured as described in 2.2.

Increasing concentrations of the 2.8KDa LMWH fraction appeared to exert an inhibitory effect on thrombin-mediated endothelial barrier permeability with a statistically significant attenuation of endothelial barrier dysfunction observed at a concentration of 20µg/mL (approximately equivalent to a standard tinzaparin concentration of 2IU/mL) (Figure 4.9). At this concentration, the 2.8KDa LMWH fraction attenuated thrombin-induced endothelial permeability to 64±9% of baseline (p<0.05).

The degree of attenuation of thrombin-mediated endothelial permeability observed with the 2.8KDa LMWH fraction was less marked than that observed with equivalent concentrations of standard tinzaparin, however the in vitro anticoagulant activity of the 2.8KDa LMWH fraction (and consequently the predicted associated haemorrhage risk) was also diminished relative to the anticoagulant activity of the standard tinzaparin preparation (Figures 4.3-7), suggesting that the endothelial barrier protective properties of LMWH are retained by LMWH preparations with limited anticoagulant activity.
Figure 4.9: A 2.8KDa tinzaparin-derived LMWH fraction attenuates thrombin-induced endothelial permeability.

Incubation of EA.hy926 monolayers with increasing concentrations of a 2.8KDa LMWH fraction (0-20µg/mL) prior to treatment with thrombin (1nM) leads to an attenuation of thrombin-induced endothelial barrier permeability. Experiments were performed in duplicate and results represent the mean±SEM of 6 independent experiments (One-Way ANOVA with Bonferroni’s multiple comparison test; *=p<0.05).
4.8 The 2.8KDa LMWH tinzaparin fraction attenuates tumour cell trans-endothelial migration

Confluent endothelial cell monolayers were incubated with the 2.8KDa tinzaparin fraction (0-20µg/mL) prior to incubation with a suspension of calcein blue AM-labelled DU145 prostate carcinoma cells and the degree of transmigration of the carcinoma cells through the endothelial cell layer was determined as described in 2.14.

A significant reduction in tumour cell transmigration was observed following pre-incubation of endothelial cell monolayers with the LMWH fraction, with a reduction in mean fluorescence intensity in the trans-well outer chamber to 76.4±4.7% and 63.9±3% of baseline fluorescence intensity in the presence of 5µg/mL and 20µg/mL of the 2.8KDa fraction respectively (p<0.001; Figure 4.10)
Figure 4.10 A 2.8KDa LMWH tinzaparin fraction attenuates tumour cell trans-endothelial migration

EA.hy926 endothelial cell monolayers were pre-treated with 0-20µg/mL of a 2.8KDa LMWH tinzaparin fraction prior to incubation with 200µL of a calcein blue AM-labelled DU145 carcinoma cell suspension (100x10⁴ cells/mL). DU145 cell migration through the endothelial monolayer was attenuated in monolayers which had been pre-incubated with the LMWH fraction as demonstrated by the reduction in fluorescence intensity in the outer chamber of the trans-well as a consequence of diminished migration of the labelled carcinoma cells. Experiments were performed in duplicate and the results are expressed as the mean±SEM of 5 independent experiments (One way ANOVA with Bonferroni multiple comparisons test, ***=p<0.001).
4.9 Simvastatin attenuates thrombin-induced endothelial barrier permeability

Statins, a class of commonly prescribed lipid-lowering drugs, have been previously shown to support endothelial barrier function.

Simvastatin mediated attenuation of thrombin-induced endothelial permeability was assessed as described in 2.17 using a range of concentrations of simvastatin which would be predicted to be equivalent to peak plasma simvastatin concentrations achieved in the setting of recommended statin dosing strategies in clinical practice (oral administration of 10-40mg simvastatin would be predicted to achieve peak plasma simvastatin levels of 5-20nM).

In the presence of increasing concentrations of simvastatin (0-20nM), a progressive attenuation of thrombin-mediated permeability was observed, with permeability attenuated to 48.67±12.5% of baseline thrombin-induced permeability following incubation of EA.hy926 monolayers with a 20nM concentration of simvastatin (p<0.05) (Figure 4.11).
Figure 4.11 Simvastatin attenuates thrombin-induced endothelial barrier permeability

Monolayers of EA.hy926 endothelial cells were incubated with simvastatin (0-20nM) for 24 hours prior to incubation with thrombin (1nM). An attenuation of thrombin-induced monolayer permeability was observed with increasing concentrations of simvastatin. Experiments were performed in duplicate and results are expressed as the mean±SEM of at least 3 independent experiments (One-way ANOVA with Bonferroni multiple comparisons test; *=p<0.05).
4.10 Co-incubation of EA.hy926 cells with simvastatin potentiates the endothelial barrier protective properties of LMWH tinzaparin

The cytoprotective activity of LMWH tinzaparin in opposing thrombin-induced endothelial barrier dysfunction is most marked at LMWH concentrations which, if replicated in vivo in plasma, would be predicted to confer a significant risk of haemorrhage. Given that simvastatin (at clinically relevant concentrations) also exerts a similar but less pronounced effect on thrombin-induced endothelial permeability, the effect of incubation of endothelial monolayers with a lower concentration of tinzaparin than would be predicted to confer a bleeding risk following incubation with simvastatin was investigated in order to determine if an additive effect existed which would permit the use of lower LMWH concentrations without losing the marked cytoprotective effect observed with LMWH concentrations in the anticoagulant range.

As outlined in 2.18, endothelial monolayers were incubated with simvastatin (0-20nM) for 24 hours prior to incubation with tinzaparin at a concentration of 0.1IU/mL, which does exert an anticoagulant effect in vitro (Figures 4.3, 4.4, 4.7) but which is below the target plasma range for anticoagulant therapy and which would not be predicted to confer a bleeding risk.

In the presence of increasing concentrations of simvastatin, the endothelial barrier protective effect of tinzaparin was greatly enhanced. In endothelial monolayers treated with 20nM simvastatin, incubation with tinzaparin (0.1IU/mL) led to an attenuation of thrombin-induced endothelial permeability to 7.9±2% (p<0.05) (Figure 4.11 A). The magnitude of the effect observed with this co-incubation strategy is greater than that observed with the use of simvastatin or tinzaparin in isolation Figure 3.6; 4.9).

A similar additive effect, although less marked, was observed when EA.hy926 monolayers were incubated with tinzaparin at a concentration range of 0-0.5IU/mL following a 24 hour incubation period with a fixed simvastatin concentration (5nM) (Figure 4.11 B).
A

Simvastatin (nM) vs. Thrombin-Induced permeability (%)

B

Tinzaparin (IU/mL) vs. % Thrombin-induced permeability
Figure 4.12 The endothelial barrier protective properties of LMWH tinzaparin are enhanced following pre-treatment of endothelial cells with simvastatin

EA.hy926 monolayers were incubated with simvastatin (0-20nM) for 24 hours prior to incubation with LMWH tinzaparin (0.1IU/mL; □) for a further 3 hours. Tinzaparin-mediated protection against thrombin-induced (1nM) endothelial permeability was more pronounced where cells received pre-treatment with increasing concentrations of simvastatin (A). Endothelial monolayers were also incubated with a fixed concentration of simvastatin (5nM; ∆) prior to incubation with a range of tinzaparin concentrations (0-0.5IU/mL) (B). Experiments were performed at least in triplicate and results represent the mean±SEM of 3 independent experiments (*=p<0.05, **=p<0.01; One-way ANOVA with Bonferroni multiple comparisons test).
4.11 Simvastatin enhances the endothelial barrier protective properties of the 2.8KDa LMWH fraction

The effect of incubation of endothelial cells with simvastatin prior to treatment with the 2.8KDa LMWH fraction was also investigated. Following a 24 hour incubation period with 0-20nM simvastatin, endothelial monolayers were incubated with the 2.8KDa fraction at a concentration of 5µg/mL (which exerts a minimal in vitro anticoagulant effect) (Figure 4.5-7) but which also exhibits limited endothelial barrier protective activity when used in isolation (Figure 4.9).

Pre-treatment of endothelial monolayers with simvastatin potentiated the barrier protective effect of the 2.8KDa fraction. At a simvastatin concentration of 20nM, the 2.8KDa fraction at a concentration of 5µg/mL attenuated thrombin-induced permeability to 29±11.1% (p<0.05) in contrast to treatment with the LMWH fraction alone which only suppressed thrombin-induced barrier permeability to 75.66±15% (Figure 4.12).
Figure 4.13 Simvastatin enhances the endothelial barrier protective properties of the 2.8KDa LMWH fraction

EA.hy926 monolayers were incubated with simvastatin (0-20nM) for 24 hours prior to incubation with the 2.8KDa LMWH fraction (5µg/mL; ○). The LMWH-mediated attenuation of thrombin-induced (1nM) endothelial permeability was enhanced in the presence of increasing concentrations of simvastatin. Experiments were performed at least in triplicate and results represent the mean±SEM of 3 independent experiments (*=p<0.05; One-way ANOVA with Bonferroni multiple comparisons test).
4.12 Summary of key findings

Therapeutic strategies which would permit the exposure of patients with cancer to LMWH without conferring a bleeding risk would be predicted to be of enormous potential clinical benefit in view of the observation that LMWH appears to mediate an anti-metastatic effect which is independent of its anticoagulant activity.

The findings described in this chapter suggest that LMWH fractions with diminished *in vitro* anticoagulant activity retain endothelial-barrier protective properties, although to a lesser extent than that observed with standard commercial LMWH preparations.

Simvastatin was also found to exert a limited protective effect on endothelial barrier function at concentrations which would be predicted to be in the range of peak plasma concentrations achieved in clinical practice. Remarkably, co-incubation of endothelial cells with LMWH at sub-anticoagulant concentrations and simvastatin at a clinically relevant concentration was found to lead to a marked suppression of agonist-induced endothelial permeability.

Collectively, these findings suggest that the risk of haemorrhage associated with LMWH use may be abrogated through strategies which include either the use of fractions of LMWH composed of largely non-anticoagulant heparin polysaccharides or the use of sub-anticoagulant concentrations of LMWH in combination with other endothelial barrier protective agents. Crucially, these strategies can be implemented in the *in vitro* setting without the loss of the beneficial cytoprotective activity associated with LMWH use, a finding which is likely to be of significant translational relevance if replicated *in vivo*.

The interpretation of these findings is limited by the lack of in vivo evidence to confirm a potential additive effect when combining a statin and LMWH, although future in vivo work using a murine model of human metastasis is planned. Similarly, the translational relevance of these results would have been enhanced if replicated using a primary endothelial cell line. While our experiments focused on the effects of simvastatin, it is plausible that other statins would elicit similar endothelial barrier protective effects however we did not specifically investigate if the cytoprotective properties of simvastatin reflected a class effect. In addition, we investigated the endothelial barrier protective properties of statins in the context of agonist-induced endothelial barrier permeability alone and did not assess the effects of statins on basal permeability.
CHAPTER 5: TO CHARACTERIZE PLASMA THROMBIN GENERATION IN EISENMENGER SYNDROME AND TO EXPLORE THE EFFECT OF DUAL ENDOTHELIN-1 RECEPTOR ANTAGONIST THERAPY ON DERANGEMENTS OF BLOOD COAGULATION AMONG AFFECTED INDIVIDUALS

5.1 Introduction

Eisenmenger syndrome (ES) is an extremely rare but devastating complication of congenital cardiac disease with pulmonary hypertension. It is characterised by irreversible, progressive pulmonary vascular disease arising as a result of the presence of a systemic-to-pulmonary shunt through a congenital cardiac defect. In ES, the pulmonary vascular disease progresses to the point that pulmonary vascular resistance matches or exceeds the pressures in the systemic circulation leading to the reversal of blood flow through the cardiac defect and the return of deoxygenated blood to the systemic circulation (26, 30, 109, 119).

ES has an estimated prevalence of just three to six per million adults in the developed world (although the prevalence is likely to be higher in the developing world where access to pre-emptive surgical treatment in childhood is limited) (30). Currently, in countries such as the Republic of Ireland, ES most frequently arises among individuals born with Trisomy 21 as this high-risk population would previously have been considered to be poor candidates for any potentially preventative surgical intervention. However, notwithstanding the current scarcity of ES in clinical practice, the complications associated with the onset of the syndrome present substantial clinical dilemmas and therapies which specifically address these complications are urgently needed (26, 30, 119).

The onset of ES is associated with a marked reduction in quality of life and shortened survival, with progressive cardiac failure and sudden cardiac death representing the most frequently reported causes of mortality within this group. Unlike other forms of pulmonary hypertension, ES is characterised by a significant burden of multi-system morbidity, with an increased thrombotic risk considered a hallmark of this disorder. Interestingly, a risk of major haemorrhage has also been reported within this group (and also represents a leading cause of early mortality), however the molecular mechanism underlying the competing thrombotic and haemorrhagic risks remain poorly understood and have represented a source of significant scientific and clinical interest (29, 111, 117, 119, 123, 131).
The aims of the experiments presented in the following chapter were to explore potential mechanisms underlying the thrombotic and haemorrhagic risks in ES and, moreover, to investigate if novel targeted therapies which lack a direct anticoagulant activity might ameliorate these derangements of haemostasis. We hypothesised that the molecular mechanism underlying the thrombotic and haemorrhagic risks were likely to be linked to the underlying vascular pathology and that consequently therapies which target these signalling pathways may indirectly normalise the observed derangements in coagulation. Given the breadth of evidence to date implicating the role of platelets in both the thrombotic and haemorrhagic tendencies in this disorder (27, 28, 115, 118, 128), we aimed to specifically investigate the activity of platelets in supporting both abnormal coagulation activation and also in impairing normal haemostasis in ES.
5.2 Recruitment of subjects

In collaboration with the national adult congenital cardiac disease service of the Republic of Ireland (led by Dr Kevin Walsh) 15 individuals with previously untreated Eisenmenger syndrome were identified during the period February to November 2015. Of these 15 individuals, 9 met eligibility criteria for recruitment and were invited to participate. In parallel, 9 healthy volunteers matched for age and gender were also recruited. Sampling of whole blood from each individual subject with ES was performed at the same time as that of their matched volunteer and paired samples were then processed and analysed simultaneously as outlined in 2.17 in order to minimise the impact of pre-analytical variables on the data generated from the subsequent experiments.

As a result of the erythrocytosis present among the ES subjects, the volume of PRP obtained by centrifugation was limited (relative to healthy volunteers) and consequently plasma thrombin generation experiments aimed at characterising TF-stimulated coagulation activation (the primary physiological procoagulant pathway) were prioritised.

The demographic profiles of the matched ES subjects and healthy volunteers, as well as their baseline haematological parameters are described in table 1. Two male and 7 female subjects were recruited to both groups. The mean age in the ES subject group was 33.2±2.6 years and 33±2.7 years in the healthy volunteer group (p=0.95).

Predictably the mean haemoglobin levels and mean haematocrit levels in the ES group was significantly higher than that observed among the healthy volunteers, reflecting the marked secondary erythrocytosis which frequently arises in the context of progressive congenital cardiac disease with pulmonary hypertension. The mean platelet count in whole blood samples was significantly lower among subjects with ES (123±13 vs 249±18.2 x10^9/L; p<0.001) and a significantly increased mean platelet volume was also observed within this cohort (8.5±0.5 vs 7.2±0.3fL; p=0.04), findings which are suggestive of increased platelet activation and consumption(236-239). In order to maximise the volume of PRP available for thrombin generation analysis, platelet counts were not determined in PRP samples obtained from all of the matched pairs of ES subjects and their controls, however in the 4 matched pairs where PRP platelet counts were determined, the PRP counts were lower among the ES subjects relative to the healthy volunteers (133.5±65.6 vs 259.3±68 x10^9/L). While PRP platelet counts are usually adjusted to specific pre-defined levels in assays such as platelet aggregation studies, previous studies have demonstrated that the absolute platelet count across a
range of approximately 100 to 400x10^9/L does not significantly affect the parameters of thrombin generation in PRP and therefore PRP platelet counts were not adjusted by dilution with autologous PPP for the purposes of these experiments (240).

None of the subjects in either group was receiving treatment with an anticoagulant agent at the time of recruitment however one subject in the ES group was receiving anti-platelet therapy (aspirin 75mgs daily). Each of the 9 ES subjects had developed ES in the context of a ventricular septal defect or atrio-ventricular septal defect and all but one had been born with Trisomy 21.
### Table 5.1 Demographic profile of matched ES subjects and healthy volunteers

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<th>Healthy Volunteers (n=9)</th>
<th>p value</th>
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<td>33±2.7</td>
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<tr>
<td><strong>Gender</strong></td>
<td>2:7</td>
<td>2:7</td>
<td></td>
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<tr>
<td><strong>Haemoglobin (g/dL)</strong></td>
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<td><strong>Haematocrit</strong></td>
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<td>0.49</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Platelet count (x10^9/L)</strong></td>
<td>123±13</td>
<td>249±18.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Platelet volume (fL)</strong></td>
<td>8.5±0.5</td>
<td>7.2±0.3</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Data expressed as mean±SEM
5.3 TF-independent thrombin generation in platelet-rich plasma is enhanced in Eisenmenger syndrome

The normal haemostatic response to tissue injury consists of the activation of procoagulant pathways leading to the generation of a fibrin clot, the purpose of which is to seal vascular defects in order to prevent bleeding. The primary activator of physiological procoagulant pathways involved in the generation of the fibrin clot is tissue factor (TF), a glycoprotein which is normally restricted to the sub-endothelium but when exposed to the circulating blood following injury initiates a series of reactions resulting ultimately in the generation of thrombin and the cleavage of soluble fibrinogen to insoluble fibrin.

In order to assess blood coagulation activation occurring in the absence of exogenous TF, plasma thrombin generation was assessed in recalcified platelet-rich plasma obtained from subjects with ES (and their matched healthy volunteers).

Thrombin generation was found to be significantly accelerated in PRP obtained from subjects with ES relative to that observed among the matched healthy volunteer with shorter mean lagtime to the onset of thrombin generation (15.5±1.9 vs 25.7±2.6 mins, p=0.007; Figure 5.1A), shorter time to peak thrombin generation (20.7±2.2 mins vs 32.4±2.3 mins, p=0.005; Figure 5.1B) and a higher thrombin generation velocity index present among the ES cases (38.3±5.8 vs 20.6±4, p=0.03; Figure 5.1C). Peak thrombin generation was also greater in PRP obtained from ES subjects relative to that observed among healthy volunteers (185.8±19.4 vs 127.4±20.8nM; p=0.07, Figure 5.1D), as was the mean ETP (1843±258 vs 1488±247.5nM*min; p=0.37, Figure 5.1E) however these differences did not reach statistical significance.
Figure 5.1 Plasma thrombin generation is significantly accelerated in platelet-rich plasma obtained from subjects with Eisenmenger syndrome despite the absence of exogenous tissue factor.

Plasma thrombin generation was assessed by CAT in recalcified PRP obtained from subjects with ES (n=7) and matched healthy volunteers (n=7), without the addition of exogenous TF to initiate coagulation activation. Thrombin generation was significantly accelerated among the ES subjects relative to the healthy volunteers with a significantly shortened mean lagtime to thrombin generation (A) and time to peak thrombin generation (B) and significantly greater thrombin generation velocity index (C) observed. Higher mean peak thrombin levels (D) and mean ETP (E) were also detected in the ES PRP. All experiments were performed in duplicate and data are expressed as mean±SEM (n=9, **=p<0.01; *=p<0.05).
5.4 TF-stimulated plasma thrombin generation in platelet-rich plasma is significantly enhanced in Eisenmenger syndrome

TF-initiated blood coagulation activation is the primary procoagulant pathway in vivo. Under physiological conditions this pathway is only activated in response to vascular injury, with the exposure of sub-endothelial TF to the circulating blood. In order to assess TF-dependent plasma thrombin generation in ES, PRP was incubated with an exogenous source of TF (which is absent from whole blood and plasma under physiological conditions) and plasma thrombin generation was then assessed by CAT as described previously.

Plasma thrombin generation was again significantly accelerated among the ES subjects (n=9) when compared with the healthy volunteers (n=9), with a shorter mean lagtime to thrombin generation (6.1±0.3 vs 7.8±0.5mins, p=0.005; Figure 5.2A), shorter time to peak thrombin generation (11.5±0.5 vs 15.2±0.6mins, p=0.0003; Figure 5.2B) and greater thrombin generation velocity index (43.9±3.7nM/min, p=0.001; Figure 5.2C) observed. In addition, the mean peak thrombin generation reached was also significantly greater in the ES group (230.6±10.9 vs 188.3±13.1nM, p=0.025; Figure 5.2D). TF-dependent mean ETP was found to be similar in both groups (2317±133.9 vs 2319±167.2nM*min, p=0.88; Figure 5.2E).
Figure 5.2 TF-stimulated plasma thrombin generation in platelet-rich plasma is enhanced in Eisenmenger syndrome

Plasma thrombin generation was assessed by CAT in recalcified PRP from subjects with ES (n=9) and healthy volunteers (n=9) which had been incubated with exogenous TF (1pM) as previously described. Lagtime to thrombin generation (A) and time to peak thrombin generation (B) were significantly shorter in the ES group and thrombin generation velocity index and peak thrombin generation were also significantly enhanced (C&D). ETP was similar in both groups. All experiments were performed in duplicate and data are expressed as mean±SEM (n=9, ***=p<0.001; **=p<0.01; *=p<0.05).
5.5 The enhanced plasma thrombin generation observed in PRP obtained from subjects with ES is not mediated through enhanced activity of the contact pathway of coagulation activation.

The contact pathway of coagulation activation consists of the coagulation factors XI and XII (FXI and FXII), high molecular weight kinogen and pre-kallikrein. The contact pathway does not play a significant role in physiological blood coagulation however it can become activated in vitro, often at the time of blood sampling or when blood comes into contact with artificial surfaces such as plastic tubing, a phenomenon which can affect the accuracy of data generated by in vitro assays of blood coagulation. Moreover, recent data suggests that pathological activation of the contact pathway may occur in vivo in the context of specific disease states (18, 20).

In order to determine whether in vitro or pathological activation of the contact pathway contributes to the differences in plasma thrombin generation observed between subjects with ES and matched healthy volunteers, CAT experiments were repeated using PRP extracted from whole blood which had been collected into tubes supplemented with CTI, an inhibitor of FXII activation. As a consequence of the relatively low yield of PRP from whole blood (particularly in ES) and the volume of plasma required to conduct all the PRP thrombin generation experiments, sufficient PRP to conduct these additional experiments was only obtained from 6 of the ES subjects and 4 matched healthy volunteers.

Notwithstanding the smaller sample size, significant differences in parameters of plasma thrombin generation were still observed between the ES group and controls despite contact pathway inhibition, with a shorter mean lagtime to thrombin generation (6.4±0.3 vs 9.9±2mins, p=0.026; Figure 5.3A) and time to peak thrombin generation detected (13.9±0.3 vs 19.5±2.2mins, p=0.01; Figure 5.3B). Higher peak thrombin generation (163.9±13.8 vs 134±15.9nM; Figure 5.3C), mean ETP (2075±142.5 vs 1835±110.6nM*min; Figure 5.3D) and thrombin generation velocity index (22±2.3 vs 14.8±3.2nM/min; Figure 5.3E) were also observed although these differences did not reach statistical significance.
Figure 5.3. The enhanced plasma thrombin generation observed in platelet-rich plasma in ES is not mediated through increased activation of the contact pathway of blood coagulation activation.

Plasma thrombin generation was assessed by CAT in PRP collected into CTI (final concentration 50µg/mL) from patients with ES and matched healthy controls and initiated with exogenous TF (1pM). Due to limitations in sample size and yield of PRP from whole blood samples, CTI-supplemented PRP was only available from 6 ES subjects and 4 healthy volunteers, a factor which limits the interpretation of the results. However, lagtime to thrombin generation (A) and time to peak thrombin generation (B) were significantly accelerated while peak thrombin (C), ETP (D) and thrombin generation velocity index (E) also appeared to be enhanced despite contact pathway inhibition. All experiments were performed in duplicate and data are expressed as mean±SEM (*=p<0.05).
5.6 Enhanced plasma thrombin generation in Eisenmenger syndrome is platelet-dependent

In order to determine the relative contribution of platelet activity to patterns of plasma thrombin generation in ES relative to healthy volunteers, CAT experiments were repeated in platelet-poor plasma, where exogenous anionic phospholipid (final concentration 4µM) was incubated with plasma as a substitute for platelet membrane phospholipid (a key component of the procoagulant extrinsic tenase, intrinsic tenase and prothrombinase enzyme complexes).

In contrast to that which was observed in the experiments exploring plasma thrombin generation in PRP, no significant differences in any of the thrombin generation parameters were seen between the ES subjects and their matched healthy volunteers in PPP.

In the recalcified PPP in the absence of an exogenous TF stimulus, lagtime to thrombin generation (15.2±1.7 vs 15.1±3.3mins; Figure 5.4A), time to peak thrombin (19.2±1.8 vs 19.1±3.3mins; Figure 5.4B), peak thrombin (246.6±32.9 vs 231.7±36.2nM; Figure 5.4C), ETP (1963±168 vs 1789±164nM*min; Figure 5.4D) and thrombin generation velocity index (65.9±13.3 vs 60.9±12.5nM/min; Figure 5.4E) were similar between both groups with no significant differences observed.

Similarly, TF-activated thrombin generation in PPP was also similar among the ES cohort and the matched volunteers with no significant differences in lagtime to thrombin generation (6.3±0.2 vs 6.7±0.4mins; Figure 5.5A), time to peak thrombin generation (11.2±0.3 vs 11.7±0.7mins; Figure 5.5B), peak thrombin (235.6±17.6 vs 203.3±23.4nM; Figure 5.5C), ETP (2126±114.1 vs 1867±154.7nM*min; Figure 5.5D) and thrombin generation velocity index (50.3±6.1 vs 44±8nM/min; Figure 5.5E) detected.

Following inhibition of the contact pathway of coagulation activation, similar patterns were observed, with no significant differences detected in thrombin generation parameters between the ES subjects and controls (lagtime: 6.8±0.4 vs 7.2±0.7mins; time to peak thrombin: 14.3±1.2 vs 13.5±1; peak thrombin: 143.2±34.8 vs 156.2±17.4nM; ETP: 1817±124 vs 1976±80.2; thrombin generation velocity index: 23.7±10.4 vs 20.6±5.7nM/min; Figure 5.6 A-E).
Figure 5.4 Plasma thrombin generation in platelet-poor plasma in the absence of exogenous tissue factor is not enhanced in Eisenmenger syndrome.

Plasma thrombin generation was assessed by CAT in PPP in the absence of exogenous TF but which had been incubated with exogenous PL (4µM) as a substitute for platelet phospholipid. No significant difference in lagtime to thrombin generation (A), time to peak thrombin generation (B), peak thrombin (C), ETP (D) or thrombin generation velocity index (E) were observed. All experiments were conducted in duplicate. Data are expressed as mean±SEM (n=4).
Figure 5.5 TF-stimulated plasma thrombin generation in platelet-poor plasma is not enhanced in Eisenmenger syndrome

Plasma thrombin generation was assessed by CAT in PPP in the presence of exogenous TF (1pM) and exogenous anionic PL (4µM). No significant difference in lagtime to thrombin generation (A), time to peak thrombin generation (B), peak thrombin (C), ETP (D) or thrombin generation velocity index (E) were observed. All experiments were conducted in duplicate. Data are expressed as mean±SEM (n=9).
Figure 5.6 Plasma thrombin generation in platelet-poor plasma is not enhanced in Eisenmenger syndrome relative to healthy volunteers following inhibition of the contact pathway of coagulation activation.

Plasma thrombin generation in the presence of exogenous TF (1pM) and PL (4µM) was assessed by CAT in PPP obtained from samples of whole blood collected into citrated tubes which had been additionally supplemented with CTI (final concentration, 50µg/mL). No significant difference in lagtime to thrombin generation (A), time to peak thrombin generation (B), peak thrombin (C), ETP (D) or thrombin generation velocity index (E) were observed. All experiments were conducted in duplicate. Data are expressed as mean±SEM.
5.7 The anticoagulant activity of activated protein C is diminished in healthy volunteer platelet-rich plasma in the absence of exogenous anionic phospholipid.

The anticoagulant activity of APC is partially dependent on the presence of anionic PL, which provides a negatively charged surface for the assembly of the complex of APC, its co-factors and its target substrates (in a similar manner to which the procoagulant enzyme complexes, such as the extrinsic tenase, require a negatively charged surface for their assembly). Activated platelet membranes are an important source of anionic PL in this context(5).

In the CAT thrombin generation assay, where exogenous APC is incubated with PPP in the presence of exogenous anionic PL, there is a marked concentration-dependent suppression of plasma thrombin generation observed, with peak thrombin suppressed to 15.7±3.4% (p<0.0001) and 5±1.3% (p<0.0001) of baseline in the presence of APC 2.5nM and 5nM respectively (Figure 5.7A). Conversely, when APC is incubated with PRP (where no exogenous PL is added as the subjects own platelets provide a source of PL) the anticoagulant activity of APC was greatly diminished with peak thrombin generation only suppressed to 89±14% and 67.1±3.8% of baseline in the presence of 2.5 and 5nM of APC respectively. However when PRP obtained from the same subjects was incubated with PL prior to incubation with APC, the anticoagulant activity of APC was enhanced (although not to the same extent as that observed in PPP supplemented with exogenous PL), with a reduction in peak thrombin generation to 46.1±17.9% in the presence of 2.5nM APC and to 4.7±0.1% in the presence of 5nM APC (which represented a significant reduction in baseline peak thrombin relative to that which was observed prior to the addition of exogenous PL (p=0.003) (Figure 5.7B).
Figure 5.7 The anticoagulant activity of activated protein C is diminished in healthy volunteer platelet-rich plasma in the absence of exogenous anionic phospholipid.

TF-stimulated (1pM) plasma thrombin generation was assessed by CAT in PPP and PRP obtained from healthy subjects in the presence or absence of PL (4µM) and APC (2.5-5nM). Observed data were normalised such that peak thrombin generation in the absence of APC were defined as 100%. In PPP in the presence of APC there was a marked suppression of thrombin generation as predicted (A). Conversely, in PRP (B) without exogenous PL, there was limited APC-mediated suppression of thrombin generation observed (○), however APC anticoagulant activity was partially restored following the addition of exogenous PL (∆). All experiments were performed in duplicate and the data represent the mean±SEM of 3 independent experiments (**=p<0.01).
5.8 The anticoagulant activity of APC is abnormally enhanced in Eisenmenger syndrome and is platelet-dependent

The anticoagulant activity of the APC pathway in ES was assessed by CAT, where samples of PRP were incubated with APC (2.5-5nM) prior to initiation of thrombin generation with TF.

Remarkably, the effect of APC in suppressing plasma thrombin generation was markedly enhanced in PRP obtained from subjects with ES relative to that which was observed among the matched healthy volunteers, with residual peak plasma thrombin generation (Figure 5.8A; 2.5nM APC: 46.8±5.3 vs 89.2±4.5%, p<0.001; 5nM APC: 28.2±4.9 vs 77±6.5%, p<0.001) and residual ETP (Figure 5.8B; 2.5 nM APC: 51.26±6.3 vs 81.1±3.2%, p=0.007; 5nM APC: 32.1±5 vs 63.7±9.7%, p=0.01) found to be significantly lower in the ES cohort.

To address the hypothesis that enhanced APC sensitivity in ES PRP is platelet-dependent, and not mediated by differences in the phospholipid composition of extracellular vesicles in plasma, platelets were removed by centrifugation and thrombin generation stimulated with PRP reagent (containing TF but no exogenous PL). Upon removal of platelets, the effect of APC on peak thrombin generated and ETP in platelet-poor plasma was similar following incubation of plasma with 2.5nM APC (normalised peak thrombin, 58.4±8.5 vs 78.3±10.7%, p=0.2; normalised ETP, 72.2±10 vs 70±6.7%, p=0.8) and with the higher APC concentration of 5nM (normalised peak thrombin generation, 37.1±6.2 vs 40.2±10.6%, p=0.8; normalised ETP, 44.9±6.9 vs 37.2±10%, p=0.7) (Figure 5.8C-D). These findings suggest that in ES, platelets support abnormally enhanced sensitivity to the anticoagulant activity of APC. Due to constraints on available PRP, the relative contributions of platelet phospholipid exposure and other platelet components to this phenomenon could not be determined.
Figure 5.8 APC sensitivity in PRP is enhanced in ES relative to healthy volunteers and is platelet-dependent

TF-stimulated (1pM) plasma thrombin generation was assessed by CAT in PRP and PPP obtained from ES subjects (●) and healthy volunteers (□) in the presence of APC (2.5-5nM). Observed data were normalised such that peak thrombin generation in the absence of APC were defined as 100%. In PRP, sensitivity to the anticoagulant activity of APC was enhanced in ES relative to that observed in the healthy volunteers with a marked suppression of peak thrombin generation (A) and ETP observed (B). Following removal of platelets by centrifugation, and in the absence of exogenous PL, sensitivity to the APC was similar between the two groups (C-D). All experiments were performed in duplicate and the data expressed as the mean±SEM (n=6; ***=p<0.001; **=p<0.01).
5.9 Platelet-mediated enhanced plasma thrombin generation in ES is attenuated following dual endothelin-1 receptor antagonist therapy

Of the 9 individuals with ES who were initially recruited to provide samples of blood for analysis at baseline, 7 were available to provide a second sample following completion of 6 months of therapy with macitentan (a novel dual endothelin-1 receptor antagonist). Three of the healthy volunteers also provided a second sample of blood for analysis following the 6 month interval in order to ensure reproducibility of the PRP thrombin generation results.

In the healthy volunteer PRP, the pattern of plasma thrombin generation in the individual subjects was similar at 6 months to that which had been observed at baseline (Figure 5.9 A-C).

Among the subjects with ES, a significant reduction in mean peak thrombin generation was observed following 6 months of therapy (234.5±9.8 vs 204.6±14.1nM, p=0.002; Figure 5.10 A). In addition, ETP was also lower at 6 months than had been observed at baseline (2426±143.7 vs 2283±260.3nM*min; Figure 5.10 B), lagtime to thrombin generation was more prolonged (6.3±0.3 vs 6.8±0.4mins; Figure 5.10 C), time to peak thrombin generation was more prolonged (11.8±0.5 vs 13±1.1mins; Figure 5.10 D) and thrombin generation velocity index was reduced (43.6±3.3 vs 35.4±3.9nM/min; Figure 5.10 E) although these differences did not achieve statistical significance.

In PPP, no significant difference in any parameter of plasma thrombin generation was observed following 6 months of macitentan therapy (lagtime to thrombin generation: 6.3±0.2 vs 6.1±0.2mins; time to peak thrombin generation: 10.8±0.3 vs 10.2±0.3mins; thrombin generation velocity index: 56.9±7 vs 70±7nM/min; peak thrombin generation: 251.3±20.6 vs 277.6±18.2nM; ETP: 2098±144.4 vs 2156±126.9nM*min; Figure 5.11).
Figure 5.9 Plasma thrombin generation in PRP obtained from healthy volunteers was similar when re-assessed following a 6 month interval.

Measurement of plasma thrombin generation (1pM TF) in PRP by CAT was repeated in three of the healthy volunteers, in parallel with the ES subjects, following a 6 month interval. The thrombin generation curves generated in the individual subjects at baseline (red) was found to be similar to that which was observed at 6 months (blue). All experiments were conducted in duplicate.
Figure 5.10 Plasma thrombin generation in PRP is attenuated in ES following therapy with macitentan.

Plasma thrombin generation in ES PRP was re-assessed by CAT following 6 months of macitentan therapy. Peak thrombin generation (1pM TF) was significantly lower at 6 months (A; △, n=7) than had been observed at baseline (●, n=7) although remained greater than that which was observed among the matched healthy volunteers at baseline (□). ETP was also lower (B) and lagtime to thrombin generation (C), time to peak thrombin generation (D) & thrombin generation velocity index (E) were more prolonged than observed at baseline although these differences did not reach statistical significance. (**=p<0.01). Experiments were performed in duplicate.
Figure 5.11 No significant difference in any parameter of plasma thrombin generation in PPP was observed in ES following macitentan therapy

Plasma thrombin generation in ES PPP was re-assessed by CAT following 6 months of macitentan therapy. Peak plasma thrombin generation (A), ETP (B), lagtime to thrombin generation (C), time to peak thrombin generation (D) and thrombin generation velocity index (E) were not significantly different at 6 months to that which was observed at baseline. All experiments were conducted in duplicate.
5.10 Platelet-mediated enhanced APC sensitivity in ES is attenuated following dual endothelin-1 receptor antagonist therapy

The anticoagulant activity of APC in ES PRP and PPP was re-assessed by CAT following completion of 6 months of macitentan therapy, at which point the abnormal enhanced sensitivity to APC which was observed in ES PRP at baseline was found to be partially reversed. Under the same experimental conditions as utilised at baseline, the residual peak thrombin generation in the presence of 2.5nM APC was significantly higher at 6 months than had been observed at baseline (81.5±4.7 vs 54.2±3.4%, p=0.03; Figure 5.12 A). Residual mean ETP in the presence of 2.5nM APC was also higher following macitentan therapy (87.9±6.8 vs 61.1±6.6%; Figure 5.12 B) and, similarly, a reduction in the abnormal enhanced APC sensitivity was also observed in the presence of 5nM APC (residual peak thrombin generation: 46.9±16.3 vs 32.4±6.4%; residual ETP: 57.1±14.5 vs 37.5±6.2%), although these differences did not achieve statistical significance (Figure 5.12 A-B).

In PPP, where the addition of exogenous PL to plasma as a substitute for endogenous platelet membrane phospholipid supports the activity of endogenous procoagulant and anticoagulant pathway activity, patterns of sensitivity to APC were similar at baseline and at 6 months following macitentan therapy (Figure 5.12 C-D).
A

B

C

D
The anticoagulant activity of APC (2.5-5nM) was re-assessed by CAT (1pM TF) following 6 months of macitentan therapy in ES PRP (A-B) and PPP (C-D). Observed data were normalised such that peak thrombin generation in the absence of APC were defined as 100%. In PRP, the normalised mean peak thrombin generation was significantly higher following macitentan therapy (□), than had been observed at baseline (●) in the presence of 2.5nM APC (A) suggesting reduced APC sensitivity. The patterns of APC sensitivity in PPP following macitentan therapy were largely unchanged. All experiments were performed in duplicate and data are described as the mean±SEM (n=5; *=p<0.05).
5.11 Summary of results

The novel data presented in this chapter suggest that derangements in platelet activity in ES modulate the activity of procoagulant and anticoagulant pathways and therapies which target specific pathways implicated in the pathogenesis of this disease attenuate these platelet-mediated derangements in a manner which would not be predicted to confer a risk of bleeding.

Platelets mediate their haemostatic activity at the site of tissue injury through the formation of platelet aggregates (primary haemostasis) and, following initial activation, by the expression of specific anionic PL to support the procoagulant activity of the extrinsic tenase, intrinsic tenase and prothrombinase enzyme complexes, leading ultimately to the generation of thrombin and a fibrin clot(3). In our experiments, enhanced plasma thrombin generation was observed in platelet-rich plasma in the absence of an exogenous TF stimulus (the primary activator of physiological blood coagulation), in the presence of exogenous TF and in the presence of CTI, an inhibitor of contact pathway coagulation activation among ES subjects. However, plasma thrombin generation was not observed to be enhanced in platelet-poor plasma, where platelet membrane phospholipid is substituted with exogenous anionic PL, suggesting that the enhanced blood coagulation activation which arises in ES is platelet-dependent and is likely to be directly linked to patterns of platelet membrane expression of anionic PL.

The APC pathway is one of the key physiological anticoagulant pathways. It acts by limiting the extent of thrombin generation through the cleavage and inactivation of the activated coagulation factors V and VIII (which are integral components of the prothrombinase and intrinsic tenase complexes respectively)(5). Resistance to the anticoagulant activity of APC is associated with an increased risk of thrombosis(241). However, previous investigators have demonstrated evidence of platelet-mediated APC resistance in healthy individuals and have postulated that in the platelet-rich environment which arises at the site of vessel injury that platelets attenuate APC sensitivity in order to ensure sufficient thrombin generation occurs in order to promote clot formation to seal vascular defects(129, 242, 243). Similarly, in the CAT PRP experiments described in this chapter, we demonstrated evidence of platelet-mediated APC resistance among our healthy volunteers. Interestingly, this APC resistant phenotype in PRP was not observed among the subjects with ES. Following removal of platelets and in the absence of exogenous PL, plasma thrombin generation in the presence of APC was similar in the ES subjects and the healthy volunteers, suggesting
that the observed differences in APC sensitivity are platelet-dependent and may be related to patterns of platelet PL exposure. Whether this disparity in platelet-modulated APC sensitivity between ES subjects and healthy volunteers in vitro is representative of APC activity in vivo at the site of vascular injury is unknown, however, increased APC anticoagulant activity at the site of injury would be predicted to impair haemostasis. Therefore the observations presented in this chapter may represent a possible mechanism through which derangements in platelet activity in ES could confer a risk of both thrombosis and impaired haemostasis concurrently, reflecting the clinical phenotype observed in this disorder.

Enhanced endothelin-1 activity within the pulmonary vasculature has been implicated in the pathogenesis of pulmonary hypertension and ES. Recently, dual endothelin-1 receptor antagonism has been shown to improve survival in pulmonary hypertension and its efficacy is currently being investigated in the setting of ES specifically (204, 209). Platelets express endothelin-1 receptors and while the effects of endothelin-1 receptor antagonism on platelet function are unknown, the endothelin receptor antagonists have not been shown to mediate any direct anticoagulant activity or antiplatelet activity (27, 28).

Given that platelet activation is thought to occur in the abnormal pulmonary vasculature in ES, we hypothesised that endothelin-1 receptor antagonism might ameliorate the derangements of haemostasis which arise in ES by indirectly attenuating the ES-associated platelet activity. The results presented in this chapter demonstrate that following a 6 month period of treatment with macitentan, a novel dual endothelin-1 receptor antagonist, that significant improvements in both the abnormal hypercoagulability and the abnormally enhanced APC sensitivity in PRP occur among ES subjects.

Although limited by the small sample size, these results suggest that the haemostatic derangements occurring in ES are platelet-mediated and that specific therapies targeted against the underlying vascular pathology may represent a novel approach to the attenuation of these haemostatic derangements despite lacking a direct anticoagulant mode of action. These findings are also somewhat limited by the fact that almost all the ES cases had a co-existing diagnosis of trisomy 21, a potential confounding factor. Currently, the majority of cases of ES arise in this population and so it would be very difficult to recruit a large cohort of ES subjects without this comorbidity. Including an additional control group comprising of individuals with trisomy 21 without a diagnosis of congenital cardiac disease could be helpful in this regard,
however this was outside the scope of the approval granted to us to conduct this study by the research ethics committee of our institution.
CHAPTER 6: DISCUSSION OF RESULTS AND FUTURE WORK

6.1 Endothelial barrier protective properties of LMWH: a novel potential tool in the prevention of cancer metastasis?

6.1.1 LMWH supports endothelial barrier function and attenuates agonist-induced endothelial permeability

The barrier function of vascular endothelium plays a key role in maintaining normal homeostasis and in regulating the passage of various solutes and cellular elements from the circulation and into the adjacent tissues. Under physiological conditions, the barrier formed by contiguous endothelial cells (and particularly by the inter-endothelial junctions formed between these cells) prevents the spontaneous movement of most circulating molecules from the intra-vascular space(49, 194).

Disassembly of inter-endothelial cell junctions resulting in increased endothelial barrier permeability can arise under certain physiological circumstances, such as in the setting of inflammatory responses where pro-inflammatory mediators (such as bradykinin, histamine etc.) promote vascular permeability in order to facilitate immune cell migration into the adjacent tissues. However, dysregulated endothelial barrier function is also a feature of several pathological states, with abnormally increased endothelial permeability (and the resulting organ and tissue dysfunction) considered a hallmark of processes such as sepsis, acute lung injury and anaphylaxis(49, 194).

In addition to their anticoagulant activity, heparins (particularly LMWH) have been reported to influence the activity of various biological processes and, in particular, have been shown to mediate anti-inflammatory and anti-cancer effects(136, 180, 187, 188, 244). Moreover, heparins have previously been reported to attenuate abnormal vascular permeability in animal models of sepsis and acute lung injury(199, 201).

As described in chapter 3, we found that the LMWH tinzaparin supported the barrier function of endothelium in vitro, as demonstrated by the diminished trans-migration of an albumin solution through a tinzaparin-treated endothelial cell monolayer relative to that which was observed in untreated endothelial cell layers. Moreover, the endothelial barrier protective effect of tinzaparin was also observed in the context of agonist-
induced endothelial permeability, a key mechanism underlying abnormal vascular permeability in pathological states. Crucially, this cytoprotective effect was observed within a clinically relevant tinzaparin concentration range (0.25-2IU/mL). In addition, this effect was observed in the setting of both VEGF and thrombin-induced permeability, suggesting that the protective effect of LMWH in the setting of agonist-induced endothelial permeability is not limited to the effects of a single agonist.

While all LMWH formulations are derived from the same parent compound (UFH), the various formulations differ in terms of their method of manufacture and, as a consequence, they exhibit significant structural heterogeneity. While all commercial LMWH formulations appear to exhibit equivalent anticoagulant efficacy, several investigators have demonstrated that significant differences exist between LMWH formulations in terms of their non-anticoagulant functions(134, 137, 179, 245). In the experiments described in chapter 3, we demonstrated that endothelial barrier protection appears to be a feature of several different LMWH preparations, suggesting that LMWH-mediated endothelial barrier protective activity may be a class effect.
6.1.2 LMWH-mediated attenuation of tumour cell trans-endothelial migration: a potential mechanism underlying the anti-metastatic activity of LMWH

The migration of circulating tumour cells through vascular endothelium, a process which is supported by tumour-induced vascular permeability, is a key ‘rate-limiting’ step in the process of cancer metastasis. Moreover, inhibition of tumour-induced vascular permeability has been shown to attenuate tumour dissemination in vivo(191, 192).

As demonstrated by the experiments described in 3.15, LMWH attenuates tumour cell trans-endothelial migration in vitro at concentrations within a clinically relevant range. In these experiments, metastatic prostate carcinoma cells were found to migrate through endothelial monolayers over time in the absence of any additional endothelial permeability-inducing agonist. However, following pre-treatment of the endothelial monolayer with tinzaparin, migration of these cells was significantly diminished.

To date the majority of data generated by experiments investigating the potential anti-metastatic properties of LMWH have been derived from experiments exploring the effects of LMWH in suppressing tumour angiogenesis(24, 81, 176-179, 182, 246). Angiogenesis is a key step in tumour growth and proliferation and synthetic anti-angiogenic agents are widely used in the treatment of various solid tumours(171, 247-250). LMWH has been shown to modulate angiogenesis however we hypothesise that the effect of LMWH in suppressing metastasis is mediated through the ability of LMWH to inhibit tumour cell trans-endothelial migration, a hypothesis which would contend that LMWH can only exert a survival benefit in vivo if administered prior to the extravasation of tumour cells from the circulation (and the seeding of micro-metastatic deposits at secondary sites). Consequently, LMWH would not be anticipated to be effective in individuals with advanced disease or with established metastases, as at this stage of disease significant tumour cell trans-endothelial migration would be predicted to have already occurred. This hypothesis is supported by the results of clinical trials to date which demonstrate a survival advantage in cancer patients with early disease stage.

Tumour cells can be detected in the systemic circulation of patients with cancer, including patients without clinically detectable metastatic tumours and among patients who are eventually cured (although the burden of circulating tumour cells increases with stage), suggesting that the key determinants of whether an incipient metastatic cell within the primary tumour bulk will succeed in establishing a metastatic focus are the events which occur after the tumour cell has already reached the systemic circulation(251-254). The primary events which occur in the metastatic process at this stage are the adhesion and extravasation of tumour cells through the vascular
endothelium at the target site and the formation of a micro-metastatic deposit in the foreign micro-environment of the target tissue with the subsequent proliferation of a metastatic tumour (which is dependent on effective angiogenesis)(141, 171). In animal models of metastasis, tumour cells are either directly injected into the systemic circulation or implanted into subcutaneous tissues(187, 223, 255). In experiments where the anti-cancer effects of LMWH have been explored, the first exposure to LMWH has generally either preceded or coincided with the inoculation of the animal with tumour cells(187, 223, 255). In this setting, LMWH is already present in the systemic circulation at the time of the first tumour cells reaching the vasculature of their target tissue. In these animal models, the administration of LMWH has been consistently shown to mediate an anti-metastatic effect(187, 223, 255).

In contrast to these pre-clinical animal models of metastasis, in the clinical studies many of the recruited patients had already developed metastatic tumours at the time of recruitment(39, 139). Moreover, circulating tumour cells and clinically undetectable micro-metastases would be predicted to be present among some of the recruited subjects, particularly those with more advanced disease, prior to commencing LMWH (despite the absence of clinically detectable metastatic tumours)(251, 252, 254, 256). Therefore, a key difference in the study of metastasis in clinical versus pre-clinical studies in the context of LMWH use is the phenomenon of micro-metastasis formation, an event which is not dependent on angiogenesis but which is dependent on vascular permeability to tumour cells(191, 197).

Reports from several studies including the FAMOUS trial and the post-hoc analysis of the CLOT trial specifically demonstrated a survival benefit associated with LMWH use which was only observed among patients with non-metastatic or less advanced disease(138, 139). The recent FRAGMATIC study in which the majority of subjects had advanced or metastatic disease at recruitment did not demonstrate a survival benefit associated with LMWH use(39). These results are in contrast to numerous studies of synthetic anti-angiogenic agents (such as bevacizumab) which have been consistently reported to prolong survival in metastatic cancers(248-250). Therefore, these observations do not support the hypothesis that the anti-cancer activity of LMWH is mediated through an inhibition of angiogenesis, as if this were the case, the anti-cancer effect of LMWH would not be expected to be limited to patients with early stage disease.

In contrast, some of the most striking results in terms of a LMWH-associated anti-cancer effect in humans were generated in the ABEL trial. This study limited
recruitment to subjects with very early stage lung tumours and reported very marked improvements in progression-free survival and overall survival (140). While the interpretation of the results of the ABEL trial is clearly limited by the small sample size relative to FRAGMATIC, it does highlight the key factor of disease stage in determining response to adjunctive LMWH therapy in cancer, a factor which would not alone be predicted to determine response if the underlying LMWH mechanism of action was solely the inhibition of angiogenesis.

In summary, tumour cell trans-endothelial migration is a step in the progression of metastasis which precedes micro-metastatic tumour formation and tumour angiogenesis in the metastatic target site. Agents which inhibit tumour-induced vascular permeability and trans-endothelial migration attenuate metastasis in vivo (191). However, among patients with cancer, such agents would need to be administered at an early stage of disease in order to be effective as the enhancement of endothelial barrier function would not affect the proliferation of tumour cells which have already breached the endothelial barrier and have begun to proliferate in the target tissue.

Given the existing evidence to support the hypothesis that LMWH mediates an anti-metastatic effect in vivo, the additional evidence we have reported in chapter 3 demonstrating that LMWH attenuates tumour cell trans-endothelial migration and the conflicting evidence reported in clinical trials (and in particular the lack of a consistent survival benefit in trials which have included patients with established metastatic disease), we hypothesise that the anti-metastatic effect of LMWH is mediated, at least in part, through the enhancement of endothelial barrier activity.
6.1.3 The LMWH-mediated endothelial cytoprotective effect is not mediated through the inhibition of agonist-induced receptor activation and appears to be independent of its anticoagulant activity

While the molecular mechanism underlying the anticoagulant activity of LMWH has been well characterised, the mechanisms underlying its additional biological functions have not been fully elucidated(46). LMWH has been shown to inhibit the pro-angiogenic signalling activity exhibited by various growth factors such as VEGF and FGF-2 by binding to them and preventing their interaction with their receptors at the surface of vascular cells(176, 182, 183). The LMWHs have also been reported to bind various adhesion molecules, such as the selectins, thereby inhibiting important adhesion interactions between cells expressing ligands for these adhesion molecules with platelets, leucocytes and endothelial cells(187, 188). The mechanism underlying the effect of LMWH on vascular barrier function has not been established although previous investigators have postulated that it may be mediated through an effect on the phosphorylation status of cytoskeletal regulatory proteins. These investigators demonstrated in vitro evidence to suggest that both heparin and a chemically-modified non-anticoagulant heparin compound appear to reduce thrombin-mediated myosin light chain phosphorylation and actin cytoskeleton re-arrangement in human endothelial cells and hypothesised that this effect arose through an interaction between the heparin compound and the thrombin receptors(257).

In chapter 3, we describe experimental results exploring the mechanism underlying the endothelial barrier protective effect of LMWH as described in 3.8-12. For the purposes of these experiments we focused on the effects of LMWH in inhibiting thrombin-induced endothelial permeability. Thrombin is a potent physiological inducer of vascular permeability and, in addition, is the key effector protease of the blood coagulation cascade(13, 50, 194). It also represents one of the key targets of the anticoagulant activity of LMWH, which is mediated through the plasma glycoprotein antithrombin, as outlined in 1.3.2.2. Thrombin mediates its procoagulant activity through the proteolytic cleavage and activation of the co-factors FVIII and FV, the serine protease FXI and through the cleavage of fibrinogen to fibrin, as outlined in 1.1.3. The cell signalling properties of thrombin on endothelial cells are mediated primarily through the PAR family of protease activated receptors(50). PAR receptors are G-protein coupled receptors possessing an extra-cellular domain which, when cleaved by thrombin or other proteases, releases a tethered ligand. Binding of the ligand to the main body of the receptor activates PAR-1 mediated intra-cellular signalling events. Under the experimental conditions as outlined in 2.11, we found that thrombin-induced endothelial
barrier permeability was entirely dependent on thrombin-mediated cleavage and activation of PAR-1, as evidenced by the complete inhibition of thrombin-induced endothelial permeability which was observed either in the presence of an antibody which prevented thrombin-induced proteolytic PAR-1 cleavage or in the presence of a PAR-1 signalling inhibitory peptide (3.9).

Endothelial cell expression of PAR-1 was not altered by incubation with LMWH. Moreover, LMWH did not impair thrombin-mediated proteolytic cleavage of PAR-1, as evidenced by flow cytometry using a fluorescence-labelled antibody directed against the PAR-1 intact cleavage site. This key finding suggests that LMWH-mediated inhibition of thrombin-induced endothelial permeability is not mediated through an inhibition of its proteolytic activity. Furthermore, in an assay where abnormal endothelial permeability was induced using a PAR-1 activating peptide (which activates PAR-1 signalling independent of receptor cleavage), LMWH was again found to support endothelial barrier function. These findings suggest that LMWH opposes the activity of thrombin-activated signalling pathways but in a manner which does not appear to be mediated through the inhibition of the procoagulant activity of thrombin.

Heparan sulphate proteoglycans are widely expressed on the cell surface and in the extra-cellular matrix of human tissues and play key roles in regulating various physiological and pathological processes including blood coagulation, inflammation, angiogenesis and metastasis(137, 144, 258). Commercial heparins are structurally similar to these endogenous HSPGs and interactions between heparin molecules and HSPG or HSPG ligands is thought to represent one of the mechanisms through which heparins influence cell signalling activity(180, 181). In 3.12, we demonstrated that while HSPG exhibit co-factor activity in promoting thrombin-induced endothelial permeability (as previously described(258)), cleavage of HSPG from the endothelial cell surface did not influence the degree of LMWH-mediated endothelial barrier protection in the presence of thrombin, suggesting that the mechanism of LMWH cytoprotective function in the context of endothelial barrier activity is not mediated through an inhibition of agonist interaction with HSPG.

Interestingly, heparins have previously been shown to directly exhibit signalling activity on a variety of tissue types and recently a putative endothelial cell heparin receptor has been identified(244, 259, 260). In these experiments reported by Farwell et al, anti-inflammatory properties of UFH in endothelial cells were studied and were found to be dependent on the presence of a specific transmembrane protein present on the endothelial cell surface (TMEM184A). Among other findings, the interaction between
UFH and this cell surface receptor limited TNF-α induced cytoplasmic actin stress fibre formation (a key component of actin cytoskeleton contraction and endothelial barrier dysfunction)(244). It has yet to be established whether LMWH fractions interact with specific heparin receptors on the endothelial cell surface in a similar manner.

In summary, the results described in 3.8-12 suggest that the cytoprotective properties of LMWH are mediated by direct LMWH signalling activity on the endothelial cell surface. In particular, this hypothesis is supported by the finding that LMWH-mediated inhibition of thrombin-induced PAR-1 signalling is not mediated through an inhibition of the interaction between thrombin and the PAR-1 receptor or its HSPG co-factor. While the precise mechanism underlying LMWH cytoprotective signalling in this context remains to be elucidated, the findings of other investigators which have recently identified an endothelial cell UFH receptor provide a novel insight into the nature of the interaction between heparin compounds and vascular cells.
6.1.4 The relative anticoagulant and cytoprotective properties of LMWH can be harnessed in vitro in a manner which minimises the anticoagulant activity of LMWH while retaining endothelial barrier protective properties

Among the general population, the risk of major haemorrhage associated with therapeutic anticoagulation has been estimated at 1-2% per year. However, among patients with cancer this risk would be predicted to be significantly higher in view of the additional risk factors for bleeding which frequently arise among these patients (68, 261). The findings described in chapter 3 suggest that the cytoprotective activity of LMWH may not be linked to its indirect, antithrombin-mediated, anticoagulant activity. This is an important finding as the primary obstacle to the use of LMWH for the purposes of its anti-cancer activity is the risk of haemorrhage associated with its administration (143). In chapter 4 we explored potential strategies in vitro, which if replicated in vivo, might represent a means of delivering the endothelial cytoprotective effects of LMWH without conferring a bleeding risk.

As outlined in 1.3.2.2, the anticoagulant activity of LMWH is mediated through a specific pentasaccharide sequence which is present in a minority of heparin polysaccharide chains (primarily larger polysaccharides) (46). This pentasaccharide binds antithrombin, leading to a conformational change in the structure of the serpin which accelerates its rate of inhibition of thrombin and FXa. LMWH formulations comprised primarily of shorter heparin polysaccharides would be predicted to contain fewer heparin chains bearing the intact pentasaccharide sequence and so would be anticipated to exhibit diminished anticoagulant activity. However, given that the cytoprotective properties of LMWH are not necessarily dependent on anticoagulant activity, we hypothesised that a LMWH tinzaparin fraction comprised of shorter heparin polysaccharide chains relative to the standard commercial formulation (mean molecular weight 2.8KDa vs 6.5KDa) would retain cytoprotective activity while exhibiting limited anticoagulant function.

Using CAT, a relatively novel technique which assesses parameters of plasma thrombin generation (and represents a more accurate method of investigating hypercoagulability or hypocoagulability than conventional clot based assays given that thrombin is the key effector of the blood coagulation pathway activity), we demonstrated in 4.4 that the 2.8KDa LMWH fraction exhibits a minimal inhibitory effect on plasma thrombin generation compared to that which is observed using equivalent concentrations of the standard 6.5KDa tinzaparin formulation (262, 263). Similar findings were observed using the chromogenic plasma anti-factor Xa activity assay.
which is widely used in clinical practice for determining intensity of anticoagulant effect among patients receiving therapy with LMWH (235). Using this assay, at a plasma concentration of 5µg/mL, the 2.8KDa fraction exhibited 0.18±0.01 IU/mL of anti-FXa activity, a value which would not be expected to confer a significant bleeding risk (the equivalent concentration of tinzaparin would be predicted to confer an anticoagulant effect associated with an anti FXa activity level within the therapeutic range of 0.5-1IU/mL).

Remarkably, despite diminished in vitro anticoagulant activity, the 2.8KDa fraction significantly attenuated thrombin-induced endothelial permeability. Moreover, LMWH fraction also inhibited tumour cell trans-endothelial migration at concentrations which would not be anticipated to confer a bleeding risk if replicated in vivo.

In addition to exploring potential strategies involving LMWH fractions as outlined above, we also investigated the additive or synergistic endothelial cytoprotective effects exhibited by combinations of standard commercial LMWH formulations (but at sub-anticoagulant concentrations) with simvastatin, a commonly used lipid lowering agent. The statins have been previously shown to exhibit endothelial barrier protective properties in vitro and in vivo in models of hyperglycaemia and thrombin-induced endothelial barrier dysfunction (although at concentrations which are in excess of the therapeutic range in humans). Statins regulate cholesterol biosynthesis by acting as inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme which functions by catalysing the conversion of HMG-CoA to mevalonic acid, a key intermediate in the synthesis of cholesterol and other lipid metabolites. Their actions in regulating endothelial barrier function appear to be mediated through the inhibition of the biosynthesis of specific lipid metabolites such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate which are required for the lipidation (and subsequent binding to the plasma membrane) of specific proteins which are involved in regulating endothelial barrier function, such as components of the RhoA GTPase signalling pathway. Statin-mediated inhibition of these processes appears to diminish agonist-induced endothelial barrier dysfunction and endothelial inflammatory responses (216, 217).

In humans, the use of statin therapy has been shown to significantly reduce cardiovascular mortality. However, the presence of plasma statin concentrations in excess of the standard therapeutic range is associated with potentially life-threatening adverse effects (264). In 4.9, we demonstrated that simvastatin appeared to attenuate thrombin-induced endothelial barrier dysfunction in vitro, although this observation only
reached statistical significance at a simvastatin concentration similar to that which would be anticipated to be achieved in clinical practice among individuals receiving a simvastatin dose which is approaching the maximum recommended daily intake (40mg). However, we demonstrated in 4.10, that a synergistic effect on endothelial barrier function is observed when simvastatin and LMWH are co-incubated with endothelial cell monolayers. Moreover, we observed that almost complete protection against thrombin-induced endothelial barrier dysfunction can be achieved in vitro when combining a concentration of LMWH tinzaparin which would not be predicted to confer a risk of haemorrhage in vivo (0.1IU/mL) and a concentration of simvastatin which would be consistent with peak simvastatin levels achieved in vivo with the daily oral administration of a 40mg dose.

In summary, the observations described in chapter 3 which suggested that the endothelial cytoprotective properties of LMWH were not dependent on anticoagulant activity prompted us to explore potential strategies to optimise the relative anticoagulant and cytoprotective properties of LMWH formulations in a manner which could allow the exposure of cancer patients to the potential beneficial anti-metastatic effects of LMWH without conferring a bleeding risk. In chapter 4 we demonstrated that a 2.8KDa fraction of LMWH derived from commercially-utilised tinzaparin exhibits diminished anticoagulant efficacy but retains endothelial barrier protective properties and inhibits tumour cell trans-endothelial migration in vitro. Furthermore, we explored strategies to enhance the endothelial barrier protective properties of standard tinzaparin but at a sub-anticoagulant concentration and found that combining low dose tinzaparin with a clinically relevant simvastatin concentration leads to a marked protection against agonist induced endothelial barrier dysfunction.
6.1.5 Limitations, conclusions and future work

This results outlined in chapters 3 and 4 must be interpreted in the context of several limiting factors. Firstly, while the work of other investigators has recently generated evidence to suggest that heparin compounds activate intra-cellular signalling by directly interacting with an endothelial cell ‘heparin receptor’, the putative receptor and signalling pathway involved specifically in LMWH-mediated endothelial barrier protection remain to be fully characterised. Secondly, in vivo data supporting our hypothesis that LMWH mediates an anti-metastatic effect by inhibiting tumour-induced vascular permeability, and that a synergistic role for LMWH and simvastatin in enhancing endothelial barrier function exists, are currently lacking. However, work aimed at exploring and replicating these findings in a murine model of cancer metastasis is currently planned. In order to achieve this we have established a collaboration to allow us to explore these observations specifically in an established murine model of human breast carcinoma metastasis, where we will investigate the endothelial barrier protective effects of the standard LMWH, the LMWH fraction and the LMWH/statin combination. Moreover, we will seek to specifically quantify the in vivo haemorrhagic risk which might be associated with both the LMWH fraction and the low-dose LMWH combination (which exhibited minimal anticoagulant activity in vitro). Thirdly, we are yet to explore the role of LMWH in preventing endothelial barrier destabilization induced by other tumour-derived agonists, including tumour-derived extracellular vesicles.

Moreover, the clinical implications of the findings reported in chapters 3 and 4 are also somewhat limited by the conflicting reports from clinical studies to date, some of which failed to detect a survival benefit following LMWH administration to cancer patients. However, as discussed in 6.1.2, in contrast to animal models of metastasis (which have consistently demonstrated a LMWH-mediated anti-tumour effect), a significant proportion of participants in clinical trials would be predicted to have already developed clinically undetectable micro-metastases by the time of recruitment (a clear confounding factor), particularly in the sub-groups of patients with more advanced disease. We would therefore hypothesise that LMWH is likely to only be effective in preventing metastasis in the sub-group of patients with very early, limited-stage tumours prior to development of established micrometastases. Consequently, the magnitude of the LMWH anti-metastatic effect in humans is likely to only become apparent in the context of more targeted studies of homogenous patient groups. As discussed in 6.1.2, circulating tumour cells can be detected in the systemic circulation of patients with cancer (including patients who are eventually cured) and the burden of
these circulating malignant cells appears to correlate with patient stage and so could therefore be predicted to represent a potential means of more accurately identifying subjects with early stage disease who are unlikely to have developed metastases, including clinically undetectable micro-metastatic deposits (one of the key confounding factors in clinical studies to date). We hypothesise that the use of this marker of tumour burden could be utilised to identify cancer patients who would be most likely to benefit from the endothelial barrier protective properties of LMWH i.e. subjects with a low burden of circulating tumour cells, which have yet to successful breach the endothelial barrier to form micro-metastatic deposits. In addition, increasing evidence supports the hypothesis that the process of cancer metastasis varies from tumour to tumour and the relative importance of the respective obstacles to metastasis (e.g. endothelial barrier, inhibition of angiogenesis, circulatory shear forces etc.) may differ depending on the tumour type and the site of potential metastasis(186). Specific lung and breast tumours have been shown to directly promote vascular permeability in the vasculature beds of their metastatic target tissues as discussed in 1.3.2.7. Moreover, the role of endothelial barrier stabilisation is likely to be more important for metastasis prevention in organs such as the brain (where endothelial cells form a tight, impenetrable barrier at baseline) rather than in an organ such as the liver, where at baseline the endothelium in parts of the hepatic vasculature is already highly-permeable and non-contiguous(186). Therefore selecting subjects based on specific tumour type or predicted site of metastasis might also represent a means of more accurately identifying the sub-group of cancer patients who might benefit from LMWH therapy.

In conclusion, the role of LMWH therapy in the prevention of cancer metastasis in clinical practice remains to be determined although compelling evidence has been generated from in vitro, pre-clinical and clinical studies to suggest that LMWH mediates a clinically relevant ant-cancer effect which is independent of its anti-thrombotic activity. We have demonstrated that LMWH supports endothelial barrier function and attenuates agonist-induced endothelial permeability, pathways which are key to the progression of metastasis. Crucially, we have also described strategies which would be predicted to limit the risk of LMWH-associated haemorrhage without diminishing these LMWH-mediated cytoprotective activities; findings which, if replicated in vivo, would likely be of significant translational value. To date, these findings represent the first instance in which endothelial barrier stabilisation has been explored as a possible mechanism underlying the anti-metastatic properties of LMWH and these results are the first to demonstrate that LMWH attenuates tumour cell trans-endothelial migration.
Moreover, we have also characterised for the first time the endothelial barrier protective properties of a LMWH-derived fraction which is not currently commercially available as an isolated product (although it is contained within the heterogeneous mixture of heparin polysaccharides which make up commercial grade LMWH tinzaparin).
6.2 Derangements of blood coagulation in Eisenmenger syndrome: underlying mechanisms and potential novel therapeutic strategies

6.2.1 Platelet procoagulant activity is a key determinant of enhanced plasma thrombin generation in Eisenmenger syndrome

As outlined in 1.2.5, ES is a rare complication of congenital cardiac disease with pulmonary artery hypertension associated with shortened survival and a substantial burden of multi-system morbidity. It is well recognised that derangements in haemostasis are common in ES and contribute substantially to the observed burden of morbidity and early mortality(26, 119).

Individuals with ES and other forms of pulmonary vascular disease have been shown to be at significant risk of both thromboembolic complications (such as stroke and pulmonary artery thrombosis) and haemorrhagic complications (such as haemoptysis and spontaneous pulmonary haemorrhage)(34, 111, 112). Thrombosis within the pulmonary vasculature not only represents a potentially life-threatening event but also contributes to the progression of pulmonary vascular disease, an observation which is reflected in current management algorithms which recommend consideration of the use of anticoagulant therapy among patients with idiopathic pulmonary hypertension(265, 266). However, the role of anticoagulant therapy in ES remains controversial in view of the associated bleeding diathesis, with severe bleeding events including fatal haemorrhages having been reported in the context of anticoagulant therapy among individuals with this disorder(112).

The molecular mechanisms underlying these competing thrombotic and haemorrhagic tendencies in ES remain poorly understood. Activated platelets play a key role in maintaining in both primary and secondary haemostasis. As outlined in 1.1.2, during the process of primary haemostasis platelets interact with VWF and sub-endothelial tissues to form a temporary platelet plug at the site of vascular injury (a process which is dependent on platelet activation, degranulation and aggregation). During the process of secondary haemostasis (where the initial platelet plug is replaced by a stable fibrin clot) platelets exhibit procoagulant activity by providing an anionic phospholipid surface (the activated platelet membrane) which promotes the activity of the procoagulant enzyme complexes generated during the process of blood coagulation. Evidence of enhanced platelet activation has been previously reported among individuals with ES but conversely thrombocytopenia and impaired platelet
aggregation responses *in vitro* have also been described in this setting(118, 128, 130, 131).

The investigation of blood coagulation derangements in ES is limited by the inability of conventional coagulation assays such as platelet aggregometry and clotting time assays (which assess various components of coagulation in isolation) to accurately reflect the complex nature of the interaction between soluble and cellular elements of the blood coagulation system. CAT is a research tool which permits the assessment of thrombin generation in plasma. The generation of this enzyme occurs in physiological coagulation activation, is a key regulator of clot formation and correlations between thrombin generation and thrombotic risk have been reported(233, 234, 267). Crucially, the CAT assay assesses thrombin generation in a manner which takes into account the activity of procoagulant and anticoagulant pathways in parallel as well as the influence of platelets on these pathways(235, 262, 263).

In chapter 5, we describe parameters of plasma thrombin generation using CAT in PRP obtained from subjects with ES, revealing an overall hypercoagulable state. In these experiments, plasma thrombin generation was found to be significantly accelerated and higher peak thrombin concentrations were generated among ES subjects relative to that which was observed among a group of age and gender-matched healthy volunteers. This pattern of accelerated, enhanced thrombin generation was detected in PRP in the presence and absence of exogenous TF and CTI (an inhibitor of contact pathway activation). Interestingly, no significant difference in any parameter of plasma thrombin generation between the cohort of subjects with ES and the healthy volunteers was detected in PPP, suggesting that abnormal platelet procoagulant activity plays a key mechanistic role in the ES-associated prothrombotic phenotype.
6.2.2 Platelets modulate the anticoagulant activity of APC in Eisenmenger syndrome

In 5.7-8, we describe patterns of sensitivity to the anticoagulant activity of APC as determined using CAT in plasma obtained from healthy volunteers and individuals with ES. The APC pathway is a key endogenous anticoagulant pathway which mediates its effect through the cleavage of activated coagulation factors V and VIII (129). The anticoagulant activity of the APC pathway is partially platelet-dependent as the platelet membrane represents a source of phospholipid which is crucial to the activity of APC(268, 269).

Resistance to the anticoagulant activity of APC is associated with an increased risk of thrombosis however a number of investigators have demonstrated in vitro evidence of platelet-mediated APC resistance in healthy individuals in response to various agonists associated with vascular injury (129, 242, 243). These investigators have postulated that in normal individuals, in the platelet-rich environment which arises at the site of vessel injury, that platelets attenuate the anticoagulant activity of APC in order to promote haemostatic clot formation.

We have demonstrated evidence of this APC resistant phenotype among our healthy volunteers, where the ability of exogenous APC to attenuate thrombin generation was found to be significantly diminished in platelet-rich plasma (PRP). However within our cohort of ES subjects we have detected the loss of this pro-haemostatic phenotype, with abnormally enhanced sensitivity to the anticoagulant activity of APC detected in PRP obtained from subjects with this disorder. To address the hypothesis that enhanced APC sensitivity in ES PRP is platelet-dependent and not mediated by differences in the phospholipid composition of extracellular vesicles also present in PRP, platelets were removed by centrifugation and thrombin generation was stimulated with TF in the absence of any exogenous phospholipid. Upon removal of platelets, the effect of APC on peak thrombin generation and ETP (in platelet-poor plasma without added PL) was now similar in cases and controls. These findings suggest that differences in ES patients’ platelets compared with controls supports abnormally enhanced sensitivity to the anticoagulant activity of APC. The precise component of platelet function which promotes enhanced APC anticoagulant function is unknown but patterns of platelet membrane lipid exposure may contribute to the abnormal APC sensitivity. Other potential mechanisms may include derangements in platelet coagulation factor V expression, however due to constraints on available PRP the
relative contributions of platelet phospholipid exposure and other platelet components to the observed blood coagulation derangements could not be determined.

To our knowledge, this represents the first instance in which the loss of platelet-mediated APC resistance has been described in the context of a disorder associated with an acquired bleeding diathesis. Moreover, this novel observation may reflect a mechanism through which physiological platelet haemostatic activity may be impaired despite abnormal platelet-mediated procoagulant activity being enhanced in parallel (as suggested by the findings discussed in 6.2.1), reflecting the clinical phenotype in ES where haemorrhagic and thrombotic tendencies co-exist.
6.2.3 Dual endothelin-1 receptor antagonism attenuates platelet mediated derangements in procoagulant and anticoagulant pathways in Eisenmenger syndrome

Macitentan, a dual endothelin-1 receptor antagonist has been shown to reduce morbidity and mortality in PAH (120). While both endothelium and platelets express endothelin-1 receptors, the implications of endothelin-1 signalling for platelet function and coagulation activation are unknown (27, 28). Remarkably, within our cohort of ES subjects, we observed a significant reduction in peak thrombin generation in PRP following completion of 6 months of macitentan therapy. No differences in parameters of plasma thrombin generation in PPP were observed between the two groups. In addition, the abnormally enhanced APC sensitivity in PRP among the ES cohort was also observed to become less marked.

The precise mechanism through which platelet-mediated derangements in these procoagulant and anticoagulant pathways may become attenuated in response to therapy remains to be elucidated. We hypothesise that the inhibition of endothelin-1 mediated endothelial dysfunction and vascular re-modelling within the pulmonary vasculature may lead to attenuated abnormal platelet activation. As a consequence of the limited PRP available for analysis, the direct effect of macitentan on various components of platelet function or membrane phospholipid exposure was not determined.
6.2.4 Limitations, conclusions & future work

The findings described in chapter 5 must be interpreted in the context of a number of limiting factors. Firstly, the size of our cohort sample is small. However, ES is extremely rare (with an estimated prevalence of just 2-6 per million adults in the developed world)(30, 270) and it is unlikely that a larger cohort of treatment-naïve individuals could have been recruited during this timeframe in another population similar in size to that of the Republic of Ireland. Secondly, as a consequence of the cohort size and as a result of the relatively short period of follow-up, it was not possible to correlate changes in parameters of plasma thrombin generation with thrombotic events. Thirdly, the CAT assay is a relatively novel research tool and is not validated for use in clinical practice to monitor hypercoagulability longitudinally or to guide therapy and so the clinical implications of the changes in plasma thrombin generation in response to dual endothelin-1 antagonist therapy over time would need to be defined in a larger clinical trial.

Notwithstanding the obvious limitations of this work, these novel observations suggest that platelet activity contributes significantly to hypercoagulability in ES but also modulates the activity of endogenous anticoagulant pathways in a manner which could also promote bleeding, reflecting the clinical phenotype where competing thrombotic and haemorrhagic risks frequently co-exist. Moreover, our findings suggest that targeted pharmacological agents which are directed against the underlying vascular pathology such as the dual endothelin-1 antagonists (which, unlike conventional anticoagulants, do not confer a bleeding risk) may attenuate these platelet-mediated derangements in blood coagulation. Given the significant clinical dilemmas posed by the presence of competing thrombotic and haemorrhagic tendencies in ES, these finding are likely to be of significant translational relevance.

While the scope of this study has been limited to individuals with ES, similar findings may also exist in other types of PAH and further study of platelet-mediated derangements of blood coagulation in these related disorders may be warranted. Furthermore, exploring the direct effects of agents such as macitentan on platelet function may shed further light on the precise mechanism underlying its effect on parameters of hyper- and hypocoagulability.
7.0 BIBLIOGRAPHY


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8.0 APPENDICES

Appendix 1

Research ethics committee notice of approval to proceed with a clinical study investigating derangements of blood coagulation in Eisenmenger syndrome, the accompanying patient information leaflet and consent form.
Mater Misericordiae
University Hospital
Sisters of Mercy
Eccles Street, Dublin 7, Ireland

Tel: +353 1 8032000  Fax: +353 1 8032404  Email: mmh@mater.ie  Web: www.mater.ie

Not for prescription purposes
Dr Fionnuala Ni Áinle
Consultant Haematologist
Mater Misericordiae University Hospital
Eccles Street
Dublin 7

9th January 2015

Our Ref: 1/378/1666

RE: To characterise plasma thrombin generation in patients with pulmonary hypertension due to congenital cardiac disease

Research protocol, Version 1.0: August 2014
Patient Information Leaflet and Consent Form, Version 2.0: November 2014
Consent Form (Patient), Version 2.0: November 2014
Withdrawal of Consent form (Patient), Version 2.0: November 2014
Parent guardian Information Leaflet and Consent Form, Version 2.0: November 2014
Consent Form (Parent guardian), Version 2.0: November 2014
Withdrawal of Consent form (Parent guardian), Version 2.0: November 2014
Patients aged 16-18 years: Information sheet and Assent form, Version 1.0: August 2014
Assent form (Patients aged 16-18 years), Version 1.0: August 2014

Dear Dr Ni Áinle

I acknowledge receipt of your correspondence dated 03/12/2014 addressing points of clarification and enclosing a revised Standard Application Form (Version 2.0, November 2014), revised Patient Information Leaflet, Consent Form, Withdrawal of Consent form (Patient) (Version 2.0, November 2014), revised Parent/Guardian Information Leaflet and Consent Form, Consent Form (Parent/Guardian) and Withdrawal of Consent form (Parent guardian) (Version 2.0, November 2014) as requested by the Mater Misericordiae University Hospital and Mater Private Hospital Research Ethics Committee for the above research study to be carried out at the Mater Misericordiae University Hospital (MMUH).

This correspondence has been noted and the revised documents have been approved. Approval to proceed with this research study at the MMUH is granted; this approval is valid until 22nd October 2016.

It is your responsibility to adhere to the approved study protocol and ensure that all investigators involved with the research only use the approved documents without deviation (unless they have been approved by the Research Ethics Committee), to submit annual reports setting out the progress of the research (giving details of the number of participants who have been recruited, the number who have completed the study and details of any adverse events etc.) and to notify the Research Ethics Committee when the research is concluded.

The Mater Misericordiae University Hospital and Mater Private Hospital Research Ethics Committee would like to remind all investigators involved in research of their legal obligations under the law on Data Protection.

Yours sincerely

Prof Malcolm Bell
Chairman
Research Ethics Committee

cc. Dr Barry Kevane, Research Registrar

Mater Misericordiae University Hospital
Sisters of Mercy
Eccles Street
Dublin 7

Not for prescription purposes
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Yours sincerely

Prof Malcolm Bell
Chairman
Research Ethics Committee

cc. Dr Barry Kevane, Research Registrar
To characterise plasma thrombin generation in patients with pulmonary hypertension due to congenital cardiac disease.

What is the purpose of the research study?

Previous research studies have suggested that an increased risk of abnormal blood clotting and bleeding can occur in patients with certain forms of heart and lung disease, particularly in the condition known as pulmonary hypertension due to congenital heart disease. It has also been suggested that abnormal activation of blood clotting may contribute in part to the development and progression of this condition. The exact factors which drive these abnormalities of blood clotting have not been determined and the degree of abnormal blood clotting activity seen in different forms of this condition, as well as the best way to treat these problems, have also yet to be determined.

We aim to use a recently developed, advanced technique (calibrated automated thrombography) to assess multiple aspects of blood clotting in patients with various forms of these heart & lung conditions. This will include patients who are being treated with a drug called macitentan, as some studies have suggested that this and similar drugs that are used to treat some of these conditions may also affect blood clotting. We aim to compare results between patients with various forms of these conditions and to compare results to that seen among healthy volunteers. We also want to compare blood results before and during treatment with the drug macitentan in patients who are being started on this drug for management of their disease.

Who is being asked to participate?

Your child is being invited to participate in this study if:

1) They have a history of congenital cardiac disease with pulmonary hypertension.  
   Or,
2) They have a history of congenital cardiac disease without any pulmonary hypertension.  
   Or,
3) They have a history of pulmonary hypertension without congenital cardiac disease  
   Or,
4) They are being invited to participate as a ‘normal control’ (healthy volunteer).

Who is organising the Research study?
This research study is being co-ordinated by Dr. Fionnuala Ní Áinle, Consultant Haematologist, Mater Misericordiae University Hospital and Dr. Kevin Walsh, Consultant Cardiologist, Mater Misericordiae University Hospital, Dublin. The study will be run through Dr. Walsh’s cardiology outpatient clinic.

**What will happen to participants if they decide to take part?**

If you are happy for your child to participate, a member of the research team will discuss the study with you in detail. In particular they will outline that this is a voluntary study and should you decline to consent to participate in this study, this will not affect your child’s current or future care in any way. In addition, should you initially decide to allow participation in the study, you are free to withdraw from the study at any point in the future if you wish and this will not affect future medical care in any way.

You will be given an opportunity to ask any questions which you might have prior to signing the attached consent form.

Should you agree to participation in this study, you will be asked to sign this form, a copy of which will be given to you for your own records.

**The study will involve taking a sample of blood** – where possible this will be done as part of routine blood sampling as part of your child’s usual medical care. The sample of blood will be taken by a trained phlebotomist/nurse/doctor that is experienced in taking blood. For optimum testing we would hope to collect 65 mls of blood. In the case of study participants who are taking the drug macitentan, this blood test will be repeated in 6 months’ time also.

**For all other participants, there will be no further blood sampling or any other procedure of any kind required for the purposes of this study.**

The samples will be processed initially in the hospital laboratory and will then be brought to a research laboratory in University College Dublin where the blood clotting analysis will be carried out. The samples will be anonymised and all the results generated from the samples will be anonymised. All the results will be kept in a secure, password-protected computer system and access to this will be restricted to the principal investigators (Dr. Fionnuala Ní Áinle and Dr. Kevin Walsh) and the research doctor (Dr. Barry Kevane). All results which may eventually be presented at medical conferences or in medical publications will not contain any information that could possibly identify you as a participant. As soon as the final analysis is completed, any remaining blood samples will be destroyed.

**Are there any disadvantages/risks in taking part in this research study?**

As with any blood test, there may be some discomfort however, the blood test will be taken by an experienced phlebotomist/nurse/doctor and every effort will be made to minimise discomfort. There are no other specific disadvantages or risks of taking part in this study.
What are the potential benefits to taking part in this study?

You and your child may or may not receive any direct benefit from taking part in this study. However, information obtained during the course of this research study may help us to improve our understanding of these conditions, particularly related to blood clotting abnormalities, which are a source of complications in pulmonary hypertension. This may help improve our treatment of patients with these conditions in the future, however this cannot be guaranteed.

Is my doctor being paid to include me in the research study?

No specific fee will be paid to the doctor for including your child in this research study.

Will patient expenses be met?

As blood testing will take place during routine follow-up appointments, no specific additional patient expenses will arise.

What happens when the study ends?

You will continue your usual medical care with your own doctor.

Are there any restrictions to what I might eat or do?

No.

What if something goes wrong?

The Research team will not alter your treatment in any way during the conduct of the study and so we do not anticipate that you will experience any harm from taking part.

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

Confidentiality
The principal investigators (Dr. Fionnuala Ní Áinle and Dr. Kevin Walsh) and the research registrar (Dr. Barry Kevane), will require limited access to your medical records during the course of the research study. All information obtained from your medical records will be kept strictly confidential and anonymised so that you cannot be identified from it.

**Hospital Research Ethics Committee Approval**

The Mater Misericordiae University Hospital and Mater Private Hospital Research Ethics Committee has granted ethical approval for this research study to be undertaken.

**What will happen to the results of the research study?**

The results of this study will be submitted for publication in international peer-reviewed medical journals and presented at medical research meetings.

**Procedure to be used if assistance or advice is required**

If you have any further queries regarding this study, you may contact Dr Barry Kevane (087-6449612) or Dr Fionnuala Ní Áinle (01-8034429).

**Voluntary participation**

It is up to you to decide whether or not you would like your child to participate in the study or not. Even if you do decide for your child to participate, you are still free to withdraw them from the study at any point and without giving a reason. This will not affect the standard of care which your child receives.
CONSENT FORM

Title of Research Study: To characterise plasma thrombin generation in patients with pulmonary hypertension due to congenital cardiac disease.

Patient Name:……………………………………

Parent/Guardian Name:………………………………

Name of Doctor and Telephone Number:…………………………………………

1. I confirm that I have read and understood the information leaflet dated………. for the above research study and received an explanation of the nature, purpose, duration, and foreseeable effects and risks of the research study and what the involvement of the participants will be

☐

2. I have had time to consider whether to agree to allow my child to take part in this research study. My questions have been answered satisfactorily and I have received a copy of the Patient Information Leaflet.

☐

3. I understand that participation is voluntary and that I am free to withdraw my child from the study at any time without their medical care or legal rights being affected

☐

4. I have to the best of my knowledge informed the investigator of my child’s previous or present illnesses and medications.

☐

5. I am willing to allow access to medical records for the purposes of this study but understand that strict confidentiality will be maintained

☐

6. I agree to my child taking part in the above study

☐

…………………………………………………………

Name of Participant (in block letters) Date Signature

…………………………………………………………

Doctor/Researcher Date Signature

1 copy for patient, 1 copy for researcher, 1 copy to be inserted in hospital notes
TO CHARACTERISE PLASMA THROMBIN GENERATION IN PATIENTS WITH PULMONARY HYPERTENSION DUE TO CONGENITAL CARDIAC DISEASE

Withdrawal of Consent form

- I wish to withdraw consent for my child’s (…………………………….) participation in the above study.
- I have been able to ask any questions that I have about this study and the doctor has answered these for me.
- I understand that withdrawing from the study will not affect the future medical care of my child in anyway.
- I allow the blood samples which have already been taken to be included in the results of this study.

YES  NO  (please circle)

Signature:  ______________________

Name:  ______________________

Date:  ______________________

STATEMENT OF INVESTIGATOR’S RESPONSIBILITY

I have explained the nature, purpose, procedures, benefits, risks and alternatives to this research study. I have offered to answer any questions that the participant may have. I believe that the participant understands my explanation and has freely given informed consent to withdraw from this study.

DOCTOR’S SIGNATURE  DATE: