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To Mam and Dad
DECLARATION

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Catriona Keenan M.Sc.

January 2003
ABSTRACT

The molecular and cellular pathways of coagulation and inflammation have traditionally been regarded as separate entities, however recent research has demonstrated that they are closely linked through endothelial and monocyte cell surfaces. This link is most apparent during severe sepsis, associated with multiple organ failure as a result of the dysregulation of procoagulant, anti-inflammatory, pro-inflammatory and fibrinolytic mechanisms. Also, the high incidence of vasculopathies seen in inflammatory and autoimmune disorders has further strengthened the relationship between these two mechanisms in the pathogenesis of other human disease states. In this thesis, I endeavoured to determine the link between coagulatory and inflammatory events in disease states traditionally associated solely with either pathway and this also included the non-pathogenic state of normal pregnancy.

Pregnancy is regarded as a hypercoagulable state, with alterations in coagulation proteins that result in the maintenance of a healthy foetus and mother, from conception through to childbirth. Pregnancy is also considered an immunological paradox due to the acceptance of paternal antigens by the maternal immune system. Moreover, control mechanisms operate throughout normal pregnancy that facilitate a homeostatic balance between coagulation and inflammatory events. To the best of our knowledge, this is the first longitudinal study of an Irish cohort of pregnant women, assessing changes in both coagulatory and inflammatory markers. We have demonstrated changes in several haematological and haemostatic factors throughout gestation including alterations in haemoglobin concentrations, factor VIII (FVIII) and fibrinogen concentrations and platelet counts, all of which return to within normal ranges post-partum. Investigation of cytokine profiles throughout gestation revealed global downregulation of inflammatory markers (IL-10, IL-6, TNFα and IFNγ) during pregnancy compared to the non-pregnant state. These findings suggest tight control over these mechanisms during pregnancy, and the need for downmodulation of inflammatory processes to maintain a viable foetus. Furthermore, regulation of cytokine concentrations during pregnancy may occur at the genetic level, since IL-6 and TNFα concentrations were found to be influenced by polymorphisms within these genes. This thesis demonstrates that in vitro, steroid hormones have a pro-inflammatory effect on endometrial cells that may be mediated via monocytes, since similar responses were observed in a monocytic cell line. Therefore, monocytes may be the link between coagulation and inflammation regulation in the pregnant uterus. In addition, heparin treatment of hormone stimulated endometrial cells
resulted in downregulation of the pro-inflammatory response, indicating both anticoagulant and anti-inflammatory roles for heparin during pregnancy. Recurrent foetal loss may result from an imbalance in procoagulant and anticoagulant factors, or dysregulation of immune responses during pregnancy. We have demonstrated similar changes in haemostatic and inflammatory markers throughout pregnancy in patients with a history of RFL compared to normal pregnancy. Both the IL-6 -174 and the TNFα -308 polymorphic markers were found at significantly different frequencies in RFL patients when compared with normal controls, indicating possible roles for these genes in the regulation of cytokine concentrations and in the maintenance of pregnancy.

Venous thromboembolism (VTE) and arterial disease (Stroke) are major complications of several disease states. We demonstrate that elevated fibrinogen, activated protein C resistance (APCR) in association with FV Leiden and the prothrombin gene variant are predisposing risk factors for VTE in an Irish cohort of patients with a personal or family history of VTE. At the same time, we found no association with any of the known thrombophilic gene mutations, whether it be coagulatory or fibrinolytic, with cerebrovascular accident (Stroke). Interestingly, we found several fibrinolytic polymorphisms (fibrinogen, tissue plasminogen activator, and angiotensin converting enzyme) with an increased frequency in patients with inflammatory bowel disease (IBD) implying a possible role in the pathophysiology of this disease.
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ABBREVIATIONS

A
Ab
ACE
ACL
Ang II
APAs
APC
APCR
APS
APTT
Arg (R)
AT
α₂AP
α₂M
β2-GP1
bp
C
C4bBP
C p s
CVA
DHPLC
DMSO
dNTP
DVT
E
EDTA
EPCOT
EPCR
ER
F 1+2
FBC
FDP
FII
fPS
FV
FVa
FVL
G
Hb
HCG

Adenine
Antibody
Angiotensin Converting Enzyme
Anticardiolipin
Angiotensin II
Antiphospholipid Antibodies
Activated Protein C
Activated Protein C Resistance
Antiphospholipid Syndrome
Activated Partial Thromboplastin Time
Arginine
Antithrombin
α₂-antiplasmin
α₂-macroglobulin
β 2-glycoprotein 1
Base pairs
Cytosine
C4b Binding Protein
Cystathionine β-synthase
Cerebrovascular Accident
Denaturing High Performance Liquid Chromatography
Dimethyl sulphoxide
deoxyribonucleoside triphosphate
Deep Vein Thrombosis
Estrogen
EthyleneDiamineTetraacetic Acid
European Prospective Cohort on Thrombophilia
Endothelial Protein C Receptor
Estrogen Receptor
Prothrombin fragments 1 + 2
Full Blood Count
Fibrinogen Degradation Products
Factor two (prothrombin)
Free Protein S
Factor five (etc.)
activated PV (etc.)
Factor V Leiden
Guanine
Haemoglobin
Human Chorionic Gonadotrophin
HCT Haematocrit
HK Hageman Factor
IBD Inflammatory Bowel Disease
IBTS Irish Blood Transfusion Service
ICH Intracerebral Haemorrhage
IFNγ Interferon gamma
IL Interleukin
IUFD Intrauterine Foetal Death
IUGR Intrauterine Growth Restriction
kb Kilobases
LAC Lupus Anticoagulant
LETS Leiden Thrombophilia Study
LMWH Low Molecular Weight Heparin
Ltα Lymphotoxin alpha
MCH Mean Cell Haemoglobin
MCHC Mean Cell Haemoglobin Concentration
MCV Mean Cell Volume
MI Myocardial Infarction
MTHFR Methylene-tetrahydrofolate Reductase
NCHCD National Centre for Hereditary Coagulation Disorders
NIBTS Northern Ireland Blood Transfusion Service
NK cells Natural Killer cells
OCP Oral Contraceptive Pill
P Progesterone
PAF Platelet Activating Factor
PAI-1 Plasminogen Activator Inhibitor-1
PBMC’s Peripheral Blood Mononuclear Cells
PC Protein C
PCI Protein C Inhibitor
PE Pulmonary Embolism
PF Purpura Fulminans
PIBF Progesterone Induced Blocking Factor
PR Progesterone Receptor
PROC Protein C gene
PROS Protein S gene
PS Protein S
PT Prothrombin Time
RS06Q Arginine-506-Glutamine
RAS Renin-Angiotensin System
RCC Red Cell Count
RDW Red cell distribution
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>RFL</td>
<td>Recurrent Foetal Loss</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SVT</td>
<td>Superficial Vein Thrombosis</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin Activable Fibrinolysis Inhibitor</td>
</tr>
<tr>
<td>TAT</td>
<td>Thrombin-Antithrombin complexes</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TIA's</td>
<td>Transient Ischaemic Attacks</td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>TMHA</td>
<td>Temperature Modulated Heteroduplex Analysis</td>
</tr>
<tr>
<td>t-MTHFR</td>
<td>Thermolabile MTHFR</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor alpha</td>
</tr>
<tr>
<td>TPA</td>
<td>Tissue Plasminogen Activator</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UFH</td>
<td>Unfractionated Heparin</td>
</tr>
<tr>
<td>UPA</td>
<td>Urokinase-type Plasminogen Activator</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous Thromboembolism</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
</tr>
<tr>
<td>WCC</td>
<td>White Cell Count</td>
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CHAPTER 1

INTRODUCTION
Chapter 1

1.1.0: THE COAGULATION & INFLAMMATION INTERFACE

Vascular disease is the primary cause of morbidity and mortality in developed countries with over half of deaths resulting from coronary thrombosis, peripheral vascular disease, deep vein thrombosis (DVT) and pulmonary embolism (PE). The high morbidity and mortality are due to the occurrence of thrombosis, the development of irregular blood clots in veins or arteries, where the vessels become partially or completely obstructed as a result of impairments in haemostasis. If neighbouring vessels do not maintain the circulation in the local environment, tissue necrosis may occur. There is also the risk of embolism, the migration of clot fragments to critical sites removed from the original clot, as in the lungs during PE (Cooper and Krawczak, 1997a).

Traditionally the processes of coagulation and inflammation have been regarded as separate entities. However research over recent years has clearly indicated that they are tightly linked and influenced by each other. Furthermore, it is believed that these pathways have emerged from the same evolutionary lineage (Krem and Cera, 2002). For many years, there has been speculation about the high incidence of vascular complications in inflammatory and immune disorders. Thrombotic disease in the form of disseminated intravascular coagulation (DIC), a paradox of haemorrhage and thrombus formation, has been the result of many clinical situations such as acute septic shock, surgical trauma, cancer and autoimmune disease (Stewart, 2001). Severe sepsis has been associated with multiple organ failure due to powerful microcirculation disturbances and enhanced activation of coagulation (Dosquet et al., 1995). The acute inflammatory and thrombotic reactions that occur during sepsis have further supported the hypothesis that coagulatory and inflammatory processes are linked. Furthermore, the multifactorial nature of thrombotic disease suggests that other processes, along with dysregulation of the coagulation pathway, contribute to disease pathogenesis. An understanding of the interplay between coagulation and inflammation mechanisms will allow greater insight to the processes involved in thrombotic disease pathogenesis.
1.2.0: Thrombophilia

Central to the pathogenesis of thrombosis in both arteries and veins is the disturbance in haemostasis. Thrombophilia is defined as a ‘tendency to clot’ and is considered a multifactorial disease since both environmental and genetic factors have a profound effect on the development of a thrombus. Environmental influences on haemostasis can be transient and include events such as pregnancy and childbirth, diet, smoking, surgery and hormone intake. Other environmental effectors with a longer lasting influence include conditions such as diabetes mellitus, hyperhomocysteinemia, hypertension, dyslipidemia and malignancy. Genetically determined disturbances in haemostasis can have a life-long impact on the development of thrombotic episodes and may therefore play a more profound role than environmental influences. Thrombophilia in the absence of genetic factors tends to occur in older populations while familial or inherited thrombophilia, due to genetic mutations, is associated with early onset of disease with environmental influences having a lesser effect. However, the late onset of many thrombotic episodes suggests that genetic influences are not the sole determinants, indicating the importance of gene-environment interactions in the development of thrombotic disease (Lane and Grant, 2000). To understand thrombotic disease and the role of thrombosis in disease pathophysiology, it is firstly important to understand haemostasis and the processes that regulate the fine balance between procoagulant and anticoagulant responses.

1.3.0: The Coagulation Cascade

Blood coagulation is one of the many mechanisms by which the body defends itself against host invasion and bleeding (Roberts and Tabares, 1995). It is a finely balanced system of signal amplification, referred to as the coagulation cascade, due to the stepwise activation of multiple zymogens to their proteolytically active forms. The resulting proteins generate thrombin and ultimately allow the lysis of fibrinogen to fibrin monomers, forming the basis of the fibrin clot (Roberts and Tabares, 1995). Anticoagulant and procoagulant mechanisms ensure tight regulation of the cascade, carefully balancing circulating concentrations of activated clotting factors. Furthermore, under normal conditions, anticoagulant pathways prevail over procoagulant mechanisms, and disruption of this fine balance between the two pathways as a result of genetic or environmental factors may lead to bleeding or thrombotic disease (Dahlback, 2000).
Thrombin plays a pivotal role in the orchestration of the coagulation cascade having many biologically important functions. Among them are activation of platelets, conversion of fibrinogen to fibrin monomers (forming the clot itself) and both positive and negative feedback amplification properties (Dahlback, 2000). Thrombin generation can be arrived at by either the intrinsic or extrinsic systems of the classic coagulation cascade, otherwise known as the contact phase or Tissue Factor (TF) pathways of initiation respectively (figure 1.1) (Cooper and Krawczak, 1997a).
Figure 1.1: The Coagulation Cascade as activated via the contact phase (A) and the tissue factor pathways (B), and amplified via the common pathway (C). Broken red arrow indicates positive feedback loops within the cascade. Solid blue arrows represent activation processes [adapted from Roberts & Tabares (1995). Molecular Basis of Thrombosis & Hemostasis. Marcel Dekker Inc, New York].
1.3.1: The Contact pathway of activation

The contact pathway of coagulation activation is initiated via the kallikrein-kinin system of contact activation which consists of three main proteins, coagulation factor XII (FXII) (Hageman factor), prekallikrein (Fletcher factor) and high molecular weight kininogen (HK). FXII can be activated upon interaction with negatively charged surfaces such as contact proteins assembled on cell membranes (endothelial cells and platelets). Activated FXII (FXIIa) converts the zymogen prekallikrein to kallikrein, which digests high molecular weight kininogen forming Bradykinin. This protein is vasoactive and acts as a proinflammatory mediator. Moreover, it is a potent stimulator of tissue TPA on endothelial cells, thus promoting fibrinolysis. Simultaneously, FXIIa proteolyses FXI liberating its active form FXIa, which activates FIX (figure 1.1.A) (Sugi and Makino, 2000). The production of FXIa permits it to autocatalytically activate its inactive self (FXI), which in turn activates FIX, FX and ultimately FVIII, initiating the common coagulation pathway. The contact factors may be bypassed via the activation of FXI by thrombin on a negatively charged surface (Cooper and Krawczak, 1997a). This mechanism of coagulation activation is not involved in trauma associated vessel damage and therefore plays an assisting role in activation via the TF pathway. However, it has an important role in the positive feedback loop, amplifying protein activation via thrombin and FXII.

1.3.2: The Tissue Factor pathway of activation

The tissue factor (TF) pathway is initiated via the exposure of the epithelial lining of the blood vessel wall upon injury, promoting TF expression. This is the main pathway for coagulation activation in damaged blood vessels and deficiencies or excesses in proteins of the TF pathway can result in both bleeding and thrombotic disorders (Dahlback, 2000). TF is a transmembrane glycoprotein that exhibits an extracellular tail similar to cytokine receptors and a unique short cytoplasmic tail. Although not ubiquitously expressed in vascular endothelial cells or monocytes, TF can be induced in these cells following inflammatory cytokine stimulus, for instance upon tissue damage (Riewald and Ruf, 2002). It acts as a cofactor to both FVII and FX (Cooper and Krawczak, 1997a) and can bind both the zymogen and active forms of FVII (Dahlback, 2000). A small amount of activated FVII (FVIIa) circulates in the blood and it is this active protease that binds TF, promoting the initiation of the TF pathway. FVIIa that is not bound to TF has very little activity but once the complex is formed it gains its full catalytic capacity, therefore the protease function of FVII in vivo is entirely TF
dependent (Dahlback, 2000; Riewald and Ruf, 2002). The TF-FVIIa complex activates FIX and FX forming FIXa and FXa respectively, providing stimulation of the common pathway (figure 1.1.C) (Roberts and Tabares, 1995). Feedback amplification occurs when FVIIa, FIXa and FXa activate the zymogen FVII that is bound to TF augmenting the coagulation process (Dahlback, 2000). Activated factors IX and X may then remain associated with the TF bearing cell or dissociate into the blood and bind to activated platelets (Dahlback, 2000). The next series of reactions lead to the amplification of coagulation on the surface of platelets, which accumulate around the cells that express TF during initiation. The exposure of negatively charged phospholipids (phosphatidylserine) on the surface of platelets gives them a high affinity for coagulation factors and cofactors necessary for efficient propagation of the cascade (Dahlback, 2000).

1.3.3: The Common Pathway

FVIII circulates in the blood in its inactive form bound to a carrier protein, von Willebrand Factor (vWF). While bound to vWF, FVIII is prevented from interacting with the phospholipid membrane. It is only in the presence of thrombin that FVIII is released from vWF and becomes activated (FVIIIa), allowing it to associate with the negatively charged surface of activated platelets (Dahlback, 2000). Therefore, the common pathway amplifies the clotting process via positive feedback loops created by the initial thrombin formation. FXa or thrombin activates FVIII to FVIIIa on the platelet surface, promoting the formation of the trimolecular ‘Tenase’ complex between FVIIIa, FIXa and FX (figure 1.2). Here FVIIIa increases the proteolytic potential of FIXa allowing activation of more FX. Simultaneously, thrombin or FXa activate FV to FVa allowing the formation of the ‘Prothrombinase’ complex of FXa, FVa and prothrombin (Factor II) (figure 1.2) (Cooper and Krawczak, 1997a). In the prothrombinase complex, FVa binds to the activated platelet surface and accelerates the activation of prothrombin by FXa to thrombin. Thrombin cleaves fibrinogen into α and β fibrin monomers. These monomers build up a lattice of fibres resulting in a cross-linked clot (Roberts and Tabares, 1995). At this stage the clot is very unstable and must be converted to a covalently linked form by FXIII, a transglutaminase that circulates in its inactive state until fibrin concentrations increase (Dahlback, 2000). Thrombin is then prompted to activate FXIII to FXIIIa which covalently links the α and β fibrin chains forming a solid clot (Roberts and Tabares, 1995). Increased thrombin formation
Chapter 1

causes the protein to promote its own amplification by activating the positive feedback mechanisms discussed previously.

1.4.0: Anticoagulant pathways

The process of blood coagulation is an organised cascade of reactions in which proteases secrete inactive zymogens and subsequently activate upstream proteases. Such enzyme cascades are characterised by regulation from within, creating both positive and negative feedback loops (Krem and Cera, 2002). The positive feedback properties of thrombin, FVII and FVIII have been illustrated previously. Three proteins exert potent negative feedback effects on the coagulation cascade. They are the inhibitors Tissue Factor Pathway Inhibitor (TFPI), Antithrombin (AT) and Activated Protein C (APC) (Butenas et al., 1999). TFPI binds FVIIa and FXa in the TF complex causing inhibition of activation of FX, and prevents the positive feedback mechanism of FVII, blocking amplification of the cascade (Dahlback, 2000; Riewald and Ruf, 2002). AT is a crucial serine protease inhibitor that acts on all of the proteins of the propagation phase of coagulation in particular factors IXa, Xa, XIa and thrombin itself (Wiedermann and Romisch, 2002). It quenches the activity of the enzymes involved, once they have become active (Butenas et al., 1999). AT prevents liberated enzymes from entering the circulation in intact vessels and limits coagulation to areas of vascular damage (Dahlback, 2000). Its ability to form such stable complexes with these enzymes is greatly enhanced in the presence of heparin, which is not naturally present in the circulation, but similar endogenous proteins are the glycosaminoglycans that are present on the surface of the intact endothelium. The specific binding of heparin to AT causes conformational changes in the inhibitor, heightening its interaction with the target protease (Cooper and Krawczak, 1997a). This is the basis for heparin anticoagulant therapy.

On undamaged endothelium, thrombin acts out another of its many functions as an anticoagulant by activating the protein C (PC) pathway. This system of anticoagulation regulates the activity of the cofactors FVa and FVIIIa via the activation of PC (Dahlback, 2000). At high thrombin concentrations, an endothelial cell transmembrane protein, Thrombomodulin (TM), binds with high affinity to thrombin. This causes a conformational change in the active site of thrombin so that PC can bind, resulting in its activation to APC, and the formation of the TM/T/APC complex (figure 1.3). Thrombin alone can activate APC in vitro but this rate of
activation is enhanced 1000 to 2000 fold in the presence of TM and calcium ions (Suzuki, 1995). APC exerts its anticoagulant activity by complexing with its cofactor Protein S (PS) on the endothelial membrane, targeting FVa and FVIIIa, resulting in cleavage of the proteins, causing their inactivation and downregulation of the coagulation cascade (Roberts and Tabares, 1995). Another receptor for PC has been shown to exist on the surface of endothelial cells, designated the endothelial protein C receptor (EPCR) (Fukudome and Esmon, 1995), which is similar to a family of proteins that are involved in inflammation (Esmon et al., 1999). The receptor has properties that are analogous to the TM/T complex and can bind to both PC and APC. Although its role is still emerging, recent studies indicate that EPCR prevents APC from inactivating FVa, possibly through cross competition between EPCR and FVa for APC binding (Esmon et al., 1999).

PC deficiency in vivo has been associated with thromboembolic disease in both humans and mice (Dahlback, 2000). Inherited PC deficiency with recurrent thrombosis was first reported in humans in 1981 and since then many studies have confirmed this association (Bucciarelli et al., 1999; Cooper and Krawczak, 1997b; Pabinger and Schneider, 1996; Shen et al., 2000). Heterozygotes are reported to have an increased risk of developing DVT and PE (Cooper and Krawczak, 1997b). Homozygous PC deficiency has been associated with neonatal thrombosis and purpura fulminans, an acute syndrome of DIC and skin necrosis (Branson et al., 1983; Seligsohn et al., 1984; Smith and White, 1999). PC infusion has proven to reverse the acute lethal thrombotic effect of its deficiency, restricting thrombin production and preventing subsequent DIC in patients suffering from purpura fulminas (PF) as a result of meningococcaemia and severe acquired and inherited PC deficiency (Dreyfus et al., 1991; Smith et al., 1997).
Figure 1.2: Thrombin production from tenase and prothrombinase complexes. [Adapted from Dahlback (2000). Blood Coagulation. The Lancet. 355: 1627-1632.]
Figure 1.3: Inhibition of FVa and FVIIIa by the PC anticoagulant pathway. TM= Thrombomodulin; PC= protein C; APC= activated protein C; PS= protein S; FVa= activated FV; FVIIIa= activated FVIII; FVi= inhibited FV; FVIIIi= inhibited FVIII. [Adapted from Dahlback (2000). Blood Coagulation. The Lancet. 355: 1627-1632.]
1.5.0: Fibrinolysis

The Fibrinolytic pathway is yet another system of regulating blood coagulation. It involves a series of events that act to break down the fibrin clot and is distinct from the coagulation pathway albeit not unconnected. The main players in this pathway are plasminogen, TPA, plasmin and fibrin. Although a separate process from coagulation, its effector is the end product of the cascade, fibrin (Lijnen and Collen, 1995).

Plasminogen is a proenzyme that is activated by TPA and urokinase-type plasminogen activator (uPA) to plasmin. Plasminogen activation via TPA is mainly concerned with dissolution of fibrin in the circulation, while uPA activation is cell-associated and involved in processes of tissue remodelling and repair, macrophage function, ovulation, embryo implantation and tumour invasion (Roberts and Tabares, 1995). The plasminogen protein consists of five homologous triple-loop motifs or ‘kringles’ that contain lysine-binding sites and aminohexyl-binding sites. It is at these sites on plasminogen that specific binding to fibrin occurs and on plasmin that binding to its inhibitor, α2-antiplasmin occurs (Lijnen and Collen, 1995).

Plasminogen is activated to plasmin via cleavage of the Arginine561-Valine562 bond by TPA. This exposes the active site of plasmin allowing it to degrade fibrin monomers (Lijnen and Collen, 1995). In the absence of fibrin, TPA has a very low affinity for plasminogen which increases two fold upon fibrin introduction into the circulation. This affinity is unaffected by fibrinogen, due to the masking of the two active sites involved (Mosesson et al., 1998). The plasmin protein is a two-chain trypsin-like serine protease comprised of a heavy chain containing the five ‘kringle’ domains of plasminogen (amino terminal) and a light chain containing the active site (carboxy terminal). Addition of lysine and arginine residues to the carboxy terminal causes conformational changes in the active site of plasmin, exposing it and allowing the dissipation of the cross-linked fibrin monomers to fibrin fragments, inactivating fibrin (figure 1.4) (Lijnen and Collen, 1995).

1.5.1: Regulation of Fibrinolysis

Similar to the coagulation cascade, the fibrinolytic pathway has many influential regulatory elements, preventing over dissolution of the clot and subsequent bleeding. Three main inhibitors of fibrinolysis exist that are directly linked to the pathway itself, plasminogen activator inhibitor-1 (PAI-1), α2-antiplasmin (α2AP) and α2-
macroglobulin ($\alpha_2$M). PAI-1 is the most prominent of these inhibitors and can be found in plasma, platelets, placenta and the extracellular matrix, however it is not stored in these cells but is synthesised and secreted as an active inhibitor. One exception to this is in platelets where it is stored in $\alpha$-granules, which secrete PAI-1 upon platelet activation (Lijnen and Collen, 1995). PAI-1 initially forms a 1:1 reversible complex with TPA, followed by a covalent complex, where the hydroxyl group of TPA's active site forms an ester bond with the carboxyl group of PAI-1's active centre. Hydrolysis of the ester bond causes cleavage and inactivation of the TPA molecule, inhibiting the process of clot dissolution and promoting coagulation (Rijken, 1995). PAI-1 has its own positive and negative regulators originating from the coagulation pathway and the renin-angiotensin system (RAS). The RAS involves the conversion of Angiotensin I to Angiotensin II (Ang II) on the endothelium, a reaction that is catalyzed by the enzyme Angiotensin Converting Enzyme (ACE) (Dzau, 2001). ACE is also involved in the catabolism of Bradykinin, one of the proteins of the contact pathway of the coagulation cascade (Hassan and Markus, 2000). The RAS exerts its effects on fibrinolysis via Ang II, a vasoconstrictor that functions at the local vascular level by influencing endothelial function and smooth muscle proliferation and regulating vascular tone. It causes the release of PAI-1 inhibiting fibrinolysis and promoting coagulation (Vaughan, 2002). Positive regulation occurs as a result of increased coagulation via the production of Bradykinin that induces the release of TPA, activating the system of clot degradation. However, ACE acts to inhibit this process by degrading Bradykinin and preventing the release of TPA. Therefore, increased ACE levels have the potential to downregulate fibrinolysis and promote coagulation. In fact ACE inhibitors have been used to induce fibrinolysis in patients suffering from ischemic events such as stroke and cardiovascular injury (Vaughan, 2002).

If the fibrinolytic pathway proceeds beyond the point of plasmin formation, $\alpha_2$-antiplasmin ($\alpha_2$AP) and $\alpha_2$-macroglobulin ($\alpha_2$M) provide further regulation. The $\alpha_2$AP glycoprotein contains a region capable of reacting with the lysine-binding sites of both plasminogen and plasmin, forming tight bonds with these proteins and preventing exposure of the active site (Lijnen and Collen, 1995). $\alpha_2$M also inhibits plasmin but it does not have as high an affinity for its active site as $\alpha_2$AP does, making it less specific (Roberts and Tabares, 1995). Further regulation is achieved by the protein Thrombin Activable Fibrinolysis Inhibitor (TAFI), which is similar to PC in that it is a macromolecular substrate for the T/TM complex, competing with PC for binding. It is
upon binding to this complex that TAFI becomes activated and downregulates fibrinolysis, by removing the lysine and arginine residues from the carboxy terminal of fibrin, those that are pivotal to plasmin binding and subsequent fibrin degradation (Nesheim et al., 1997). Protein C inhibitor (PCI) or plasminogen activator inhibitor-3 (PAI-3) is a serine proteinase that functions as an anticoagulant by downregulating thrombin, FXa and FXIa and as an procoagulant by inhibiting both PC and APC and by inhibiting the T/TM complex forming. As an antifibrinolytic agent it has been reported to inhibit TPA and UPA as well as preventing TAFI activation via the T/TM complex, therefore having profibrinolytic properties also. However, its physiological role and mode of action is not fully understood but its action has been reported as enhanced in the presence of heparin and other glycosaminoglycans (Meijers et al., 2002).
Figure 1.4: The Fibrinolytic pathway and its regulators. uPA/tPA = urokinase/ tissue plasminogen activator; $\alpha_2$AP=$\alpha_2$Antiplasmin; $\alpha_2$M=$\alpha_2$Macroglobulin; PAI-1=plasminogen activator inhibitor-1.
1.6.0: The coagulation & inflammation crosstalk

The endothelium and the monocyte link coagulation and inflammation by several connecting points joining them in a unique host defence mechanism. Upon tissue damage, the surfaces of endothelial cells and monocytes express cytokines that have many functions. Pro-inflammatory cytokines induce TF production on these cell surfaces upon tissue damage, initiating the extrinsic pathway of the clotting cascade. Such cytokines also downregulate the expression of TM inhibiting the anti-coagulant pathway. Coagulation products themselves can influence inflammatory mechanisms, with platelet adhesion promoting the production of substances involved in maintaining tissue integrity and cytokines at the site of inflammation. Moreover, thrombin has been found to be chemotactic for monocytes and neutrophils, and to date there have been numerous reports of it stimulating the expression of several cytokines on the surface of monocytes and endothelial cells (Cicala and Cirino, 1998). Therefore cytokines of the inflammatory response impair the natural balance within coagulation and products of the coagulation pathway influence the expression and secretion of inflammatory proteins at the site of tissue damage.

1.6.1: The Immune system

The immune system is a complex defence mechanism protecting the host from invading pathogens, tumours, non-self molecules, infections and tissue damage, via two processes, innate immunity and acquired or antigen-specific immunity (Kuby, 1994). The effector cells of an innate immune response include natural killer (NK) cells, neutrophils, eosinophils, basophils, mast cells and professional antigen-presenting cells (A-PCs), that fight pathogens and upregulate the activation of antigen-specific or acquired immune responses (Hale and Haynes, 1999). The acquired or antigen-specific immune response involves a system that generates an immunological memory against the invading agent and is mediated by T and B lymphocytes, T lymphocytes orchestrating the cellular immune response and B lymphocytes functioning in humoral immunity (Hale and Haynes, 1999; Kuby, 1994).

1.6.1.1: T cells & Immunity

Armed effector T cells produce two types of effector molecules following cellular activation, cytotoxins and cytokines. Cytotoxins are released by cytotoxic CD8+ T cells (Tc cells) and are stored in specialised granules, while cytokines and related membrane-associated proteins are synthesised de novo by all effector T cells and are
the key mediators of CD4⁺ T cell or T helper (Th) cell effector actions (Janeway et al., 2001). Cytokines are polypeptide molecules that exhibit pleiotropic activity by acting on several types of cells, stimulating various cellular functions (Krasnow et al., 1996; Lim et al., 1996; Sundy et al., 1999). They control local and systemic immune responses, inflammatory reactions, healing and haematopoiesis and are found in practically every nucleated cell in the body, although they are mainly produced by cells of the immune system (Krasnow et al., 1996; Lim et al., 1996).

Two types of T helper cells were found to exist after murine CD4⁺ Th cells were stimulated in vitro with particular antigens. Th cells produced a restricted and typical pattern of cytokine secretion allowing for the designation of type 1 T helper (Th1) cells and type 2 T helper (Th2) cells (Mosmann et al., 1986). Type 0 T helper (Th0) cells have been observed to possess cytokine patterns similar to both Th1 and Th2 cells and are thought to be partially differentiated T helper cells (Romagnani, 1999) or T cells at different stages of differentiation (Mosmann and Sad, 1996). Moreover, cells other than lymphocytes produce many of these cytokines, complicating the matter further. This raises the question, does the cytokine secretion pattern of T cells represent different sub-sets of cells, or are they merely a continuum of cells at different stages of differentiation with different combinations of cytokine secretion? Regardless of the true answer, there is no doubt that a dichotomy exists between IL-2/IFNγ/TNFβ secretion and IL-4/IL-5/IL10 secretion since these groups of cytokines induce opposite effects. For this reason the nomenclature Th1 and Th2 is oversimplified, therefore these cell types are referred to as type 1 (Th1-type) and type 2 (Th2-type) cells (Mosmann and Sad, 1996).

1.6.1.ii: T cell cytokine secretion

Cytokines secreted by type 1 cells mediate macrophage activation and delayed-type hypersensitivity (DTH) reactions or cellular immunity, while those secreted by type 2 cells act as growth and differentiation factors for B cells. Therefore, it has been suggested that a type 1 response is indicative of cellular immunity while a type 2 response is characteristic of humoral immunity (Romagnani, 1999). Cytokines and chemokines (molecules that attract neutrophils and monocytes from the bloodstream), released by macrophages in response to host invasion, induce a process known as inflammation. Inflammation is traditionally defined in Latin by the words calor, dolor, rubor and tumor, meaning heat, pain, redness and swelling. All of these events are
typical of the effects that cytokines and inflammatory mediators have on the blood vessels. The inflammatory response induces dilation and increased permeability of blood vessels, causing increased blood flow and leakage of fluid, which is responsible for the heat, redness and swelling. The pain is due to the migration of leukocytes across the epithelial wall to the site of infection in response to cytokine and chemokine stimulus. Lymphocytes are employed late in the inflammatory response, secreting cytokines that can be either pro-inflammatory, ant-inflammatory or both (Janeway et al., 2001).

Inflammatory responses are not just limited to host invasion by exogenous pathogenic microorganisms. Tissue damage also elicits an inflammatory response where there has been no exogenous invasion of bacteria. During such damage, the vascular epithelial wall becomes exposed allowing opportunistic endogenous microorganisms to infect the blood vessels. If this occurs the serious medical condition of sepsis, or blood poisoning, may ensue. This is a classic situation where by the inflammatory pathway becomes activated. It is widely accepted that fibrin accumulation occurs during cell-mediated immune reactions as a result of coagulation activation via TF. TF is not endogenous to endothelial cells but upon tissue damage, they are induced to express TF on their surface, giving monocytes and macrophages procoagulant activity (del Prete et al., 1995). Furthermore, sepsis has been associated with increased activation of the coagulation pathway (ten Cate et al., 1997) and cytokines have been implicated in the progression of thrombotic disease where there is a tendency to clot (Dosquet et al., 1995).
Figure 1.5: T helper cell differentiation and typical cytokine release pattern. Red links indicate inhibition. Black arrows represent activation pathways. A-PC = Antigen presenting cells; IL-4R = IL-4 receptor; IFNγR = IFNγ receptor [adapted from Theofilopoulos et al. (2001). The role of IFNγ in systemic lupus erythematosus: a challenge to the Th1/Th2 paradigm in autoimmunity. *Arthritis Research*: 3; 136-141].
1.6.2: The Sepsis model

The toxic qualities of *E. coli* lipopolysaccharide (LPS) have been exploited to induce sepsis *in vitro* and *in vivo* and used as a model of disease. Sepsis by *E. coli* stimulus is in fact an acute inflammatory disease of the vascular endothelium whereby the endothelium itself participates in the progression of disease. Pro-inflammatory markers override the normal anticoagulant protection of the endothelium causing it to develop a procoagulant phenotype (Taylor, 1994). In this respect sepsis induces an autoimmune condition of the endothelium.

Human studies of endotoxemia using non-lethal doses of LPS as a stimulant have revealed that both the coagulation cascade and the fibrinolytic pathway are dysregulated. Early studies of endotoxemia in baboons using lethal and non-lethal doses of *E. coli* derived toxin found consumptive coagulopathy in the form of decreased fibrinogen concentrations (Pixley *et al.*, 1992; Taylor, 1994). It became apparent that TF may be the route of coagulation activation in sepsis, since antibodies against TF and its associated proteases (FVII and TFPI) when administered to baboons, resulted in no coagulation activation, no development of DIC and complete protection from sepsis lethality, while FXII antibodies did not infer this protection (Creasey *et al.*, 1993; Taylor *et al.*, 1991). In similar experiments, when FXa antibodies were administered, DIC was inhibited but lethality still occurred suggesting that TF influences other inflammatory responses that lead to lethality in baboons (ten Cate *et al.*, 1997). Therefore, TF appears to be a key mediator in the coagulatory/inflammatory response. Furthermore, inflammatory cytokines have been found at increased levels during systemic infection in both human and animal experimental studies, with TNFα predominating and inducing the production of IL-1β and a number of other cytokines (pro- and anti-inflammatory). Together these two pro-inflammatory cytokines have been shown to induce the expression of TF on the surface of monocytes and endothelial cells in response to tissue damage (Mantovani *et al.*, 1997). In addition, a number of investigations have demonstrated varying effects on coagulation and fibrinolytic proteins in response to inflammatory cytokine stimulus, including decreased TPA and TM and increased PAI-1 (table 1.1). These dysregulated processes cause decreased fibrinolysis and decreased anticoagulation, creating a potentially lethal prothrombotic environment. This effect is particularly potent with the downmodulation of TM transcription by TNFα, preventing activation of the PC anticoagulant pathway in an already prothrombotic setting (Okajima, 2001).
1.6.3: Influence of coagulatory proteins on inflammation

Coagulation proteins are capable of regulating inflammatory proteins, since thrombin itself potentiates pro-inflammatory effects by activating platelets, which in turn promote TNFα and IL-1 production. Thrombin has been shown to be chemotactic for monocytes and neutrophils and it increases TNFα and IL-1 induced neutrophil chemotaxis. It has also been shown to have vasoactive properties. Therefore thrombin is an important mediator of cellular inflammatory responses (Cicala and Cirino, 1998). The anticoagulant effects of AT also have knock on effects towards inflammation. This is apparent via the action of FXa, which has pro-inflammatory effects by stimulating the production of IL-6, IL-8, and adhesion molecules (Senden et al., 1998). Inhibition of FXa by AT may inhibit these pro-inflammatory reactions. Recently there has been evidence of the anti-inflammatory properties of AT independent of its role in inhibiting fibrin formation by inhibiting activation of the transcription factor NFκB, involved in the regulation of cytokine gene expression, and also inhibiting LPS induced TNFα and IL-6 production in monocytes (Oelschlager et al., 2002; Okajima and Uchiba, 1998). In addition, heparin, the potent enhancer of AT anticoagulant processes, has also been shown to have anti-inflammatory properties by reducing the enzyme heparinase, which is secreted by leukocytes to enable attachment to the endothelial surface (Bannon et al., 1995), and inhibiting the synthesis of pro-inflammatory cytokines (Cahalon et al., 1997; Salas et al., 2000; Wan et al., 2002).

APC is also emerging as having anti-inflammatory properties as well as anti-coagulant functions. It has been reported to inhibit the inflammatory response in THP-1 monocyteic cells by preventing the nuclear translocation and subsequent activation of NFκB and resulting TNFα production (White et al., 2000). In addition, several studies have demonstrated reduced lethality in induced sepsis after administration of APC (Taylor et al., 1984; Taylor et al., 1991; Taylor et al., 2000). Furthermore, recombinant human soluble TM has been shown to inhibit LPS-induced pulmonary injury via activation of APC and subsequent inhibition of leukocyte activation as a result of TNFα inhibition on monocytes. This effect was not observed when the APC activation ability of TM was blocked (Murakami et al., 1997; Uchiba et al., 1997). Therefore APC has a pivotal role to play in the progression of inflammatory disease independent of its role in anticoagulation. Furthermore, administration of TFPI to rabbits subjected to lethal doses of E.coli exhibited reduced mortality and reduced coagulation abnormalities (Carr et al., 1994).
The natural anticoagulants have been shown to have both anti-inflammatory abilities as well as coagulation regulation properties, displaying specific cellular interactions that may be involved in controlling cell activation processes (figure 1.6). They have potential as therapeutic agents in the treatment of diseases associated with thrombosis and inflammation whereby the interaction of the coagulation and inflammation pathways leads to progression of disease and subsequent morbidity and mortality (Esmon, 2001).
Table 1.1: Studies of cytokine influence on coagulation and fibrinolytic factors in sepsis. TPA= tissue plasminogen activator; PAI-1= plasminogen activator inhibitor 1; F1+2= Prothrombin fragments 1+2; PCP= Protein C activation peptide; FPA= Fibrinopeptide A; TM= Thrombomodulin; PAP= Prothrombin activation peptide; ↑ = increase; ↓ = decrease.

<table>
<thead>
<tr>
<th>Study</th>
<th>Cytokine</th>
<th>Haemostatic Factor effected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schleef et al. (1988)</td>
<td>TNF &amp; IL-1</td>
<td>tPA ↓, PAI-1↑</td>
</tr>
<tr>
<td>Bauer et al. (1989)</td>
<td>TNF</td>
<td>F1+2, PCP, FPA ↑</td>
</tr>
<tr>
<td>van Hinsbergh et al. (1990)</td>
<td>TNF</td>
<td>uPA ↑</td>
</tr>
<tr>
<td>Lentz et al. (1991)</td>
<td>TNF</td>
<td>TM ↓</td>
</tr>
<tr>
<td>Niedbala &amp; Picarella (1992)</td>
<td>TNF</td>
<td>uPA ↑</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>uPA ↓</td>
</tr>
<tr>
<td>Prete et al. (1995)</td>
<td>Type 1 cells (proinflam)</td>
<td>TF ↑</td>
</tr>
<tr>
<td></td>
<td>Type 2 cells (anti-inflam)</td>
<td>TF ↓</td>
</tr>
<tr>
<td>Arnman et al. (1995)</td>
<td>TNF/LPS</td>
<td>PAI-1/2 ↑, uPA ↑</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>PAI-2 ↑↑, uPA ↓</td>
</tr>
<tr>
<td>Gallicchio et al. (1996)</td>
<td>TNFα &amp; IL-1α</td>
<td>PAI-1↑</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>PAI-1↓ (independent of TNF)</td>
</tr>
<tr>
<td>Pajkrt et al. (1997)</td>
<td>IL-10</td>
<td>TPA, PAP, PAI-1 ↓</td>
</tr>
</tbody>
</table>
Figure 1.6: Regulation of inflammation and coagulation by natural anticoagulants. [Taken from Okajima (2001). Regulation of inflammatory responses by natural anticoagulants. *Immunological Reviews* :184; 259-274].
1.7.0: The Crosstalk in Thrombotic Disease

The increasing association of vascular complications with inflammatory diseases has generated interest in the interaction between these processes in the development of specific diseases. To investigate the mechanism of this crosstalk in thrombotic disease, it is first necessary to understand the normal non-pathogenic interactions that occur between coagulation and inflammation pathways \textit{in vivo}. A good model for such a study is pregnancy. Not only is the pregnant state a non-pathogenic hypercoagulable one, but it is also an immunological phenomenon, with the acceptance of foreign material within the maternal immune system for an extended period of time and no resulting pathological phenotype. Furthermore, recurrent foetal loss has been associated with gestational thrombophilia and imbalances in the immune response during pregnancy (Hill \textit{et al.}, 1995; Preston \textit{et al.}, 1996). Therefore, knowledge of coagulatory and inflammatory processes occurring in normal pregnancy will enable investigation of dysregulation leading to the development of pathogenic states such as recurrent miscarriages.

Venous thromboembolism is a multifactorial disorder and is a major complication of many other multifactorial disease states that are more traditionally associated with disturbances in inflammatory processes. Such diseases are therefore influenced by both environmental and genetic factors, with inherited factors having a life long impact on disease susceptibility. A number of genes have been implicated in the predisposition to thrombotic disease including those encoding coagulatory and fibrinolytic proteins. Similar markers have been located within genes encoding inflammatory proteins, and have been implicated in the progression of inflammatory diseases. However, recent studies have limited investigations of such genetic markers to within the more traditional thrombotic and inflammatory diseases respectively. This study aims to assess such parameters in several disease states complicated by thrombotic episodes, namely recurrent foetal loss, venous thromboembolism, ishemic stroke and inflammatory bowel disease. It is hoped that these investigations will shed greater light on the phenomenon of coagulatory and inflammatory interactions.

However, to study the role that genetic markers play in disease predisposition and pathophysiology, it is first important to understand the mode of inheritance of such genetic markers, and how these markers can be investigated.
1.8.0: Inheritance & Genetic Polymorphism

Our extensive knowledge of genes, chromosomes and modes of inheritance all stems from the original works of the Austrian monk, Gregor Mendel, who in 1865 published his cross-breeding experiments on the garden pea (Mueller and Young, 1995). Through his work and the subsequent investigations of other Scientists later in the 1900’s, the ‘laws of inheritance’ for many organisms were established. The discovery of chromosomes and the genes they house led to the explosive study of inheritance or Genetics (Brown, 1996). One of the laws that Mendel speculated upon was the law of segregation which stated that each individual organism possesses two genes for a particular trait or characteristic (e.g.: tallness and shortness) and that only one of these genes can be transmitted at any one time. We now know that each gene within eukaryotic organisms can exist in two or more forms called alleles. During cell replication, the splitting of the chromosome dictates which allele will be transmitted to the progeny’s cell and subsequently which trait will be expressed by the offspring. Furthermore, the laws of inheritance suggest that siblings will have related alleles, creating only four possible alleles in one nuclear family for a given gene, two from the maternal X chromosomes and one each from the paternal X and Y chromosomes. In addition, an allele for a particular genetic trait may be expressed as dominant or recessive, leading to two possible types of allele pairs, homozygotes (two of the same alleles) or heterozygotes (two different alleles) (Brown, 1996). In the case of recessive alleles, an individual may appear not to be carrying that allele type, since the dominant allele is being phenotypically expressed, which is often the case in heterozygote genotypes (Mueller and Young, 1995).

These basic laws of inheritance have led to the discovery that the human genome is highly variable as no two individuals have identical genomic sequences, due to this phenomenon of two or more allelic variants at a particular genetic locus. Today, these variants in allele type are referred to as polymorphic regions of the gene. A genetic polymorphism is defined as ‘...the occurrence in a population of two or more genetically determined forms, in such frequencies that the rarest of them could not be maintained by mutation alone’ (Mueller and Young, 1995). These variants are often due to single nucleic acid differences between individuals, insertion or deletion of nucleic acids, or the occurrence of a repeat region of a particular segment of nucleotides at a given genetic locus. If these differences occur within a region of the genomic sequence that encodes the site of action of enzymes that cut DNA (restriction
enzymes), then exposure to such enzymes will produce different sized fragments of DNA for each genotype. This method of genome analysis is called Restriction Fragment Length Polymorphism (RFLP) analysis, and is exploited in molecular genetics, when such RFLP patterns occur at a mutation site, that is responsible for altering protein production (Mueller and Young, 1995). In these types of tests, three genetic profiles are created from the different patterns of enzyme cutting. Homozygote wild types (WT) occur when both alleles are the same possessing the normal genetic sequence, while heterozygote individuals possess two different alleles, one representing a base pair or sequence difference. Homozygote mutant individuals are those with both alleles the same but representing the changed sequence. This is the basis of genotyping a population or a family for a given genetic variant to determine its role in the health of these individuals.

1.9.0: Genetic Epidemiology

Such advances in human genetics, the increasing information emerging from the Human Genome Project, and the ever changing environment, are all factors that are today influencing the extraordinary changes occurring in the health sector in relation to how diseases are being treated and studied. To date, thousands of gene variants associated with both rare diseases and with susceptibility to common chronic diseases have been discovered, resulting from inherited gene interactions and gene environment interactions. These discoveries have led to the development of a unique medical discipline called genetic epidemiology (Khoury et al., 2000). Genetic epidemiology is the study of the familial distribution of traits, and how they interact with the environment to produce various disease phenotypes in humans, in particular in the case of multifactorial disorders such as thrombotic disease (Washington, 2003). It endeavours to elucidate the potential influence of gene-gene and gene-environment interactions, determining the cause, incidence, distribution and control of disease in groups of relatives and of inherited causes of disease in populations (Kaprio, 2000).

With the increasing knowledge emerging from genetic research, there is a greater need for an understanding of how to use this genetic information to promote health and prevent disease, the ultimate goal of public health initiatives. However, in certain disciplines and for some disease types, this goal is difficult to achieve. This may be due to a lack of population-based information about distribution of genotypes in and between different populations and a lack of knowledge about the significance of these
genetic variants in relation to the disease in question. Genetic epidemiology studies provide a link between the public health sector and the science sector and this area is often referred to as ‘...the scientific core of public health’ (Khoury et al., 2000). For these reasons, population based epidemiology studies are required to quantify the impact of genetic variants on the risk of disease occurrence, morbidity and mortality. These studies are also necessary to identify and quantify the impact of modifiable risk factors that interact with gene variants, and that may be used to help preventative interventions. In addition, such information has the potential to help medical professionals better target behavioural and environmental interventions, aiding in achieving public health goals.

### 1.10.0: Patient sampling & statistical analysis in population-based epidemiology studies

Two major types of population-based studies exist that provide the information necessary to perform genetic epidemiological studies. They are cohort studies and case-control studies. A cohort study is one where two or more groups of patients are studied over a period of time, measuring parameters in each group before the disease has affected the study group, or while the disease phenotype is beginning to manifest itself. These types of studies are often termed prospective. In contrast, a case control study involves the sampling of patients after they have been exposed to the disease, making them retrospective in nature. In this thesis, both types of studies will be employed. The cohort study will be used to compare the changes throughout pregnancy in pregnant women with a history of recurrent foetal loss and in pregnant women with no history of previous losses. The case control type of study design will be used to assess the frequency of genetic variants in disease populations and compare them to those in control populations. For each study type, the sample size and the study type dictate the kind of statistical analysis that will be performed. In cohort studies, the sample size is usually several orders of magnitude higher than case-control studies, and often sampling spans a number of years. Cohort sample numbers must be large enough to allow a sufficient number of end points to develop in all groups being studied (i.e.: a sufficient number of disease affected individuals). Ideally a cohort study will have numbers in the thousands, but this is often difficult to achieve when performing clinically based research, in particular in the case of rare diseases. In contrast, case-control studies only require sample numbers in the hundreds, as these are merely representative of the disease population, measuring disease risk based on frequency.
differences (Daly and Bourke, 2000). The most commonly encountered problem in increasing and maintaining sample sizes in cohort studies is the losses that occur due to patient drop out, or inconsistent involvement in the sampling procedure. In addition, the unsuitability of certain patients for inclusion in such studies often only becomes apparent at the time of sampling, which can be the case when patients react badly to blood sampling by fainting or when the patients veins are not sufficiently stable to withstand repeat sampling. For these reasons, statistical methods exist that account for this eventuality in clinical research.

Another problem with clinical research data is the behaviour of the results. The majority of statistical tests presume that the data will be distributed normally, with a representative bell shaped curve indicating the range of data increasing and subsequently decreasing over a given population. These tests are referred to as parametric tests of significance (analysis of variance, ANOVA). However in clinical studies, in particular with cohort studies, the data does not always conform to this assumption and is not normally distributed. Non-parametric tests of significance allow for this and make no assumptions about the performance of the data. These types of ANOVA methods also allow the analysis of data when the sample size is very small. This is particularly useful for clinical studies, when sample sizes are difficult to increase due to the reasons mentioned previously. In this way, clinical data from both cohort and case-control studies can be analysed more accurately. In this thesis, the Two-Way Chi square ($\chi^2$) non-parametric test will be used to create a cross-tabulation of independent variables (disease and control group) against dependent variables (genotype or allele). This method will enable the determination of the significance of the difference between the frequencies of genotypes and alleles in the disease group and those frequencies in the control group. The greater the difference between these frequencies, the higher the significance will be, represented by a value denoted the $p$ value. In situations where the expected or control frequency falls below a certain figure during cross-tabulation, then the Fischer’s Exact test is employed, which is thought to be more accurate under these circumstances. The Chi square test is used extensively in statistics as it is undemanding on the data and does not assume normal distribution. However, it does not work well for small sample sizes so will be limited to the case-control studies. For the cohort study, where sample sizes will be limiting, a similar analysis of variance method will be performed which will take into account the small sample size and the rank of the individual data points. This is important when data is
spread out over a wide range deviating largely from the mean, which is often the case in clinical studies. The Kruskal-Wallis One-Way ANOVA by Ranks test is such a test that examines the median of data points between independent samples. It is used when there are at least six cases in each group to be studied. This test takes into consideration the rank of each point and how far these points deviate from the mean value, indicating if the ranked values for a variable are equally distributed throughout the samples studied (Levin and Fox, 2000). The final type of analysis that will be employed in this study is one that is used when a parameter has been measured on the same patient over a period of time. This is called a Repeated Measures ANOVA test. In this type of statistical analysis, the equality of the mean value for a given patient over time is tested as normal. However, this test accounts for the variance that may occur between patients being tested for the same parameter repeatedly, thus reducing the error variance between samples, which would ordinarily be large if the standard ANOVA methodology were used. The repeated measures ANOVA test is particularly useful for longitudinal studies such as the cohort study in this thesis. This is because it is especially designed to account for difficulty in subject recruitment, allowing an economical method of testing each patient under all conditions studied (i.e.: at each time point throughout pregnancy) (StatisticalServices, 1997).

By utilising such statistical tools, clinical research can be transformed into genetic epidemiological data, providing the necessary link between science, medicine and the public health sector. In this way, the impact of both genetic and environmental factors on disease pathophysiology can be determined accurately, allowing the development of more personalised medicine. Therefore, this thesis aims to investigate the genetic epidemiology of the aforementioned coagulatory and inflammatory disorders, to contribute to the overall knowledge of how these systems interact in pathophysiological circumstances, and to pave the way for the development of greater therapeutic care.
AIMS OF STUDY

• To assess changes in haemostatic and immunological factors throughout normal pregnancies, determining the normal pregnant range for such parameters.

• To evaluate differences in haemostatic and immunological factors throughout pregnancies of individuals with previous pathological pregnancies to ascertain areas of dysregulated control.

• To determine the incidence of inherited thrombophilias in an Irish population suffering venous thromboembolism and to compare the prevalence of polymorphisms within coagulatory genes in such patients with the Irish population.

• To evaluate the incidence of polymorphisms within coagulatory and fibrinolytic genes in a cohort of patients suffering cerebrovascular accident.

• To determine the prevalence of coagulatory and fibrinolytic polymorphisms within a cohort of patients suffering inflammatory bowel disease.
CHAPTER 2

MATERIALS & METHODS
2.1.0: PATIENT SAMPLING & PROCESSING

2.1.1: Blood Donor Controls

Ethical approval was obtained from all donor clinics and hospitals. Irish population control samples were obtained from the Irish Blood Transfusion Service (IBTS) and the Northern Ireland Blood Transfusion Service (NIBTS). 390 whole blood samples were collected from donor clinics in Dublin, Cork, and mobile units throughout the Republic (n=330), and clinics throughout all counties of Northern Ireland (n=60). The female to male distribution of samples was 164: 226 respectively (42%: 58%). The mean age of samples was 37 years with a range of 18-65 years of age.

2.1.2: Guthrie Card Controls

A second Irish control population was obtained via the National Newborn Screening Program for Inherited Metabolic Disorders. This program routinely samples all newborns from all maternity hospitals across the country. Heel prick blood samples were collected from 1000 newborns on Guthrie cards (blotting paper) using a random number generator, taking into consideration the proportion of births at different hospitals. Almost 100% of newborns in Ireland are included in this program so a representative sample of the entire Irish population was collected for the years 1984 and 1994.

2.1.3: Venous Thromboembolic (VTE) cohort

VTE study patients were collected from the thrombosis clinic at the National Centre for Hereditary Coagulation Disorders (NCHCD), St. James’s Hospital, Dublin 8. 238 VTE patients (DVT and PE) were collected from the clinic between 1998 and 2001. Venous puncture peripheral blood samples were taken in EDTA and citrate anticoagulated vacutainers and transported immediately to the laboratory. EDTA samples were stored at -20°C for DNA extraction. Citrated bloods were spun immediately at 2000g and plasma aliquoted for phenotype (hypercoagulation screens) analysis. Genetic and biochemical data were analysed retrospectively.
2.1.4: Stroke cohort

103 patients suffering from stroke were recruited from St Vincent’s Hospital, Dublin. The stroke study was approved by the hospital ethical committee, and consent was obtained from all participating subjects. Hypertension, smoking status and a history of previous myocardial infarction or stroke with and without residual disability was recorded for each patient. Venous puncture peripheral blood samples were taken in EDTA anticoagulated vacutainers.

2.1.5: Inflammatory Bowel Disease cohort

IBD patients were recruited from St. James’s Hospital, Dublin 8. 120 patients with Crohn’s Disease (n=46) and Ulcerative Colitis (n=74) were collected and analysed for homocysteine, plasma folate and vitamin B\textsubscript{12} levels. Venous puncture peripheral blood samples were taken in EDTA and citrate anticoagulated vacutainers. Citrate samples were transported to the routine folate laboratory immediately where homocysteine and folate measurements were recorded.

2.1.6: Recurrent Foetal Loss patients (St. James’s Hospital)

Recurrent foetal loss patients were recruited from the thrombosis clinic at the NCHCD, St. James’s Hospital, Dublin 8. Venous puncture peripheral blood samples were taken from 73 patients in EDTA anticoagulated vacutainers and transported to the research laboratory for genetic analysis. DNA was extracted and stored at $-20^\circ\text{C}$ while the remaining blood was stored at $-70^\circ\text{C}$ for future use.

2.1.7: Prospective Sampling for Pregnancy Study

Two Obstetric and Gynaecology specialists, a dedicated midwife, a Haematology Consultant and the researcher undertook a three year study of pregnancy. Ethical approval was obtained and a patient information sheet and approval of consent form were drawn up and handed to the participant upon attending the clinic. A patient questionnaire was designed to obtain the personal and familial thrombotic history of each participant at each visit to the clinic [Appendix figures A.1.1 and A1.2].

To ensure the samples were collected, processed and stored correctly, it was necessary for the researcher to attend the Pregnancy Loss Clinic at the Rotunda Hospital once weekly for the duration of the clinic and the study. Once the patient had been examined by the clinician and it was decided that they were eligible and willing to join the study
for its duration, the designated phlebotomist took the appropriate blood samples from each patient. Immediately after sampling, the blood samples were removed to the haematology laboratory at the Rotunda Hospital.

Venous puncture peripheral blood samples were collected from patients and controls attending a Pregnancy Loss Clinic from January 1999 until January 2002. Clotted blood was collected along with EDTA and citrate anticoagulated samples from each individual at approximately 8 week intervals from week 8 of gestation through to week 36 and finally at three months post-partum. Patient questionnaires and samples were labelled with the corresponding laboratory number. Clotted samples were centrifuged immediately @ 2000RPM for 10 minutes and the serum supernatant removed, aliquoted and placed on dry ice (-80°C). Citrate anticoagulated blood samples were double centrifuged @ 4000RPM and the plasma aliquoted and transferred to dry ice for rapid freezing and transportation. EDTA blood was aliquoted for genetic and routine haematology analysis. Haematology tests were performed on the day of sampling. Samples were labelled by a number only system once aliquoted. All samples were stored @ -70°C until analysed. A database was updated weekly with the questionnaire information obtained from each patient. The previous week's results were logged into the database and all questionnaires and results were filed securely.

15 pregnant women with a history of RFL (≥ 3 consecutive losses) and 66 pregnant women with no previous history of pregnancy loss were recruited at the Rotunda Maternity Hospital from January 1999 until June 2002. Due to the small number of RFL patients it was not possible to age match patients and pregnant controls. Of the 15 patients studied, only 2 (13%) were of non-Irish nationality (both Eastern European). Two patients experienced second trimester losses while the remainder experienced previous first trimester losses. Nine of the 15 patients had a family history of either arterial or venous thrombosis while eight of these included a family history of one or more spontaneous foetal losses. The average age was 31 years for RFL patients (range 27-41 years) and 29 years for pregnant controls (range 17-42 years). All patients recruited with a history of RFL carried their pregnancies to full term within this study and gave birth to healthy babies. Blood samples were analysed for hypercoagulability by testing at each visit for the following coagulation markers; AT, FVIII, PC, PS, APCR:FV ratio, Fibrinogen, activated partial thromboplastin time (APTT), prothrombin time (PT) and lupus anticoagulants. Full blood counts (FBC) were performed on all
patients at each visit, indicating the following cellular levels: white and red cells, haemoglobin, haematocrit ratio (HCT), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red cell distribution (RDW) and platelet counts.

Non-pregnant female controls were recruited (n=65) from volunteers at the Rotunda Maternity Hospital and the Genetics Department, TCD (range 21-40 years). All three patient groups involved in the prospective pregnancy study were analysed for the presence of inflammatory markers using cytokine immunoassays for IL-10, IL-6, TNFα and IFNγ (see ELISA assays section later).

2.2.0: DNA EXTRACTION

2.2.1: Crude Extracts for IBTS controls

5ml of whole blood was lysed and incubated overnight with proteinase K and 10% SDS. DNA was extracted by phenol/chloroform precipitation and resulting pellets were washed in 70% ethanol (Appendix A.2.1.1). Samples were stored at -20°C.

2.2.2: Phenol/Chloroform extracts for Guthrie cards and whole blood patient/control samples.

One punch from a Guthrie card or 200μl of EDTA anticoagulated whole blood was incubated with 0.5% SDS TNE lysis buffer (Appendix table A.2.2) and proteinase K (20mg/ml) overnight in a 37°C water bath (Grant Instruments Ltd., Cambridge). DNA was extracted by phenol/chloroform precipitation and incubated with NaAc (pH 5.2) and ice-cold 100% ethanol at -20°C overnight or -70°C for 30 minutes. DNA pellets were washed twice with 80% ethanol and stored at -20°C until use (Appendix 2.1.2.i).

2.2.3: QIAamp™ DNA Mini kit for DNA extraction of VTE patient samples (Qiagen, Crawley, West Sussex).

200μl of whole blood was lysed in the presence of proteinase K (20mg/ml Qiagen) and lysis buffer (Buffer AL) by incubation at 70°C for 10 minutes. 70% Ethanol was added and DNA collected in a Qiagen spin column by centrifugation at 12,000xg. DNA was washed (Buffer AW) and collected in an elution buffer (Buffer AE). Samples were stored at -20°C until use (Appendix 2.2.1.ii).
2.3.0: ROUTINE HAEMATOLOGY & COAGULATION TESTS

All routine haematology tests (full blood counts) were carried out by staff of the Department of Haematology, St. James’s Hospital. Hypercoagulation screens were carried out by staff of the Coagulation laboratory at the National Centre for Hereditary Coagulation Disorders (NCHCD), St. James’s Hospital, Dublin.

2.3.1: Full blood Counts

All tests were performed automatically using the Sysmex 2100 instrumentation. Blood tubes were agitated on a rocker before direct insertion into the instruments blood tube racks.

2.3.1.i: Leukocyte counts

White cells (WCC) were counted by firstly removing the red cells from EDTA anticoagulated peripheral blood samples using a lysing agent. Normal adult ranges for the WCC are 4.0-11.0 x 10^9 cells per litre. Differential leukocytes were counted by flow cytometry. A differential cell count includes neutrophils, lymphocytes, monocytes, eosinophils and basophils. The normal adult ranges of each cell type are as follows: neutrophils = 2.0-7.5 x 10^9 cells per litre; lymphocytes = 1.5-4.0 x 10^9 cells per litre; monocytes = 0.2-0.8 x 10^9 cells per litre; eosinophils = 0.0-0.4 x 10^9 cells per litre; basophils = 0.0-0.1 x 10^9 cells per litre.

2.3.1.ii: Haemoglobin & Red Cell Count

The haemoglobin (Hb) content of a blood sample gives an estimation of the oxygen capacity of red cells while the red cell count (RCC) gives an estimation of the oxygen capacity of the blood. The RCC and Hb content of blood samples were obtained directly from the automated system. Normal adult ranges of red cells are 3.8-5.8 x 10^{12} cells per litre. Normal adult Hb ranges are 11.5-16.5g/dL.

2.3.1.iii: Haematocrit

The haematocrit (HCT) test measured the packed cell volume (PCV) of a blood sample and provided a simple screening test for anaemia. Blood samples were centrifuged in haematocrit tubes and the height of the column of red cells was taken as the packed cell volume. This was the volume occupied by the red cells expressed as a fraction of the total volume of the blood. Normal adult ranges of HCT are 0.37-0.47 ratio. The mean cell volume (MCV), mean cell haemoglobin (MCH) and the mean cell Hb
concentration (MCHC) are all tests used for the classification of anaemia and are calculated from the RCC, Hb and HCT counts. Normal adult ranges are as follows: \( MCV = 80-96fL \) (fentolitres); \( MCH = 27-32pg \); \( MCHC = 30-36g/dL \).

2.3.1.iv: Red Cell Distribution

The red cell distribution (RDW) was measured automatically on the Sysmex 2100. This assay measures the volume of each red cell generating a histogram of small, medium and large cell volumes. The RDW measurement increases in increments of ten percentiles from the baseline and the width of the cells is measured. The normal adult range for red cell distribution is 11-15 units.

2.3.1.v: Platelets

Platelets were lysed from red cells and counted automatically on the Sysmex 2100. The normal adult range of platelets in peripheral blood is \( 140-450 \times 10^9 \) cells per litre.

2.3.2: Hypercoagulation Screens

2.3.2.i: Prothrombin Time (PT)

PT is a measurement of the clotting time of plasma (seconds) using the IL Test™ PT kit (Instrumentation Laboratory, Milan, Italy) in the presence of an optimal concentration of a tissue extract called thromboplastin (highly sensitive calcium thromboplastin used). The PT reflects the overall efficiency of the extrinsic or tissue factor pathway of coagulation. A prolonged clotting time occurs when there are low plasma levels of prothrombin (FII), fibrinogen, FV, FVII and FX. Citrated blood was separated by centrifugation at 2880g (3333rpm) for 4 minutes at room temperature and the plasma collected. Thromboplastin was automatically added to the test plasma on the ACL Futura/Advance instrument where the PT was measured in seconds. Normal adult ranges of PT are 11.5-15.0 seconds (S.O.P. C3.2, Coagulation laboratory, NCHCD, St. James’s Hospital, 2002).

2.3.2.ii: Activated Partial Thromboplastin Time (APTT)

The APTT assay measures the clotting time (seconds) of plasma after the activation of the contact factors using the IL Test™ APTT kit (Instrumentation Laboratory, Milan, Italy) and the ACL Futura/Advance instrument. It reflects the overall efficiency of the intrinsic pathway or contact phase of coagulation. It indicates changes in the contact factors and in the clotting factors FII, XI, IX VIII. It is also sensitive to changes in
factors X, V, II and fibrinogen and the presence of anticoagulants and heparin. Citrated blood was separated by centrifugation at 2880g (3333rpm) for 4 minutes at room temperature. A contact activator was used to maximise the activation of FXII, providing a surface for the function of High Molecular Weight Kininogen, kalikrein and FXIIa. Phospholipids were added to the assay system to form complexes that activate FX and prothrombin. This contact activation was allowed to proceed at 37°C for a specific period of time, after which calcium was added, triggering further clotting reactions. The time required for clot formation was measured in seconds and reported directly by the instrumentation. Normal adult ranges of APTT are 25.0-36.0 seconds (S.O.P. C3.2, Coagulation laboratory, NCHCD, St. James’s Hospital, 2002).

2.3.2.iii: Antithrombin

Antithrombin (AT) or Heparin Cofactor I is the major inhibitor of blood coagulation and is essential for effective heparin therapy. AT levels were measured using the IL Test™ Antithrombin chromogenic assay (Instrumentation Laboratory, Milan, Italy) on the ACL Advance instrument. Citrated blood was separated by centrifugation at 2000g (3333rpm) for 20 minutes at 4°C and the plasma collected. Plasma was incubated with a FXa reagent in the presence of excess heparin, allowing the formation of the AT/FXa/heparin complex. Residual FXa activity was measured by a chromogenic substrate. This reaction releases paranitroaniline, which was monitored kinetically at 405nm, and is inversely proportional to the AT level in the test sample. AT levels were reported from the ACL Advance and no calculations were necessary. Normal adult ranges of AT are 0.76-1.2 IU/ml (S.O.P. C3.38, Coagulation laboratory, NCHCD, St. James’s Hospital, 2002).

2.3.2.iv: FVIII

The principle of this one-stage assay is based on the ability of diluted samples containing Factor VIII:C to shorten the clotting time of FVIII:C deficient plasma. The limiting factor is the FVIII:C from the test plasma, indicating an inverse relationship between the clotting time and FVIII concentration. Abnormalities of factors involved in the contact phase of coagulation were determined by a modified APTT, reflected by prolongation of the APTT. Citrated blood was collected and the plasma separated as before. Control and patient samples were diluted 1/5 and 1/10 with factor diluent and vortexed. The 1/10 diluted sample reading was multiplied by two and an average taken for both the 1/5 and 1/10 readings. Results were displayed by the ACL instrumentation
in percentage units and divided by 100 to obtain a measurement in IU/ml (e.g. 95%=0.95 IU/ml). A reference plasma containing a defined amount of FVIII:C (Sigma Diagnostics, St. Louis, MO, USA and Baxter AG, Vienna, Austria) was used to construct a standard curve on the ACL 300/3000 instrument. The exact sample activity was interpolated from this standard calibration curve. Normal adult ranges of FVIII:C are 0.50-2.0 IU/ml (S.O.P. C3.14, Coagulation laboratory, NCHCD, St. James’s Hospital, 2002).

2.3.2.v: Fibrinogen

Fibrinogen levels were measured using the Clauss technique, based on a clotting time using thrombin. Addition of thrombin to a plasma sample promotes the enzymatic conversion of fibrinogen to fibrin. Fibrin undergoes polymerisation forming a fibrin network, which is stabilised by FXIII (activated by thrombin), constructing a visible clot. The clotting time is inversely proportional to the fibrinogen concentration in the test plasma. Measurements were carried out on the ACL Advance instrument as before. Fibrinogen concentrations were determined from a standard curve, prepared from a fibrinogen standard of known concentration using the Fibriquik® kit (Organon Teknika, Durham, NC. USA). Plasma was collected from citrated blood as previously described and loaded onto the ACL instrument where thrombin was automatically added to the test sample and the fibrinogen concentration determined. Normal adult ranges of fibrinogen are 1.5-4.0g/L (S.O.P. C3.8, Coagulation laboratory, NCHCD, St. James’s Hospital, 2002).

2.3.2.vi: Protein C

PC is activated by thrombin in the presence of thrombomodulin, causing the inactivation of FVa and FVIIIa, in the presence of calcium ions and PS. The anticoagulant effect of PC is prolongation of the clotting time. PC was quantitatively measured using an automated assay on the ACL 300/3000 instrument and the IL Test™ ProClot kit (Instrumentation Laboratory, Milan, Italy). This kit is a functional PC test based on the prolongation of an APTT assay in the presence of APC and is used due to the sensitivity of the APTT test to FV and FVIII levels. APC was generated in plasma samples by a rapid in vitro PC activator, Protac®, derived from the venom of the copperhead snake *Agkistrodon contortrix contortrix*. Citrated blood was separated and the plasma collected as previously described. Plasma samples were positioned on the
automated carousel for analysis. PC normal adult ranges are 0.71-1.45 IU/ml (S.O.P. C3.40, Coagulation laboratory, NCHCD, St. James’s Hospital, 2002).

2.3.2.vii: Protein S

PS acts as a cofactor to APC during inactivation of FVa and FVIIIa. Two forms of PS are present in plasma, 40% free PS and 60% PS linked to the complement C4b-binding protein. Only free PS has functional cofactor activity and it is this that is measured by the automated quantitative assay using the ACL 9000 instrument and the IL Test™ Protein S assay kit (Instrumentation Laboratory, Milan, Italy). PS functional activity was determined by measuring the degree of prolongation of prothrombin time in the presence of bovine thromboplastin, calcium ions and APC. PS activity was proportional to the prolongation of the PT of a PS deficient plasma to which diluted sample was added. PS deficient plasma was incubated with PC activator as before (Protac®) to generate APC and therefore stimulate PS activity. Results were reported from the ACL 9000 in percentage units and divided by 100 to give measurements in IU/ml. Normal adult ranges of PS activity are 0.71-1.45 IU/ml (S.O.P. C3.42, Coagulation laboratory, NCHCD, St. James’s Hospital, 2002).

2.3.2.viii: Activated Protein C Resistance (APCR)

The APC resistance phenotype is characterised by an abnormally low anticoagulant response in human plasma on addition of human APC. Greater than 90% of cases of APCR are due to the FV Leiden mutation. Coatest® APC™ Resistance (Chromagenix-Instrumentation Laboratory, Milan, Italy) provides an APTT based assay for the determination of the response towards human APC. The prolongation of the basal APTT after addition of APC is shorter in plasma from patients with the APCR phenotype than from those with a normal response to APC. An abnormally reduced APC ratio (<2.0) is suggestive of Factor V Leiden mutation carrier status that is then confirmed by mutation analysis. The APCR ratio was calculated as follows:

\[
\text{APCR ratio} = \frac{\text{APTT (APC added)}}{\text{APTT (no APC added)}}
\]

Citrate anticoagulated blood was processed as previously discussed to separate the plasma. Plasma was incubated with APTT reagent for a standard period of time. Coagulation was triggered by the addition of CaCl₂ in both the absence and presence of
human APC, and the time for clot formation was recorded. The APC ratio was then calculated as above.

2.3.2.ix: Lupus Anticoagulant (LAC)

The LA screen is comprised of two tests. The first is the Kaolin Clotting Time (KCT), which is an APTT test without the presence of added phospholipid (Thrombosis Reference Centre, Withington Hospital, Manchester, UK). Clotting is therefore dependent upon the phospholipid present in the patient's blood sample. This increases the sensitivity of the test to the presence of anti-phospholipid antibodies (APA's). APA's are associated with LAC. In the presence of APA's, the KCT will be prolonged. Plasma was pre-incubated with kaolin (Kaoclot, Gradipore Ltd., North Ryde, Australia) to standardise activation of the contact factors. Calcium was then added to the reaction mixture. This allowed coagulation to proceed to completion. The KCT test is also sensitive to factor deficiencies. To improve its specificity for circulating inhibitors, the KCT was carried out on mixtures of patient and normal plasma. A KCT ratio was obtained using the patient plasma and normal control plasma. An abnormally high ratio (>1.2) was indicative of a positive KCT.

The second test in the LAC screen is the Dilute Russell Viper Venom Time (DRVVT). It is based on the activation of FX in the presence of FV, FII, phospholipid (PL) and calcium. LAC prolongs the clotting time by binding to phospholipids and preventing the action of RVV. Dilution of the venom and phospholipid makes it particularly sensitive to the detection of lupus anticoagulant. The DRVVT was combined with a concentrated phospholipid neutralisation procedure step (CRVVT) to ensure specificity for LA. The concentrated PL will absorb out the LA and correct the prolongation of the DRVVT. A correction step with normal plasma (CNP) was also included in both the dilute and concentrated phospholipid tests in order to rule out any effect due to factor deficiencies. The times obtained for the four tests; DRVVT neat, DRVVT with CNP, concentrated RVVT neat and concentrated RVVT with CNP, were compared with normal plasma by calculating ratios and also using a mathematical formula to determine the degree of correction between dilute and concentrated phospholipid. A positive DRVVT was reported when there was >65% correction. Together these two tests reported a positive or negative LAC screen.
2.4.0: POLYMERASE CHAIN REACTION (PCR)

2.4.1: Coagulation and Fibrinolytic polymorphisms

Standard PCR reactions were performed for all polymorphic markers as set out for Factor V Leiden and changes made where applicable. Table 2.1 lists the primer sequences, 5' position of primers and restriction sites for each polymorphism studied within genes encoding proteins of the coagulation and fibrinolytic pathways. Table 2.2 lists the differing PCR cycling conditions for each polymorphism. The standard PCR reaction mix is listed in Appendix A.3.1.

2.4.1.1: Factor V Leiden (FVL G1691A)

The FV Leiden gene polymorphism is a result of a single base substitution of guanine (G) with adenine (A) at nucleotide position 1691 in the FV gene. This base change predicts the loss of the APC cleavage site at Arginine (Arg) 506, the site that is necessary for cleavage at the two subsequent sites (Arg 306 and 679). Loss of this cleavage site renders the FV protein resistant to inhibition by APC (Bertina et al., 1994). This polymorphic marker was demonstrated using the Polymerase Chain Reaction (PCR), by designing primers that flank the region of interest, and subsequently cutting the amplified product with a restriction enzyme, resulting in a particular pattern of fragments depending on genotype. PCR reactions were performed in 50μl volumes of PCR buffer of 50mM KCl, 10mM Tris-HCl pH 9.0, Triton X-100, 1.5mM MgCl₂, containing 0.4units of DNA Taq polymerase (Promega), 2μl of genomic DNA, 4% dimethyl sulphoxide (Sigma Aldrich), 30mM each of deoxyribonucleoside triphosphates (Boehringer Mannheim) and 0.25mM of sense primer (5'-AGCCAGGAGACCTAACATGTTC-3') and antisense primer (5'-GGAACAACACCATGATCAGAGCA-3') (Sigma Genosys). Amplification for 30 cycles was performed using a thermocycler (Peltier Thermal Cycler, PTC-100/200, MJ Research) with the following cycling parameters: denaturation at 94°C for 60 seconds, primer annealing at 62°C for 30 seconds, and an extension step at 72°C for 90 seconds and yielded a product of 287 base pairs (bp). PCR products were digested using the restriction enzyme Mnl-I (New England BioLabs) according to the manufacturer's guidelines. Wild type homozygotes yielded 3 DNA fragments of 37bp, 93bp, and a control 157bp; FV Leiden homozygotes (rare) yielded two fragments of 157bp (control) and 130bp; heterozygotes yielded all 4 fragments. Restriction digest products were visualised on a 3% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6μg/ml
Ethidium Bromide (Sigma Aldrich). Figure 2.1 illustrates the typical DNA banding patterns obtained by this method.

2.4.1.ii: Prothrombin (Factor II G20210A)

Similar to FV Leiden, there exists a polymorphic marker within the prothrombin gene that has been associated with increased plasma prothrombin levels. This polymorphism involves a single nucleotide transition from G to A at position 20210 in the 3' untranslated region of the prothrombin gene (Poort et al., 1996), which was easily detected using PCR as described previously. PCR reactions were performed as above with 0.25mM of sense primer (5'-AGCACAGACGGCTGGTTCTCT-3') and a mutagenic antisense primer (5'-ATAGCAGACGGCTGGATTGAACGC-3') (Sigma Genosys). The nucleotide underlined is not present in the normal sequence but introduces a Hind III digest site when the A20210 allele is present. This primer pair spans an invariant Hind III control digest site and the A20210 specific site. Amplification for 30 cycles was performed as before (section 2.1.4.i) but with a primer annealing temperature of 53°C for 45 seconds and yielded a PCR product of 507bp. Restriction enzyme digestion was performed using the enzyme Hind III (New England BioLabs) according to the manufacturer’s guidelines. Wild type homozygotes yielded 2 DNA fragments of 408bp, and 99bp (control fragment); prothrombin A20210 homozygotes yielded three fragments of 385bp, 99bp (control fragment), and 23bp; heterozygotes yielded all 4 fragments. Restriction digest products were visualised on a 3% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6µg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.2 illustrates the typical DNA banding patterns obtained by this method.
Figure 2.1: Agarose gel separation of FV Leiden PCR fragments illustrating wild type homozygote (GG) and heterozygote (GA) genotypes.
**Figure 2.2:** Agarose gel separation of prothrombin PCR fragments illustrating wild type homozygote (GG) and heterozygote (GA) genotypes.
2.4.1.iii: Methylene TetraHydroFolate Reductase (MTHFR C677T)

A cytosine (C) to thymine (T) nucleotide transition has been reported at nucleotide 677 of the MTHFR gene that results in the production of a thermolabile enzyme with 50% activity (t-MTHFR). Homozygosity for the T allele has been suggested to infer a greater requirement for folate in effective individuals, increasing the risk of thrombosis (D'Angelo and Selhub, 1997). An RFLP assay exists to test for this genotype. PCR reactions were performed as above with 0.25mM of sense primer (5'-TGAAGGAGAAGGTGTCTGCGGGA-3') and antisense primer (5'-AGGACGGTGCGGTGAGAGTG-3') (Sigma Genosys). Amplification for 40 cycles was performed as previously described (section 2.1.4.i) but with an annealing temperature of 62°C for 1 minute and yielded a PCR product of 198bp. The amplified fragment was digested with Hinf I and allele specific fragments (C, 198bp and T, 175bp and 23bp) were produced. Restriction digest products were visualised on 3% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6µg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.3 illustrates the typical DNA banding patterns obtained by this method.

2.4.1.iv: Fibrinogen (G-455A)

A polymorphism within the promoter region of the β fibrinogen gene at position -455 predicts a guanine (G) to adenine (A) base transition, which is thought to increase circulating fibrinogen concentrations (Humphries et al., 1995). This polymorphism was detected using PCR techniques as follows. PCR reactions were performed as above with 0.25mM of sense primer (5'-CCTGATTGCAACACAAGT-3') and antisense primer (5'-ACACAAGCTCCGAAAGAATA-3') (Sigma Genosys). Primers were designed so that the amplicon also contained a natural Hae III site to act as a restriction control. Amplification for 40 cycles was performed with the following cycling parameters: denaturation at 94°C for 60 seconds, primer annealing at 62°C for 60 seconds, and an extension step at 72°C for 90 seconds and yielded a product of 607bp. The amplified product was digested with Hae III: wild-type homozygotes (G/G) yielded three fragments of 73bp, 151bp, and 383bp; homozygotes (A/A) yielded two fragments of 73bp and 534bp; heterozygotes (G/A) yielded all four fragments of 73bp, 151bp, 534 and 383bp. Restriction digest products were visualised on 4% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6µg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.4 illustrates the typical DNA banding patterns obtained by this method.
Figure 2.3: Agarose gel separation of MTHFR PCR fragments illustrating wild type homozygote (CC), heterozygote (CT) and homozygote mutant (TT) genotypes.
Figure 2.4: Agarose gel separation of Fibrinogen PCR fragments illustrating wild type homozygote (GG), heterozygote (GA) and homozygote mutant (GG) genotypes.
2.4.1.v: Plasminogen Activator Inhibitor – 1 (PAI-1 –675 deletion 4G/insertion 5G)

A single guanine insertion/deletion (4G/5G) polymorphism in the promoter region of the PAI-1 gene at position –675 has been associated with increased plasma PAI-1 levels with the 4G/4G genotype (Dawson et al., 1993). An RFLP assay was developed to detect this genotype as follows. PCR reactions were performed as above with 0.06mM of sense (5’-AGAGAGAGTCTGGACACGTGGGT*-3’) and antisense primer (5’-CCCGGTGCTCTGGACCACCTCCAGG-3’). The sense primer was designed incorporating a mutation (G to T*) at the most 3’ G of the 4G allele. The mutated nucleotide ensures that the restriction enzyme Hinf I specifically cleaves only when a guanine residue is inserted (5G allele). Primers were designed so that the amplicon also contained a natural Hinf I site to act as a restriction control. Amplification for 40 cycles was performed with the following cycling parameters: denaturation at 94°C for 40 seconds, primer annealing at 64°C for 60 seconds, and an extension step at 72°C for 70 seconds and yielded a product of 216 bp. The amplified product was digested with Hinf I: 4G homozygotes yielded two DNA fragments of 133bp (control fragment) and 83bp; 5G homozygotes yielded three fragments of 133bp (control), 60bp and 23bp; heterozygotes yielded all 4 fragments. Restriction digest products were visualised on 4% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6μg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.5 illustrates the typical DNA banding patterns obtained by this method.

2.4.1.vi: Tissue Plasminogen Activator (TPA Alu insertion/deletion)

An Alu repeat insertion (I)/deletion (D) polymorphism has been located in intron 8 of the TPA gene. The I allele is thought to influence the release rates of TPA and increase the risk of VTE in affected individuals (Hooper et al., 2001; Jern et al., 1999). The Alu repeat was detected using the following RFLP assay. PCR reactions were performed as above with 0.2μM sense primer (5’- TCCGTAACAGGACACCTCA-3’) and antisense primers (5’- TCAGTCAACCAAATGAAAACC-3’) (Sigma Genosys). Amplification for 40 cycles was performed as previously described (section 2.1.4.i) but at an annealing temperature of 52°C for 60 seconds. The TPA gene contains a 297bp Alu repeat insertion/deletion polymorphism, and a 135bp partial Alu repeat sequence in intron 8 of its sequence. Amplification of the insertion allele at the known polymorphic Alu repeat site yields a 485bp product, while the deletion allele generates a 188bp fragment. Amplification products were visualised on 3% Agarose gels (Pronadisa, Madrid) and
stained with 1.6μg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.6 illustrates the typical DNA banding patterns obtained by this method.

2.4.1.vii: Angiotensin Converting Enzyme (ACE Alu insertion/deletion)

Angiotensin converting enzyme (ACE) is a vasoconstricting enzyme with many roles including influencing TPA production and PAI-1 release. An Alu repeat insertion (I)/deletion (D) polymorphism exists in the ACE gene that has been associated with considerable differences in serum and cellular ACE concentrations, with increased levels being associated with the D allele. The following RFLP assay was used to determine the ACE genotype. PCR reactions were performed as above with 0.25mM sense primer (5'-CTGGAGACCCTCCCATCCTTTCT-3') and antisense primers (5'-GATGTGGCCATCACATTCGTCAGAT-3') (Sigma Genosys). Amplification for 40 cycles was performed with identical parameters the fibrinogen assay above (section 2.4.1.vii). The ACE gene contains a 287bp inverted Alu repeat insertion/deletion polymorphism in intron 16 of its sequence. Amplification of the insertion allele with these primers gave rise to a 479bp fragment, while the deletion allele yielded a 191bp fragment. Amplification products were visualised on 3% Agarose gels (Pronadisa, Madrid) and stained with 1.6μg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.7 illustrates the typical DNA banding patterns obtained by this method.

Table 2.1 lists the primer sequences for each polymorphism, their location, the restriction enzymes used and the sites at which they cut. Table 2.2 illustrates the PCR cycling parameters for each polymorphism.
Figure 2.5: Agarose gel separation of PAI-1 PCR fragments illustrating wild type homozygote (5G/5G), heterozygote (4G/5G) and homozygote mutant (4G/4G) genotypes.
Figure 2.6: Agarose gel separation of TPA PCR fragments illustrating wild type homozygote (DD) and homozygote mutant (II) genotypes.
Figure 2.7: Agarose gel separation of ACE PCR fragments illustrating wild type homozygote (II), heterozygote (ID) and homozygote mutant (DD) genotypes.
### Table 2.1: Coagulation and fibrinolytic PCR primer sequences with position of 5’ base, restriction enzymes and cut sites.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
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<th>Restriction Sites</th>
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Table 2.2: Coagulation and Fibrinolytic Polymerase chain reaction cycling parameters

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</table>
2.4.1. viii: Allelic Discrimination confirmation assays

FV Leiden G1691A, Prothrombin G20210A and MTHFR C677T genetic assay results for venous thromboembolic patients were confirmed by an allelic discrimination approach using the ABI 7700 instrument (Applied Biosystems) by Dr. Helen Egan of the NCHCD, St. James’s Hospital, Dublin. PCR primers and fluorogenic probes were designed using Primer Express software (ABI) and synthesised by Applied Biosystems for each of the following polymorphisms: Factor V Leiden (G1691A), Prothrombin (G20210A) and MTHFR (C677T). The probes were designed for the specific target polymorphic regions for each of the three mutations. Probes were engineered with a reporter fluorescent dye at the 5’ end (FAM: 6-carboxyfluorescein, 6FAM or VIC™) and a non-fluorescent quencher dye at the 3’ end. The addition of a minor groove binder at the 3’ end increases the melting temperature of probes allowing use of shorter probes and more accurate allelic discrimination. PCR amplification reactions were carried out in 25µl volumes. Each run included 8 No Template Controls (NTCs), 8 Allele 1 Controls (AL1), and 8 Allele 2 Controls (AL2). Final concentrations used in each reaction were 50-100ng of DNA, 200nM of each probe, 900nM of forward and reverse primers and IX Taqman® Universal Master Mix (ABI part 4304437). AmpErase ®UNG is a component of Taqman® Universal Master Mix and prevents amplification of carryover-PCR products. Thermal cycling conditions consisted of an initial cycle of 2 min at 50°C and 95°C for 10 mins, then 40 amplification cycles of 92°C for 15 sec and 62°C for 1 min.
2.4.2: Inflammatory Polymorphisms

2.4.2.i: IL-10 -1082

Two polymorphisms exist within the promoter region of the IL-10 gene. The first occurs at position -1082 in the promoter, and involves a G to A transition, with the G allele being associated with increased IL-10 and the A allele being associated with decreased IL-10 secretion (Turner et al., 1997). The -1082 polymorphism was detected using the following RFLP assay. PCR reactions were performed in 50μl volumes of PCR buffer of 50mM KCl, 10mM Tris-HCl pH 9.0, Triton X-100, 1.5mM MgCl₂, containing 0.4 units of DNA Taq polymerase (Promega), 2μl of genomic DNA, 4% dimethyl sulphoxide (Sigma Aldrich), 30mM each of deoxyribonucleoside triphosphates (Boehringer Mannheim), and 0.2μM of sense primer (5’-TCTGAAGAAGTCCTGATGTC-3’) and antisense primers (5’-CTCTTACCTATCCCTACTTCC-3’) (Sigma Genosys). Amplification for 40 cycles was performed using a thermocycler (Peltier Thermal Cycler, PTC-100/200, MJ Research) with the following cycling parameters: denaturation at 94°C for 60 seconds, primer annealing at 58°C for 60 seconds, and an extension step at 72°C for 60 seconds and yielded a product of 190 base pairs (bp). PCR products were digested using the restriction enzyme Mnl-I (New England BioLabs) according to the manufacturer’s guidelines. Wild type homozygotes (GG) yielded DNA fragments of 15bp, 17bp, 91bp, and control fragments of 2bp, 20bp and 45bp; IL-10 -1082 homozygotes (AA) yielded fragments of 123bp, and 2bp, 20bp and 45bp (controls); heterozygotes (GA) yielded all 7 fragments although the 2bp and 20bp were not clearly visible on agarose gels. Restriction digest products were visualised on a 3% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6μg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.8 illustrates the typical DNA banding patterns obtained by this method.

2.4.2.ii: IL-10 -592

The IL-10 -592 polymorphism involves the substitution of C with A at position -592 in the promoter region of the gene (Crawley et al., 1999). To date, no functional significance of the transition to the A allele genotype has been found. The IL-10 -592 genotype was detected using the following method. PCR reactions were performed as above with 0.2μM of sense primer (5’-GACTCCAGCCACAGAAGCTTA-3’) and antisense primers (5’- ATATCCTCAAAGTTCCCAAGC-3’) (Sigma Genosys). Amplification for 40 cycles was performed as previously described (section 2.4.2.i) but with an annealing temperature of 64°C for 1 minute and yielded a PCR product of
The amplified fragment was digested with *Rsa I* and allele specific fragments were produced as follows: wild type homozygous (CC) produced a 302bp fragment, and control fragments of 85bp, 42bp and 8bp. Restriction digest products were visualised on 3% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6μg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.9 illustrates the typical DNA banding patterns obtained by this method.

2.4.2.iii: *IL-6* –174

The *IL-6* gene contains a promoter polymorphism at position –174, that predicts the substitution of G with C. This base change lies within a transcription regulatory region, and the C allele is thought to negatively affect transcription of the *IL-6* gene, producing decreased levels of the *IL-6* protein (Fishman *et al.*, 1998; Terry *et al.*, 2000). We have used the following RFLP assay to detect this marker. PCR reactions were performed as in section 2.4.2.i with 0.2μM of sense primer (5’-ATGACTTCAGCTTTACTCTT-3’) and antisense primers (5’-ATAAATCTTTGTTGGAGGGT-3’) (Sigma Genosys). Amplification for 40 cycles was performed as before (section 2.4.2.i) but with an annealing temperature of 58°C for 1 minute and an elongation temperature of 72°C for 1 minute, yielding a PCR product of 243bp. The amplified fragment was digested with *Hsp92 II* and allele specific fragments were produced as follows: wild type homozygous (GG) produced 2 fragments of 32bp (control) and 212bp; -174C/C homozygotes produced 3 fragments of 32bp, 90bp and 122bp; heterozygotes produced all four fragments of 32bp, 90bp, 122bp and 212bp. Restriction digest products were visualised on 3% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6μg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.10 illustrates the typical DNA banding patterns obtained by this method.
Figure 2.8: Agarose gel separation of IL-10 -1082 PCR fragments illustrating wild type homozygote (GG), heterozygote (GA) and homozygote mutant (AA) genotypes.
Figure 2.9: Agarose gel separation of IL-10 -592 PCR fragments illustrating wild type homozygote (CC), heterozygote (CA) and homozygote mutant (AA) genotypes.
Figure 2.10: Agarose gel separation of IL-6-174 PCR fragments illustrating wild type homozygote (GG), heterozygote (GC) and homozygote mutant (CC) genotypes.
2.4.2.iv: TNFa-308

The G to A transition mutation at position -308 in the promoter region of the TNFa gene predicts the TNF A1 (G) allele or the A2 (Carp et al., 1992) allele (Wilson et al., 1992). The more rare A2 allele has been associated with higher endogenous and inducible levels of TNFa secretion (Warzocha et al., 1998). For this reason we have used the following assay to detect for the TNFa-308 genotype. PCR reactions were performed as before (section 2.4.1.i) with 0.2μM of sense primer (5'-GGAGGCAATAGGTTTTGAGGG-3') and antisense primers (5'-CTGTCTCGGTTTCTTCTCCATGGCC-3') (Sigma Genosys). Amplification for 40 cycles was performed as previously described (section 2.4.2.i) but with an annealing temperature of 65°С for 1 minute and an elongation temperature of 72°С for 1 minute, yielding a PCR product of 195bp. The amplified fragment was digested with Nco I and allele specific fragments were produced as follows: wild type homozygous TNFA1/TNFA1 (GG) produced a 151bp fragment and 2x 22bp fragments (control); TNFA2/2 (AA) homozygotes produced a 173bp fragment and a 22bp fragment; TNFA1/2 heterozygotes (GA) produced all three fragments of 173bp, 151bp and 22bp. Restriction digest products were visualised on 3% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6μg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.11 illustrates the typical DNA banding patterns obtained by this method.

2.4.2.v: TNFB

Similar to TNFa, the TNFB gene (also known as lymphotoxin α) contains a polymorphic site at position +252 of intron 1 of the gene. Here, there is a G to A transition that predicts the TNFB1 (G) allele or the TNFB2 (Carp et al., 1991) allele (Messer et al., 1991). The effect of this polymorphism on both TNFa and TNFB concentrations remains unknown, but the close proximity of these genes suggests a possible effect on either protein. We have used the following RFLP assay to determine the TNFB genotype. PCR reactions were performed (section 2.4.1.i) with 0.2μM of sense primer (5'-CCCTCCTGCACCTGCTGCCTGG-3') and antisense primers (5'-AGAGGGGTGGATGCTTGGGTTC-3') (Sigma Genosys). Amplification for 37 cycles was performed as before (section 2.4.2.i) but with an annealing temperature of 68°С for 1 minute and an elongation temperature of 74°С for 42 seconds, yielding a PCR product of 782bp. The amplified fragment was digested with Hinf I and allele specific fragments were produced as follows: wild type homozygous TNFB1/TNFB1 (GG)
produced a 637bp fragment and a 145bp fragment (control); TNFB2/2 (AA) homozygotes produced fragments of sizes 582bp, 145bp and 55bp; TNFB1/2 heterozygotes (GA) produced all four fragments of 637bp, 582bp, 145bp and 55bp. Restriction digest products were visualised on 3% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6μg/ml Ethidium Bromide (Sigma Aldrich). Figure 2.12 illustrates the typical DNA banding patterns obtained by this method.

Table 2.3 lists the inflammatory polymorphic primer sets, restriction enzymes and the sites at which they cut. Table 2.4 illustrates the differing cycling parameters for each polymorphism.
Figure 2.11: Agarose gel separation of TNFα -308 PCR fragments illustrating wild type homozygote [A1/1 (GG)], heterozygote [A1/2 (GA)] and homozygote mutant [A2/2 (AA)] genotypes.
Figure 2.12: Agarose gel separation of TNFβ +252 PCR fragments illustrating wild type homozygote (B1/1), heterozygote (B1/2) and homozygote mutant (B2/2) genotypes.
<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Cycles</th>
<th>Denaturation</th>
<th>Primer Annealing</th>
<th>Elongation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10 -1082</td>
<td>40</td>
<td>94°C, 1min</td>
<td>58°C, 1min</td>
<td>72°C, 1 min</td>
</tr>
<tr>
<td>IL-10 -592</td>
<td>40</td>
<td>94°C, 1min</td>
<td>64°C, 1min</td>
<td>72°C, 1 min</td>
</tr>
<tr>
<td>IL-6 -174</td>
<td>40</td>
<td>94°C, 1min</td>
<td>58°C, 1min</td>
<td>72°C, 1min30sec</td>
</tr>
<tr>
<td>TNFα -308</td>
<td>40</td>
<td>94°C, 1min</td>
<td>65°C, 1min</td>
<td>72°C, 1 min</td>
</tr>
<tr>
<td>TNF B</td>
<td>37</td>
<td>95°C, 1min</td>
<td>68°C, 1min</td>
<td>72°C, 42 sec</td>
</tr>
</tbody>
</table>

Table 2.4: Inflammatory Polymerase chain reaction cycling parameters
2.4.3: Statistical Analysis

For each study, allele and genotype frequencies were compared between patient and control groups by means of Contingency analysis by the chi-squared test and Fischer’s exact test when appropriate, using the Statview statistical program (Abacus Concepts Inc., version 4.57) and the JMP statistical package (SAS Institute Inc., version 4.0.5). A $\chi^2$ p-value of <0.5 was considered statistically significant. Allele frequencies were determined by gene counting and statistical significance was estimated by cross-tabulation. Genotype distributions were tested using the Hardy-Weinberg equilibrium. Repeated measures ANOVA tests were performed on longitudinal study data using SPSS for Windows (SPSS Inc.) and JMP statistical packages.

2.5.0: ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA’s)

The ELISA sandwich assay is a method of detecting protein levels in serum and plasma samples. Polystyrene plates are coated with a primary antibody (Ab), specific for the protein of interest. Samples are incubated with this antibody and any protein present in the sample will bind with high affinity to the primary Ab. A secondary Ab specific to the said protein is added to the system, which binds to the protein-primary antibody complex. An enzyme specific for the secondary antibody is labelled with a colorometric tag and added to the assay. This binds to the primary Ab-protein-secondary Ab complex. Upon addition of a substrate for the labelled enzyme, a colour is emitted from the well of the plate, which is proportional to the concentration of protein in the sample.

2.5.1: ELISA assays for IL-10, IL-6, IFN$\gamma$ and TNF$\alpha$

ELISA Duoset development kits were used for the measurement of serum cytokine concentrations in the peripheral blood of patients and controls. IL-10 (DY217), IL-6 (DY206), IFN$\gamma$ (DY285) and TNF$\alpha$ (DY210) cytokines levels were measured using a standard ELISA protocol as recommended by the manufacturers (R&D Systems, Minneapolis, USA). Primary or capture antibody was diluted in Phosphate Buffered Saline (Gibco-BRL) to the desired concentration (Appendix A.5.1.1). 96-well plates were coated with 100$\mu$l of capture antibody using a repeater pipette (Eppendorf Repeater® Plus), sealed with an adhesive acetate sheet and incubated overnight at RT$^\circ$C (in dark). Plates were washed and aspirated several times using an automatic plate washer (Anthos Labtec) with Wash Buffer (PBS/Tween 20, Appendix A.4.1.2) to remove unbound antibody. Plates were incubated for 1 hour with Blocking buffer
(PBS/BSA, Appendix A.4.1.3) to block non-specifically bound antibody. Blocking buffer was removed from plates by washing and aspirating as before. Reagent Diluent (RD) (Appendix A.4.1.4) was filtered (0.2μm) and used for the dilution of cytokine standards and secondary antibodies. 100μl of serially diluted standards (Appendix A.5.1.3) and undiluted samples were added to each well, the plate was sealed with an adhesive acetate sheet and incubated in the dark for 2 hours to allow the proteins to bind to the antibodies. Washing and aspiration of samples and standards was performed as before and excess liquid blotted off plates. Detection or secondary antibody was diluted in reagent diluent (Appendix A.5.1.2) and 100μl was added to each well of the plate, sealed and incubated for 2 hours in the dark. Plates were washed and aspirated as before. 100μl working dilution of HRP-Streptavidin (Appendix A.4.1.6) was added to each well, sealed and incubated for 20 minutes. Plates were washed again and 100μl of TMB substrate solution (Appendix A.4.1.7) was added to each well and incubated. Depending on the assay in question and the relative levels of proteins present, this incubation step was performed from between 5 minutes and 20 minutes. 50μl of Stop solution (Appendix A.4.1.8) was added to the substrate solution in each well, turning any blue emission to yellow. Optical density was determined at 450nm (Rosys Anthos 2010, Anthos Labtec) and a standard curve was created. Sample cytokine concentrations were determined by interpolating absorbance data from the standard curves.

2.5.2: β-2 Glycoprotein-1 (β2GP1) Assay

This assay was performed by Frederick Lins, PhD student, of the Department of Immunology, St. James’s Hospital and Trinity College Dublin. Nunc Maxisorp™ (Roskilde, Denmark) ELISA plates were incubated overnight at 4°C with 100μl (0.32μg) of mouse anti-human β2GP1 (Chemicon International Inc., Temecula, CA, USA) in coating buffer (50mM carbonate buffer, pH 9.6). Wells were washed four times with PBS-Tween (149.2mM, pH 7.2, 0.05% v/v Tween 20). 100μl of serially diluted pooled standard serum (diluted from 1:320 to 1:20480 in PBST) and test samples (at 1/3000 in PBST) were added in duplicate. Two wells with PBST were used as negative controls. The plates were incubated for 1 hour at 37°C. Following washes with PBST, 100μl of horseradish peroxidase-conjugated rabbit polyclonal anti-human β2GP1 (Dako A/S, Glostrup, Denmark) diluted at 1/1000 in PBST was added. Plates were incubated for 1 hour at 37°C. Plates were washed again and 100μl of a hydrogen
peroxide substrate solution was added and incubated for 5 minutes at room temperature. 100µl of 2.5M H₂SO₄ stop solution was added to all wells to stop the colour development. Optical density was measured at 492nm.

2.6.0: DENATURING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (dHPLC) USING THE TRANSGENOMIC WAVE® DNA FRAGMENT ANALYSIS SYSTEM

The existence of novel gene mutations within the IFNγ gene in patients with RFL and non-pregnant controls was investigated to facilitate the generation of a restriction fragment length polymorphic assay, in order that the genotype/phenotype relationship could be determined. The Transgenomic WAVE® DNA fragmentation system was used to screen all areas of the IFNγ genome (NM_000619) in a sub-set of patients and controls, before direct sequencing of individual samples. The theory of this technique is explained in chapter 4.

The human interferon gamma gene was located on the Human Genome Browser web site (http://genome.ucsc.edu/cgi-bin/hgGateway?db=hgl2). Several gene sequences have been submitted to this database over the years with differing degrees of completion. In order that the most up to date and complete sequence was chosen, sequences were compared to each other for homology using the Clustalw homology service from the European Bioinformatics Institute (EBI: http://www.ebi.ac.uk/clustalw). The gene sequence chosen was submitted to the database on 31 October 2000 with the accession number NM_000619 and entitled ‘Homo sapiens interferon, gamma (IFNG), mRNA’.

The human IFNγ gene (NM_000619) is comprised of 4 exons interrupted by 5 introns with a 108bp 5’ untranslated region (UTR). Primers were designed spanning all four exons and approximately 600bp of the promoter region using the Primer 3 service from Massachusetts Institute of Technology. Care was taken to ensure that there was at least 40bp between the region of interest and the primers since areas located near the primers are hard to analyse using the dHPLC instrument. Primers were then compared to the genome database to ensure their specificity for the human IFNγ gene using the BLAST service from the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Table 2.5. lists the primer sequences for each exon and the promoter region studied.
2.6.1: PCR Amplification of IFNγ gene regions

PCR reactions were performed in 50µl volumes of PCR buffer containing 50mM KCl, 10mM Tris-HCl pH 9.0, Triton X-100, 1.5mM MgCl₂, 1 unit (U) of DNA Taq polymerase (Promega), 2µl of genomic DNA, 200µM each of deoxyribonucleoside triphosphates (Boehringer Mannheim) and 0.25mM of sense and antisense primers (table 2.5) (Sigma Genosys). Clean PCR products were difficult to amplify for Exon 1 so a HotStarTaq™ (Qiagen) was used to increase the specificity of the PCR reaction. 1U of HotStarTaq™ was used per reaction with 10µl of Q solution (provided with the kit). Amplification was performed using a thermocycler (Peltier Thermal Cycler, PTC-100/200, MJ Research) under specified conditions for each primer pair (table 2.6). PCR products were visualised on 3% w/v Agarose gels (Pronadisa, Madrid) and stained with 1.6µg/ml Ethidium Bromide (Sigma Aldrich).

2.6.2: Heteroduplex processing of PCR products

For mutation detection using the dHPLC instrumentation, it was necessary to allow the samples to form hetero- and homoduplexes as described in Chapter 4. PCR products were heated to 95°C for 5 minutes then allowed to cool to 25°C at a rate of 1.5°C per minute. This was achieved using the Peltier Thermocycler. Processed PCR products were then transferred to 0.5ml thin walled eppendorf tubes for use in the dHPLC instrument.

The WAVE® DNA fragment analysis system comprises a sensitive capillary column for DNA mutation detection. Buffers were prepared and added (Appendix A.6.1.0), the column primed and the oven equilibrated at 50°C.
Table 2.5: Human IFNγ gene promoter and exonic primer sequences. S = Sense primer; As = Antisense primer; bp = base pairs.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Position of 5' Base (bp)</th>
<th>Product Size (bp)</th>
</tr>
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<tr>
<td>Promoter 3 S</td>
<td>CCTGTGTGCTTTGATTTGTATT</td>
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<tr>
<td>Promoter 3 As</td>
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<tr>
<td>Promoter 2 S</td>
<td>TGCCCCTTTTGTAAAGGTTTG</td>
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Table 2.6: Interferon γ gene PCR cycling parameters

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<th>Gene Region</th>
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<th>Elongation</th>
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<tr>
<td>Promoter region 3</td>
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<td>Promoter region 2</td>
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<td>94°C, 1 min</td>
<td>60°C, 45 sec</td>
<td>72°C, 45 sec</td>
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<td>94°C, 1 min</td>
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<td>72°C, 45 sec</td>
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<tr>
<td>Exon 1</td>
<td>25</td>
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<td>63°C, 2 min</td>
<td>72°C, 3 min</td>
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<tr>
<td>Exon 2</td>
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<td>94°C, 1 min</td>
<td>59°C, 2 min</td>
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<td>Exon 4</td>
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</table>
2.6.3: Analysis parameters for mutation detection on the Transgenomic WAVE® instrument.

Sequences of individual regions of the IFNγ gene were stored on the instrumentation software and analysis parameters were determined as set out in Appendix A7.1.5. Melting temperatures of each strand analysed were determined by comparing recommended temperatures offered by the Transgenomic software and the on-line program from Stanford University (http://insertion.stanford.edu/melt.html). Briefly, Transgenomic software offers a graphical representation of the strand of DNA to be analysed suggesting temperatures capable of melting each region. Figure 2.13 demonstrates how 75% helical fractionation is required to ensure analysis within a particular region, illustrating how more than one temperature is often required ensuring the whole strand is analysed. The Stanford melting program offered the particular temperature for each region also but included the percentage of buffers for the particular temperature. The predicted separation pattern for the gene sequence was then displayed, ensuring the elution peak occurred between 3 and 4 minutes for accurate detection (Figure 2.14). Samples were eluted at their particular temperature and percentage of elution buffer and results were determined from graphical chromatogram outputs.
Figure 2.13: Typical temperature profiles obtained from the Transgenomic software for promoter region 2 of the IFNγ gene. At 60°C the above 280bp DNA fragment will only be analysed between 170bp and 240bp. Therefore further temperatures are required to ensure the entire DNA strand is analysed. Use of all three temperatures ensured analysis of the entire gene region within the promoter 2 area.
Figure 2.14: Transgenomic software prediction pattern of the promoter 2 region of the IFNγ gene. The DNA fragment of 280 base pairs was predicted to elute between 3 and 4 minutes (as desired) at 60°C, as shown by the position of the red and blue bars either side of the thin blue line. The red peak represents the buffer peak that occurs at the end of every run. % = % Helical Fraction; Minutes = elution time in minutes.
2.7.0: TISSUE CULTURE

The human endometrium tumourogenic cell line Ishikawa (ECACC # 99040201) along with ZR-75-1 (ECACC # 87012601), Jurkat (ECACC # 88042803) and THP-1 cells (ECACC # 88081201) were obtained from the European Collection of Cell Cultures (ECACC). The identity and tissue origin of all cells is listed in Table 2.4. Cells were grown in Minimum Essential Medium (MEM) and RPMI 1640 medium (Gibco-BRL) as indicated in Table 2.7. All cell lines were supplemented with 10% foetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine for growth (Gibco-BRL) (Appendix table A.7.1). Ishikawa cells were further supplemented with 1% Non-Essential Amino Acids (NEAA) (Gibco-BRL).

Suspension cell lines were aseptically diluted 1/10 for growth with supplemented medium and grown in sterile 75cm² tissue culture flasks. Adherent cell lines were aseptically passed, washing twice with phosphate buffered saline (PBS) (Gibco-BRL) to remove residual serum, then by incubation in 5ml 0.25% Trypsin (Gibco-BRL) for 5-10 minutes to remove cells from the surface of the flask. Cells were recovered by centrifugation at 400g for 5 minutes, fresh media added and transferred to sterile 75cm² tissue culture flasks for further growth. Cells were counted using a haemacytometer (Neubauer) and trypan blue staining (Sigma) (Appendix A.8.1.0).

2.7.1: Hormone Experiments

Estrogen (17β-estradiol) and Progesterone (Sigma) were diluted with RPMI 1640 medium (Appendix A.7.1.1) without supplements within a Category II safety cabinet (Holten Lamin Air, 1.2). 2ml of adherent cells were plated onto 6-well tissue culture plates (Iwaki) at a concentration of 1x10⁵ cells/ml, 24 hours before use to ensure complete adherence and growth. Media was removed from the adhered cells and 1ml of fresh media containing a reduced concentration of FCS (0.5%) was added, prior to hormonal additions. 1ml of suspension cells were plated on the day of the experiment at a concentration of 1x10⁶ cells/ml in media containing reduced FCS (0.5%).

Dose-response curves were performed for each cell line and each hormone to determine the optimal concentration of hormone to be used, using the following concentrations: 1x10⁻⁶M; 1x10⁻⁸M; 1x10⁻¹⁰M; 1x10⁻¹²M (see Appendix A.7.1.1 for dilutions). The concentration of 10⁻⁸M of each hormone was determined to be optimal for all cell lines. Optimal cytokine production in response to hormonal addition was determined to be 12
hours. Cells were incubated with Estrogen, Progesterone, and Low Molecular Weight Heparin (LMWH) (Innohep, 500 units) (Appendix A.7.1.2) to determine the effect of anticoagulation on the production of cytokines in response to hormonal stimulus.
### Table 2.7: Panel of cell lines, their origins and growth medium.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>Features</th>
<th>Growth Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ishikawa</td>
<td>Endometrial Carcinoma</td>
<td>Epithelial</td>
<td>MEM</td>
</tr>
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<td>Epithelial</td>
<td>RPMI 1640</td>
</tr>
<tr>
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<td>T cells</td>
<td>Suspension</td>
<td>RPMI 1640</td>
</tr>
<tr>
<td>THP-1</td>
<td>Monocytes</td>
<td>Suspension</td>
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</tr>
</tbody>
</table>
CHAPTER 3

THE COAGULATION & INFLAMMATION INTERFACE IN NORMAL PREGNANCY
3.1.0: HAEMOSTASIS DURING PREGNANCY

Pregnancy is regarded as a hypercoagulable state where the body prepares for blood loss occurring during labour. Regulation of this procoagulant environment is crucial for the maintenance of placental vasculature and successful pregnancy outcome. Many pathological mechanisms can interfere with this process resulting in gestational thrombosis, intrauterine growth restriction (IUGR) and foetal death (IUFD), preeclampsia, placental abruption and recurrent foetal loss (RFL) (Brenner and Blumenfeld, 1997; Walker, 2000). A ‘normal’ pregnancy is characterised by a healthy mother throughout gestation and the birth of a healthy baby, with no complications such as the pathological events mentioned above. Normal pregnancy is associated with changes in the haemostatic environment, changing venous stasis considerably. Within the first trimester, veins enlarge and gain greater capacity resulting in decreased blood flow in the lower limbs (Bates and Ginsberg, 1997). Plasma blood volume and red cell volumes increase while haemoglobin concentrations and platelet counts decrease (Hayashi et al., 2002; Hytten and Lind, 1973). Furthermore, the increase in maternal blood volume that is necessary to sustain sufficient oxygen supply to the developing foetus is seen to remain slightly increased during the post-partum period. Procoagulant clotting factors tend to increase in concentration, while some natural anticoagulant clotting factors decrease along with downregulation of the fibrinolytic pathway, a process that prevails for some time post-partum (Walker, 2000).

3.1.0.1: Changes in haemostatic parameters during pregnancy

To date, studies analysing haemostatic parameters during normal pregnancy have reported conflicting findings. FVIII, FVII, prothrombin fragments 1 + 2 (F1+2), FV, fibrinogen, PAI-1 and D-dimers have been found to increase progressively throughout gestation, with increases remaining during the post-partum period for FVIII, FV and F1+2 (Cerneca et al., 1997; Clark et al., 1998; Comeglio et al., 1996; de Moerloose et al., 1998; Kjellberg et al., 1999; Paniccia et al., 2002; Stirling et al., 1984; Wright et al., 1998). Decreases have been reported in PS, activated protein C resistance (APCR) and platelet counts (Cerneca et al., 1997; Clark et al., 1998; Faught et al., 1995; Hayashi et al., 2002; Kjellberg et al., 1999). AT and PC have been detected as unchanged compared to the non-pregnant state by many investigators (Cerneca et al., 1997; Clark et al., 1998; Faught et al., 1995; Kjellberg et al., 1999). However, contradictory studies have illustrated a slight decrease in AT concentrations (Stirling et
Thrombomodulin (TM) has been reported as both increased and unchanged throughout gestation (Hayashi et al., 2002; Kjellberg et al., 1999), while tissue plasminogen activator (TPA) levels have been found as both increased and decreased (de Moerloose et al., 1998; Kjellberg et al., 1999). Interestingly, the report of increases in all three of these proteins (TM, tPA and PAI-1) throughout gestation found each factor to decrease during the post-partum period, indicating prevention of both anticoagulant and fibrinolytic mechanisms three days post delivery (de Moerloose et al., 1998). The overall efficiency of the tissue factor pathway of coagulation, measured by the prothrombin time (PT), has been reported to decrease progressively and remain decreased in the puerperium. Conversely, the contact pathway of activation, measured by the activated partial thromboplastin time (APTT), has remained constant throughout all trimesters (Cerneca et al., 1997). These findings illustrate conflicting evidence for the theory of absolute increased coagulation and decreased fibrinolysis during pregnancy (table 3.1). However, one thing that remains clear is the dramatic haemodynamic changes that occur during the post-partum period. Plasma volume decreases by almost one litre due to the blood loss that occurs during labour, but increases again three days post-partum by the same amount. Haematocrit measurements (test for anaemia) have also been reported to decrease in patients that have undergone cesarean delivery. Although controversy still surrounds the coagulatory events throughout pregnancy, common sense suggests that increased coagulation is imperative for sustaining the excessive clotting required at the time of delivery and beyond, even as late as eight weeks post-partum. This is apparent when one considers that women can experience the expulsion of blood clots vaginally up to six weeks post delivery, clots which are thought to originate from the placental implantation site (Tula State University, 2003). Due to the existing controversies, we have performed prospective standardised measurements of these parameters throughout normal pregnancies to investigate the procoagulant state of gestation and the post-partum period, and to enable comparisons with pathological pregnancies in future work (chapter 4).

The haemostatic fluxes that occur during pregnancy have not been fully elucidated. By comparing complicated pregnancies with normal gestational outcomes, more insight may be gained into the normal pregnant haemostatic environment and an understanding of the underlying defects causing the problems experienced by some women during
pregnancy. Such an understanding will hopefully lead to the development of more efficient therapeutic modalities for these obstetrical pathologies (Brenner and Blumenfeld, 1997). One such pathology is gestational thrombophilia.

3.1.0.ii: Thrombophilia in pregnancy

The disruption of the natural balance of the coagulation and fibrinolytic pathways in conjunction with decreased blood flow in the lower limbs during pregnancy increases the risk of developing a thrombotic event both during the peripartum and post-partum periods, with a five-fold increased risk of venous thrombosis during gestation (McColl et al., 1997) and a ten-fold increased risk post-partum (Tula State University, 2003). Thromboembolism is still the leading cause of maternal morbidity and mortality in the developed world (Bates and Ginsberg, 1997; McColl et al., 1997). Diagnosis can be difficult as procedures are often damaging to the foetus, while treatment with anticoagulants can have serious foetal and maternal side effects (Bates and Ginsberg, 1997). The true incidence of thromboembolism (DVT, PE) during pregnancy has not been fully elucidated due to difficulties in diagnosis, with methods involving high radiation risks to the foetus. However, figures reported range from 0.05% to 0.07% (Bates and Ginsberg, 1997; McColl et al., 1997).

Gerhardt et al (1999) reported a family history of thrombosis to be an independent risk factor predicting a thrombotic event during pregnancy. Furthermore, a genetic predisposition to thromboembolic disease during pregnancy has been suggested, with the emergence of familial deficiencies of proteins involved in haemostasis. These include mutations in the Factor V gene (FV Leiden), the prothrombin gene (G20210A), antithrombin (AT) deficiency and protein C (PC) & protein S (PS) deficiencies (McColl et al., 1997; Walker, 2000). Moreover, inherited thrombophilias are increasingly being associated with an increased risk of developing pre-eclampsia (high blood pressure, swelling, proteinuria), placental abruption and poor obstetric outcome.
Table 3.1: Haemostatic changes throughout pregnancy. A review of the literature. N= number of patients in study.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Origin (N)</th>
<th>Increased</th>
<th>Decreased</th>
<th>Unchanged</th>
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</thead>
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<td>Antithrombin (AT) (slight decrease)</td>
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<td>Faught et al., 1995</td>
<td>Canada (91)</td>
<td>Fibrinogen, FII fragments 1 + 2 (F 1+2)</td>
<td>Free Protein S (PS)</td>
<td>Protein C (PC)</td>
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<td>Comeglio et al., 1996</td>
<td>Italy (104)</td>
<td>Fibrinogen, PC (post-partum), TPA, PAI-1, F 1+2</td>
<td>Prothrombin Time (PT) PS</td>
<td>Activated Partial Thromboplastin Time (APTT)</td>
</tr>
<tr>
<td>Cerneca et al., 1997</td>
<td>Italy (239)</td>
<td>Fibrinogen, FVII, FV, PC (post-partum), F 1+2</td>
<td>PS</td>
<td>AT</td>
</tr>
<tr>
<td>Clark et al., 1998</td>
<td>UK (239)</td>
<td>Fibrinogen, FVII, FV, PC (post-partum), F 1+2</td>
<td>PS</td>
<td>AT</td>
</tr>
<tr>
<td>de Moerloose et al., 1998</td>
<td>France (100)</td>
<td>TPA, PAI-1, TM</td>
<td>PS</td>
<td>PC</td>
</tr>
<tr>
<td>Kjellberg et al., 1999</td>
<td>Sweden (48)</td>
<td>Fibrinogen, Fibrin, FVII, FII, F 1+2, PAI-1, D-dimers</td>
<td>PS</td>
<td>TPA APCR</td>
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<td>Wright et al., 1998</td>
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<td>Fibrinogen, FVII, D-dimers</td>
<td>Platelets</td>
<td>TM</td>
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<td>D-dimers</td>
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<td>Hayashi et al., 2002</td>
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<td>Thrombin-antithrombin complexes (TAT)</td>
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</table>
3.1.0.iii: Acquired Thrombophilias

The antiphospholipid syndrome

The antiphospholipid syndrome (APS) is an acquired thrombophilic disorder associated with both venous and arterial thrombosis (Cuadrado et al., 1997), and Systemic Lupus Erythematosus (SLE), an autoimmune disorder (Kutteh, 1997). More recently it has been described as a disorder of recurrent thrombosis, pregnancy loss and thrombocytopenia (Cuadrado et al., 1997). The pathophysiological mechanism by which APA’s induce a procoagulant state is multifactorial and includes binding to phospholipids on the surface of platelets and the vascular endothelium, decreasing production of prostacyclin by endothelial cells, increasing thromboxane production by platelets and decreasing PC activation (Franklin et al., 2000; Kutteh, 1997). Furthermore, the thrombogenicity of APA’s is counteracted by the binding of a potent anticoagulant, Annexin-V to these antibodies. Annexin-V binding to APA’s has been reported as disrupted in trophoblast and endothelial cells causing increased thrombosis, and may be pivotal to development of thrombosis and pregnancy loss in the APS (Rand, 2000). Therefore, the regulation of APA’s may be crucial for the maintenance of a viable foetus.

The APA’s are a family of immunoglobulins of which anticardiolipin (ACL) and lupus anticoagulants (LAC) are the best known (Blumenfeld and Brenner, 1999; Franklin et al., 2000). APA frequency in the general population is thought to be between 1 and 2%, while the frequency in patients with venous thromboembolism is estimated to be between 5 and 15%, inferring a nine-fold increased risk of developing thrombosis (Walker, 2000). In a US study, the incidence of women positive for APA’s in normal obstetric patients was 5.3% (n=7,278), compared to 20% in RFL (n=2,226) (Kutteh, 1997). APA’s are as frequent in normal pregnancy as they are in thromboembolic disease, supporting the observation that pregnancy is a hypercoagulable state. Antibodies to β2-glycoprotein 1 (β2-GP1) have also been implicated in the pathogenesis of the antiphospholipid syndrome and RFL, since β2-GP1 is thought to have anticoagulant properties. Therefore, the higher the level of β2-GP1 antibodies, the greater the thrombogenicity (Samarkos et al., 2001; Visvanathan and McNeill, 1999).
Acquired activated protein C resistance (APCR)

Activated protein C resistance (APCR) usually occurs as a result of a polymorphism in the factor V gene called Factor V Leiden R506Q. Under normal circumstances, APC acts as a potent anticoagulant, cleaving activated FV along with its co-factor protein S. The FVL genotype changes one of the APC cleavage sites within the FVa molecule, thus markedly reducing APC’s ability of inactivating factor V’s procoagulant activity. The end result is increased thrombin generation raising the risk of thrombosis (Clark et al., 1999). Acquired APCR, or APCR in the absence of FV Leiden, results from elevated FVIII levels and is commonly found in patients with lupus anticoagulant or antiphospholipid syndrome (Brenner and Blumenfeld, 1997; Walker, 2000). An increase in APCR (decrease in APCR:FV ratio) has been observed with increasing age and in women taking estrogens as well as with advancing gestation (Clark et al., 1999; Walker, 2000). A progressive increase in APCR has been detected in multiple studies of women throughout normal pregnancy with a peak in the third trimester (Benedetto et al., 2002; Clark et al., 1998; Cumming et al., 1995; Mathonnet et al., 1996; Walker et al., 1997).

3.1.0.iv: Inherited Thrombophilias – Natural Anticoagulants

Antithrombin deficiency

Antithrombin (AT) deficiency was originally described in 1965 by Egeberg who studied a family with a high incidence of VTE, finding levels of antithrombin in affected family members to be half that of unaffected siblings (Greer et al., 1992). AT is a glycoprotein acting as a natural anticoagulant by inactivating thrombin along with other serine proteases of the coagulation cascade (Blumenfeld and Brenner, 1999; Greer et al., 1992). Inhibition of factors II, IXa, Xa, XIa and XIIa decreases coagulation activity and is enhanced 40,000 fold in the presence of glycosaminoglycans especially heparan sulphate (Blumenfeld and Brenner, 1999). AT deficiency is an autosomal dominant heterogenous disorder with more than eighty separate mutations documented, the main forms being Type I (quantitative, lack of protein production) and Type II (qualitative, non-functional protein) (High and Roberts, 1995). Heterozygous AT deficiency infers a 50% increased risk of developing a thrombotic episode over a lifetime and a 51% increased risk of developing thrombosis during pregnancy (Blumenfeld and Brenner, 1999). In contrast, the deficiency has also been shown to have no association with adverse pregnancy outcome with concentrations remaining
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constant and within normal ranges throughout pregnancy (Cerneca et al., 1997; Clark et al., 1998; Gris et al., 1999).

_Protein C & Protein S deficiencies_

PC and PS deficiencies have been observed in pregnancy associated venous thromboembolism (VTE) but their incidence is much lower than that observed for AT deficiency (Walker, 2000). PC deficiency is usually inherited in an autosomal dominant manner. It has been categorised into type I and type II deficiencies, with reduced functional and immunological PC levels and greater functional PC reduction than immunological reduction respectively. Type I PC deficiency is characterised by mutations in the PC gene (PROC) (Cooper and Krawczak, 1997a). Heterozygous symptomatic PC deficiency has a frequency of 0.3-0.5% in the general population (Seligsohn and Lubetsky, 2001).

PS deficiency, first described in 1984, has been associated with both arterial and venous thrombosis. PS circulates in two forms, as free PS (40%) and as that which is bound to C4b Binding Protein (C4bBP) (60%), a component of the complement system. Only free PS possesses APC cofactor activity in the anticoagulant pathway. PS deficiency can be categorised into type I (reduced free and total PS levels and activity), type II (normal free and total PS levels but reduced activity) and type III (normal total PS levels with reduced free PS and activity) (Cooper and Krawczak, 1997b). PS levels can be influenced by both the plasma concentrations of C4bBP and inherited defects (Cooper and Krawczak, 1997b). Two genes for PS have been mapped to chromosome 3, PROS1 the active gene and PROS2 the pseudogene. Due to the heterogeneity of mutations within the PROS gene, the prevalence of polymorphisms resulting in PS deficiency in the general population is largely unknown. It is estimated that PS deficiency in European populations with recurrent VTE has a prevalence of 1 to 5% and patients deficient in PS have a 2.4-fold increased risk of developing thrombosis (Faioni et al., 1997).

The risk of thrombosis during pregnancy has been found to increase in the presence of deficiencies of the natural anticoagulants, with AT deficiency presenting the greatest risk (Bates and Ginsberg, 1997). In a comprehensive UK study of 50 women with gestational thrombosis, AT deficiency was detected in 12%, and PC deficiency in 2%
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of patients with VTE, with the majority of cases of DVT presenting in the antenatal period, the time of greatest thrombogenic potential (McColl et al., 1997). The heterogeneity of PS deficiency makes it difficult to determine the risk of its deficiency in promoting thrombosis during pregnancy (Walker, 2000), however it has been estimated to be between 0-6%, while the risk with PC deficiency has been estimated as 3-10% (Eldor, 2001).

3.1.0.v: Inherited Thrombophilies – Procoagulants

Fibrinogen

Fibrinolytic activity, whilst decreased during pregnancy, rapidly returns to normal levels after delivery. This is manifested by increased fibrinogen concentrations throughout gestation, which have been demonstrated by a number of studies (table 3.1). Fibrinogen as an acute phase protein may be increased as a result of inflammation or infection during pregnancy, however since pregnancy is an acute phase state, progressive increased fibrinogen levels may be a direct result of this response (Lane and Grant, 2000). A polymorphism within the β fibrinogen gene at position –455 in the 5′ promoter region has been associated with increased plasma fibrinogen concentrations (Humphries et al., 1995). This guanine (G) to adenine (A) transition is close to an IL-6 responsive element, which is thought to mediate cytokine upregulation of fibrinogen (Franco and Reitsma, 2001; Lane and Grant, 2000). Recently the G-455A fibrinogen polymorphism was reported to have similar allelic distribution in a cohort of pre-eclamptic patients and controls (Laasanen et al., 2002), but to date, the G-455A polymorphism has not been investigated in patients suffering from gestational thrombosis.

APCR & FV Leiden

Inherited APCR is the result of a single point mutation of G to A at nucleotide 1691 in the factor V gene called the FVL polymorphism (Kutteh et al., 1999), destroying one of the APC cleavage sites, and substituting a glutamine for arginine at amino acid 506 (Younis et al., 1997). There are three APC cleavage sites on membrane bound activated factor V (FVα), Arginine (Arg) 306, Arg 506 and Arg 679. The cleavage that occurs at Arg 506 is required for subsequent cleavage at Arg 306 and 679. In the presence of the FVL polymorphism, this important site is lost leaving the FVα molecule intact and active, promoting thrombosis (Cooper and Krawczak, 1997b). The European
prevalence of FVL is about 4% with frequencies reported to range from 1% in a French population to 11% in a Swedish cohort (Gandrille et al., 1995; Holm et al., 1996; Lane and Grant, 2000). It is the most common inherited cause of thrombophilia in certain populations, with a prevalence of 20-60% in patients with venous thromboembolism (Bates and Ginsberg, 1997). The risk of developing thrombosis is increased 5-10 fold for heterozygous individuals and 50-100 fold for homozygous individuals, although these are rare (Younis et al., 1997). Furthermore, FVL has been found in approximately 60% of cases of gestational thrombosis (Bates and Ginsberg, 1997; Blumenfeld and Brenner, 1999; Brenner and Blumenfeld, 1997; Kutteh et al., 1999). For these reasons we determined to evaluate the prevalence of this polymorphism in an Irish population of pregnant women as a preliminary step to investigating the same in an Irish cohort of RFL patients, to evaluate the pathogenicity of this procoagulant polymorphism in women with a history of RFL. Furthermore, it is hoped that investigation of this polymorphism will demonstrate the multifactorial nature of RFL, since women possessing FV Leiden have maintained normal pregnancies.

**Prothrombin**

The zymogen prothrombin or Factor II (FII) is activated by FXa to thrombin, which cleaves fibrinogen to fibrin monomers. A defect in the gene for FII could potentially disrupt the fine balance of the coagulation cascade and promote thrombosis (Lane and Grant, 2000). Poort et al (1996) reported the presence of a single nucleotide transition from G to A at position 20210 in the 3' untranslated region of the prothrombin gene, subsequently called the prothrombin G20210A variant. Eighteen percent of VTE patients had the polymorphism compared with 1% of healthy controls, increasing the risk of VTE 3-fold (odds ratio 2.8). Moreover, the A allele is associated with increased plasma prothrombin levels which is most likely the cause of increased thrombosis (Poort et al., 1996). The prevalence of the G20210A prothrombin polymorphism in the general Caucasian population is said to be 2% (Lane and Grant, 2000; Rosendaal et al., 1998), with European studies showing prevalence ranges from 1% in a French population to 7.8% in a German cohort, while the prevalence in the UK has been reported at 3.5% (Beauchamp et al., 1994; Gandrille et al., 1995; Schroder et al., 1996). Southern European populations have a higher prevalence of the A allele than northern populations, with non-Caucasian ethnic groups lacking this gene variant (Lane and Grant, 2000; Rosendaal et al., 1998; Rosendaal et al., 1997). The prothrombin
variant has been associated with an increased risk of gestational thrombosis in a group of German patients (16.9%, n=119) compared to non-thrombotic controls (1.3%, n=233, p=0.001) (Gerhardt et al., 2000).

**Homocysteine & MTHFR**

Homocysteine is a non-protein forming sulphur amino acid derived from dietary methionine, and has a normal concentration range in plasma of 5-15μmol/L. (Blumenfeld and Brenner, 1999; D'Angelo and Selhub, 1997). Homocysteine is trans-sulphurated to cystathionine by cystathionine β-synthase (Cβs) or remethylated to methionine by a mechanism involving methylenetetrahydrofolate reductase (MTHFR) and methylene synthase. Hyperhomocysteinemia may be caused by inherited defects in these enzymes. When present in the homozygous state (especially Cβs) plasma homocysteine concentrations may exceed >50μmol/L, this elevation having been associated with VTE and atheroma progression in childhood and early adulthood (Blumenfeld and Brenner, 1999). A cytosine (C) to thymine (T) transition has been described at nucleotide 677 in the MTHFR gene. The polymorphism results in a thermolabile enzyme with 50% activity (t-MTHFR). Homozygosity (TT: thymine base on both alleles of the gene) has been reported in 5-15% of normal populations studied depending on ethnicity (Blumenfeld and Brenner, 1999). The enzymatic cofactors folic acid, vitamin B₆ and vitamin B₁₂, have an important role in the regulation of homocysteine metabolism. In addition, nutritional deficiencies of the enzymatic cofactors can cause acquired hyperhomocysteinemia. It has been suggested that individuals with homozygosity for the t-MTHFR variant may have a higher requirement for folate than those with the wild type genotype (CC) (D'Angelo and Selhub, 1997). Indeed folic acid deficiency and the t-MTHFR variant have been associated with an increased risk of neural tube defects, VTE, arterial thrombosis and early pregnancy loss (D'Angelo and Selhub, 1997; den Heijer et al., 1998; Margaglione et al., 1998; Nelen et al., 1997; Steegers-Theunissen et al., 1994). However, the prevalence of MTHFR in normal controls and thrombosis has been determined but demonstrated as highly variable. Further studies included in this thesis (chapter 5, VTE) reveal a higher prevalence of homozygosity for the t-MTHFR variant in the general Irish population (T allele frequency 0.41) than previously published (T allele frequency 0.29) (Whitehead et al., 1995). This has been supported by a similar Irish population study reporting a T allele frequency of 0.38 (Harmon et al., 1997). These
conflicting reports suggest non-standardised assay conditions or the necessity for very large control populations, making this gene variant unreliable in disease association studies.

**Tissue plasminogen activator & Plasminogen activator inhibitor**

Tissue plasminogen activator (TPA) stimulates the activation of the fibrinolytic pathway via activation of plasmin. Although increased TPA concentrations have been associated with a 2-fold increased risk of myocardial infarction (MI) (van der Bom et al., 1997), controversy surrounds the role of TPA in gestational thrombosis. Whilst increased plasma concentrations of TPA have been reported in normal pregnancy by a number of investigators (table 3.1) (Cerneca et al., 1997; de Moerloose et al., 1998), others have reported decreased concentrations in pregnant women (Kjellberg et al., 1999; Wright et al., 1988). An Alu repeat insertion (I)/deletion (D) polymorphism has been located in intron 8 of the TPA gene (Tishoff et al., 1996). The I allele has been reported to influence the release rates of TPA (Jern et al., 1999) and has recently been associated with an increased risk of gestational VTE in a cohort of Caucasian women (Hooper et al., 2001). Plasminogen activator inhibitor-1 (PAI-1) is a potent inhibitor of TPA whose increased concentrations have been implicated in impaired fibrinolysis during pregnancy and subsequent gestational thrombosis (Bellart et al., 1998). A single nucleotide insertion/deletion (4G/5G) polymorphism in the promoter region of the PAI-1 gene has functional importance in regulating expression (Dawson et al, 1993). The 5G, but not the 4G, allele contains a transcriptional repressor binding site (Dawson et al, 1993). Individuals homozygous for the 4G allele have higher PAI-1 plasma levels compared to individuals with the 5G/5G genotype, with heterozygotes showing an intermediate level (Eriksson et al, 1995; Grubic et al, 1996). The presence of the 4G allele has been associated with venous and arterial thrombosis (Eriksson et al, 1995) and with poor outcome and the development of septic shock following meningococcal infection (Westendorp et al, 1999; Hermans et al, 1999). To the best of our knowledge, the prevalence of these polymorphisms in obstetric patients has not yet been evaluated.

**Angiotensin Converting Enzyme**

Angiotensin converting enzyme (ACE) catalyses the conversion of Angiotensin I to Angiotensin II which functions as a vasoconstrictor, regulator of endothelial function and smooth muscle cell proliferation (Hassan and Markus, 2000). In addition, it
Consequently, the immune response during normal pregnancy has gained great attention in recent years.

The uterus is part of the common mucosal immune system and is unique in its adaptation to support pregnancy, while continuing to retain the barrier function characteristic of all mucosal tissues. The most notable difference between uterine and other mucosal surfaces is the lack of highly organised secondary lymphoid nodules that aid in the presentation of antigens to the mucosal surface (Kuby, 1994; Robertson, 2000). At the feto-maternal interface, T-lymphocytes secrete specific cytokines that are characteristic of specific types of immune responses (Robertson, 2000). Vassiliadis et al. (1998) studied the serum levels of cytokines in non-pregnant women and throughout normal gestation, reporting stable TNF-α and IL-12 levels throughout normal pregnancy and labour, while IFN-γ levels were found to exhibit a steady increase after the first trimester of pregnancy. IL-4 and IL-10 were consistently present throughout gestation with IL-10 concentrations peaking during labour (Vassiliadis et al., 1998). Krasnow et al. (1996) analysed the secretion of cytokines from the endometrium and decidual stroma cells. They found that IL-10 levels increased greater than 10-fold from the peri-implantation period to the first trimester of pregnancy with a further increase in the second trimester (Krasnow et al., 1996). Therefore, there appears to be a specific pattern of production and release of cytokines throughout gestation.

3.1.1 Type 1/Type 2 immunity and pregnancy

The first report on a type 2 immune response bias in pregnancy came from Wegman et al. (1993) who postulated the existence of a bi-directional system of cytokine production during pregnancy in murine models. It was found that feto-placental tissue spontaneously released the type 2 cytokines IL-4, IL-5 and IL-10, with IFN-γ present in almost undetectable levels during the early stages of gestation. Further analysis showed that abortion prone mice predominantly secreted the type 1 cytokines IL-2, TNF-α and INFγ. It was then postulated that the secretion of type 2 cytokines, particularly IL-10, is the most beneficial to reproductive fitness (Wegman et al., 1993). This prompted the first human study of cytokine profiles in peripheral blood mononuclear cells (PBMC’s) in women with a history of RFL. It was reported that women with no history of RFL and male controls failed to produce a type 1 immune response, but instead exhibited a type 2 bias in contrast to those with a history of RFL whose immune response was
predominantly type 1 (Hill et al., 1995). Subsequent human studies have reported a similar type 2 response in successful pregnancies (Gratacos et al., 1998; Lim et al., 1998; Rezaei, 2002). Furthermore, in normal pregnancy, the type 2 cytokines IL-6 and IL-10 have been reported at a significantly higher concentration than in patients with a history of RFL (Jenkins et al., 2000; Lim et al., 2000; Makhseed et al., 2000). It is now widely accepted that type 1 cytokines and maternal cell-mediated anti-foetal immunity are detrimental to successful pregnancy, while normal pregnancy is characterised by a localised humoral immune response secreting type 2 cytokines (Raghupathy, 1997). Figure 3.1 demonstrates the delicate balance of cytokine expression required for successful pregnancy.

3.1.1.ii: Type 1/type 2 cellular effects on procoagulant activity

The presence of a humoral immune response has further implications for the maintenance of successful pregnancy by preventing the development of a procoagulant environment within the uterus. Induction of TF expression and subsequent coagulation has been shown to be a specific function of Th1 cells and Th0 cells, but not Th2 cells (Fan et al., 1990). IFNγ was found to play a major role in this TF induction by type 1 cells, with anti-IFNγ antibody administration reducing TF synthesis. In addition, type 2 cytokines IL-4, IL-13 and in particular IL-10 were reported to exert a dose-dependent inhibitory effect on type 1 induced monocyte TF production, while anti Th2-type cytokine treatment resulted in reduced inhibition of TF synthesis (del Prete et al., 1995). It has recently been shown that patients with APS, a condition associated with recurrent thrombosis and pregnancy loss, have increased TF synthesis in response to stimulation by β2GP1, a phospholipid binding protein thought to be the target of antiphospholipid binding assays (Lockshin, 1999). This response is thought to occur via β2GP1-specific CD4+ T cells, that proliferate and produce IFNγ when stimulated with β2GP1 (Visvanathan et al., 2000). Production of IFNγ by activated β2GP1-specific T cells may contribute to the high frequency of pregnancy failure in the APS. Furthermore, type 1 cytokines may be detrimental to pregnancy by upregulating coagulation, since impaired coagulatory mechanisms have been associated with 50% of cases of RFL (Lane and Grant, 2000).
We endeavoured to determine the concentrations of type 1 cytokines (TNFα, IFNγ) and type 2 cytokines (IL-6, IL-10) throughout normal pregnancy, to determine the normal cytokine profile throughout gestation and to elucidate which type of immune response is more beneficial to reproductive fitness.
Figure 3.1: The T helper 1 and T helper 2 type reactivity balance in pregnancy (taken from Raghupathy, *Immunology Today*. 1997; 18(10): 478-482). PIBF = Progesterone induced blocking factor; NK = Natural killer cells.
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3.1.2: Genetic regulation of cytokine concentrations in pregnancy

A recent review of all cytokine gene polymorphisms in human disease provides a comprehensive list of 32 cytokine gene regions corresponding to 110 polymorphic markers. These include polymorphisms in the genes for the classic type 1 and type 2 cytokines discussed previously (Bidwell et al., 1999). Although a comprehensive list, three years have passed since its generation and a multitude of research into inflammatory mechanisms and polymorphic loci has been undertaken, yielding no independent immunological genetic risk factor for RFL. To investigate the frequency of such polymorphisms in RFL patients, it was firstly necessary to determine their frequency in the normal pregnant population and in non-pregnant female controls, an exercise that also enabled the determination of a correlation between genotype and phenotype in this longitudinal study.

3.1.2.i: Interleukin-10

Secretion of IL-10 has been demonstrated as a necessary component for successful pregnancy by many investigators (Hill et al., 1995; Lim et al., 1996; Makhseed et al., 2000; Wegman et al., 1993), and increases have been found throughout pregnancy (Jenkins et al., 2000; Krasnow et al., 1996), and at low levels during gestation with a peak during labour (Makhseed et al., 2000; Vassiliadis et al., 1998). Two polymorphisms within the promoter region of the IL-10 gene have been reported (Turner et al., 1997). The –1082 polymorphism is located within a transcription factor binding site while the –592 polymorphism lies within a negative regulatory region of the promoter (Crawley et al., 1999; Turner et al., 1997). The IL-10 –1082 G allele has been associated with increased IL-10 concentrations, while the A allele has been associated with decreased concentrations (Turner et al., 1997). As yet, no functional significance has been found for the C-A transition at position –592. Both polymorphisms are reported to vary significantly between different ethnic populations. The IL-10 –1082 G allele was detected on average in 50% of European Caucasian individuals studied compared to 6% in a Chinese population. The –592 C allele occurs most frequently in Europeans (80%) compared to other populations such as the Chinese (33%) (Reynard et al., 2000). No studies to date have investigated the effect of these polymorphisms on IL-10 levels throughout normal pregnancy.
3.1.2.ii: Interleukin-6

IL-6 is a type 2 cytokine produced by many cell types with many diverse functions including roles in haematopoietic stem cell differentiation, stimulation of acute phase responses to infection and injury, differentiation and/or activation of macrophages and T cells and growth of B cells. IL-6 is not endogenously expressed but is induced by various stimuli such as IL-1, TNFα, platelet-derived growth factor, bacterial endotoxin and viral infections. One of its main functions is in the acute phase response where it acts as a mediator for T cell-dependent antibody responses (Terry et al., 2000). Moreover, IL-6 mediates fibrinogen changes via an IL-6 responsive element within the promoter of the β chain gene of fibrinogen (Franco and Reitsma, 2001; Lane and Grant, 2000). The IL-6 gene contains a promoter polymorphism in the 5' flanking region of the gene predicting a G to C transition at position −174 (Olomolaiye et al., 1997; Olomolaiye et al., 1998), which lies within the region from −180 to −123 bases that has been found to be crucial for transcription induction (Terry et al., 2000). The IL-6 −174 polymorphism negatively affects the transcription of the IL-6 gene and homozygotes for the CC genotype have been reported to have decreased plasma levels of IL-6 after exposure to inflammatory stimuli such as IL-1 and LPS (Fishman et al., 1998). An investigation of patients with systemic-onset juvenile chronic arthritis reported the CC genotype at a reduced frequency in patients compared to controls. Individuals with the GG genotype had IL-6 concentrations twice that of the CC genotype, suggesting a protective effect of the C allele in these patients (Fishman et al., 1998). Increased IL-6 concentrations are thought to be beneficial to successful pregnancy since both estrogen and progesterone regulate IL-6 expression, suggesting a role for this anti-inflammatory cytokine in implantation and the continuation of early pregnancy (Lim et al., 1996). IL-6 levels have been reported as stable throughout pregnancy and decreased in patients with RFL (Makhseed et al., 2000). To date there have been no reports of an association of the IL-6 −174 polymorphism with levels of the protein in normal pregnant patients.

3.1.2.iii: Tumor Necrosis Factor (TNF) α and β (Lymphotoxin α)

TNF is a pro-inflammatory cytokine capable of upregulating both inflammatory and coagulation mechanisms. It is the primary cytokine produced in the early stages of an inflammatory reaction promoting the production and secretion of a number of other cytokines involved in eliciting the inflammatory response, including IL-1β, IL-6, IL-10 and platelet activating factor (PAF) (Mantovani et al., 1997). As a procoagulant TNF
increases thrombin generation \textit{in vivo}, induces TF production on the surface of monocytes and endothelial cells, and inhibits the transcription and expression of thrombomodulin, downregulating the PC anticoagulant pathway (Lentz \textit{et al.}, 1991; Mantovani \textit{et al.}, 1997; Okajima, 2001; ten Cate \textit{et al.}, 1997). A G to A transition mutation at position $-308$ in the promoter region of the TNF$\alpha$ gene predicts the TNF A1 (G) allele or the TNF A2 (A) allele (Wilson \textit{et al.}, 1992). This polymorphism has been suggested to influence TNF gene expression and the more rare TNF 2 allele has been associated with higher endogenous and inducible levels of TNF$\alpha$ secretion (Warzocha \textit{et al.}, 1998).

The TNF $\beta$ gene, also known as lymphotoxin $\alpha$ (LT$\alpha$), has been found to have a polymorphic marker at position $+252$ of intron 1 of the gene. This G to A transition predicts the TNFB1 (G) and TNFB2 (A) alleles respectively (Messer \textit{et al.}, 1991). The effect of this polymorphism on concentrations of both TNF$\alpha$ and TNF$\beta$ is still largely undetermined due to conflicting reports (Lee \textit{et al.}, 1997; Majestschak \textit{et al.}, 1999; Messer \textit{et al.}, 1991; Pociot \textit{et al.}, 1993; Pociot \textit{et al.}, 1991; Stuber \textit{et al.}, 1996; Whichelow \textit{et al.}, 1996). Controversy continues to surround these TNF polymorphisms with regard to their influence on TNF concentrations, an area that requires further investigation.

We endeavoured to elucidate the prevalence of polymorphisms within these inflammatory genes in normal pregnant patients to determine the genotype/phenotype relationship between cytokine serum concentrations and genotypes during normal pregnancy, in the hope of revealing inter-individual differences in cytokine production regulated at the genetic level.
AIMS

- To determine the normal range of haemostatic components throughout pregnancy by examining their changes longitudinally.

- To determine the normal concentrations of inflammatory cytokines throughout pregnancy.

- To elucidate the prevalence of polymorphisms within genes encoding coagulatory, fibrinolytic and inflammatory proteins in pregnant patients and to determine their influence on protein expression during pregnancy.
3.2.0: MATERIALS & METHODS

3.2.1: Subjects and sample processing
65 pregnant women with no previous history of pregnancy loss were recruited at the Rotunda Maternity Hospital from January 1999 until June 2002. The average age of pregnant patients was 29 years (range 17-42 years). Clotted blood was collected along with EDTA and citrate anticoagulated samples from each individual at approximately 8 week intervals from week 8 of gestation through to week 36 and again at three months post-partum. The post-natal samples were difficult to obtain as very few patients and controls returned following phone requests, and therefore the post-natal numbers dropped to 18 patients (chapter 2.1.7). 65 non-pregnant control samples were collected from female volunteers and all blood samples were processed on site of collection (chapter 2.1.7). DNA was extracted from EDTA anticoagulated whole blood by phenol/chloroform treatment and ethanol precipitation (chapter 2.2.2).

3.2.2: Standard Full Blood Counts and Thrombophilia Screens
All routine measurements were carried out by staff of the Haematology Department, St James’s Hospital, and the coagulation laboratory of the National Centre for Hereditary Coagulation Disorders (NCHCD), St. James’s Hospital, Dublin 8. Full blood counts (FBC) included the measurement of the following parameters at each time interval throughout pregnancy: Hb concentrations; HCT including MCV, MCH and MCHC; RCC and RDW; platelet counts (Chapter 2.3.1.ii-v). Differential leukocytes were counted using automated flow cytometry techniques or by manual cell counting under a microscope when automation was not possible (Chapter 2.3.1.i). The differential cell count included assessment of the following cell types: total WCC; neutrophils; lymphocytes; monocytes; eosinophils; basophils. Thrombophilia screens included the measurement of the following functional coagulation assays at each time interval: PT; APTT; AT; FVIII; fibrinogen; PC; PS; APCR and LAC (Chapter 2.3.2.i-ix).

3.2.3: PCR analysis of polymorphic markers
PCR amplification of all polymorphic sites was performed in a total reaction volume of 50μl. The standard PCR reaction mix (Appendix table A.3.1) consisted of Taq DNA Polymerase buffer with MgCl₂ (Promega) [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 1.5 mM MgCl₂], 0.4 units of DNA Taq polymerase, 2μl of genomic DNA, 4% dimethyl sulphoxide (DMSO, Sigma), 30μM each of
deoxyribonucleoside triphosphates (Boehringer Mannheim), and specific concentrations of each sense primer and antisense primer (Sigma Genosys) depending on assay conditions (chapter 2.4.1-2.4.2). The cycling parameters for each assay are listed in chapter 2, table 2.2 and 2.4. The FV Leiden, PAI-1, prothrombin G20210A, beta fibrinogen, and the cytokine gene amplicons were digested with the appropriate enzyme (chapter 2, table 2.1 and 2.3) overnight at 37°C according to manufacturers instructions (New England BioLabs). The ACE and TPA PCR products were visualised directly on agarose gels (Pronadisa, Madrid). Restriction digest/PCR products were run on the appropriate percentage of agarose gel containing 1.6μg/ml ethidium bromide (Sigma). Typical DNA banding patterns of PCR amplicons and restriction digest products for each polymorphism are illustrated in chapter 2, figures 2.1-2.12.
3.3.0: RESULTS

3.3.1: Full Blood Count changes throughout pregnancy

3.3.1.i: Haemoglobin

The mean Haemoglobin (Hb) concentration decreased from 14.6g/dL at 8 weeks to 11.7g/dL throughout pregnancy with a slight increase at 3 months post-partum to 12.8g/dL (table 3.2, figure 3.2). There was no significant variation in the change in concentrations over time in this cohort of patients (p=0.92).

3.3.1.ii: Haematocrit, MCV, MCH & MCHC

The HCT ratio (volume of red cells expressed as fraction of total blood volume) is a simple screening test for anaemia along with the MCV, MCH and MCHC measurements. The HCT ratio was seen to fluctuate over time during pregnancy increasing post-partum, but remaining consistently within normal ranges (ratio 0.37-0.47). Repeated measures ANOVA revealed a significant variation in HCT ratio over time (p=0.04). There was no significant variation detected over time for the MCV, MCH and MCHC measurements throughout pregnancy (table 3.2, figure 3.2).

3.3.1.iii: Red cell parameters

The Red cell count (RCC) revealed a significant decrease over time during pregnancy (p=0.015) ranging from 4.2-3.8 x 10^{12} cells/L and increasing to 4.6 x 10^{12} cells/L post-partum, values within the normal range (3.8-5.8 x 10^{12} cells/L) (table 3.2, figure 3.2). The red cell distribution (RDW) test measures the red cell volume in units, a parameter which increased throughout pregnancy and remained increased post-partum. No significant difference in this change was detected over time (p=0.12).

3.3.1.iv: Platelet Counts

A small decrease in platelet counts was detected between 8 and 16 weeks gestation (228-217 x 10^{9} cells/L), with a gradual increase to 258 x 10^{9} cells/L post-partum (normal range 140-450 x 10^{9} cells/L) (table 3.2, figure 3.2). These changes in platelet counts were found to be significantly variable over time (p=0.012).
Table 3.2: Mean concentrations of haemostatic components of the standard full blood count at time points throughout pregnancy and postpartum. 3 months PN = 3 months post natal.

<table>
<thead>
<tr>
<th>Haemostatic Parameter</th>
<th>Normal Range</th>
<th>8 weeks</th>
<th>16 weeks</th>
<th>26 weeks</th>
<th>36 weeks</th>
<th>3 months PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (Hb)</td>
<td>11.5-16.5g/dL</td>
<td>14.6</td>
<td>12.0</td>
<td>11.4</td>
<td>11.7</td>
<td>12.3</td>
</tr>
<tr>
<td>Haematocrit (HCT)</td>
<td>0.37-0.47 ratio</td>
<td>0.38</td>
<td>0.36</td>
<td>0.39</td>
<td>0.34</td>
<td>0.38</td>
</tr>
<tr>
<td>Mean Cell Volume (MCV)</td>
<td>80-96 fL</td>
<td>88.9</td>
<td>89.9</td>
<td>91.1</td>
<td>90.0</td>
<td>84.5</td>
</tr>
<tr>
<td>Mean Cell Hb (MCH)</td>
<td>27-32pg</td>
<td>30.3</td>
<td>30.5</td>
<td>31.4</td>
<td>35.8</td>
<td>28.3</td>
</tr>
<tr>
<td>MCH concentration (MCHC)</td>
<td>30-36g/dL</td>
<td>34.0</td>
<td>33.7</td>
<td>34.2</td>
<td>34.1</td>
<td>33.5</td>
</tr>
<tr>
<td>Red Cell Count (RCC)</td>
<td>3.8-5.8 x10^12 cells/L</td>
<td>4.2</td>
<td>4.0</td>
<td>3.7</td>
<td>3.8</td>
<td>4.6</td>
</tr>
<tr>
<td>Red Cell Distribution (RDW)</td>
<td>11-15 units</td>
<td>12.7</td>
<td>13.1</td>
<td>12.9</td>
<td>13.0</td>
<td>13.9</td>
</tr>
<tr>
<td>Platelets</td>
<td>140-450 x10^9 cells/L</td>
<td>228.0</td>
<td>216.6</td>
<td>221.0</td>
<td>221.8</td>
<td>257.8</td>
</tr>
</tbody>
</table>
Figure 3.2: Longitudinal assessment of haemostatic changes by Full Blood Count measurement on Sysmex 2100 instrumentation. (a) Haemoglobin; (b) Haematocrit; (c) Mean cell volume; (d) Mean cell haemoglobin; (e) Mean cell haemoglobin concentration; (f) Red cell count; (g) Red cell distribution; (h) Platelets.
3.3.2: Coagulation changes throughout pregnancy

3.3.2.i: Prothrombin Time
The Prothrombin Time (PT) was found to remain relatively stable over time throughout pregnancy with a slight increase in the mean at 26 weeks, probably due to a spurious increase in one patient only. Consequently, there was no significant variation in the clotting time throughout pregnancy (p=0.42).

3.3.2.ii: Activated Partial Thromoplastin Time
The activated partial thromboplastin time (APTT) was found to remain stable throughout pregnancy, with no significant variation over time detected (p=0.59).

3.3.2.iii: Antithrombin
AT concentrations were detected within the normal adult non-pregnant range (0.76-1.2 IU/ml) throughout pregnancy with no significant variation over time detected (p=0.31).

3.3.2.iv: Factor VIII
Factor VIII (FVIII) concentrations were found to vary significantly over time within normal pregnancy (p=0.023), increasing progressively from 1.29 IU/ml at 8 weeks to 2.35 IU/ml at 36 weeks gestation. A decrease in concentration to within normal ranges (0.5-2.0 IU/ml) was observed post-partum (1.25 IU/ml).

3.3.2.v: Fibrinogen
Fibrinogen concentrations were increased above the normal range (1.5-4.0g/L) throughout pregnancy, with a significant variation over time detected (p=0.0013). Concentrations increased progressively from 8 weeks (4.2 g/L and 4.4g/L) to 36 weeks (5.4g/L and 5.5g/L) gestation, returning to within normal ranges post-partum (3.5g/L).

3.3.2.vi: PC
PC concentrations exhibited a slight fluctuation throughout pregnancy, increasing from 1.1 IU/ml at 8 weeks to 1.2 IU/ml at 26 weeks, with a further decrease to 1.09 IU/ml at 36 weeks gestation. Subsequently, concentrations returned to 1.1 IU/ml post-partum, all of which were within normal non-pregnant ranges (0.71-1.45 IU/ml). Consequently, there was no significant variation in concentration over time (p=0.90).
3.3.2. PS

PS concentrations decreased from 0.66 IU/ml at 8 weeks gestation to a low of 0.59 IU/ml at 36 weeks gestation, falling below normal non-pregnant ranges (0.71-1.45 IU/ml) throughout normal pregnancy. This variation was not statistically significant over time (p=0.19). Concentrations returned to within the normal range post-partum (0.93 IU/ml).

3.3.2. APCR

APCR was measured at each stage during pregnancy with no acquired resistance detected (in the absence of FV Leiden). The mean APCR ratio was 2.5 in non-FV Leiden carriers and 1.6 in carriers of the mutation (see FV Leiden results below). No significant variation in APCR was detected over time throughout pregnancy (p=0.07).

3.3.2. Beta-2 Glycoprotein 1 & LAC

β2GP1 concentrations were found to decrease throughout pregnancy from 181µg/ml at 8 weeks to 159µg/ml at 36 weeks gestation, with a slight increase post-partum (163µg/ml). Repeated measures ANOVA analysis revealed no significant variation in β2GP1 concentrations over time (p=0.63). All patients in this study were found to be negative for the presence of lupus anticoagulants.
Table 3.3: Mean concentrations of components of the coagulation thrombophilia screen test at time points throughout pregnancy and postpartum. 3 months PN = 3 months post natal; PT = prothrombin time; APTT = activated partial thromboplastin time; AT = antithrombin; FVIII = factor VIII; PC = protein C; PS = protein S; APCR = activated protein C resistance; β2GP1 = β-2 Glycoprotein 1.

<table>
<thead>
<tr>
<th>Coagulant Parameter</th>
<th>Normal Range</th>
<th>8 weeks</th>
<th>16 weeks</th>
<th>26 weeks</th>
<th>36 weeks</th>
<th>3 months PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT</td>
<td>11.5-15.0 seconds</td>
<td>13.7</td>
<td>12.9</td>
<td>15.4</td>
<td>12.8</td>
<td>13.2</td>
</tr>
<tr>
<td>APTT</td>
<td>25.0-36.0 seconds</td>
<td>33.0</td>
<td>33.1</td>
<td>32.5</td>
<td>32.6</td>
<td>32.2</td>
</tr>
<tr>
<td>AT</td>
<td>0.76-1.2 IU/ml</td>
<td>1.0</td>
<td>0.94</td>
<td>0.96</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>FVIII</td>
<td>0.5-2.0 IU/ml</td>
<td>1.29</td>
<td>1.76</td>
<td>2.04</td>
<td>2.35</td>
<td>1.25</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1.5-4.0 g/L</td>
<td>4.2</td>
<td>4.1</td>
<td>4.7</td>
<td>5.4</td>
<td>3.5</td>
</tr>
<tr>
<td>PC</td>
<td>0.71-1.45 IU/ml</td>
<td>1.1</td>
<td>1.1</td>
<td>1.2</td>
<td>1.09</td>
<td>1.1</td>
</tr>
<tr>
<td>PS</td>
<td>0.71-1.45 IU/ml</td>
<td>0.66</td>
<td>0.65</td>
<td>0.60</td>
<td>0.5</td>
<td>0.93</td>
</tr>
<tr>
<td>APCR</td>
<td>&gt;2.0 ratio</td>
<td>2.5</td>
<td>2.4</td>
<td>2.8</td>
<td>2.3</td>
<td>2.5</td>
</tr>
<tr>
<td>β2GP1</td>
<td>undetermined</td>
<td>181</td>
<td>171</td>
<td>166</td>
<td>159</td>
<td>163</td>
</tr>
</tbody>
</table>
Figure 3.3: Longitudinal study of coagulation proteins throughout pregnancy. Citrate anticoagulated blood was collected at 8, 16, 26, and 36 weeks gestation and 3 months post-partum (3mo PN). Plasma was collected and stored at -20°C until batch analysis was performed on the ACL Futura/Advance instrumentation. (A) Protein S; (B) Factor VIII; (C) Fibrinogen.
3.3.3: Coagulation & Fibrinolytic gene polymorphisms

3.3.3.i: FVL, Prothrombin and t-MTHFR polymorphisms

Heterozygosity for the FV Leiden polymorphism was detected at a frequency of 1.5% in pregnancy patients (1/66) with the A allele at a frequency of 0.01. No homozygotes for the polymorphism were detected (table 3.4). The prothrombin variant was not detected in any individual in this study. Heterozygosity for the MTHFR thermolabile variant (C677T) was detected in 15.1% of pregnant patients (10/66) with the T allele occurring at a frequency of 0.35 (table 3.4).

3.3.3.ii: Fibrinogen G-455A

Heterozygosity for the fibrinogen G-455A polymorphism was detected in 1.5% of pregnant patients (1/66) (table 3.4). There was no significant variation in fibrinogen concentrations at each time point in relation to genotype (p>0.05) by One way ANOVA analysis indicating that this mutation did not dictate the increase in fibrinogen observed during pregnancy.

3.3.3.iii: PAI-1, TPA & ACE

Homozygosity (4G/4G) for the PAI-1 polymorphism was detected in 33.3% of pregnant patients (22/66) while the TPA II genotype was detected in 24.2% of patients (16/66). Homozygotes for the ACE DD genotype represented 37.9% of normal pregnant patients (25/66) (table 3.4).
Table 3.4: Coagulation and fibrinolytic genotype and allele counts in normal pregnant patients.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Raw Counts</th>
<th>Counts (%)</th>
<th>Allele</th>
<th>Frequency</th>
</tr>
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<tbody>
<tr>
<td>FV</td>
<td>AA</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>1</td>
<td>1.5</td>
<td>G</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>65</td>
<td>98.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FII</td>
<td>AA</td>
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<td>0</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>0</td>
<td>0</td>
<td>G</td>
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</tr>
<tr>
<td></td>
<td>GG</td>
<td>66</td>
<td>100</td>
<td></td>
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</tr>
<tr>
<td>MTHFR</td>
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<td>45.5</td>
<td>C</td>
<td>0.65</td>
</tr>
<tr>
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<td>39.4</td>
<td>T</td>
<td>0.35</td>
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<td>TT</td>
<td>10</td>
<td>15.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
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<td>1</td>
<td>1.5</td>
<td>A</td>
<td>0.19</td>
</tr>
<tr>
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<td>GG</td>
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<td></td>
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<td>PAI-1</td>
<td>4G/4G</td>
<td>22</td>
<td>33.3</td>
<td>4G</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>4G/5G</td>
<td>38</td>
<td>57.6</td>
<td>5G</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>5G/5G</td>
<td>6</td>
<td>9.1</td>
<td></td>
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</tr>
<tr>
<td>TPA</td>
<td>DD</td>
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<td>D</td>
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<tr>
<td></td>
<td>II</td>
<td>16</td>
<td>24.2</td>
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<td></td>
</tr>
<tr>
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<td>DD</td>
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</tr>
<tr>
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<td>I</td>
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</tr>
<tr>
<td></td>
<td>II</td>
<td>16</td>
<td>24.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.4: Differential leukocyte counts throughout pregnancy

3.3.4.i: White Cell Counts

White cell counts (WCC) progressively increased from $8.3 \times 10^9$ cells/L to a peak of $11.5 \times 10^9$ cells/L at 36 weeks gestation, which was above the normal range (4.0-11.0 $\times 10^9$ cells/L). WCC returned to within the normal range post-partum ($7.1 \times 10^9$ cells/L) (table 3.5, figure 3.4). The variation in WCC during pregnancy did not achieve statistical significance ($p=0.09$) using repeated measures ANOVA.

3.3.4.ii: Neutrophils

Neutrophil counts increased during pregnancy from 8 weeks ($6.1 \times 10^9$ cells/L) right through to 36 weeks ($7.5 \times 10^9$ cells/L) with a peak observed at 26 weeks gestation ($7.8 \times 10^9$ cells/L). Counts decreased post-partum to $4.3 \times 10^9$ cells/ml (table 3.5, figure 3.4). All of these changes were within the normal non-pregnant ranges for neutrophils (2.0-7.5 $\times 10^9$ cells/L). Repeated measures ANOVA analysis revealed no significant variation in neutrophil counts over time ($p=0.20$).

3.3.4.iii: Lymphocytes

Lymphocyte counts were detected within the normal range ($1.5-4.0 \times 10^9$ cells/L) during pregnancy. Counts decreased between 8 ($1.8 \times 10^9$ cells/L) and 16 weeks ($1.5 \times 10^9$ cells/L) with a subsequent increase at 26 weeks ($2.6 \times 10^9$ cells/L) and a further decrease at 36 weeks ($1.9 \times 10^9$ cells/L) gestation, increasing post-partum ($2.1 \times 10^9$ cells/L) (table 3.5, figure 3.4). No significant variation in counts throughout gestation was observed in this cohort ($p=0.29$).

3.3.4.iv: Monocytes

Monocyte counts exhibited a similar pattern of expression to lymphocytes but with a peak at 36 weeks gestation ($0.64 \times 10^9$ cells/L). All monocyte counts were within the normal range (0.2-0.8 $\times 10^9$ cells/L) (table 3.5, figure 3.4) and no significant variation was detected over time ($p=0.70$).
3.3.4.v: Eosinophils

Eosinophil counts were detected within the normal range (0.0-0.4 \times 10^9 \text{ cells/L}) at all stages during pregnancy and post-partum. Patients demonstrated consistent concentrations right throughout pregnancy until three months post-partum where concentrations increased (0.23 \times 10^9 \text{ cells/L}), remaining within normal ranges (table 3.5, figure 3.4). Eosinophil counts were not significantly variable throughout pregnancy (p=0.39).

3.3.4.vi: Basophils

Basophil counts were consistently detected within the normal range (0.0-0.1 \times 10^9 \text{ cells/L}) during pregnancy. Counts decreased throughout gestation up until 36 weeks where they increased to levels similar to 8 weeks gestation, with a subsequent decrease post-partum (table 3.5, figure 3.4). No significant variation in basophil counts was detected throughout pregnancy (p=0.53).
Table 3.5: Mean concentrations of differential cell counts at time points throughout pregnancy and post-partum. 3 mth PN = 3 months post natal.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Normal Range x 10^3 cells/L</th>
<th>8 weeks</th>
<th>16 weeks</th>
<th>26 weeks</th>
<th>36 weeks</th>
<th>3 mth PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total White Cell Count (WCC)</td>
<td>4.0-11.0</td>
<td>8.3</td>
<td>9.0</td>
<td>10.2</td>
<td>11.5</td>
<td>7.1</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2.0-7.5</td>
<td>6.1</td>
<td>7.8</td>
<td>7.8</td>
<td>7.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.5-4.0</td>
<td>1.8</td>
<td>1.5</td>
<td>2.6</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.2-0.8</td>
<td>0.54</td>
<td>0.47</td>
<td>0.56</td>
<td>0.64</td>
<td>0.50</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.0-0.4</td>
<td>0.14</td>
<td>0.12</td>
<td>0.13</td>
<td>0.12</td>
<td>0.23</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.0-0.1</td>
<td>0.04</td>
<td>0.03</td>
<td>0.02</td>
<td>0.04</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Figure 3.4: Longitudinal study of leukocyte counts during pregnancy (x10^9/L). Whole blood samples were collected in EDTA anti-coagulated tubes at 8, 16, 26, and 36 weeks gestation. A further sample was collected 3 months post partum (3mo PN). These samples were analysed using a Sysmex 2100 and total white cell counts determined. (a) Total white cells; (b) Neutrophils; (c) Lymphocytes, (d) Monocytes; (e) Eosinophils; (f) Basophils.
3.3.5: Cytokine concentrations throughout pregnancy

3.3.5.i: Interleukin-10 (IL-10)
Non-pregnant female controls had highly variable IL-10 concentrations with a mean of 53.5 pg/ml. IL-10 concentrations decreased throughout gestation from 8 weeks (6.5 pg/ml) through to 36 weeks gestation (5.2 pg/ml), with an increase post-partum (8.5 pg/ml) (table 3.6, figure 3.5). Repeated measures ANOVA revealed no significant variation in IL-10 concentrations throughout pregnancy (p=0.4).

3.3.5.ii: Interleukin-6 (IL-6)
IL-6 concentrations in non-pregnant controls were detected at highly variable concentrations with a mean of 99.8 pg/ml. Pregnant patients demonstrated considerably decreased IL-6 concentrations compared to non-pregnant controls, but without statistical significance (Kruskal-Wallis p=0.66). IL-6 levels remained stable throughout pregnancy (31.3-34.2pg/ml) until a decline post-partum (19 pg/ml) (table 3.6, figure 3.5). Repeated measures ANOVA analysis revealed no significant variation in IL-6 concentrations throughout pregnancy (p=0.07).

3.3.5.iii: Tumour Necrosis Factor-α (TNFα)
TNFα concentrations were also detected at highly variable concentrations in the female non-pregnant control cohort with a mean TNFα concentration of 273.5 pg/ml. All pregnant patients exhibited significantly decreased concentrations of TNFα compared to non-pregnant controls (Kruskal-Wallis p=0.008) (table 3.6, figure 3.5). TNFα levels remained stable until 26 weeks (47.5-53.2 pg/ml), remaining increased until 36 weeks gestation, and decreasing post-partum (37.9 pg/ml). There was no significant variation detected in TNFα concentrations throughout pregnancy (p=0.18).

3.3.5.iv: Interferon-γ (IFNγ)
Similar to all other cytokines in this study, IFNγ concentrations were detected at highly variable concentrations in the non-pregnant control cohort with a mean concentration of 51.9 pg/ml. IFNγ concentrations in pregnant patients were considerably lower compared to non-pregnant controls although these differences did not achieve statistical significance (p=0.34). Fluctuating IFNγ concentrations were observed until the post-partum period, when a marked increase was observed (table 3.6, figure 3.5), however,
there was no significant variation detected in IFNγ concentrations throughout pregnancy (p=0.21).
Table 3.6: Mean concentrations of pro- and anti-inflammatory cytokines at time points throughout pregnancy. 3 months PN = 3 months post natal.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>General Inflammatory Action</th>
<th>Control Level (pg/ml)</th>
<th>8 weeks (pg/ml)</th>
<th>16 weeks (pg/ml)</th>
<th>26 weeks (pg/ml)</th>
<th>36 weeks (pg/ml)</th>
<th>3 months PN (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>Anti</td>
<td>53.5</td>
<td>6.5</td>
<td>5.6</td>
<td>6.0</td>
<td>5.2</td>
<td>8.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>Anti/Pro</td>
<td>99.8</td>
<td>31.3</td>
<td>31.4</td>
<td>32.4</td>
<td>34.2</td>
<td>19.0</td>
</tr>
<tr>
<td>TNFα</td>
<td>Pro</td>
<td>273.5</td>
<td>47.5</td>
<td>47.7</td>
<td>53.2</td>
<td>52.3</td>
<td>37.9</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Pro</td>
<td>51.9</td>
<td>4.9</td>
<td>3.9</td>
<td>4.5</td>
<td>4.2</td>
<td>6.5</td>
</tr>
</tbody>
</table>
Figure 3.5: Longitudinal study of inflammatory cytokine concentrations throughout pregnancy. Clotted blood samples were collected at 8, 16, 26, 36 weeks gestation and three months post-partum (3mo PN). Cytokine concentrations were determined by ELISA quantitation. (a) Interleukin 10 (IL-10); (b) Interleukin 6 (IL-6); (c) Tumour necrosis factor alpha (TNFα); (d) Interferon gamma (IFNγ).
3.3.6: Cytokine gene polymorphisms

3.3.6.i: IL-10-592
The IL-10-592 AA genotype was detected in 7.6% of pregnant patients (5/66) and 10.8% of non-pregnant controls (7/65). Comparison of IL-10 concentrations with genotypes revealed inconsistent variation in IL-10 levels between pregnant and non-pregnant individuals (table 3.7). There was no statistically significant variation detected in IL-10 levels for each genotype at each time point throughout pregnancy (p>0.05) and in non-pregnant controls (p=0.92).

3.3.6.ii: IL-10-1082
The IL-10-1082 AA genotype was detected in 24.6% of normal pregnant patients (16/66) and 26.2% of non-pregnant controls (7/65). Assessment of IL-10 concentrations for each genotype revealed a trend toward lower IL-10 concentrations with the AA genotype in pregnant patients but this trend was not observed in non-pregnant controls (table 3.7). Statistical analysis revealed no significant variation in IL-10 concentration with genotypes at each time point throughout pregnancy (p>0.05) and in non-pregnant controls (p=0.55).

3.3.6.iii: IL-6-174
The IL-6-174 CC genotype was detected in 1.5% of normal pregnant patients (1/66) and 10.8% in non-pregnant controls (7/65). Comparison of IL-6 concentrations with genotypes during pregnancy revealed decreased concentrations with the CC genotype at each time point but with no statistical significance (p>0.05) (table 3.7). In contrast, non-pregnant controls heterozygous for the IL-6-174 polymorphism exhibited decreased IL-6 concentrations but with no statistical significance (p=0.31).

3.3.6.iv: TNFα-308
The TNFα-308 AA genotype was detected in 4.5% of normal pregnant patients (3/66) and 12.3% of non-pregnant controls (8/65). Comparison of TNFα concentrations with genotypes revealed consistently increased levels of the protein with the AA genotype in both pregnant patients (figure 3.6.a) and non-pregnant controls (table 3.7). However, these differences did not achieve statistical significance at any time point throughout pregnancy (p>0.05) and in non-pregnant individuals (p=0.71).
3.3.6.v: TNFβ +252

The TNFB 22 (AA) genotype was detected in 40.9% of normal pregnant patients (27/66) and 38.5% of non-pregnant controls (25/65), while the TNFB 11 (GG) genotype occurred at a frequency of 15.2% and 18.5% respectively. Assessment of the relationship between the TNFB polymorphism and TNFα concentrations at each time point throughout pregnancy revealed no statistically significant variation in concentrations (p>0.05), a finding that was also detected in non-pregnant controls (p=0.58). However, the TNFB 11 genotype consistently corresponded to increased TNFα concentrations in both pregnant patients (figure 3.6.b) and non-pregnant controls.
Table 3.7: Inflammatory gene mutation genotype and allele counts in pregnant patients and non-pregnant female controls (%). Pregnant cytokine concentrations were measured relative to genotype at the maximum concentration observed in table 3.6 and figure 3.5, i.e: IL-10 = 8 weeks; IL-6 = 36 weeks; TNFα = 26 weeks; Conc = concentration; Preg = pregnancy

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Control counts</th>
<th>Mean control cytokine Conc (pg/ml)</th>
<th>Normal Pregnancy</th>
<th>Mean Pregnant cytokine Conc (pg/ml)</th>
<th>Allele</th>
<th>Control Frequency</th>
<th>Normal Preg Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10-592</td>
<td>CC</td>
<td>40 (61.5)</td>
<td>62.4</td>
<td>42 (63.6)</td>
<td>9.6</td>
<td>C</td>
<td>0.75</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>18 (27.7)</td>
<td>18.6</td>
<td>19 (28.8)</td>
<td>6.3</td>
<td>A</td>
<td>0.25</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>7 (10.8)</td>
<td>92.5</td>
<td>5 (7.6)</td>
<td>3.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10-1082</td>
<td>GG</td>
<td>16 (24.6)</td>
<td>54.1</td>
<td>18 (27.7)</td>
<td>13.0</td>
<td>G</td>
<td>0.49</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>32 (49.2)</td>
<td>35.8</td>
<td>31 (47.7)</td>
<td>7.1</td>
<td>A</td>
<td>0.51</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>7 (26.2)</td>
<td>86.4</td>
<td>16 (24.6)</td>
<td>5.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6-174</td>
<td>GG</td>
<td>27 (41.5)</td>
<td>82.6</td>
<td>23 (34.9)</td>
<td>7.6</td>
<td>G</td>
<td>0.65</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>31 (47.7)</td>
<td>20.4</td>
<td>42 (63.6)</td>
<td>49.3</td>
<td>C</td>
<td>0.35</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>7 (10.8)</td>
<td>69.3</td>
<td>1 (1.5)</td>
<td>5.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α-308</td>
<td>GG</td>
<td>34 (52.3)</td>
<td>171.1</td>
<td>38 (57.6)</td>
<td>51.9</td>
<td>G</td>
<td>0.70</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>23 (35.4)</td>
<td>375.4</td>
<td>25 (37.9)</td>
<td>42.7</td>
<td>A</td>
<td>0.30</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>8 (12.3)</td>
<td>416.1</td>
<td>3 (4.5)</td>
<td>202.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFB</td>
<td>22</td>
<td>25 (38.5)</td>
<td>189.6</td>
<td>27 (40.9)</td>
<td>60.3</td>
<td>2 (A)</td>
<td>0.60</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>28 (43.0)</td>
<td>276.6</td>
<td>29 (43.9)</td>
<td>49.6</td>
<td>1 (G)</td>
<td>0.40</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>12 (18.5)</td>
<td>441.3</td>
<td>10 (15.2)</td>
<td>55.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.6: Relationship between TNFα concentration and genotype of polymorphisms at time points during pregnancy where maximum cytokine concentrations were observed. (a) = TNFα-308; (b) = TNFβ.
3.4.0: DISCUSSION

Pregnancy is a hypercoagulable state, however the physiological basis for this increased coagulation is often difficult to define. Standard laboratory methods include reference ranges that are applicable to the non-pregnant state and often include male subjects. Furthermore, the diagnosis of coagulation disorders during pregnancy using laboratory findings is often difficult due to the lack of knowledge of normal gestational ranges. This problem is amplified when diagnosing gestational complications, which may be due to placental thrombosis. The interplay between the coagulation and inflammation pathways further complicates the understanding of such mechanisms. This longitudinal study investigated normal ranges of haemostatic parameters, full blood count assays and thrombophilia screen assays, throughout pregnancy. Inflammatory markers were also examined to determine their normal pregnant ranges. To the best of our knowledge, this investigation is the first of its kind in an Irish population, defining normal ranges of haemostatic and inflammatory parameters throughout the three trimesters of pregnancy and the puerperium.

Very little data is available for full blood count measurements during pregnancy but plasma blood volume and red cell volumes are believed to increase, probably due to the increased demand for sufficient blood supply by the foetus. It has also been reported that haemoglobin concentrations, haematocrit ratios and platelet counts decrease, which may also accommodate this demand (Hayashi et al., 2002). Here we report a progressive decrease in haemoglobin concentrations, fluctuating haematocrits and decreased red cell counts during pregnancy, suggesting a multifactorial aetiology of, rapid consumption of maternal red cells at the feto-maternal interface, a high turnover of cellular production, and an increase in volume of blood that is pumped to the placenta to maintain the nutritional demands of the foetus. A reduction in platelet counts was also observed, which has been reported previously (Hayashi et al., 2002). This reduction may represent a protective mechanism to ensure that accumulation of platelets at the placenta does not occur. Alternatively, it may indicate consumption of platelets at the feto-maternal interface, releasing phospholipids required for the induction of coagulation via the contact factors. However, we found no significant variation in PT and APTT throughout gestation, indicating that the contact pathway of coagulation activation is not greatly affected by coagulation changes during pregnancy, at least in the maternal circulation. These findings are similar to those of an Italian
study where APTT was reported as unchanged throughout normal gestations (Cerneca et al., 1997).

Antithrombin deficiency is thought to be the most thrombogenic of all the inherited thrombophilias, increasing the risk of thrombosis by 50% (Blumenfeld and Brenner, 1999). AT concentrations have previously been reported as both decreased and unchanged during normal pregnancy (Cerneca et al., 1997; Clark et al., 1998; Stirling et al., 1984). Here we report no variation in AT concentrations, findings that support the hypothesis that acquired AT deficiency as a cause of pregnancy associated thromboembolism may be overestimated due to selection bias in retrospective studies (Walker, 2000).

Factor VIII concentrations have previously been reported as increased during pregnancy in a UK study (Stirling et al., 1984). The present study revealed similar elevations in FVIII concentrations in an Irish cohort of women. Fibrinogen concentrations increase during pregnancy, with a large number of studies reporting this finding (table 3.1). We report significant increases in fibrinogen concentrations above the normal range. Increased fibrinogen may be a direct result of FVIII increases during pregnancy, supporting the concept of pregnancy as a hypercoagulable state. Increased FVIII and fibrinogen concentrations may also be a result of upregulation of the acute phase response, since pregnancy itself is an acute phase state, promoting cytokines to induce the expression of acute phase proteins such as fibrinogen and FVIII. The fibrinogen G-455A polymorphism, associated with increased fibrinogen concentrations, did not influence fibrinogen concentrations in this cohort. These findings are in contrast to previous reports of an association of the A allele with increased fibrinogen concentrations (Humphries et al., 1995). Therefore, this study found no association of the fibrinogen -455 A allele with increased fibrinogen concentrations, and suggests that genotype does not affect circulating fibrinogen concentrations during pregnancy.

PC concentrations have been reported as unchanged throughout gestation (Faught et al., 1995; Kjellberg et al., 1999) and increased post-partum (Cerneca et al., 1997; Clark et al., 1998), while PS concentrations have been reported as decreased during pregnancy (Cerneca et al., 1997; Clark et al., 1998; Faught et al., 1995; Kjellberg et al., 1999). The current investigation detected very little change in PC concentrations over time.
during pregnancy with only a slight decrease at 36 weeks gestation, increasing post-partum. This slight decrease observed at 36 weeks may reflect inactivation of the PC anticoagulant pathway in preparation for the necessary clotting required during labour. These findings support previous gestational observations, with post-partum increases noted by others also (Cerneca et al., 1997; Clark et al., 1998). PS concentrations exhibited a decrease from 8 weeks through to 36 weeks gestation, when PS levels were below the normal non-pregnant range. Once again these changes in PS along with the slight decrease in PC concentrations suggest inactivation of the PC anticoagulant pathway in preparation for labour with the resultant binding of PS to its carrier protein C4b binding protein (C4bBP), reducing levels in circulation. Prevention of activation of PC may allow C4bBP to bind to PS more readily, since C4bBP and APC compete for complexing with PS. The increase in PS to within normal ranges post-partum supports the increases in PC observed by us and others, possibly creating a mechanism of reducing the risk of bleeding during the post-partum period.

Antibodies directed against β2GP1 have been implicated in the pathogenesis of the antiphospholipid syndrome and RFL, albeit with conflicting reports (Balasch et al., 1999; Chong et al., 1998; Holmes et al., 2001; Samarkos et al., 2001; Visvanathan and McNeill, 1999). The present study found no significant variation in the concentrations of β2GP1 throughout pregnancy, however concentrations of this protein were observed to decrease until 36 weeks gestation, after which a slight increase was observed. Non-standardisation of the β2GP1 assay, the lack of knowledge of normal ranges and the possibility that there may also be population variation in this protein (Samarkos et al., 2001) suggest further studies are required to elucidate the role it plays in human reproduction.

Acquired activated protein C resistance has been observed during pregnancy where the ratio decreases transiently, and returns to normal in the post-partum period (Clark et al., 1998; Kjellberg et al., 1999). This acquired resistance is often due to the increased FVIII levels discussed previously as a result of the acute phase response occurring during pregnancy. Acquired APCR was not observed in this cohort of pregnant patients. APCR secondary to the FV Leiden polymorphism was observed in only 1.5% of patients. The decreased prevalence of this procoagulant genotype in normal pregnancy may indicate an incompatibility of this polymorphism with successful
pregnancy. There is conflicting evidence for an association of the prothrombin variant with gestational complications. The present study did not detect the polymorphism in pregnant patients. We have also demonstrated the Irish blood donor population prevalence of the G20210A mutation to be 1.8% in a larger cohort of patients (chapter 4). Therefore, a greater number of patients will be required to accurately determine the prevalence of such a rare polymorphism as the prothrombin variant. In the present study, the MTHFR C677T thermolabile variant was observed at a high frequency with TT homozygotes occurring in 15.1% of patients. This is similar to the Irish blood donor population prevalence we report in chapter 4 (16.9%). Previously, the prevalence in the Irish population has been reported as 7.3% (Mahmud et al., 1999) and 14.1% (Harmon et al., 1997), indicating that the prevalence of this variant is highly variable between cohorts of patients and cannot be regarded as a reliable tool for disease associations unless large cohorts of patients are observed.

Plasminogen activator inhibitor-1 concentrations have been reported increased during pregnancy by a number of investigators (Cerneca et al., 1997; Clark et al., 1998; de Moerloose et al., 1998). The insertion/deletion polymorphism within the PAI-1 gene has been studied in patients suffering from the antiphospholipid syndrome with no association found (Yasuda et al., 2002). The present study detected homozygosity for the PAI-1 polymorphism in 33.3% of pregnant patients. Tissue plasminogen activator has been reported as both increased and decreased during pregnancy (table 3.1). The I allele of the insertion/deletion polymorphism in the TPA gene has been reported to influence the plasma release rates of TPA (Jern et al., 1999) and has recently been associated with gestational thrombosis (Hooper et al., 2001). We detected the II genotype in 24.2% of patients. The D allele of the ACE gene polymorphism is suggested to increase serum ACE concentrations, negatively affecting activation of the fibrinolytic pathway and potentially promoting a hypercoagulable state (Costerousse et al., 1993; Mathew et al., 2001; Rigat et al., 1990). The present study detected 37.9% homozygosity for the DD genotype in pregnant patients. To the best of our knowledge, this is the first investigation of these polymorphisms in pregnancy. Each of these polymorphisms associated with fibrinolysis, occurred at high frequencies in these women with successful gestational outcomes suggesting that they may not be useful tools in predicting patients at risk of gestational thrombosis. However, the relationship between genotype and phenotype was not assessed here, and may be beneficial in
determining increased plasma concentrations and increased risks of gestational thrombosis.

Changes in cells of the immune system and cytokine production during normal pregnancies were investigated in the present study to ascertain the possibility of how the immune system is regulated and controlled during non-pathogenic pregnancies. Total white cell counts and neutrophil counts were found to increase progressively throughout pregnancy indicating an increase in the immune response, a trend that was reversed post-partum. Their increase may reflect the presence of non-maternal antigen in the immune system. The decrease in lymphocyte and monocyte counts at 16 weeks gestation may also signal the priming of the immune response, a time during which innate immunity predominates, in preparation for stimulation of T cells (Janeway et al., 2001). The basal levels of eosinophils observed here indicate that these cells are kept under tight control to prevent inappropriate toxic responses, normally occurring after antigen invasion. Low levels of eosinophils may also be necessary to prevent the induction of inflammatory cytokines during the earlier stages of pregnancy. Furthermore, the post-partum increase in eosinophil levels suggests that these responses may be repressed during normal pregnancy.

The highly variable cytokine concentrations detected in the non-pregnant cohort suggest the possibility of latent infections in asymptomatic individuals, a parameter that cannot be excluded when sampling volunteers. Concentrations of the type 2 cytokine, IL-10 have been reported as both increased from the early stages of pregnancy and absent during pregnancy, peaking at labour (Krasnow et al., 1996; Makhseed et al., 2000). The present study detected decreased IL-10 concentrations throughout pregnancy compared to the non-pregnant state, with decreases occurring throughout gestation until a subsequent increase post-partum. These findings suggest that the immune system is under tight control during pregnancy, and even cytokines that are considered anti-inflammatory are reduced to maintain a viable pregnancy. The mean increase post-partum did not reach levels observed in non-pregnant controls, possibly due to a sustained increase in hormonal levels post-partum in lactating individuals. Both IL-10 polymorphic markers (-1082, -592) revealed similar allele and genotype frequencies in pregnant patients. No relationship could be established between IL-10 concentrations and the IL-10−592 polymorphism, with inconsistent changes observed.
In contrast, the A allele of the IL-10-1082 polymorphism exhibited a trend toward decreased IL-10 concentrations, however these changes were not significantly different between genotypes. These findings suggest that the IL-10 polymorphisms may not dictate IL-10 concentration variations during pregnancy in individuals possessing these variants.

IL-6 has also been suggested to have a protective role in pregnancy as a type 2 cytokine (Vassiliadis et al., 1998). However, IL-6 concentrations have been reported at a steady state throughout pregnancy, increasing at delivery (Makhseed et al., 2000). The present study reports stable IL-6 concentrations throughout pregnancy with an increase in the later stages and a decrease post-partum, findings that are similar to previous studies (Makhseed et al., 2000). The increase in IL-6 near the end of gestation coincides with the maximum increase in both acute phase proteins fibrinogen and FVIII. This result is not surprising since IL-6 is known to upregulate the production of fibrinogen via an IL-6 responsive element located within the promoter region of the fibrinogen gene (Franco and Reitsma, 2001; Lane and Grant, 2000). Although IL-6 levels were lower than the non-pregnant control population, there was no statistically significant difference in levels between these groups. However, these findings suggest global downregulation of cytokine production during pregnancy. The IL-6 -174 polymorphism was detected in 1.5% of pregnant patients with decreased concentrations observed with the C allele, but without statistical significance. This decrease in IL-6 levels in the presence of the C allele has been noted by others and may represent a protective mechanism against acute phase reactions during gestation. However, a greater number of individuals may reveal the true relationship between genotype and IL-6 phenotype.

TNFα is a pro-inflammatory cytokine produced by type 1 cells and is indicative of cellular immunity, a process that is destructive to human pregnancy (Hill et al., 1995). Moreover, levels of TNFα have been reported higher in RFL patients compared to controls, within the first trimester of pregnancy (Makhseed et al., 2000). Here we report significantly reduced TNFα concentrations during pregnancy compared to non-pregnant controls, suggesting downregulation of TNFα responses and cellular immunity during pregnancy. Furthermore, a reduction in TF on the surface of monocytes during pregnancy has been demonstrated recently (Holmes, et al., 2002), suggesting that decreased TF may be a direct result of reduced TNFα. Homozygotes
for the TNFα -308 polymorphism represented 4.5% of the pregnant population and the AA genotype was consistently associated with increased TNFα concentrations, although without statistical significance. The A allele has previously been associated with increased TNFα levels, therefore, detection of this genotype may aid in monitoring levels of this pro-inflammatory cytokine during pregnancy. The TNF B2 allele was detected at a high frequency in pregnant patients and non-pregnant controls, but no significant variation in TNFα concentrations was observed. The theory that the TNF B polymorphism affects TNFα concentrations was given some support by the finding that the TNFB 11 (GG) genotype exhibited consistently increased TNFα concentrations but without statistical significance. Once again, knowledge of the influence of the TNF B polymorphism on TNFα levels during pregnancy may be beneficial in directing patient care in those at risk of gestational complications.

IFNγ is another pro-inflammatory cytokine produced by many cells, including type 1 cells during cellular immunity. Increased concentrations of IFNγ have been detected in patients with RFL and it has also been found to be toxic to embryonic trophoblast cells in vitro (Hill et al., 1995). In the present study, IFNγ concentrations were considerably lower than non-pregnant controls, but with no significant variation detected, suggesting downregulation of cellular immune responses during normal pregnancy. Repression of this pro-inflammatory cytokine may be a direct result of a decrease in TNFα secretion, since TNFα is the primary cytokine produced in an immune response, inducing the secretion of all other cytokines. Therefore, the global reduction in cytokines during pregnancy may be dictated by a reduction in TNFα expression by as yet some unknown mechanism.

In conclusion, pregnancy is a hypercoagulable state whereby certain clotting factor levels are globally increased in preparation for the onset of labour. Surprisingly, anticoagulant proteins do not appear to be dysregulated in response to this hypercoagulable environment, which indicates other mechanisms of regulation preventing a prothrombotic state. These mechanisms may originate with cells of the immune system such as lymphocytes, which are decreased throughout pregnancy, influencing activation of the immune response and ultimately the production of the major inflammatory mediator TNFα. The knock on effect toward production of other
cytokines and the prevention of tissue factor induction may be the key to prevention of the development of gestational thrombosis. This study has highlighted a number of areas within both coagulatory and inflammatory processes that are under tight control during pregnancy, but further specific research is necessary to elucidate the exact regulatory mechanisms in operation. However, it is our belief that the role of TNFα in the regulation of gestational inflammatory and coagulatory processes may be crucial to the maintenance of a viable pregnancy.

The longitudinal measurements of haematological and thrombophilic factors throughout pregnancy in this study now provide a standard reference range for these proteins throughout normal pregnancies. This is a unique study based on an Irish population of pregnant women, as these reference ranges have not been reported previously. The observations of increasing and decreasing concentrations of the various factors involved suggest pregnancy to be a unique non-pathogenic hypercoagulable state. Knowledge of these pregnant measurements is important when testing a woman for thrombophilia during pregnancy, as misinterpretation of such results may cause the unnecessary administration of anticoagulant therapy, an exercise that may prove lethal to the survival of the foetus and the health of the mother. Furthermore, observation of such deviations from the non-pregnant state allow a greater understanding of events during pregnancy, providing a reference range in the event of further deviations. If this occurs for a particular individual, a quicker response and greater patient care may be achieved before any effect on the foetus. Therefore, the setting of standard reference ranges of haematological and thrombophilic factors during pregnancy is crucial to improved obstetrical care.
CHAPTER 4

THE COAGULATION & INFLAMMATION INTERFACE IN
RECURRENT FOETAL LOSS
4.1.0: HAEMOSTASIS & RECURRENT FOETAL LOSS

The failure rate of all normal pregnancies is approximately 15% (Berry et al., 1995), with recurrent miscarriages affecting 1% of all women (Clifford et al., 1994). The most significant predictive factor of spontaneous abortion is the occurrence of a previous pregnancy loss. Women with previous successful pregnancies have a low incidence of abortion (4%) compared to those with only unsuccessful pregnancies (24%). Risk factors for spontaneous abortion have been evaluated such as maternal age and infertility, obstetric history, chromosomal abnormalities and carrier status, endocrinopathy, uterine anomalies, infections and metabolic dysfunction (Berry et al., 1995). In general a patient is said to be suffering from recurrent pregnancy loss when she has had three or more consecutive losses without the presence of chromosomal aberrations, infection or endocrinopathies.

In addition to these risk factors, recurrent pregnancy loss has been associated with acquired and inherited thrombophilic deficiencies. Together these thrombophilias can account for 50-65% of cases of RFL without a previous known cause (Brenner et al., 2000). The feto-maternal circulatory system must be maintained throughout gestation for sufficient supply of oxygen and nutrients to the foetus. Any disturbances in the haemostasis of this system, leading to a thrombotic environment, may compromise the survival of the foetus (Preston et al., 1996). The occurrence of microthrombi on placental bed vessels has been suggested to lead to multiple infarctions causing disruption in placental circulation and foetal distress (Younis et al., 1997). While pregnancy is a hypercoagulable state, gestational thrombophilia may be a major cause of foetal demise (Preston et al., 1996).
4.1.0.1: Acquired Thrombophilias & RFL

The antiphospholipid syndrome

The APS has been associated with placental thrombosis and subsequent recurrent pregnancy loss by a number of investigators as reviewed by Kutteh et al (1997), with 15-27% of APS patients experiencing RFL. A previous US study revealed the presence of one or more of the APA’s in 59.7% of RFL patients (n=352) compared to 4.6% of controls (n=43) (Matzner et al., 1994). There is clearly a role for APA’s in the pathophysiology of RFL but until recently the mechanism of thrombosis promotion was not well understood, and it was also unknown if APA’s were a cause or consequence of thrombosis in RFL patients (Kutteh, 1997). However, recently it has emerged that a potent anticoagulant, Annexin V, may play a crucial role in the prevention of thrombosis at the feto-maternal interface. Annexin V is found on placental trophoblast cells and is thought to protect these cells from APA binding, by themselves binding the phospholipids on the surface of trophoblasts, reducing the availability of phospholipids to APA’s. However the increase in APA’s in the APS is thought to disrupt this balance, preventing Annexin V binding, inducing a thrombogenic effect and subsequently thrombosing the placenta (Rand et al., 1997). This mechanism may explain the increased frequency of RFL in APS patients.

Acquired Activated Protein C Resistance

Only 16% of patients with acquired APCR without FVL were reported to have live births in an Israeli study (n=56 pregnancies)(Brenner et al., 1997) indicating a role for acquired APCR in gestational pathologies. Acquired APCR in the absence of FVL predisposes to thrombotic formation, particularly in the second and third trimesters, which may lead to foetal loss and other gestational pathologies such as preeclampsia.

4.1.0.ii: Inherited Thrombophilias – Natural Anticoagulants & RFL

Antithrombin deficiency

AT deficiency has been associated with recurrent pregnancy loss, placental abruption, pre-eclampsia and stillbirth in independent studies (Alfirevic et al., 2002; McColl et al., 1997; Preston et al., 1996; Sanson et al., 1996). Preston et al (1996) found a 200% increased risk of spontaneous foetal loss with AT deficiency alone compared with non-deficient controls in 1384 European women (EPCOT study). AT deficiency has also been reported in 2% of recurrent aborters in a US study (n=160) and in 13.8% of
similar patients in an Israeli cohort (n=116) (Bick, 2000; Carp et al., 2002a). However, AT deficiency has also been reported as having no adverse affect on pregnancy outcome remaining constant throughout gestation (Cerneca et al., 1997; Clark et al., 1998; Gris et al., 1999).

**Protein C & Protein S deficiencies**

AT, PC and PS deficiencies are usually analysed in tandem in studies of pregnancy and recurrent foetal loss. In 1996, the European Prospective Cohort on Thrombophilia (EPCOT) evaluated a group of women with heritable thrombophilia and foetal loss in a multicentre collaborative study. Control patients with thrombophilia were found to have a 23.5% incidence of foetal loss, while the prevalence increased in women with heritable thrombophilia, namely AT deficiency (31.5%), PC deficiency (29.6%), PS deficiency (29%) and FVL polymorphism (26.9%). The occurrence of combined defects in these patients increased the incidence of foetal loss further more to 40%. All defects except FVL proved to have statistical significance, suggesting uteroplacental insufficiency is a contributory factor to foetal demise (Preston et al., 1996). The relative risk of abortion and stillbirth in deficient patients of AT, PC and PS (n=69) compared with non-deficient patients (n=60) was reported to be 2.0 in an Italian cohort of 129 women (where a value greater than 1 indicates an increased risk exceeding that of controls). When AT and PC deficiencies were examined together, the relative risk increased to 2.5, while PS deficiency alone had a risk of 1.5 (Sanson et al., 1996). PC deficiency has been reported in 19.4% of individuals with recurrent miscarriage (n=36), while PS deficiency was found in 32.4% of recurrent miscarriage cases (n=34) (McColl et al., 1997) (Carp et al., 2002a).

4.1.0.iii: Inherited Thrombophilias - Procoagulants & RFL

**Fibrinogen**

Haverkate & Samama (1995) suggested a link between hereditary dysfibrinogenemia and miscarriages. They studied 15 women with hereditary dysfibrinogenemia associated thrombosis and found 7 (46.7%) of them to experience postpartum thrombosis while 39% of all pregnancies studied ended in miscarriage (Haverkate and Samama, 1995). The occurrence of dysfibrinogenemia without thrombosis has been observed in patients suffering from foetal loss indicating the role of other mechanisms than placental thrombosis in the pathology of these disorders (Blumenfeld and Brenner,
Chapter 4

The prevalence of the \( \beta \) fibrinogen gene polymorphism (G\(-\)455A) (Humphries et al., 1995) has not yet been investigated in a cohort of RFL patients.

**Activated protein C resistance & FV Leiden**

The FVL polymorphism is the most commonly inherited thrombophilia and has been implicated in the pathogenicity of RFL by a number of investigators. However, conflicting reports question its role in the generation of a procoagulant environment in patients with a history of RFL. The EPCOT multicentre study found no increased risk of miscarriage (any number of losses <28 weeks gestation) in 1384 European women with the FVL polymorphism (26.9%) compared to controls (23.5%), with an odds ratio of 0.9 (Preston et al., 1996). Moreover, several groups have reported no significant increase in FVL in RFL patients compared to controls including a UK study of 1111 Caucasian patients and 150 controls (3.6% vs. 4.0%) (Dilley et al., 2002; Dumwald et al., 2000; Muldoon, 1999; Pihusch et al., 2001; Rai et al., 2001). The FVL polymorphism has been reported to range from 0% to 16.3% in women with recurrent pregnancy loss compared to approximately 4% in controls, with higher frequencies occurring in women with second and third trimester losses (Brenner and Blumenfeld, 1997; Grandone et al., 1997; Kutteh et al., 1999; Pauer et al., 1998). However, a UK study found similar frequencies of the A allele in early (n=1808) and late (n=414) miscarriage patients and controls (n=300) (Rai et al., 2001). Nevertheless, although conflicting, there is evidence to suggest that the polymorphism is important in second and third trimester recurrent pregnancy losses but not in first trimester losses (Grandone et al., 1997; Pauer et al., 1998). Discrepancies are due in part to variations in ethnicity, sample size and patient selection criteria. It is clear that the FVL R506Q polymorphism plays an important role in the development of thrombosis, however carrier status does not automatically assume the imminence of a thrombotic event. We have reviewed here, publications with substantial population numbers and RFL cohort selection criteria of greater than three consecutive losses. Table 4.1 surmises the large number of studies that have investigated the role of the FVL R506Q polymorphism in gestational thrombosis, obstetrical complications and recurrent pregnancy loss. It is crucial to observe the sample size when considering these findings.
Table 4.1: Prevalence of the FV Leiden polymorphism in RFL patients and controls. 1° = primary loss (no previous pregnancies); 2° = secondary loss (≥ 1 successful pregnancy). Complications = pre-eclampsia, placental abruption, stillbirth, IUGR; Thrombosis = gestational thrombosis; N = sample size; NS = non-significant.

<table>
<thead>
<tr>
<th>References</th>
<th>Origin of Study</th>
<th>Time of loss</th>
<th>RFL % (Heterozygosity (N))</th>
<th>Controls % (Heterozygosity (N))</th>
<th>P value</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preston et al. (1996)</td>
<td>European</td>
<td>≤ 28 weeks</td>
<td>26.9 (843)</td>
<td>23.5 (541)</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Grandone et al. (1997)</td>
<td>Italy (Caucasian)</td>
<td>All trimesters</td>
<td>16.3 (43)</td>
<td>4.2 (118)</td>
<td>0.01</td>
<td>4.4</td>
</tr>
<tr>
<td>Kutteh et al. (1999)</td>
<td>USA (Caucasian)</td>
<td>&lt; 12 weeks</td>
<td>2.0 (50)</td>
<td>4.0 (50)</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Tal et al. (1999)</td>
<td>Israel</td>
<td>1\textsuperscript{st}/2\textsuperscript{nd} trimesters</td>
<td>14.4 (125)</td>
<td>5.6 (125)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Brenner et al. (1999)</td>
<td>Israel</td>
<td>1\textsuperscript{st}/2\textsuperscript{nd} trimesters</td>
<td>32.0 (76)</td>
<td>10.0 (106)</td>
<td>&lt;0.001</td>
<td>4.0</td>
</tr>
<tr>
<td>Souza et al. (1999)</td>
<td>Brazil</td>
<td>1\textsuperscript{st}/2\textsuperscript{nd} losses</td>
<td>7.1 (56)</td>
<td>1.6 (384)</td>
<td></td>
<td>4.9</td>
</tr>
<tr>
<td>Kupfermic et al. (1999)</td>
<td>Israel</td>
<td>Complications</td>
<td>20 (110)</td>
<td>6.0 (110)</td>
<td>0.003</td>
<td>3.7</td>
</tr>
<tr>
<td>Murphy et al. (2000)</td>
<td>Ireland</td>
<td>Thrombosis</td>
<td>11 (27)</td>
<td>4.2 (572)</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>Foka et al. (2000)</td>
<td>Greece</td>
<td>1\textsuperscript{st}/2\textsuperscript{nd} trimesters</td>
<td>19.0 (80)</td>
<td>4.0 (100)</td>
<td>0.003</td>
<td>5.5</td>
</tr>
<tr>
<td>Martinelli et al. (2000)</td>
<td>Italy</td>
<td>&lt; 20 weeks</td>
<td>7.0 (67)</td>
<td>3.0 (232)</td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td>Gerhardt et al. (2000)</td>
<td>Germany</td>
<td>Thrombosis</td>
<td>43.7 (119)</td>
<td>7.7 (233)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Wrambsy et al. (2000)</td>
<td>Sweden</td>
<td>&lt; 22 weeks</td>
<td>15.5 (84)</td>
<td>3.0 (69)</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td>Reznikoff-Etievant et al. (2001)</td>
<td>France (Caucasian)</td>
<td>&lt; 10 weeks</td>
<td>10.4 (260)</td>
<td>4.6 (240)</td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>Rai et al. (2001)</td>
<td>UK (Caucasian)</td>
<td>&lt; 12 weeks</td>
<td>3.6 (1111)</td>
<td>4.0 (150)</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Carp et al. (2002)</td>
<td>Israel</td>
<td>All trimesters</td>
<td>3.7 (108)</td>
<td>6.1 (82)</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Dilley et al. (2002)</td>
<td>USA</td>
<td>All trimesters</td>
<td>1.7 (60)</td>
<td>14.0 (92)</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>
**Prothrombin**

The prothrombin variant was detected at similar frequencies in a US study of 50 Caucasian RFL patients with losses within the first trimester (< 12 weeks) and 50 Caucasian controls (2% each, OR=1.0) (Kutteh et al., 1999). Similar frequencies were also reported in Swedish and British patients with no significant difference between patients experiencing losses within the first and second trimesters (3.5% and 3.3%) and controls (2.9% and 4.5%) (Pickering et al., 2001; Wrambsy et al., 2000). Interestingly, a French study reported a slightly lower frequency of the polymorphism in RFL patients (0.9%, n=232) compared to controls (1.0%, n=464) (Gris et al., 1999). In contrast to these findings, a number of European studies have reported an association of this procoagulant polymorphism with losses within both the first and second trimesters. A Greek study detected the polymorphism in 9% of all RFL patients (n=80) compared to 2% of controls (n=100, p=0.038), with a prevalence of 8.1% in patients with losses within the first trimester (p=0.06) and 10.5% in patients with losses within the second trimester (p=0.06) (Foka et al., 2000). Individuals whom experienced first trimester losses in a French study were reported to have a heterozygosity prevalence of 7.7% (n=260) compared to 2.9% in controls (n=240) (Reznikoff-Etievant et al., 2001). Non-European populations have also been investigated, with Israeli and Brazilian groups both associating the polymorphism with an increased risk of RFL (Kupfermic et al., 1999; Souza et al., 1999). Table 4.2 illustrates the numerous studies investigating the prothrombin variant for an association with an increased risk of recurrent miscarriages and obstetrical complications. Examination of the origin of these studies and the prevalence of the polymorphism indicate that the prothrombin variant has a higher frequency in southern European populations, suggesting a single origin of this procoagulant allele. These conflicting reports suggest that the prothrombin variant alone does not predispose individuals to recurrent pregnancy loss or other obstetric complications, but it may interact with other risk factors such as FV Leiden, deficiencies in the natural anticoagulants or environmental stimulus.
Homocysteine & t-MTHFR

The association of hyperhomocysteinemia with both venous and arterial thrombotic disease and the association of the t-MTHFR variant with reduced maternal folic acid concentrations in neural tube defects has generated great interest in the role the t-MTHFR variant has to play in the pathophysiology of RFL (Cattaneo, 1999; Steegers-Theunissen et al., 1994). The t-MTHFR variant has been reported as having no influence on the pathogenesis of RFL in several studies to date (Dilley et al., 2002; Foka et al., 2000; Grandone et al., 1998; Kutteh et al., 1999; Martinelli et al., 2000; Murphy et al., 2000; Pihusch et al., 2001). A recent investigation of this gene variant in RFL patients revealed a higher prevalence of the polymorphism in RFL patients compared to controls (13% vs. 8.5%), however this difference was not statistically significant (Carp et al., 2002b). In contrast, the t-MTHFR variant was found significantly increased in RFL patients compared to controls in a number of other studies (table 4.3) (Brenner et al., 1999; Dumwald et al., 2000; Kupfermic et al., 1999; Lissak et al., 1999; Nelen et al., 1997; Unfried et al., 2002). The role of the t-MTHFR variant remains controversial in relation to gestational thrombosis, obstetrical complications and recurrent pregnancy loss. For this reason we endeavoured to determine the prevalence of this common polymorphism in a cohort of patients with a history of RFL.
Table 4.2: Prevalence of the prothrombin G20210A variant in women with a history of RFL. NS= not significant where exact p values or odds ratios were not available. 1°= primary loss (no previous pregnancies); 2°=secondary loss (≥1 successful pregnancy). Complications = pre-eclampsia, placental abruption, stillbirth, IU GR; Thrombosis = gestational thrombosis; N = sample size; NS = non-significant.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Origin</th>
<th>Time of loss</th>
<th>RFL % Heterozygosity (N)</th>
<th>Controls % Heterozygosity (N)</th>
<th>P value</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kutteh et al. (1999)</td>
<td>USA (Caucasian)</td>
<td>&lt;12 weeks</td>
<td>2 (50)</td>
<td>2 (50)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Brenner et al. (1999)</td>
<td>Israel</td>
<td>1\textsuperscript{st}/2\textsuperscript{nd} trimesters</td>
<td>8 (76)</td>
<td>4 (106)</td>
<td>0.23</td>
<td>2.2</td>
</tr>
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<td>Souza et al. (1999)</td>
<td>Brazil</td>
<td>1\textsuperscript{st}/2\textsuperscript{nd} losses</td>
<td>3.6 (56)</td>
<td>1.0 (384)</td>
<td>0.03</td>
<td>3.5</td>
</tr>
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<td>Kupfermic et al. (1999)</td>
<td>Israel</td>
<td>Complications</td>
<td>10 (110)</td>
<td>3.0 (110)</td>
<td>0.03</td>
<td>3.9</td>
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<td>Gris et al. (1999)</td>
<td>France</td>
<td>≤ 22 weeks</td>
<td>0.9 (232)</td>
<td>1.0 (464)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Foka et al. (2000)</td>
<td>Greece</td>
<td>All trimesters:</td>
<td>All: 9.0 (80)</td>
<td>2.0 (100)</td>
<td>0.038</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1\textsuperscript{st} trimester: 8.1 (61)</td>
<td></td>
<td></td>
<td>0.06</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2\textsuperscript{nd} trimester: 10.5 (19)</td>
<td></td>
<td></td>
<td>0.06</td>
<td>5.7</td>
</tr>
<tr>
<td>Martinelli et al. (2000)</td>
<td>Italy</td>
<td>2\textsuperscript{nd}/3\textsuperscript{rd} trimester</td>
<td>9.0 (67)</td>
<td>3.0 (232)</td>
<td>0.001</td>
<td>3.3</td>
</tr>
<tr>
<td>Gerhardt et al. (2000)</td>
<td>Germany</td>
<td>Thrombosis</td>
<td>16.9 (119)</td>
<td>1.3 (233)</td>
<td>0.56</td>
<td></td>
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<tr>
<td>Wrambsy et al. (2000)</td>
<td>Sweden</td>
<td>&lt; 22 weeks</td>
<td>3.5 (84)</td>
<td>2.9 (69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pickering et al. (2001)</td>
<td>UK</td>
<td>1\textsuperscript{st} trimester</td>
<td>3.3 (122)</td>
<td>4.5 (66)</td>
<td>0.32</td>
<td>0.71</td>
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<td>Reznikoff-Etievant et al. (2001)</td>
<td>France (Caucasian)</td>
<td>&lt; 10 weeks</td>
<td>7.7 (260)</td>
<td>2.9 (240)</td>
<td></td>
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<tr>
<td>Carp et al. (2002)</td>
<td>Israel</td>
<td>All trimesters:</td>
<td>4.6 (108)</td>
<td>6.1 (82)</td>
<td>NS</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Table 4.3: Prevalence of the t-MTHFR C677T polymorphism in women with a history of RFL and obstetrical complications. NS = not significant where exact p values or odds ratios were not available. Complications = pre-eclampsia, placental abruption, stillbirth, IUGR; N= sample size; NS = non-significant.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Origin</th>
<th>Time of loss</th>
<th>RFL % Homozygosity (n)</th>
<th>Controls % Homozygosity (n)</th>
<th>P value</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nelen et al. (1997)</td>
<td>Holland</td>
<td>&lt;17 weeks</td>
<td>16.0 (185)</td>
<td>5.0 (113)</td>
<td></td>
<td>3.3</td>
</tr>
<tr>
<td>Grandone et al. (1998)</td>
<td>Italy</td>
<td>&lt;17 weeks</td>
<td>18.1 (94)</td>
<td>18.7 (150)</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Holmes et al. (1999)</td>
<td>UK</td>
<td>≤/&gt; 12 weeks</td>
<td>14.0 (142)</td>
<td>12.2 (49)</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Lissak et al. (1999)</td>
<td>Israel</td>
<td>1st trimester</td>
<td>9.0 (41)</td>
<td>22.0 (18)</td>
<td>&lt;0.95</td>
<td></td>
</tr>
<tr>
<td>Brenner et al. (1999)</td>
<td>Israel</td>
<td>1st/2nd trimesters</td>
<td>18.0 (76)</td>
<td>10.0 (106)</td>
<td>0.12</td>
<td>1.95</td>
</tr>
<tr>
<td>Kutteh et al. (1999)</td>
<td>USA (Caucasian)</td>
<td>&lt;12 weeks</td>
<td>8.3 (50)</td>
<td>4.0 (50)</td>
<td>2.06</td>
<td></td>
</tr>
<tr>
<td>Kupfermic et al. (1999)</td>
<td>Israel</td>
<td>Complications</td>
<td>22.0 (110)</td>
<td>8.0 (110)</td>
<td>0.005</td>
<td>3.1</td>
</tr>
<tr>
<td>Martinelli et al. (2000)</td>
<td>Italy</td>
<td>2nd/3rd trimester</td>
<td>13.0 (67)</td>
<td>20.0 (232)</td>
<td>NS</td>
<td>0.8</td>
</tr>
<tr>
<td>Gris et al. (1999)</td>
<td>France</td>
<td>≤ 22 weeks</td>
<td>0.9 (232)</td>
<td>1.0 (464)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Murphy et al. (2000)</td>
<td>Ireland</td>
<td>All trimesters</td>
<td>3.0 (40)</td>
<td>10.4 (540)</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Foka et al. (2000)</td>
<td>Greece</td>
<td>All trimesters</td>
<td>8.0 (80)</td>
<td>15.0 (100)</td>
<td>0.13</td>
<td>0.4</td>
</tr>
<tr>
<td>Wrambsy et al. (2000)</td>
<td>Sweden</td>
<td>&lt; 22 weeks</td>
<td>3.6 (84)</td>
<td>4.3 (69)</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Carp et al. (2002)</td>
<td>Israel</td>
<td>All trimesters</td>
<td>12.9 (108)</td>
<td>8.5 (82)</td>
<td>NS</td>
<td>1.59</td>
</tr>
<tr>
<td>Unfried et al. (2002)</td>
<td>Austria</td>
<td>&lt; 20 weeks</td>
<td>17.3 (133)</td>
<td>5.4 (74)</td>
<td>0.03</td>
<td>3.7</td>
</tr>
</tbody>
</table>
TPA & PAI-1

Very little is known about the effect of polymorphisms within the TPA and PAI-1 genes on the pathogenesis of RFL. To the best of our knowledge, only one report exists of an association study of the TPA insertion/deletion polymorphism and the PAI-1 polymorphism with gestational complications. The prevalence of these polymorphisms was investigated in patients suffering from APS, a syndrome often accompanied by recurrent spontaneous abortion. No increased frequency of either polymorphism was detected in APS patients compared to controls (Yasuda et al., 2002). These polymorphisms have not yet been investigated in a cohort of RFL patients. We endeavored to determine the prevalence of the TPA and PAI-1 polymorphism in a cohort of patients with a history of RFL.

ACE

The importance of ACE at the local vascular level has generated interest in the ACE gene polymorphism as having a possible role in RFL pathogenesis. A recent study investigated several inherited thrombophilias, including AT, PC and PS deficiencies, along with the ACE gene variant in a cohort of 59 women with first trimester foetal loss. It was found that 56% of patients possessed the ACE DD genotype (Fatini et al., 2000), the D allele being associated with increased ACE concentrations and the possibility of greater vasoconstriction as one consequence of this (Rigat et al., 1990) (Costerousse et al., 1993; Mathew et al., 2001). In contrast the ACE DD genotype has been reported at similar frequencies between RFL patients and controls by others, with no influence on pregnancy outcome (Hefler et al., 2002; Tamura et al., 1996). The role of the ACE polymorphism in gestational pathologies remains elusive due to the small number of studies to date, and therefore requires further extensive investigation in multiple populations. We investigated the prevalence of this polymorphism in a cohort of women in Ireland with a history of RFL to determine its relevance in the pathogenesis of RFL.
4.1.1: The immune system & Recurrent Foetal Loss

The immunology of pathological pregnancies can be divided into autoimmunity and alloimmunity. Autoimmunity refers to the production of antibodies to substances that are self, cellular or subcellular constituents. One such autoimmune response associated with complications of pregnancy is the production of APA’s as discussed in chapter 3. Alloimmunity refers to the occurrence of immunological differences between individuals of the same species. Therefore, it may be that gestational pathologies including RFL, are a result of an imbalance in alloimmune factors (Lim et al., 1996).

Several groups have studied such factors in RPL but reports suggest their frequency to be 1-40%. The variance in frequency may be a result of the difficulty in diagnosing RFL, and the complexity of alloimmune factors in the uterine environment, making detection difficult (Lim et al., 1996). Normal endometrial biopsies contain an increased number of T helper (Th) cells compared to T suppressor (Ts) cells throughout the menstrual cycle except at the onset of menstruation (Hill and Anderson, 1990). This ratio was increased in biopsies from women with idiopathic RFL compared to those with other known causes (Hill and Anderson, 1990). In addition, cytokine anomalies were reported in the serum of patients with embryotoxicity suggesting an imbalance in the immune response of women with RFL (Hill et al., 1995).

4.1.1.i: Type 1/Type 2 immunity and pregnancy

It is now widely accepted that type 1 cytokine production, representing maternal cell-mediated immunity, is detrimental to successful pregnancy, while a type 2 humoral immune response is exhibited during normal pregnancy (Raghupathy, 1997). Hill et al (1995) were the first to study PBMC’s from non-pregnant women with unexplained RFL and those with previous normal gestations. They found that women with RFL (≥3 first-trimester losses) responded to trophoblast (epithelial layer surrounding implanting foetal cells) antigens with a dichotomous (two opposing classes) T helper immune response. The type 1 cytokine IFN-γ was produced by 51.2% of RFL affected women following exposure to antigen. Conversely, women with no history of RFL and male controls failed to produce a type 1 response under identical conditions, but instead exhibited a type 2 bias (Hill et al., 1995). Moreover, IFN-γ has been shown to be toxic to embryonic and trophoblast cells in vitro suggesting its role in reproductive dysfunction in patients with RFL (Hill et al., 1995). Subsequent studies have reported
the dominance of the type 1 cytokines IFNγ, TNFα and IL-1β during pregnancies of individuals with a history of RFL (Jenkins et al., 2000; Makhseed et al., 2000; Shaaraway and Nagui, 1997). Furthermore, TF synthesis has been demonstrated as increased in the presence of type 1 immune responses and the production of type 1 cytokines, in particular TNFα, IL-1β and IFNγ (del Prete et al., 1995; Fan et al., 1990). Therefore, there lies the potential to upregulate activation of the coagulation cascade and create a procoagulant environment in the pregnant uterus, increasing the risk of placental thrombosis and expulsion of the foetus. Of particular interest is a recent contradictory report, indicating that women with a history of RFL who subsequently miscarried had significantly lower concentrations of TNFα compared to both pregnant (p=0.02) and non-pregnant (p=0.0004) controls, while RFL patients who had a successful pregnancy had similar concentrations to pregnant controls. RFL patients exhibited increased type 2 cytokines, IL-10 and IL-4 compared to pregnant and non-pregnant controls (Bates et al., 2002). Although it may be surmised that a type 2 response benefits normal pregnancy, and recurrent foetal loss is characterised by a predominant type 1 response, further analysis will be required in light of recent contradictory findings. We endeavoured to determine the concentrations of type 1 cytokines (TNFα, IFNγ) and type 2 cytokines (IL-6, IL-10) throughout pregnancies of individuals with a history of recurrent foetal loss, to elucidate a possible difference in immune response between normal pregnancy and pathological pregnancies.

4.1.2: Genetic regulation of cytokine concentrations in RFL

Many polymorphisms within cytokine genes have been linked to various disease states associated with inflammation (Bidwell et al., 1999). However, RFL may be both an inflammatory and a coagulatory disorder, and may be influenced by both envinomental and genetic factors, and is therefore considered a multifactorial disease. For these reasons there have been no reports of independent immunological genetic risk factors for RFL.

4.1.2.i: Interleukin-10

The importance of IL-10 and its promoter polymorphisms have been considered in only a handful of studies concerning gestational pathologies. Preeclamptic patients were reported to have placental deficiency of IL-10 and lower levels than controls after
mitogen stimulation (Darmochwal-Kolarz et al., 1999; Hennessy et al., 1999). Two recent studies investigating the IL-10 –1082 polymorphism with recurrent pregnancy loss revealed no significant difference in genotype and allele frequencies between patients and controls (Babbage et al., 2001; Karhukorpi et al., 2001). No studies to date have reported an association between the IL-10 –592 polymorphism and pathological pregnancies. The present study aimed to determine the frequency of the IL-10 polymorphisms in patients with a history of RFL and compare this to normal pregnancy and non-pregnant female controls sampled from the same geographical area. The correlation between IL-10 concentrations throughout pregnancy and genotype was also investigated to determine if IL-10 levels throughout pregnancy are regulated by these polymorphisms, perhaps influencing RFL pathogenesis.

4.1.2.ii: Interleukin-6
IL-6 levels have been reported as decreased in patients with RFL, supporting the hypothesis that increased IL-6 levels are beneficial to a successful pregnancy outcome (Makhseed et al., 2000). To date there have been no reports of an association of the IL-6 –174 polymorphism with levels of the protein in patients with RFL. However, a recent study of Norwegian preeclamptic patients found decreased cord plasma IL-6 concentrations in patients compared to controls (Odegard et al., 2001). Due to the lack of studies of IL-6 polymorphisms and RFL, this investigation aimed to determine the prevalence of the IL-6 –174 polymorphism in a cohort of patients with a history of RFL and compare this frequency to both pregnant and non-pregnant normal populations. We also endeavoured to determine the relationship between IL-6 genotype and phenotype throughout pregnancy in this cohort of patients to ascertain the possibility of decreased IL-6 levels as a risk factor for RFL.

4.1.2.iii: Tumor Necrosis Factor (TNF) α and β (Lymphotoxin α)
The TNFα -308 polymorphism has been associated with increased TNFα secretion in the presence of the A2 allele (Warzocha et al., 1998), while the effect of the TNFβ +252 polymorphism is still largely undetermined due to conflicting reports (Lee et al., 1997; Majestschak et al., 1999; Messer et al., 1991; Pociot et al., 1993; Pociot et al., 1991; Stuber et al., 1996; Whichelow et al., 1996). A UK study of 43 Caucasian women with RFL and 73 Caucasian age-matched controls found no significant
variation in the frequency of both genotypes and alleles of the TNF\(\alpha\) -308 polymorphism between patients and controls \((p=0.4)\) (Babbage et al., 2001). Similarly, in a separate study of 40 British women with RFL and 43 controls, no significant difference in inheritance of the TNF A2 allele was found between patients and controls \((p=0.53)\) (Reid et al., 2001). Controversy continues to surround the TNFB polymorphism with regard to its influence on TNF concentrations. To the best of our knowledge, there are no previous reports of the relationship between TNF polymorphisms and TNF\(\alpha\) levels during pregnancies of women with a history of RFL. For these reasons we aimed to determine the prevalence of these polymorphisms in a large cohort of RFL patients and to determine the genotype-phenotype relationship in a sub-set of patients throughout pregnancy.

4.1.3: Mutation detection in the IFN\(\gamma\) gene

Although there have been numerous reports of single nucleotide polymorphisms (SNP’s) within the IFN\(\gamma\) gene, many of these mismatch bases have been reported in only one individual. Furthermore, there are no studies assessing these polymorphisms in large populations. From the list of 21 SNP’s in the IFN\(\gamma\) gene, 11 were from African/African American populations, 2 were from European populations, 4 from unspecified populations and 3 from a mixture of all three population types. We aimed to determine if a reported or novel polymorphism existed in a cohort of European patients with a history of RFL. Traditionally detection of novel mutations has been achieved by sequencing all regions of the gene in all patients, a time consuming and costly exercise. The Transgenomic WAVE® denaturing high performance liquid chromatography (dHPLC) system has proved to speed up this process of gene scanning by detecting mutations in an individual sample using a method known as temperature modulated heteroduplex analysis (TMHA). We employed this technology to screen the IFN\(\gamma\) gene.

4.1.3.i: Temperature Modulated Heteroduplex Analysis (TMHA)

Variation in DNA sequence at a particular genomic site between individuals is referred to as a polymorphism, if the change is present at a frequency greater than 1% in the given population (Kuklin, 1998). As seen previously, these markers are used to indicate genes associated with certain diseases by ascertaining a gene-disease relationship or
genotype-phenotype association, allowing for rapid detection of at risk individuals and possible treatment regimes. TMHA allows the visual detection of a mutation without indicating its location, allowing subsequent sequencing of a reduced number of samples, preventing laborious unnecessary sequencing. This method relies upon the formation of conformational changes in the DNA molecule induced by mismatched heteroduplex formation during re-annealing of wild-type and mutant DNA strands (Kuklin, 1998).

This method of mutation detection is highly sensitive and specific, detecting 100% of both mutant and wild type individuals for mutations in the genes for factor IX and neurofibromatosis (O'Donovan et al., 1998). DHPLC techniques have also been successfully employed to detect novel mutations in the gene for Marfan syndrome, a connective tissue disorder that is caused by a large number of mutations scattered throughout the gene, that are quite often unique to individual families (Lui et al., 1997-98). Further identification of polymorphisms in candidate genes for multiple sclerosis have been detected using dHPLC methods (Giordano et al., 1999).

DHPLC methods will be used to determine the presence of novel or existing mutations in the interferon gamma gene in a sub-set of patients suffering recurrent pregnancy loss and a sub-set of control patients. DNA sequencing methods will be employed to elucidate the exact mutation, if one is present, and an RFLP assay will be developed to screen all patients within the study population.
4.1.4: Regulation of immunity during pregnancy by steroid hormones

During pregnancy and parturition, there is a fine balance between the immune and endocrine systems controlling and regulating intrauterine function (Denison et al., 1998). The secretion of progesterone prepares the endometrium for implantation of foetal cells or the blastocyst. Conversely, during the initial stages of menstruation, progesterone withdrawal induces a number of changes in the endometrium, including the expression of cytokines, attracting leukocytes, producing inflammatory mediators and inducing vasoconstriction of the spiral arteries within the uterus (Critchley et al., 2001). This suggests an important role for progesterone in the regulation of the immune response in the endometrium and in the maintenance of pregnancy.

Progesterone has been found to be a potent inducer of type 2 cytokine production, by inducing mRNA expression and production of IL-4 (type 2 inducer) in established Th1 cell clones. It also induces CD30 expression on the membrane of established Th1 clones, CD30 having been found to correlate with type 2 cytokine expression (Piccinni et al., 2000). In contrast, estrogen is regarded as a pro-inflammatory hormone in the uterine environment, where it causes an invasion of neutrophils and macrophages, tissue oedema, and proliferation of uterine epithelial cells. In conjunction with these functions, estrogen induces the production of both estrogen receptors (ER) and progesterone receptors (PR), priming the uterus to respond to these hormones (Tibbetts et al., 1999).

Progesterone also induces lymphocytes to produce progesterone-induced blocking factor (PIBF) that suppresses mitogen-induced lymphocyte proliferation, activation of natural killer (NK) cells and TNF production by NK cells (Raghupathy, 1997). Moreover, estrogen and progesterone regulate the expression of IL-6, which in turn induces the production of human chorionic gonadotrophin (HCG) in the endometrium (figure 4.1). This hormone is involved in the implantation period suggesting a role for IL-6 in implantation and the continuation of early pregnancy (Lim et al., 1996). Estrogen and progesterone also regulate the type 1 cytokines TNFα and INFγ. TNFα intrauterine concentrations vary throughout the menstrual cycle increasing toward the onset of menstruation, correlating with a fall in estrogen and progesterone. The induction of menstruation mimics spontaneous abortion, therefore, low TNFα
concentrations during early pregnancy may be crucial in the maintenance of a viable foetus (Lim et al., 1996). Furthermore, TNFα along with IL-2 and INFγ has been reported to terminate normal pregnancy upon injection into pregnant mice (Raghupathy, 1997). The activity of INFγ in lymphoid tissues is greatly increased by estrogen so any disturbance in this system may influence the progression of gestation. In a further interaction between steroid hormones and inflammation, INFγ has been shown to increase estrogen receptor expression by up to 50% (Lim et al., 1996). These findings suggest that regulation of progesterone and estrogen at the feto-maternal interface actually contribute to the success of pregnancy, in part influencing cytokine production (Piccinni et al., 2000).

4.1.5: Heparin therapy and RFL

The beneficial effects of heparin as an anticoagulant have been well known since its discovery in 1916 by McLean (Hirsh et al., 2001). In its synthetic therapeutic form, heparin is available as unfractionated (UFH) and low-molecular weight heparin (LMWH). UFH has pharmacokinetic properties, which require close monitoring in a hospital setting. Its side effects include bleeding, acute anaphylaxis, osteoporosis and eosinophilia. LMWH, first developed in the late 1970s, has superior pharmacokinetic properties and a more favourable benefit-risk ratio (Bannon et al., 1995). The potential beneficial effect of heparin as a therapeutic agent in RFL has been well documented in recent case studies (Bombeli et al., 2001; Empson et al., 2002; Lockwood, 2002).

Heparin not only has anticoagulant activity, but also a wide range of anti-inflammatory and immunomodulating properties (Bannon et al., 1995; Cahalon et al., 1997). An important aspect of cell migration during inflammation is the mechanism of leucocyte rolling and tethering. In order to allow leucocyte-endothelium attachment, leucocytes secrete heparinase, which cleaves glycosaminoglycans. Heparins reduce the effect of these heparinases by substituting endogenous glycosaminoglycans and inhibiting the enzyme (Bannon et al., 1995). Moreover, heparin has an inhibitory effect on the synthesis of inflammatory cytokines including IL-6, TNF-α and IL-1 (Cahalon et al., 1997; Salas et al., 2000; Wan et al., 2002). We postulate that LMWH may modulate the immune response in patients with RFL by inhibiting changes in inflammation induced by estrogen and progesterone. Therefore, we determined to elucidate the effect of optimal concentrations of these steroid hormones have on endometrial cells in
culture, and the effect of anticoagulant therapy on cytokine production in response to hormonal stimulus.

AIMS

• To determine the expression pattern of proteins of the haemostatic system (including coagulation) and the inflammatory process throughout pregnancy of individuals with a history of RFL and compare these to those observed during normal pregnancies (chapter 3).

• To determine the relationship between phenotype and genotype of polymorphisms in genes encoding these coagulatory, fibrinolytic and inflammatory proteins and to elucidate any role these polymorphisms may play in RFL pathogenesis.

• To scan the IFN\(\gamma\) gene for the presence of novel or existing mutations within coding sequences and within a region of the promoter of the gene in RFL patients with a view to the establishment of a rapid RFLP screening technique that may enable genotype-phenotype studies.

• To determine the effect of steroid hormones on the expression of inflammatory cytokines \textit{in vitro} in an endometrial cell line.

• To elucidate the role LMWH plays in the maintenance of pregnancy in women with a history of RFL by in vitro examination of the effect of LMWH on endometrial cytokine release after stimulation with steroid hormones.
Figure 4.1: Cytokine-endocrine interactions during implantation [taken from Lim et al. (1996). Cytokines and immuno-endocrine factors in recurrent miscarriage. Human Reproduction Update: 2 (6); 469-481]. HCG = Human chorionic gonadotrophin; IL-1, 6 = Interleukin-1, 6; TNFα = Tumour necrosis factor α; IFNγ = Interferon γ; PIBF = Progesterone induced blocking factor.
4.2.0: MATERIALS & METHODS

4.2.1: Subjects and sample processing

15 pregnant women with a history of RFL (≥ 3 consecutive losses) were recruited at the Rotunda Maternity Hospital from January 1999 until June 2002. Inclusion criteria were three or more consecutive spontaneous losses in the absence of infection, endocrinopathy and chromosomal abnormalities. Of the 15 patients studied, only 2 (13%) were of non-Irish nationality (both Eastern European). Two patients experienced second trimester losses while the remainder experienced previous first trimester losses. Nine of the 15 patients had a family history of either arterial or venous thrombosis while eight of these included a family history of one or more spontaneous foetal losses. The average age of patients was 31 years (range 27-41 years). All patients recruited with a history of RFL carried their pregnancies to full term within this study and gave birth to healthy babies. Clotted blood was collected along with EDTA and citrate anticoagulated samples from each individual at approximately 8 week intervals from week 8 of gestation through to week 36 and again at three months post-partum. Similar to pregnant patients sampled in chapter 3, only 5 out of 15 in the post-partum period had samples collected. All samples were processed as set out in chapter 2.1.7. A further 73 RFL patients were recruited from the thrombosis and haemostasis clinic at the NCHCD, St. James’s hospital (chapter 2.1.6) and were combined with patients from the Rotunda hospital for polymorphism analysis. Those patients recruited from St. James’s hospital consisted of 56 women with a history of first trimester losses and seventeen patients with a history of second trimester losses. Ethnic information on these patients was not available. EDTA anticoagulated blood was collected from this cohort of patients. DNA was extracted from EDTA anticoagulated whole blood by phenol/chloroform treatment and ethanol precipitation (Chapter 2.2.2). Irish population control samples were obtained from the IBTS (Chapter 2.1.1) and DNA was extracted by phenol/chloroform precipitation (Chapter 2.2.1).

4.2.2: FBC and Thrombophilia Screens

FBC and thrombophilia screens were performed as set out in chapter 2.3.3.i-ix and chapter 3.2.2 at each time interval throughout pregnancy. Mean concentrations and cell counts were analysed appropriately.
4.2.3: Cytokine concentration measurement by ELISA

Pro- and anti-inflammatory cytokine concentrations were measured in the serum of normal pregnant patients, patients with a history of RFL and in non-pregnant controls. Cytokine concentrations were also measured in tissue culture supernatants after stimulation with estrogen and progesterone. An enzyme linked immunosorbent technique was employed using DuoSet ELISA kits (R&D Systems, Minneapolis, USA) (chapter 2.5.1). Colorimetric detection was achieved by measuring the absorbance at 450nm and subtracting this from the reference wavelength of 595nm on a microplate reader (Rosys Anthos 2010, Anthos Labtec) (chapter 2.5.1). Standard curves were created and serum cytokine concentrations were measured by interpolating data.

4.2.4: PCR analysis of polymorphic markers

PCR amplification of all RFL patient DNA samples was performed as set out in chapter 2.4.1-2.4.2 and chapter 3.2.3 for coagulation, fibrinolytic and inflammatory gene polymorphisms. RFL patients from both the Rotunda Maternity Hospital and the NCHCD at St James’s were combined for these studies (n=88). The polymorphism frequencies in this combined cohort of patients with a history of RFL were compared to the Irish population frequency in each instance using the chi-squared test ($\chi^2$) (chapter 2.4.3).

4.2.5: Mutation detection in the interferon $\gamma$ gene

The IFN$\gamma$ gene was separated into promoter regions and exonic regions as illustrated in Chapter 2, table 2.5. Each region was amplified for mutation detection on the Transgenomic WAVE® DNA fragmentation system. Briefly PCR reactions were performed in 50µl volumes of PCR buffer containing 50mM KCl, 10mM Tris-HCl pH 9.0, Triton X-100, 1.5mM MgCl$_2$, 1 unit of DNA Taq polymerase (Promega), 2µl of genomic DNA, 200µM each of deoxyribonucleoside triphosphates (Boehringer Mannheim) and 0.25mM of sense and antisense primers (Sigma Genosys) (table 2.5). PCR products were visualised on 3% w/v Agarose gels gels (Pronadisa, Madrid) and stained with 1.6µg/ml Ethidium Bromide (Sigma Aldrich). The double stranded DNA amplicons were then subject to denaturation at 95°C, separating the two strands. DNA strands were then cooled slowly to allow the re-annealing of double stranded DNA forming a mixture of potentially three species, a
mutant homoduplex, a heteroduplex and a wild type homoduplex. [Mixing of homozygous mutant DNA with wild type DNA can also achieve the same result, however, DNA from a wild-type homozygous individual will only ever form one species upon re-annealing, the wild type homoduplex]. The re-annealing of mutant and wild-type DNA strands creates a heteroduplex molecule with physical alterations forming a 'bubble', exposing a short linear single strand of DNA, making the molecule larger than the homoduplex. These duplexes were then separated on a chromatography capillary column. An anion exchanger was created from the stationary phase using an ion-pairing agent (TEAA), allowing separation of DNA molecules by fragment size. The smaller positively charged homoduplex molecules have a higher affinity for the column matrix and are therefore retained longer than the heteroduplex molecules, resulting in the formation of two peaks (figure 4.2). This technique allows the separation of a single sample in seven minutes. Alternatively, up to 50 samples may be pooled and analysed in the one run. If there is no heteroduplex formation in this group of samples, all may be regarded as having no mutations. However, if a heteroduplex is found, then individual samples may be analysed (Kuklin, 1998). TMHA has detected mutations in DNA fragments of up to 1.5kb, but greater accuracy is achieved with smaller DNA fragments between 150-450bp long (Taylor and Gjerde, 1998).

4.2.6: Estrogen & Progesterone stimulation of endometrial cells

Four cell lines were grown as set out in chapter 2.6.0. These included the endometrial adenocarcinoma cell line Ishikawa, a breast cancer cell line ZR-75-1, the monocytic cell line THP-1 and a T cell line of Jurkat cells (see table 2.4). Dose response (for 48 hours) and time course experiments revealed optimal concentrations and times for stimulation with each hormone in each cell line (see chapter 2.6.1). Cells were incubated under these conditions in the presence and absence of low molecular weight heparin (Innohep). Reactions were stopped with ice cold PBS and supernatants collected for ELISA analysis of cytokine concentrations (chapter 2.6.1).
**Figure 4.2**: Chromatogram showing typical double peaks achieved between 3 and 4 minutes with mutation detection using dHPLC technology. The initial broad peak between 0.5 and 1 minute represents the injection of the sample, while the broader peak at 6 minutes represents the elution peak indicating that the entire sample has run through the capillary column.
Chapter 4

4.3.0: RESULTS

4.3.1: Full Blood Count changes throughout pregnancy in RFL

4.3.1.i: Haemoglobin (Hb)

Mean Hb concentrations decreased from 12.7 to 11.3 g/dL up to 36 weeks gestation with a small increase observed post-partum (13 g/dL) (table 4.4). There was no significant variation in Hb concentrations throughout pregnancy in this RFL cohort (p=0.92), and no significant difference between Hb concentrations in normal pregnancy and in pregnancies of individuals with a history of RFL (p=0.89).

4.3.1.ii: HCT, MCV, MCH & MCHC

Patients with a history of RFL exhibited a decrease in HCT ratio throughout pregnancy, which reached levels below that of the normal range (ratio 0.37-0.47), but increased 3 months post-partum. Repeated measures ANOVA revealed a significant decrease in HCT ratio over time in this cohort of RFL patients (p=0.04). In total, no significant variation in HCT ratios over time was detected between normal pregnancies and pregnancies of those with a history of RFL (p=0.92). At 16 weeks gestation, one-way ANOVA analysis revealed a statistically significant decrease in HCT ratio between normal pregnancies and pregnancies of individuals with a history of RFL (p=0.05). However, non-parametric analysis designed for small sample sizes revealed no significant difference between the two patient groups ($\chi^2$ p=0.12). There was no significant variation detected over time for the MCV, MCH and MCHC measurements throughout pregnancies of patients with a history of RFL, a finding similar to normal pregnancy (table 4.4).
4.3.1.iii: Red cell parameters

The RCC of women with a history of RFL exhibited a similar pattern of change as observed during normal pregnancies (chapter 3), with a significant decrease in RCC occurring throughout (p=0.015) (range 4.1-3.8 x 10^{12} cells/L and 4.7 x 10^{12} cells/L post-partum) (table 4.4). There was no statistically significant difference between normal patients and those with a history of RFL (p=0.92). The RDW also increased throughout pregnancy in RFL patients, remaining increased post-partum without statistical significance (p=0.12). Further statistical analysis revealed no significant difference in these increases between normal pregnancies and pregnancies of those with a history of RFL (p=0.88).

4.3.1.iv: Platelet Counts

Platelet counts in RFL patients decreased from 8 weeks gestation but remained decreased up until 36 weeks with counts of 204 x 10^{9} cells/L. Similar to normal pregnancy, these counts increased in post-partum samples but to a greater degree with counts as high as 302 x 10^{9} cells/L, however still within normal ranges (140-450 x 10^{9} cells/L) (table 4.4). Overall, there was no significant difference in changes over time between the two patient groups (p=0.73).
Table 4.4: Mean concentrations of haemostatic components of the standard full blood count at time points throughout pregnancy and post-partum in pregnancies of patients with a history of RFL. 3 months PN = 3 months post natal.

<table>
<thead>
<tr>
<th>Haemostatic Parameter</th>
<th>Normal Range</th>
<th>8 weeks</th>
<th>16 weeks</th>
<th>26 weeks</th>
<th>36 weeks</th>
<th>3 months PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (Hb)</td>
<td>11.5-16.5 g/dL</td>
<td>12.7</td>
<td>11.6</td>
<td>11.4</td>
<td>11.3</td>
<td>13.0</td>
</tr>
<tr>
<td>Haematocrit (HCT)</td>
<td>0.37-0.47 ratio</td>
<td>0.37</td>
<td>0.34</td>
<td>0.33</td>
<td>0.34</td>
<td>0.39</td>
</tr>
<tr>
<td>Mean Cell Volume (MCV)</td>
<td>80-96 fl</td>
<td>90.3</td>
<td>89.4</td>
<td>90.6</td>
<td>88.6</td>
<td>85.3</td>
</tr>
<tr>
<td>Mean Cell Hb (MCH)</td>
<td>27-32 pg</td>
<td>30.9</td>
<td>30.9</td>
<td>30.9</td>
<td>29.8</td>
<td>27.8</td>
</tr>
<tr>
<td>MCH Concentration (MCHC)</td>
<td>30-36 g/dL</td>
<td>34.2</td>
<td>34.5</td>
<td>34.1</td>
<td>33.6</td>
<td>32.6</td>
</tr>
<tr>
<td>Red Cell Count (RCC)</td>
<td>3.8-5.8 x10^{12} cells/L</td>
<td>4.1</td>
<td>3.8</td>
<td>3.7</td>
<td>3.8</td>
<td>4.7</td>
</tr>
<tr>
<td>Red Cell Distribution (RDW)</td>
<td>11-15 units</td>
<td>12.3</td>
<td>12.7</td>
<td>12.9</td>
<td>13.2</td>
<td>14.2</td>
</tr>
<tr>
<td>Platelets</td>
<td>140-450 x10^9 cells/L</td>
<td>230.0</td>
<td>218.2</td>
<td>217.7</td>
<td>204.1</td>
<td>301.8</td>
</tr>
</tbody>
</table>
4.3.2: Coagulation changes throughout pregnancy in RFL

4.3.2.i: PT

The PT in patients with a history of RFL was stable throughout pregnancy, with no significant variation detected between normal and RFL pregnancies (p=0.97). However, individual trimester analysis found a significant increase in PT at 16 weeks gestation in RFL patients (13.8 secs, n=11) compared to normal pregnancy (12.9secs, n=61) for both F test (p=0.008) and non-parametric $\chi^2$ analysis (p=0.015) (table 4.5, figure 4.4). Nevertheless, these clotting times remained within the non-pregnant normal reference range of 11.5-15.0 seconds.

4.3.2.ii: APTT

Similar to individuals with previous successful pregnancies, the APTT measurement in those with a history of RFL remained stable throughout gestation, reflecting no significant difference in clotting times (p=0.73) (table 4.5). A significant difference in clotting times post-partum was detected between the normal cohort (32.2 secs, n=11) and patients with previous RFL (37.1 secs, n=2) (p=0.025). However, the sample size was too small to consider these findings reliable as indicated by an increase in the p value using non-parametric analysis (p=0.075).

4.3.2.iii: AT

AT concentrations throughout pregnancies of individuals with a history of RFL did not vary significantly over time (p=0.31) and were not significantly different from normal pregnancy (p=0.29). At 26 weeks gestation, there was a significant increase in AT concentrations in the RFL patients (1.11 IU/ml, n=11) compared to the control pregnancies (0.96 IU/ml, n=56) by both F-test analysis (p=0.0001) and by non-parametric $\chi^2$ analysis (p=0.0018) (table 4.5, figure 4.4).

4.3.2.iv: Factor VIII

FVIII concentrations varied significantly over time during pregnancies of individuals with a history of RFL (p=0.023), increasing from 1.58 IU/ml at 8 weeks to 2.30 IU/ml at 36 weeks gestation, returning to within normal ranges post-partum (1.35 IU/ml) (table 4.5). These findings are similar to the FVIII pattern of variation in normal pregnancies, therefore overall, there was no significant difference in FVIII between
normal pregnancy and RFL pregnancies (p=0.8). However, at 8 weeks gestation there was a significant increase in RFL patient FVIII concentrations (1.58 IU/ml, n=13) compared to normal pregnancy (1.29 IU/ml, n=65) as detected by non-parametric χ² analysis (p=0.025) (figure 4.4).

4.3.2.v: Fibrinogen

Fibrinogen concentrations increased above the normal range throughout pregnancies of RFL patients, with a significant variation over time (p=0.0013), returning to within normal ranges post-partum (2.9g/L) (table 4.5). There was no difference in variations of fibrinogen concentrations between normal pregnancy and pregnancies of those with a history of RFL (p=0.18).

4.3.2.vi: PC

In RFL patients, PC concentrations exhibited an almost identical pattern throughout pregnancy to normal pregnancies (table 4.5). Consequently, there was no significant variation between both populations (p=0.51).

4.3.2.vii: PS

PS concentrations increased from 0.67 IU/ml at 8 weeks gestation to a high of 0.73 IU/ml at 26 weeks gestation in pregnancies of RFL patients (table 4.5). This increase was within normal ranges (0.71-1.45 IU/ml) and not statistically variable over time (p=0.19) or different from the normal pregnant population (p=0.30). However, at 26 weeks gestation there was a significant increase in PS concentrations in RFL patients (0.73 IU/ml, n=10) compared to the normal pregnant patients (0.60 IU/ml, n=54) for both F-test analysis (p=0.008) and non-parametric χ² analysis (p=0.008) (figure 4.4).

4.3.2.viii: APCR

Acquired APCR in the absence of the FV Leiden mutation was not detected during pregnancy in any patient with a history of RFL (table 4.5) and no statistically significant variation in APCR was detected in RFL patients throughout pregnancy (p=0.07) or between normal pregnancy and the present cohort (p=0.28).
4.3.2.ix: Beta-2 Glycoprotein I

The mean β2GP1 concentration at each time interval throughout pregnancy was consistently lower in RFL patients (table 4.5) than in normal pregnant patients. Concentrations increased from 165μg/ml at 8 weeks to 173μg/ml at 26 weeks gestation, with a considerable decrease at 36 weeks (131μg/ml), although there was no significant variation detected over time (p=0.63). The increase in β2GP1 expression in RFL was in contrast to the decrease in levels throughout normal pregnancy, however there was no significant difference in variation between normal and RFL pregnancies (p=0.89). This lack of significance may be due to sample size. All RFL patients were found to be negative for the presence of lupus anticoagulants.
Table 4.5: Mean concentrations of components of the coagulation thrombophilia screen test at time points throughout pregnancy and post-partum in patients with a history of RFL. 3 mth PN = 3 months post natal. PT = prothrombin time; APTT = activated partial thromboplastin time; AT = antithrombin; FVIII = factor VIII; PC = protein C; PS = protein S; APCR = activated protein C resistance; β2GP1 = β-2 Glycoprotein 1.

<table>
<thead>
<tr>
<th>Coagulant Parameter</th>
<th>Normal Range</th>
<th>8 weeks</th>
<th>16 weeks</th>
<th>26 weeks</th>
<th>36 weeks</th>
<th>3 mth PN</th>
<th>RFL vs. Normal Pregnancy</th>
<th>Time point difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT</td>
<td>11.5-15.0 seconds</td>
<td>13.7</td>
<td>13.8</td>
<td>13.7</td>
<td>13.2</td>
<td>13.2</td>
<td>p=0.97</td>
<td>16wks: p=0.02</td>
</tr>
<tr>
<td>APTT</td>
<td>25.0-36.0 seconds</td>
<td>32.8</td>
<td>32.8</td>
<td>32.2</td>
<td>33.1</td>
<td>37.1</td>
<td>p=0.73</td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>0.76-1.2 IU/ml</td>
<td>1.0</td>
<td>0.96</td>
<td>1.11</td>
<td>1.0</td>
<td>0.99</td>
<td>p=0.29</td>
<td>26wks: p=0.002</td>
</tr>
<tr>
<td>FVIII</td>
<td>0.5-2.0 IU/ml</td>
<td>1.58</td>
<td>1.50</td>
<td>1.84</td>
<td>2.30</td>
<td>1.35</td>
<td>p=0.80</td>
<td>8wks: p=0.03</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1.5-4.0 g/L</td>
<td>4.4</td>
<td>4.6</td>
<td>4.6</td>
<td>5.5</td>
<td>2.9</td>
<td>p=0.18</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>0.71-1.45 IU/ml</td>
<td>1.1</td>
<td>1.1</td>
<td>1.2</td>
<td>1.04</td>
<td>1.1</td>
<td>p=0.51</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>0.71-1.45 IU/ml</td>
<td>0.67</td>
<td>0.72</td>
<td>0.73</td>
<td>0.67</td>
<td>0.97</td>
<td>p=0.30</td>
<td>26wks: p=0.008</td>
</tr>
<tr>
<td>APCR</td>
<td>&gt;2.0 ratio</td>
<td>2.4</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.5</td>
<td>p=0.28</td>
<td></td>
</tr>
<tr>
<td>β2GP1</td>
<td>undetermined (pg/ml)</td>
<td>165</td>
<td>169</td>
<td>173</td>
<td>131</td>
<td>159</td>
<td>p=0.89</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.3: Variation in coagulatory protein concentrations throughout gestation between women with a history of RFL (red bars) compared to those experiencing only normal pregnancies (blue bars). Plasma was separated from citrated blood samples at 8, 16, 26 and 36 weeks gestation, and 3 months post-partum (3 mo PN). PS was measured using ACL Advance/Futura instrumentation.
4.3.3: Coagulation & Fibrinolytic gene mutations

4.3.3.i: Factor V Leiden

Heterozygosity for the FVL polymorphism was detected in 6.8% (6/88) of RFL patients compared to 4.4% (17/389) of Irish population controls (IBTS), the A allele occurring at a frequency of 0.02 and 0.03 respectively (table 4.6). There was no statistically significant difference in both genotype (p=0.70) and allele (p=0.90) frequencies between RFL patients and IBTS controls. No homozygotes for the mutation (AA) were detected in either group.

4.3.3.ii: Prothrombin G20210A

The prothrombin polymorphism had a heterozygosity frequency of 2.3% (2/88) in RFL patients and 1.8% (7/390) in IBTS controls with an A allele frequency of 0.01 for both groups (table 4.6). Consequently, both genotype (p=0.86) and allele (p=0.98) frequencies were not statistically significant between RFL patients and population controls.

4.3.3.iii: t-MTHFR

Homozygosity for the t-MTHFR variant occurred in 14.8% (13/88) of RFL patients and 16.9% (66/390) of controls, with similar allele frequencies detected (table 4.6). Statistical analysis revealed no significant difference in genotype (p=0.13) and allele (p=0.10) frequencies.

4.3.3.iv: Fibrinogen G-455A

Homozygotes for the fibrinogen polymorphism occurred in 2.3% (2/88) of RFL patients and 3.3% (13/390) of controls (table 4.6). Genotype (p=0.24) and allele (p=0.29) frequencies were not statistically different between the two groups of patients. One way ANOVA analysis revealed no statistically significant variation in fibrinogen concentration at each time point throughout pregnancy in relation to genotype (p>0.05) in this RFL cohort.
4.3.3.v: PAI-1, TPA & ACE

Homozygosity for the PAI-1 polymorphism was present in 37.5% (33/88) of RFL patients and 33.2% (128/386) of controls (table 4.6). There was no significant difference in both genotype (p=0.54) and allele (p=0.28) frequencies between RFL patients and population controls.

4.3.3.vi: TPA

The TPA Alu insertion variant occurred at a frequency of 27.3% homozygosity (II) in RFL patients (24/88) and 32.4% (126/389) in controls (table 4.6). No significant difference in genotype (p=0.47) and allele (p=0.06) frequencies was found between RFL patients and Irish population controls.

4.3.3.vii: ACE

Homozygosity for the ACE deletion (DD) was detected in 21.6% (19/88) of RFL patients and 24.1% (94/390) of controls (table 4.6). Genotype (p=0.40) and allele (p=0.74) frequencies were not significantly different between RFL patients and Irish population controls.
Table 4.6: Coagulation and fibrinolytic genotype and allele counts in RFL patients (n=88) and Irish population controls (IBTS, n=390). Percentages in parenthesis.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>IBTS</th>
<th>RFL</th>
<th>Allele</th>
<th>IBTS Frequency</th>
<th>RFL Frequency</th>
<th>P value RFL v IBTS Genotypes</th>
<th>P value RFL v IBTS Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV</td>
<td>AA</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>A</td>
<td>0.02</td>
<td>0.03</td>
<td>0.33</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>17 (4.4)</td>
<td>6 (6.8)</td>
<td>G</td>
<td>0.98</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>372 (95.6)</td>
<td>82 (93.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FII</td>
<td>AA</td>
<td>1 (0.3)</td>
<td>0 (0)</td>
<td>A</td>
<td>0.01</td>
<td>0.01</td>
<td>0.86</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>7 (1.8)</td>
<td>2 (2.3)</td>
<td>G</td>
<td>0.99</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>382 (97.9)</td>
<td>86 (97.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTHFR</td>
<td>CC</td>
<td>137 (35.1)</td>
<td>41 (46.6)</td>
<td>C</td>
<td>0.59</td>
<td>0.66</td>
<td>0.13</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>187 (48.0)</td>
<td>34 (38.6)</td>
<td>T</td>
<td>0.41</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>66 (16.9)</td>
<td>13 (14.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>AA</td>
<td>13 (3.3)</td>
<td>2 (2.3)</td>
<td>A</td>
<td>0.17</td>
<td>0.20</td>
<td>0.24</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>103 (26.4)</td>
<td>31 (35.2)</td>
<td>G</td>
<td>0.83</td>
<td>0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>274 (70.3)</td>
<td>55 (62.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>4G4G</td>
<td>128 (33.2)</td>
<td>33 (37.5)</td>
<td>4G</td>
<td>0.57</td>
<td>0.61</td>
<td>0.54</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>4G5G</td>
<td>183 (47.4)</td>
<td>42 (47.7)</td>
<td>5G</td>
<td>0.43</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5G5G</td>
<td>75 (19.4)</td>
<td>13 (14.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>DD</td>
<td>81 (20.8)</td>
<td>23 (26.1)</td>
<td>D</td>
<td>0.44</td>
<td>0.49</td>
<td>0.47</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>ID</td>
<td>182 (46.8)</td>
<td>41 (46.6)</td>
<td>I</td>
<td>0.56</td>
<td>0.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>126 (32.4)</td>
<td>24 (27.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE</td>
<td>DD</td>
<td>94 (24.1)</td>
<td>19 (21.6)</td>
<td>D</td>
<td>0.50</td>
<td>0.51</td>
<td>0.40</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>ID</td>
<td>200 (51.3)</td>
<td>52 (59.1)</td>
<td>I</td>
<td>0.50</td>
<td>0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>96 (24.6)</td>
<td>17 (19.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.4: Differential leukocyte counts

4.3.4.i: White Cell Counts
Mean WCC of individuals with a history of RFL revealed a similar increase throughout gestation to normal pregnancy but counts were consistently lower than normal patients, increasing from $7.8 \times 10^9$ cells/ml at 8 weeks to $9.6 \times 10^9$ cells/L at 36 weeks, decreasing post-partum ($6.9 \times 10^9$ cells/L) (table 4.7). In contrast to normal pregnancy, the peak at 36 weeks gestation did not exceed the normal ranges in RFL patients. No significant variation in WCC during pregnancies.

4.3.4.ii: Neutrophils
Neutrophil counts were consistently lower in pregnancies of those with a history of RFL compared to normal pregnancy, but still exhibited an increase from $5.6 \times 10^9$ cells/L to a peak at 36 weeks of $7.1 \times 10^9$ cells/L and a further decrease post-partum to $4.2 \times 10^9$ cells/L (table 4.7). These increases were not statistically significant over time ($p=0.20$) and no significant difference in variation of counts between normal pregnancy and pregnancies of those with a history of RFL was observed ($p=0.85$).

4.3.4.iii: Lymphocytes
Lymphocyte counts in RFL patients decreased between 8 ($1.7 \times 10^9$ cells/L) and 16 weeks ($1.6 \times 10^9$ cells/L) but did not increase again until 36 weeks gestation ($1.8 \times 10^9$ cells/L), with a further increase post-partum ($2.1 \times 10^9$ cells/L) (table 4.7). These changes throughout pregnancy were not significantly variable over time ($p=0.29$) and no significant difference in lymphocyte variability was observed between RFL patients and normal pregnancy ($p=0.40$).

4.3.4.iv: Monocytes
Similar to lymphocytes, monocyte counts exhibited a peak at 36 weeks gestation in pregnancies of individuals with a history of RFL ($0.60 \times 10^9$ cells/L) (table 4.7), with no significant variation in counts observed throughout pregnancy ($p=0.70$). RFL patients displayed similar variations in monocytes to normal pregnant patients ($p=0.87$).
4.3.4. v: Eosinophils

RFL patients were found to have higher eosinophil counts at both 8 weeks (p=0.06) and 16 weeks (p=0.04) gestation compared to normal pregnant patients, although post-partum counts were not as increased as in normal pregnancy (0.18 vs 0.23 x10^9 cells/L respectively) (table 4.7). These were not statistically significant (p=0.39) and overall there was no significant variation between RFL patients and normal pregnancy (p=0.76).

4.3.4. vi: Basophils

Basophil counts remained within the normal range (0.0-0.1 x10^9 cells/L) throughout pregnancies of individuals with a history of RFL, with a decrease at 26 weeks from 0.03-0.01 x10^9 cells/ml. Basophils increased again at 36 weeks gestation, exhibiting a slight decrease post-partum (table 4.7). No significant variation in basophil counts was detected throughout pregnancy (p=0.53) and no significant difference in variance was observed between normal pregnancy and pregnancies of those with a history of RFL (p=0.85).
Table 4.7: Mean concentrations of differential cell counts at time points throughout pregnancy and postpartum in individuals with a history of RFL. WCC = white cell count; 3 mth PN = 3 months post natal.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Normal Range x 10^3 cells/L</th>
<th>8 weeks</th>
<th>16 weeks</th>
<th>26 weeks</th>
<th>36 weeks</th>
<th>3 mth PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCC</td>
<td>4.0-11.0</td>
<td>7.8</td>
<td>8.9</td>
<td>9.3</td>
<td>9.6</td>
<td>6.9</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2.0-7.5</td>
<td>5.6</td>
<td>6.9</td>
<td>6.9</td>
<td>7.1</td>
<td>4.2</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.5-4.0</td>
<td>1.7</td>
<td>1.6</td>
<td>1.6</td>
<td>1.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.2-0.8</td>
<td>0.46</td>
<td>0.47</td>
<td>0.60</td>
<td>0.60</td>
<td>0.44</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.0-0.4</td>
<td>0.15</td>
<td>0.18</td>
<td>0.18</td>
<td>0.15</td>
<td>0.18</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.0-0.1</td>
<td>0.03</td>
<td>0.03</td>
<td>0.01</td>
<td>0.03</td>
<td>0.02</td>
</tr>
</tbody>
</table>
4.3.5: Cytokine concentrations throughout RFL pregnancies

4.3.5.i: Interleukin-10 (IL-10)
RFL patients exhibited an decrease in IL-10 from 8 weeks (9.9 pg/ml) to 16 weeks gestation (11.8 pg/ml), with subsequent decreasing concentrations until 36 weeks gestation (8.5 pg/ml). Similar to normal pregnancy, the IL-10 level increased post-partum (table 4.8). Variations in IL-10 concentrations throughout pregnancy in women with a history of RFL were approaching statistical significance (p=0.06) by non-parametric repeated measures ANOVA testing. RFL patients had consistently increased IL-10 concentrations at each time point measured compared to pregnant controls (chapter 3), with a statistically significant difference observed (p<0.0001) (figure 4.4).

4.3.5.ii: Interleukin-6 (IL-6)
RFL patients demonstrated consistently higher IL-6 concentrations than normal patients at all stages of pregnancy and post-partum (table 4.8). However, these variations between groups were not statistically significant (p=0.14). Repeated measures ANOVA analysis revealed no significant variation in IL-6 concentrations throughout RFL pregnancies (p=0.19).

4.3.5.iii: Tumour Necrosis Factor-α (TNFα)
A similar pattern of TNFα expression to normal pregnancy was observed in patients with a history of RFL, however there was a marked increase in TNFα levels at 36 weeks gestation (80.4pg/ml) (table 4.8), although this did not achieve statistical significance when compared to normal pregnancy ($\chi^2$ p=0.97). There was no significant variation detected between TNFα concentrations in normal pregnancy and pregnancies of individuals with a history of RFL (p=0.47).

4.3.5.iv: Interferon-γ (IFNγ)
IFNγ concentrations were increased in RFL patients compared to normal pregnancy but without statistical significance. Similar to normal pregnancy, IFNγ levels demonstrated a decline from 8 weeks (6.8pg/ml) to 26 weeks gestation (4.1pg/ml), increasing towards the end of pregnancy (table 4.8). There was no significant variation detected in IFNγ concentrations throughout pregnancy in RFL patients (p=0.21) and no significant
difference in concentrations between normal pregnancy and pregnancies of individuals with a history of RFL (p=0.37).

The above comparisons in cytokine concentrations proved not to be significantly different between normal pregnancy and in pregnancies of women with a history of RFL, although some time points exhibited variation. These findings may be due to the small sample size in the cohort with a history of RFL since statistical power analysis of each test revealed the least significant number to be several orders of magnitude greater than the current sample size, in order to detect any variation in these parameters between these two cohorts.
Table 4.8: Mean concentrations of pro- and anti-inflammatory cytokines at time points throughout pregnancy and post-partum in patients with a history of RFL. 3 months PN = 3 months post natal.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>General Inflammatory Action</th>
<th>Control Level (pg/ml)</th>
<th>8 weeks (pg/ml)</th>
<th>16 weeks (pg/ml)</th>
<th>26 weeks (pg/ml)</th>
<th>36 weeks (pg/ml)</th>
<th>3 months PN (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>Anti</td>
<td>53.5</td>
<td>11.8</td>
<td>9.3</td>
<td>5.3</td>
<td>8.4</td>
<td>7.7</td>
</tr>
<tr>
<td>IL-6</td>
<td>Anti/Pro</td>
<td>99.8</td>
<td>39.7</td>
<td>30.3</td>
<td>34.9</td>
<td>81.2</td>
<td>25.9</td>
</tr>
<tr>
<td>TNFα</td>
<td>Pro</td>
<td>273.5</td>
<td>49.8</td>
<td>50.1</td>
<td>54.3</td>
<td>80.4</td>
<td>22.2</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Pro</td>
<td>51.9</td>
<td>6.8</td>
<td>5.2</td>
<td>4.1</td>
<td>5.9</td>
<td>5.7</td>
</tr>
</tbody>
</table>
Figure 4.4: IL-10 concentrations throughout normal pregnancy and during pregnancies of patients with a history of RFL. For repeated measures ANOVA analysis, only patients with measurements at each time point were included in the analysis. Therefore the three month post-natal samples were not in sufficient numbers to be included in this figure. N= number of patients in each group. Normal= Normal pregnancy (n=46); Recurrent= RFL patients (n=7). IL10_8, 16, 26, 36 = Mean IL-10 concentrations at 8, 16, 26 and 36 weeks.
4.3.6: Cytokine gene mutations in RFL

4.3.6.i: IL-10 -592
Homozygosity (AA) for the IL-10 -592 polymorphism was detected in 5.7% (5/88) of RFL patients and 4.0% (15/389) of Irish population controls, with both groups exhibiting the same A allele frequency (0.22) (table 4.9). Therefore no significant difference in genotype (p=0.70) and allele (p=0.90) frequencies was observed. There was no significant variation in IL-10 concentrations throughout pregnancy with a particular genotype (p>0.05). Lower concentrations were observed with homozygosity for the polymorphism, however only one individual was homozygous within the sub-set of patients that had cytokine measurements taken throughout gestation (n=13). An increase in patient number is required for definitive comparisons.

4.3.6.ii: IL-10 -1082
The IL-10 -1082 AA genotype was detected in 21.6% (19/88) of RFL patients and 21.9% (85/388) of population controls (table 4.9). Consequently, no significant difference in genotype (p=0.17) and allele (p=0.11) frequencies was observed. Assessment of IL-10 concentrations for each genotype throughout pregnancies of patients with a history of RFL revealed no significant variation in IL-10 concentrations with each variant of the IL-10 -1082 polymorphism (p>0.05), but a trend toward increased IL-10 levels with the AA genotype was observed.

4.3.6.iii: IL-6 -174
The IL-6 -174 CC genotype was detected in 27.3% of RFL patients (24/88) and 17% of IBTS controls (68/389). Both genotype (p=0.0009) and allele (p=0.0005) frequencies were significantly different between RFL patients and population controls with the C allele occurring at an increased frequency in RFL patients (table 4.9). Within the RFL sub-population (n=13), comparison of IL-6 concentrations revealed increased levels with the GG genotype at each time point but with no statistical significance (p>0.05). However, a greater number of patients is required to elucidate the relationship between genotype and IL-6 concentrations.
4.3.6.iv: TNFα -308

Homozygosity for the TNFα -308 polymorphism was detected in 9.1% of RFL patients (8/88) and 4.0% of population controls (16/390). Genotype frequencies were significantly different in RFL patients compared to the Irish population (p=0.007), however allele frequencies were not (p=0.43, table 4.9). Comparison of TNFα concentrations and genotype in the RFL sub-group (n=12) throughout pregnancy revealed consistently lower levels of TNFα with the AA genotype (figure 4.5a) but these differences did not achieve statistical significance (p>0.05).

4.3.6.v: TNFB +252

The TNFB 22 genotype was detected in 47.7% (42/88) of RFL patients and 34.0% (132/389) of Irish population controls, while the TNFB 11 (GG) genotype occurred in 20.5% (18/88) and 13.0% (52/389) respectively (table 4.9). While allele frequencies were similar (p=0.41), genotype frequencies were significantly different in RFL patients compared to the Irish population (p=0.002). Assessment of the relationship between the TNFB polymorphism and TNFα concentrations revealed no statistically significant variations in protein levels in the presence of the polymorphism at all time points (p>0.05). However 10 of the 13 (77%) patients in this RFL sub-group were homozygous for the 22 genotype with consistently increased TNFα concentrations occurring throughout pregnancy (figure 4.5.b).
**Table 4.9:** Inflammatory gene polymorphism genotype and allele counts in RFL patients (n=88) and Irish population controls (IBTS, n=390). Percentages in parenthesis.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>IBTS</th>
<th>RFL</th>
<th>Allele</th>
<th>IBTS Frequency</th>
<th>RFL Frequency</th>
<th>P value RFL v IBTS Genotypes</th>
<th>P value RFL v IBTS Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10 -592</td>
<td>CC</td>
<td>235 (60)</td>
<td>54 (61.4)</td>
<td>C</td>
<td>0.78</td>
<td>0.78</td>
<td>0.70</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>139 (36)</td>
<td>29 (32.9)</td>
<td>A</td>
<td>0.22</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>15 (4)</td>
<td>5 (5.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10 -1082</td>
<td>GG</td>
<td>123 (31.7)</td>
<td>22 (25.0)</td>
<td>G</td>
<td>0.55</td>
<td>0.52</td>
<td>0.17</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>180 (46.4)</td>
<td>47 (53.4)</td>
<td>A</td>
<td>0.45</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>85 (21.9)</td>
<td>19 (21.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL6 -174</td>
<td>GG</td>
<td>123 (32)</td>
<td>11 (12.5)</td>
<td>G</td>
<td>0.57</td>
<td>0.43</td>
<td>0.0009</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>198 (51)</td>
<td>53 (60.2)</td>
<td>C</td>
<td>0.43</td>
<td>0.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>68 (17)</td>
<td>24 (27.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α -308</td>
<td>GG</td>
<td>234 (60)</td>
<td>62 (70.4)</td>
<td>G</td>
<td>0.78</td>
<td>0.81</td>
<td>0.007</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>140 (36)</td>
<td>18 (20.5)</td>
<td>A</td>
<td>0.22</td>
<td>0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>16 (4)</td>
<td>8 (9.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFB</td>
<td>22</td>
<td>132 (34)</td>
<td>42 (47.7)</td>
<td>2 (A)</td>
<td>0.60</td>
<td>0.64</td>
<td>0.002</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>205 (53)</td>
<td>28 (31.8)</td>
<td>1 (G)</td>
<td>0.40</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>52 (13)</td>
<td>18 (20.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.5: The relationship between TNFα concentrations and polymorphisms within the TNF gene in patients with a history of RFL. TNFα concentrations were analysed here at the maximum concentration observed throughout pregnancy, 26 weeks. (A) TNFα -308 polymorphism (AA= TNFα 22; GG= TNFα 11); (B) TNFβ +252 polymorphism (AA= TNFB 22; GG= TNFB 11).
**4.3.7: Mutation detection in the interferon γ gene**

DHPLC analysis of 15 normal pregnant patients and 15 RFL patients revealed chromatograms exhibiting only one peak for each patient sampled. Some peaks observed were wider than others with shouldering peaks on the side, leaving room for speculation and the possibility of a second peak representing a mutation (figure 4.6.b). DNA from such spurious chromatograms was sequenced revealing no mutations within these gene regions. This was assessed by comparing the resulting sequence to the original IFNγ gene sequence (NM_000619), located on the Human Genome Browser web site (chapter 2.7.0). Sequenced DNA samples from spurious chromatograms were compared to the IFNγ gene for homology using the Clustalw homology service from the European Bioinformatics Institute (EBI: http://www.ebi.ac.uk/clustalw). All sequences were identical to the original IFNγ gene sequence. Therefore, no novel mutations were detected within 600bp of the promoter region and within all four exons of the interferon γ gene in normal pregnant patients and in patients with a history of RFL.
Figure 4.6: Chromatograms illustrating a clear single peak (A) and spurious double peaks (B) obtained from patients with RFL and pregnant controls. Spurious wide peaks and similar shoulder peaks as shown here (B) were sequenced revealing no mutations within the IFNγ gene. Such spurious peaks resulted from impure DNA and PCR products.
4.3.8: Hormonal regulation of cytokine expression in an endometrial cell line

4.3.8.i: Dose-Response Curves

Stimulation of each cell line with estrogen and progesterone was carried out using increasing concentrations of each hormone. Using IFNγ expression in Ishikawa cells as an example, optimal stimulation was obtained after incubation of cells with $1 \times 10^{-8}$M of estrogen and progesterone. Each cell line exhibited similar patterns so this concentration was chosen for both hormones in each cell line.

4.3.8.ii: Time Course determination

Each cell line was incubated with $1 \times 10^{-8}$M of estrogen and progesterone over a 72 hour period, stopping reactions at the following time points post-hormone addition: 1hr; 3hrs; 6hrs; 12hrs; 24hrs; 48hrs; 72hrs. For estrogen and progesterone stimulation, the 12 hour time point demonstrated the greatest increase and decrease in cytokine production respectively in all cell lines.

4.3.8.iii: Cytokine response to estrogen, progesterone & low molecular weight heparin (LMWH)

The cell lines Ishikawa (endometrial), ZR-75-1 (breast carcinoma), Jurkat (T cells) and THP-1 (monocytes) were treated with estrogen (E), progesterone (P), and both hormones in combination (E+P). Resting cells and hormone treated cells were also co-incubated with LMWH to monitor changes in cytokine expression. IL-10, IL-6, TNFα and IFNγ measurements were made using pre-developed ELISA's from each cell culture supernatant (table 4.10 a-c). Findings for each are described below:

4.3.8.iii(A): IL-10

IL-10 measurements were consistently below 2pg/ml throughout. In Ishikawa cells, E, P, E+P, and LMWH all reduced expression of IL-10 from that observed in untouched cells. In this cell line, addition of LMWH to E/P treated cells inhibited the decrease in IL-10 by steroid hormones (figure 4.7a). In breast cancer cell carcinomas (ZR-75-1), IL-10 was slightly increased with P. LMWH reduced resting levels of IL-10 and inhibited the effects of the hormones. In Jurkat cells, all steroid hormone treatments reduced the expression of the anti-inflammatory cytokine IL-10. LMWH had no effect on P treatment but inhibited IL-10 downregulation in cells incubated with E and E+P.
In THP-1 cells, E and P increased expression of IL-10, which was undetectable in resting cells. LMWH inhibited this increase in IL-10 expression in each case. Although there is no definite trend between cell lines, it is clear that LMWH inhibits the effects of E and P on IL-10 expression (table 4.10a).

4.3.8.iii(B): IL-6
E, P and E+P treatment of Ishikawa cells resulted in decreased IL-6 expression, while LMWH inhibited this effect (figure 4.7b). In ZR-75-1 cells, no change was observed on addition of E, and in P treated cells IL-6 was undetectable. In each case, LWMH in combination with hormones appeared to inhibit the decrease in IL-6 expression. No clear trend was observed in either T cells or monocytes except the consistent inhibition of IL-6 expression by LMWH in the presence of hormones (table 4.10b).

4.3.8.iii(C): TNFα
In endometrial cells, E and P alone increased TNFα secretion whereas E+P reduced secretion considerably. Both resting cell expression and E and P induced expression of TNFα was inhibited by LMWH (figure 4.7c). In turn, LMWH inhibited E+P downmodulation of TNFα expression (i.e. increased TNFα concentrations). In breast cancer and T cells, both hormone and LMWH treatment reduced TNFα expression from that observed in resting cells. In monocytes, E, P and E+P increased TNFα expression, an effect that was inhibited by LMWH (table 4.10c).

4.3.8.iii(D): IFNγ
Very little variation was observed in IFNγ expression after stimulation with E and P in all cell types. LMWH did not have any effect on this expression also.
Table 4.10: Cytokine concentrations (pg/ml) after 12 hours of incubation with estrogen, progesterone and LMWH in four cell lines. A = IL-10; B = IL-6; C = TNFα. Ishikawa cell line = endometrial adenocarcinoma; ZR-75-1 cell line = breast carcinoma line; Jurkat cell line = acute lymphoblastic leukemia cell line (T cells); THP-1 = monocytic leukemia cell line.

A: IL-10

<table>
<thead>
<tr>
<th>Reaction contents</th>
<th>Ishikawa</th>
<th>ZR-75-1</th>
<th>Jurkats</th>
<th>THP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting: cells only</td>
<td>1.43</td>
<td>2.49</td>
<td>3.55</td>
<td>0.00</td>
</tr>
<tr>
<td>+ Estrogen (E)</td>
<td>0.37</td>
<td>3.02</td>
<td>1.43</td>
<td>2.49</td>
</tr>
<tr>
<td>+ Progesterone (P)</td>
<td>0.37</td>
<td>0.90</td>
<td>2.49</td>
<td>0.90</td>
</tr>
<tr>
<td>+ E + P</td>
<td>0.00</td>
<td>2.49</td>
<td>0.90</td>
<td>3.55</td>
</tr>
<tr>
<td>+ LMWH</td>
<td>0.90</td>
<td>0.37</td>
<td>1.96</td>
<td>2.49</td>
</tr>
<tr>
<td>+ LMWH + E</td>
<td>1.43</td>
<td>1.96</td>
<td>1.43</td>
<td>0.00</td>
</tr>
<tr>
<td>+ LMWH + P</td>
<td>0.90</td>
<td>2.49</td>
<td>0.90</td>
<td>0.37</td>
</tr>
<tr>
<td>+ LMWH + E + P</td>
<td>1.96</td>
<td>3.55</td>
<td>0.90</td>
<td>0.00</td>
</tr>
</tbody>
</table>

B: IL-6

<table>
<thead>
<tr>
<th>Reaction contents</th>
<th>Ishikawa</th>
<th>ZR-75-1</th>
<th>Jurkats</th>
<th>THP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting: cells only</td>
<td>2.54</td>
<td>1.70</td>
<td>2.76</td>
<td>2.23</td>
</tr>
<tr>
<td>+ Estrogen (E)</td>
<td>1.08</td>
<td>1.70</td>
<td>4.86</td>
<td>1.70</td>
</tr>
<tr>
<td>+ Progesterone (P)</td>
<td>1.08</td>
<td>0.00</td>
<td>2.32</td>
<td>0.00</td>
</tr>
<tr>
<td>+ E + P</td>
<td>0.79</td>
<td>2.76</td>
<td>2.23</td>
<td>2.23</td>
</tr>
<tr>
<td>+ LMWH</td>
<td>2.25</td>
<td>0.65</td>
<td>4.33</td>
<td>2.23</td>
</tr>
<tr>
<td>+ LMWH + E</td>
<td>2.83</td>
<td>2.76</td>
<td>0.00</td>
<td>0.65</td>
</tr>
<tr>
<td>+ LMWH + P</td>
<td>2.83</td>
<td>2.76</td>
<td>1.36</td>
<td>0.65</td>
</tr>
<tr>
<td>+ LMWH + E + P</td>
<td>2.25</td>
<td>3.28</td>
<td>1.70</td>
<td>0.13</td>
</tr>
</tbody>
</table>

C: TNFα

<table>
<thead>
<tr>
<th>Reaction contents</th>
<th>Ishikawa</th>
<th>ZR-75-1</th>
<th>Jurkats</th>
<th>THP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting: cells only</td>
<td>1.22</td>
<td>8.84</td>
<td>7.14</td>
<td>0.00</td>
</tr>
<tr>
<td>+ Estrogen (E)</td>
<td>2.07</td>
<td>7.99</td>
<td>0.00</td>
<td>3.76</td>
</tr>
<tr>
<td>+ Progesterone (P)</td>
<td>2.07</td>
<td>3.76</td>
<td>3.76</td>
<td>0.37</td>
</tr>
<tr>
<td>+ E + P</td>
<td>0.37</td>
<td>6.30</td>
<td>0.37</td>
<td>5.45</td>
</tr>
<tr>
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<td>2.91</td>
<td>5.45</td>
<td>1.22</td>
</tr>
<tr>
<td>+ LMWH + E</td>
<td>0.37</td>
<td>5.45</td>
<td>2.07</td>
<td>0.00</td>
</tr>
<tr>
<td>+ LMWH + P</td>
<td>0.37</td>
<td>7.99</td>
<td>1.22</td>
<td>0.00</td>
</tr>
<tr>
<td>+ LMWH + E + P</td>
<td>5.45</td>
<td>7.99</td>
<td>0.00</td>
<td>0.37</td>
</tr>
</tbody>
</table>
Figure 4.7: Concentrations of IL-10, IL-6 and TNFα in Ishikawa cells after 12 hour incubation with estrogen (E), progesterone (P) and low molecular weight heparin (LMWH). R= resting cells.
4.4.0: DISCUSSION

To date there have been extensive numbers of studies published that have failed to reach definitive conclusions on the pathophysiological events resulting in RFL. Each study uses different criteria for defining the term ‘Recurrent Foetal Loss’, some requiring only two losses, others specifying three or more. Many reports fail to recognise the difference that the stage of gestation may have made to data collected. These factors make it difficult to analyse previous studies conclusively. However, it is becoming apparent that both the haemostatic environment and the immune system behave quite differently throughout the three trimesters of pregnancy even in normal pregnancies. The present longitudinal study examined haemostatic and inflammatory mechanisms throughout all trimesters of pregnancy in women with a previous history of RFL, identifying areas of difference between these populations.

Full blood count screening of patients with a history of RFL revealed no difference in the behaviour of all factors analysed between RFL patients and normal pregnant patients. To the best of our knowledge, this study is the first of its kind comparing these parameters throughout pregnancy in two distinct patient populations. Clotting times as measured by the PT and APTT assays did not exhibit variability between normal pregnancy and pregnancies of individuals with a history of RFL when analysed in total. However, PT clotting times significantly increased at 16 weeks gestation in RFL patients compared to controls, indicating decreased concentrations of prothrombin, fibrinogen, FV, FVII and FX. The absence of similar increases at other time points throughout pregnancy and the lack of change in APTT, which is sensitive to variations in these proteins also, suggests this finding to be an anomaly and not a true representation of the PT levels at this time point. This may have occurred as a result of an increase in one patient only, when such small patient numbers are involved. However, these tests alone are not sufficient for concluding overall differences in clotting mechanisms in RFL patients.

Both acquired and inherited thrombophilias play important roles in the pathophysiology of gestational thrombosis and recurrent pregnancy loss. Reviewed literature indicates that the most commonly inherited thrombophilia apart from FV Leiden and the prothrombin variant is AT deficiency. In the present study, the variation in AT concentrations throughout pregnancies of RFL patients was not significantly different
from normal pregnancies, however at 26 weeks gestation, a significant increase in AT was observed. AT increases may be an indirect effect of the increases in acute phase proteins induced by cytokines of the acute phase response, with the possibility of a greater response occurring in RFL patients. However, this study suggests that further investigations of AT in RFL patients during and after pregnancy are necessary to elucidate the role, if any, this anticoagulant has in the maintenance of pregnancy. Significantly increased FVIII concentrations were observed in RFL patients at 8 weeks gestation only compared to normal pregnancy. This increase was not reflected by an increase in fibrinogen at this time point, as would be expected, since fibrinogen levels increase as a result of the change in FVIII.

PC concentrations have previously been found to be unchanged during pregnancy, a finding reflected in this study until a slight decrease at 36 weeks gestation in both normal patients and those with a history of RFL, with no significant difference observed between these groups. This suggests inactivation of the PC anticoagulant pathway only in the later stages in preparation for the onset of labour. The similarity in responses between normal pregnancy and RFL pregnancies suggests that RFL pathogenesis is not influenced by dysregulation of the PC pathway in this group of patients. However, at 26 weeks gestation RFL patients exhibited significantly greater PS levels compared to normal patients. This increase in PS later in gestation in patients with a history of RFL may indicate that these women have reduced control over these mechanisms, with the possibility of greater inhibition of anticoagulant responses and the release of more PS than can be complexed with C4bBP. Such a mechanism may result in a greater concentration of free PS in solution later in pregnancy in patients with a history of RFL, but is unlikely to affect a successful outcome.

β2GP1 is thought to have anticoagulant properties by binding the phospholipids that are required for activation of coagulation factors, inhibiting the activation of the coagulation pathway. Antibodies to β2GP1 have been suggested to increase the thrombogenic potential in patients with RFL (Samarkos et al., 2001; Visvanathan and McNeill, 1999). We assessed the concentration of β2GP1 throughout pregnancy in RFL patients revealing consistently lower concentrations compared to normal pregnancy, although not significantly different. The decrease in β2GP1 concentrations in RFL patients may allow greater activation of coagulation proteins in these patients,
providing a potential mechanism of increased hypercoagulability. However, since β2GP1 levels were not found to be significantly decreased, this suggests no subsequent increase in coagulation factors.

Due to the small sample size of RFL patients collected at the Rotunda hospital, these patients were combined with RFL patients from St. James’s hospital for genotype analysis, and polymorphic frequencies compared to an Irish blood donor control population, shown to be representative of an unbiased control group (Livingstone et al., 2000). We found no increased prevalence of both coagulatory and fibrinolytic polymorphisms in this RFL cohort compared to the Irish population suggesting no benefit for routine screening of these genes in patients with a history of unexplained RFL.

An imbalance between cells of the immune system, particularly T cells, is thought to mediate immune reactions at the feto-maternal interface with a bi-directional system of cytokine production postulated (Lim et al., 1996; Robertson, 2000; Wegman et al., 1993). Type 2 cytokines have been shown to be more beneficial for reproductive fitness with type 1 cytokines being detrimental to survival of the foetus in both murine and human models (Hill et al., 1995; Wegman et al., 1993). A shift from cellular immunity (type 1) to humoral immunity (type 2) has been postulated within the first trimester of pregnancy allowing a successful outcome, with the persistence of type 1 cytokines being implicated in RFL pathogenesis (Hill et al., 1995). The present study demonstrated a decrease in white cells in patients with a history of RFL compared to normal pregnancy and compared to non-pregnant control ranges, indicating an imbalance in immune responses in these patients. Concentrations of type 1 and type 2 cytokines were measured longitudinally in RFL patients revealing decreased levels of all cytokines compared to non-pregnant controls, as found in normal pregnancy. TNFα expression was similar in the RFL and normal pregnancy cohorts, while IL-6 and IFNγ were consistently increased in RFL patients, but without statistical significance. Statistical power analysis revealed higher sample numbers are required in some tests to identify a significant difference between these cohorts if one truly exists. Therefore, this study did not detect the predominance of type 1 cytokine expression during RFL pregnancies. In fact IL-10 concentrations were significantly increased in patients with a history of RFL, suggesting a protective role for this cytokine in the maintenance of
pregnancy. Increased IL-10 may have acted to decrease the levels of type 1 cytokines such as TNFα and IFNγ, as IL-10 has been shown to oppose the pro-inflammatory effect of type 1 responses (Mosmann and Sad, 1996). The protective role of IL-10 has been postulated previously, and has been suggested as the most beneficial cytokine to reproductive fitness (Wegman et al., 1993). The current findings support these postulations in relation to IL-10 since all of these patients experienced successful outcomes. Furthermore, our findings are in agreement with a recent report also detecting increased IL-10 along with IL-4 in RFL patients (Bates et al., 2002). Genetic analysis of two polymorphisms within the IL-10 gene (-592, -1082) revealed no increased prevalence of these markers in RFL patients compared to the Irish population. Moreover, no significant variation in IL-10 concentrations was detected in the presence of a particular genotype in RFL pregnancies. These findings coupled with the inconsistent changes in IL-10 observed with these polymorphisms in normal pregnancy and non-pregnant controls indicate that these polymorphisms do not dictate the changes in IL-10 that occur during pregnancy. This finding has also recently been reported by others (Babbage et al., 2001; Karhukorpi et al., 2001).

In contrast to coagulator and fibrinolytic polymorphisms, polymorphisms within the genes for the inflammatory cytokines IL-6 and TNF were significantly increased in RFL patients compared to the control Irish population. A significant increase in the C allele of the IL-6 -174 polymorphism was observed in the larger RFL cohort. An increase in IL-6 concentration was observed with the GG genotype, which is in agreement with a previous report that noted decreased IL-6 production with the CC genotype in response to inflammatory stimuli (Fishman et al., 1998). Therefore the predominance of the G allele in this cohort may contribute to increased IL-6 levels, and may be one factor promoting maintenance of pregnancy in these patients, since IL-6 has been implicated in the implantation process (Lim et al., 1996). Furthermore, increased IL-6 may be the major link between the coagulatory and inflammatory mechanisms in pregnancies of patients with a history of RFL, since IL-6 induces the expression of fibrinogen during the acute phase response (Franco and Reitsma, 2001; Lane and Grant, 2000). Both polymorphisms in the TNF gene (α-308 A, β+252 A/2) were significantly increased in RFL patients compared to population controls. Sub-set analysis of the protein levels detected consistently decreased TNFα concentrations with the 22 (AA) genotype in individuals with a history of RFL. This is contrary to
previous report that suggests the 22 genotype to be associated with increased TNFα concentrations (Warzocha et al., 1998). However, the present study only detected one individual with this genotype, which does not allow definitive comparisons to be made. Homozygosity for the 22 genotype of the TNFB (β+252) polymorphism occurred in 77% of sub-set RFL patients with consistently increased TNFα concentrations, although with no significance. Controversy surrounds this polymorphism in relation to its influence on TNFα concentrations. The current findings indicate increased protein expression in the presence of the 2 (A) allele, which to the best of our knowledge, has not been shown previously in a cohort of patients with a history of RFL. Further longitudinal studies are required in a greater number of individuals to conclusively determine the role these markers play in cytokine expression during pathological pregnancies. In addition, polymorphisms within the IFNγ gene were not detected in patients with a history of RFL and normal pregnant controls using denaturing high performance liquid chromatography.

Investigation of haemostatic and inflammatory mechanisms in pregnant women with a history of RFL revealed potential areas of dysregulation in these processes. The occurrence of successful pregnancies in all patients may also have masked further changes or more dramatic variations in certain factors between these patients and normal pregnancy. However, due to the small number of patients included in the entire study and the high drop out rate of participants, it is suggested that further collection of patients be performed over an extended time period to obtain greater numbers, increasing the power of these longitudinal studies. However, it is our belief that IL-6 may have important roles to play in the maintenance of pregnancy, and also has a great influence over coagulatory processes via the regulation of fibrinogen transcription in the acute phase response. IL-6 has previously been suggested as being involved in implantation by inducement of HCG in the endometrium, as a result of IL-6 regulation by the steroid hormones (Lim et al., 1996). Therefore, IL-6 concentrations at the time of implantation may be crucial to the successful anchorage of trophoblast cells. The finding that all patients with a history of RFL achieved successful pregnancies in this study, may indicate that stress factors are important in the maintenance of a viable foetus in these individuals. Throughout these investigations, patients were examined by the same doctor at more regular intervals than normal, a factor that may have helped to decrease the stress levels of the patient. Moreover, IL-6 has been demonstrated as the
predominant cytokine involved in processes of stress response in mice models and in exercising individuals, and exercise induced IL-6 expression has been suggested as a good model of sepsis (Shephard, 2002; Hale et al., 2003). Therefore, the decreased concentrations of IL-6 observed throughout these pregnancies may be a direct result of reduced stress levels in this cohort.

In addition to interactions between coagulation and inflammation processes, further mechanisms of regulating intrauterine functions occur via the immune and endocrine systems (Denison et al., 1998). These pathways of regulation are predominantly governed by the steroid hormones estrogen and progesterone, which have been found as having pro-inflammatory and anti-inflammatory roles respectively (Critchley et al., 2001; Piccinni et al., 2000). We assessed these hormones for their effect on inflammatory cytokine expression in an endometrial cell line (Ishikawa), breast cancer cells (ZR-75-1), T cells (Jurkats) and monocytes (THP-1), with surprising results. Both estrogen and progesterone demonstrated pro-inflammatory properties in all cell lines examined by reducing IL-10 production. IL-6 levels were also found to decrease, while TNFα secretion increased, with little effect on IFNγ release. Therefore, decreased IL-6 levels during pregnancy may be in part regulated by these hormones. The pro-inflammatory effect of progesterone was unexpected since it has previously been demonstrated as having anti-inflammatory properties in the uterine environment (Piccinni et al., 2000). Another surprising result is the response of both T cells and monocytes to hormonal regulation, since neither of these cell lines are known to express estrogen (ER) and progesterone receptors (PR). However, estrogen has been reported to stimulate the expression of both ER and PR, priming the uterus for further hormonal exposure (Tibbetts et al., 1999). Consequently, T cells and monocytes may be induced to express these receptors in response to estrogen. However, it should be noted that these cell lines are tumourogenic cells, with the capability of behaving in unexpected manners that are not necessarily indicative of normal human responses. The steroid hormones may exert different effects in different tissues, since breast carcinoma cells exhibited increased IL-10 in response to progesterone stimulation. These in vitro systems were exposed to heparin to examine their response to anticoagulant therapy, which has been used successfully in the treatment of pregnant patients with a history of RFL. Heparin inhibited hormonal regulation of cytokines in all cell lines investigated, in particular in endometrial cells where heparin inhibited the downmodulation of IL-10 and IL-6 by E and P, and inhibited the increase in TNFα by these hormones. These
findings suggest that heparin prevents the steroid hormones from exerting their immunomodulatory function in the uterus, and may be one mechanism by which heparin maintains the foetus in individuals with a history of RFL. Furthermore, this indicates that heparin has both anticoagulant and anti-inflammatory properties that may be exploited in the treatment of RFL.

In summary, inflammatory cytokines clearly have a role to play in the maintenance of pregnancy. We observed decreased white cell counts and cytokine production in individuals with a history of RFL compared to pregnant and non-pregnant controls. Genetic studies revealed the regulation of these cytokines by polymorphisms within genes for these proteins, which have the potential to dictate the cytokine profile of an individual exacerbating the effects of pathological mechanisms during pregnancy. Moreover, we have demonstrated in an in vitro model, that cytokine secretion is also regulated by steroid hormones in the endometrium, a process that can be modulated by treatment with heparin anticoagulant and anti-inflammatory therapy.
CHAPTER 5

VENOUS THROMBOEMBOLIC DISEASE
5.1.0: VENOUS THROMBOEMBOLIC DISEASE

Venous thromboembolism is a complex disease defined as '...the obstruction of the circulation by clots resulting from an increased acquired or inherited tendency to hypercoagulability' (Nowak-Gottl et al., 2001b). Thrombus formation is a result of local coagulation activation and disruption of the normal balance between coagulation and fibrinolysis (Nowak-Gottl et al., 2001b). The overall incidence of venous thromboembolism per year in Western countries is about 1 in 1,000 individuals (De Stefano et al., 2002; Heit et al., 2001). VTE can result in several types of thrombotic states of increasing severity including superficial vein thrombosis (SVT), deep vein thrombosis (DVT) and pulmonary embolism (PE) (Cooper and Krawczak, 1997; Martinelli et al., 1999). SVT is the least severe manifestation of VTE but 7-46% of cases develop into DVT (Martinelli et al., 1999). A recent US study revealed the incidence of VTE in a Caucasian population to be 201,000 new cases annually, of which 107,000 were DVT alone and 94,000 were PE, with or without DVT (Heit et al., 2001). These figures are probably an underestimate since only a Caucasian population was studied, but they still give some indication as to the seriousness of VTE in the general population. It is estimated that about 10% of cases of DVT result in death from PE within hours of thrombus manifestation (Cooper and Krawczak, 1997). The risk of early death following PE is suggested to be 18-fold higher than with DVT alone. Survival rates one and seven days post VTE were reported as 97% and 96.2% respectively for DVT, while these figures decreased dramatically to 63.6% and 71.1% for PE (Heit et al., 2001).

In addition to morbidity and mortality in the general population, VTE is the leading cause of both maternal death in the UK (Falter, 1997) and preventable death in hospitalised patients (Meissner, 1998; Tanios et al., 2001). Furthermore, thrombosis is the most frequent complication and the second cause of death in patients with overt malignant disease (Levitan et al., 1999; Rickles and Levine, 1998). Thrombotic episodes may also precede the diagnosis of cancer by months or years, representing a potential marker for malignancy (Clark, 1998; Rickles and Levine, 1998). Pulmonary embolism (PE) accounts for up to 25% of early deaths after stroke (Wijdicks and Scott, 1997). Moreover, congenital and acquired thrombophilias can contribute to the pathophysiological processes that underlie miscarriage, intrauterine growth restriction, and pre-eclampsia (Preston et al., 1996; Sanson et al., 1996). For these reasons,
strategies to predict and prevent thrombus formation have resulted in a plethora of coagulation protein association studies, providing insight into the inherited and acquired predisposition to clot formation.

The pathogenesis of thrombosis is a complex mechanism and involves the interaction of environmental, acquired and inherited risk factors that contribute to the multifactorial basis of disease. Until 1993, the cause of thrombophilia was understood in only a small proportion of patients and centred on acquired risk factors such as age, surgery, concurrent illness and hormonal contraception. Hereditary defects were confined to deficiencies of the natural anticoagulants protein C, protein S and antithrombin, amounting to between 5-10% of all cases (table 5.1) (Goldhaber, 1998). However, discovery of prothrombotic genetic polymorphisms along with evidence that high levels of Factor VIII and homocysteine are associated with VTE has led to a greater degree of interest and understanding of this common clinical problem.

<table>
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<tr>
<th>PERSISTENT</th>
<th>TRANIENT</th>
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<tr>
<td><strong>Inherited</strong></td>
<td><strong>Acquired</strong></td>
</tr>
<tr>
<td>Antithrombin</td>
<td>Age</td>
</tr>
<tr>
<td>Protein C &amp; S</td>
<td>Malignancy</td>
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<tr>
<td>Factor V Leiden (FVL)</td>
<td>Antiphospholipid antibodies</td>
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<tr>
<td>Prothrombin G20210A</td>
<td>History if VTE</td>
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- Surgery & major trauma
- Pregnancy & puerperium
- Oral contraceptive use
- Hormone replacement therapy
- Prolonged immobilisation
- Smoking status
5.1.1: Acquired risk factors for VTE

Acquired risk factors may be transient and involve alterations in the circulating levels of proteins involved in coagulation and fibrinolysis. These include increasing age, surgery and trauma, immobilisation (hospitalisation), malignancy and a personal or familial history of thrombosis. Among women, risk factors include pregnancy, oral contraceptive use and hormone replacement therapy (Heit et al., 2001). The FV Leiden polymorphism results in resistance of FVa to inactivation by APC, inferring a procoagulant state and the APC phenotype as discussed in chapter 3. In greater than 95% of cases, APCR is accompanied by FV Leiden and therefore is an inherited risk factor. However decreased APCR ratios (increasing resistance to APC) have been reported without the FV Leiden polymorphism, therefore changing its status to an acquired risk factor for VTE. The most prominent procoagulant state associated with acquired APCR is pregnancy and PE is the most common cause of maternal death during pregnancy (Cumming et al., 1995). Gestational acquired APCR can occur without the presence of FV Leiden and has been found in 42% (14-20 weeks gestation), 55% (28 weeks) and 44% (at delivery) of pregnant women (Cumming et al., 1995; Mathonnet et al., 1996). Furthermore, acquired APCR has been reported with a concomitant increase in thrombin generation (Clark et al., 1999). Estrogen in the form of the oral contraceptive pill has also been reported to increase APCR without FV Leiden (Clark and Walker, 2001).

The APS is an autoimmune disorder that manifests as recurrent venous and arterial thrombosis, pregnancy loss and thrombocytopenia. It has also been called an acquired thrombophilic disorder due to the thrombotic complications associated with the disease. APL's are produced by patients suffering from APS and have been indicated as acquired risk factors for developing thrombosis. Moreover, APS has been associated with the autoimmune condition SLE (Arnout, 2001) and LAC antibodies have been found to be significantly associated with acquired APCR in patients with SLE (Male et al., 2001). More recently this has been confirmed by the association of both LAC and anti-prothrombin antibodies with acquired APCR in 59.2% of SLE patients studied (Nojima et al., 2002).

Elevated plasma fibrinogen concentrations have been associated with an increased risk of VTE. The Leiden Thrombophilia Study (LETS) noted a positive association between
plasma fibrinogen levels and the risk of thrombosis, with levels greater than 5g/L (normal levels 1.45-4.0g/L) estimated to increase the risk of VTE 4-fold (Koster et al., 1994; Martinelli, 2001). Swiatkiewicz et al (2002) also reported a significant increase in fibrinogen concentrations between DVT patients and controls.

5.1.2: Inherited risk factors for VTE

An important independent risk factor for predicting thrombosis is a positive personal or familial history of VTE (Gerhardt et al., 1999). Between 20-30% of patients presenting with a first episode of DVT report at least one relative with thrombotic disease. AT, PS and PC deficiencies are considered the more rare genetic risk factors for VTE and all result in the loss of function of the protein or the production of a protein with reduced activity. The more common genetic defects that infer an increased risk of VTE are a result of single point mutations that cause plasma protein levels to change in concentration or to alter in function. These include FV Leiden and the prothrombin G20210A variant. Interestingly, the mutations that result in enhanced protein production are in genes involved in procoagulant pathways while those deficiencies that result in decreased protein production are involved in anticoagulant pathways (Bertina, 2001). However, all these genetic defects have the same net result, enhanced thrombin generation (Bertina, 1997).

5.1.2.i: Deficiencies in natural anticoagulants

Deficiencies in the natural anticoagulants AT, PS and PC have been studied extensively for their involvement in the risk of VTE since their original association with thrombotic disease (Egeberg, 1965; Griffin et al., 1981; Schwarz et al., 1984). The prevalence of mutations within the genes of these proteins in the general population is thought to be 1% while in thrombotic patients is estimated to range from 5-10%. Individuals with heterozygote deficiencies of the natural anticoagulants are thought to have an increased risk of developing a thrombotic episode before the age of 45 without environmental stimulus. Such mutations also infer a higher risk of recurrent VTE and there is often a positive family history of thrombosis (Martinelli, 2001). Patients with deficiencies in all three proteins, AT, PC and PS were found to have a 13-fold increased risk of developing SVT compared to controls, while pregnancy associated SVT occurred in 38% of women studied (Martinelli et al., 1999).
Recently the risk of thrombosis in AT deficient individuals was found to be 20-fold higher than in those without the deficiency (Boven et al., 1999). Females with AT deficiency have been found to develop thrombosis earlier in life than those with PC and PS deficiency since AT deficiency infers a 40% increased risk of VTE during pregnancy and oral contraceptive pill (OCP) use (Pabinger and Schneider, 1996). The incidence of PS deficiency ranges from 1-5% in European populations with thrombosis (Borgel et al., 1997). Studies of patients with recurrent unexplained thrombosis and low PS levels revealed a mutation in 70% of cases (Borgel et al., 1997). However, no association between PS deficiency and thrombosis was found in the Leiden Thrombophilia study (Koster et al., 1995b). This same study found a 3-fold increased risk of thrombosis in patients with PC deficiency and a similar increased risk with AT deficiency compared to controls (Koster et al., 1995b). It appears that AT deficiency carries a greater risk of developing a thrombotic event than PC and PS deficiency since the lifetime risk was reported to be 2-fold greater than PC deficiency and 3-fold greater than PS deficiency (Bucciarelli et al., 1999). However, this may not be applicable to all populations since a recent Japanese study reported no association of AT deficiency with VTE while PC and PS deficiencies were the most common risk factors (Shen et al., 2000).

5.1.2.ii: Procoagulant gene defects

5.1.2.ii.a: FV Leiden G1691A

APCR due to the FV Leiden polymorphism (as described in chapter 3) has been associated with elevated levels of thrombin in patients heterozygous or homozygous for the mutation (Bertina et al., 1994; Martinelli et al., 1996). There have been many studies associating FV Leiden with thrombotic disease (table 5.2). The Leiden Thrombophilia study performed both case-control and familial studies of FV Leiden and revealed a 7-fold increased risk of VTE in heterozygotes increasing to an 80-fold increased risk in homozygotes (Rosendaal et al., 1995). The European population frequency of FV Leiden is about 4% (range 3.5-11%; table 5.3) (Rees et al., 1995) with frequencies increasing to approximately 20% in patients with VTE (Alhenc-Gelas et al., 1999). Individuals homozygous or heterozygous for FV Leiden have experienced VTE at a younger age than controls (31 v 44 years). Moreover, most homozygote individuals experience at least one thrombotic episode in their lifetime (Rosendaal et al., 1995). This polymorphism has been found to have a high prevalence in Caucasian
populations but is completely absent (along with a lack of APCR) from other races such as Chinese (Shen et al., 1997), African Blacks (Hira et al., 2002; Zivelin et al., 1997), Japanese, native Americans and Greenland Inuit sub-populations (Zivelin et al., 1997). FV Leiden is considered the most important risk factor for thrombosis in Caucasian populations. However, cases of family members carrying the mutation without a thrombotic phenotype underline the importance of considering environmental influences and also the concomitant expression of other procoagulant mutations (Lane and Grant, 2000).

5.1.2.iib: Prothrombin (Factor II) G20210A

The prothrombin G20210A polymorphism has been implicated as the second most important inherited risk factor for VTE next to FV Leiden (Poort et al., 1996). The Leiden Thrombophilia study found 18% of VTE patients to be heterozygous for the prothrombin variant compared to 1% of controls and reported the A allele to infer a 3-fold increased risk of thrombosis in this Dutch population (table 5.2) (Poort et al., 1996). A similar incidence of 18.5% (n=162) was found in a recent investigation of Israeli VTE patients compared to 5.4% (n=334) in controls (Salomon et al., 1999). A recent French study reported slightly lower frequencies of the prothrombin variant with frequencies of 10.2% in VTE patients (n=205) compared to 2.8% in controls (n=398) (Alhenc-Gelas et al., 1999). The prevalence is highly variable between populations since only 3.2% of VTE patients were reported to have the polymorphism compared to 1.3% of controls in a US study (Hessner et al., 1999). However, this study combined different Caucasian racial groups as controls therefore, it is possible that the VTE group were of mixed origin also. Nonetheless, the risk of VTE was determined as 2.4-fold for carriers of the prothrombin variant (Hessner et al., 1999) indicating its importance in the progression of thrombotic disease.

5.1.2.iic: MTHFR C677T & Homocysteinemia

Interest has been mounting in recent years on the role the MTHFR polymorphism has to play in thrombosis, since hyperhomocysteinemia has been associated with arterial thrombotic disease (Cattaneo, 1999) and more recently with venous thrombosis (Cattaneo et al., 1998). Mild hyperhomocysteinemia has been reported as a risk factor for VTE since 10-25% of patients were found to be have reduced concentrations of homocysteine compared to only 5% of Caucasian controls (Cattaneo et al., 1998). Due
to the role of MTHFR in folate and homocysteine metabolism, the t-MTHFR gene variant has been investigated and implicated in thrombosis (Froost et al., 1995) with conflicting results (table 5.2). Several studies have found no significant difference in the prevalence of the t-MTHFR variant between VTE patients and control subjects (Alhenc-Gelas et al., 1999; Cattaneo et al., 1999; De Stefano et al., 2000; Hessner et al., 1999). However an increased frequency of TT homozygotes has been reported in VTE patients (22.8%) compared to controls (14.3%, OR=2.1) (Salomon et al., 1999). The role of the t-MTHFR variant and homocysteine in venous thrombosis remains controversial primarily due to the variation in study designs and the type of populations studied. Clearly, their roles in the progression of VTE requires further investigation.

5.1.2. ii.d: Idiopathic VTE

Although there exists a number of acquired and inherited risk factors for VTE, and many patients can be diagnosed on the basis of the occurrence of some of these factors, there remains a proportion of patients that experience idiopathic VTE episodes (i.e.: unexplained VTE). These individuals have a strong personal history of VTE but have no family history of the disorder, and have no obvious prothrombotic or genetic defects. Therefore the cause of VTE in these cases is unknown. The reason for this lies in the multifactorial nature of thrombotic disease, and the lack of understanding of all the pathways and systems that may be involved in such processes. Such idiopathic VTE cases may be due to a number of factors, including dysregulation of the crosstalk between coagulation and inflammation, as discussed in chapters 3 and 4. They may also be influenced by environmental risk factors such as cigarette smoking and decreased folate intake, since folic acid deficiency as been associated with an increased risk of thrombosis (den Heijer et al., 1998). Furthermore, such idiopathic cases may be the result of underlying pathologies that have not yet been diagnosed, as can often happen in the presence of malignancies. Therefore, an idiopathic thrombotic episode can be the warning sign that some other pathology is present (Rickles and Levine, 1998).

Debate remains on whether the discovery of an underlying thrombophilic defect should change the management of anticoagulation, except in specific circumstances. The APS carries a high risk of recurrent VTE, whereas patients with hyperhomocysteinemia may benefit from long term folate supplementation. The presence of a thrombophilic trait can certainly influence a physician’s management of a patient in high-risk
circumstances such as pregnancy, surgery or concurrent illness. Discovery of a hereditary thrombophilia also allows screening of other family members. This is particularly important for young women who are likely to use hormonal contraception or become pregnant.

Awareness of the concept of thrombophilia has led to a significant increase in the number of patients referred to the haematology service for assessment and management of possible underlying defects of thrombotic disease. This increased workload has necessitated the application of a strategic plan to develop a Comprehensive Care Centre for the management of individuals with a personal or family history of venous thrombosis. Over the past three years the NCHCD (St James’s Hospital, Dublin 8) has developed a team consisting of specialist consultants, medical registrars and senior house officers, nurse practitioners, genetic and family counsellors along with dedicated diagnostic and research laboratory scientists in a purpose built centre. The development of a routine genetic screening service for inherited thrombotic risk factors was achieved over a three year period, from research based assays using standard molecular techniques, to the creation of a permanent hospital based molecular screening lab using advanced instrumentation and allelic discrimination techniques (ABI TaqMan). Working as part of this comprehensive service, we endeavoured to elucidate the prevalence of inherited thrombotic risk factors in the Irish population and compare these, along with acquired risk factors, to the prevalence in a cohort of venous thromboembolic patients attending the thrombosis and haemostasis clinic.

AIMS

- To assess the success of the NCHCD in providing a comprehensive and specialised service of patient care for VTE patients and their families.
- To determine the prevalence of inherited thrombophilias in an Irish population of patients suffering VTE.
- To develop a routine genetic screening program for recently identified polymorphisms within genes encoding coagulation proteins.
- To determine the frequency of such polymorphisms in a VTE population compared to an Irish blood donor control population.
Table 5.2: Association of gene polymorphism with venous thromboembolic disease.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Effect on Expression</th>
<th>Association with VTE</th>
<th>No Association with VTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV Leiden</td>
<td>+1691 G→A</td>
<td>A: substitutes Arg506 with Gln (R506Q), at one of three APC cleavage sites causing APCR</td>
<td>(Beauchamp et al., 1994)</td>
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<td></td>
<td></td>
<td>(Bertina et al., 1994).</td>
<td>(Rosendaal et al., 1995)</td>
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<td></td>
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<td>(De Stefano et al., 1998)</td>
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<td>(Cattaneo et al., 1999)</td>
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<td></td>
<td>(Nowak-Gottl et al., 2001)</td>
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<td></td>
<td></td>
<td></td>
<td>(Salomon et al., 1999)</td>
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<tr>
<td>Prothrombin</td>
<td>+21210 G→A</td>
<td>A: Elevated prothrombin levels</td>
<td>(Poort et al., 1996)</td>
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<td></td>
<td>(Poort et al., 1996)</td>
<td>(Makris et al., 1997)</td>
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<td>(Souto et al., 1998)</td>
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<td>(Alhenc-Gelas et al., 1999)</td>
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<td>(Salomon et al., 1999)</td>
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<td></td>
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<td>(Martinelli et al., 1999)</td>
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<tr>
<td>MTHFR</td>
<td>+677 C→T</td>
<td>T: allele creates a thermolabile enzyme with reduced activity for metabolising homocysteine, increasing plasma homocysteine concentrations</td>
<td>(Koch et al., 1999)</td>
<td>(Alhenc-Gelas et al., 1999)</td>
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<td>(Salomon et al., 1999)</td>
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<td>(De Stefano et al., 2000)</td>
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<td>(Hessner et al., 1999)</td>
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Table 5.3: Frequency of the FV Leiden polymorphism in Previously Published European Control Populations (Number of samples in control population in paranthesis).

<table>
<thead>
<tr>
<th>Population</th>
<th>% Heterozygosity</th>
<th>Reference</th>
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<tbody>
<tr>
<td>British</td>
<td>3.5% (144)</td>
<td>(Beauchamp et al., 1994)</td>
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<tr>
<td>Danish</td>
<td>6.6% (4188)</td>
<td>(Larsen et al., 1998)</td>
</tr>
<tr>
<td>Dutch</td>
<td>3.0% (414)</td>
<td>(Rosendaal et al., 1995)</td>
</tr>
<tr>
<td>Finish</td>
<td>2.1% (262)</td>
<td>(Helio et al., 1999)</td>
</tr>
<tr>
<td>French</td>
<td>1.0% (104) - 2.6%</td>
<td>(Gandrille et al., 1995)(Bathelier et al., 1998)</td>
</tr>
<tr>
<td>German</td>
<td>7.1% (814) - 7.8%</td>
<td>(Braun et al., 1996; Schroder et al., 1996)</td>
</tr>
<tr>
<td>Hungarian</td>
<td>6.5% (665)</td>
<td>(Stankovics et al., 1998)</td>
</tr>
<tr>
<td>Italian</td>
<td>3.2% (416)</td>
<td>(Cattaneo et al., 1999)</td>
</tr>
<tr>
<td>Polish</td>
<td>5.0% (200)</td>
<td>(Herrmann et al., 1997)</td>
</tr>
<tr>
<td>Spanish</td>
<td>3.3% (150)</td>
<td>(Garcia-Gala et al., 1997)</td>
</tr>
<tr>
<td>Swedish</td>
<td>11.0% (101)</td>
<td>(Holm et al., 1996)</td>
</tr>
</tbody>
</table>
5.2.0: MATERIALS & METHODS

A thorough medical evaluation of VTE patients was carried out and a comprehensive laboratory thrombophilia screen undertaken. Patient (n=238) blood samples were collected at the NCHCD (chapter 2.1.3). Tri-sodium citrated (9:1), clotted and EDTA anticoagulated blood samples were taken from patients requiring hypercoagulation screening and genetic analysis. Citrated and clotted blood samples were centrifuged immediately at 2000g for 20 minutes. Plasma and serum were separated, aliquoted and stored at -80°C until batch analysis. DNA was extracted from EDTA treated blood using the QIAmp™ DNA Mini kit (Qiagen, Crawley, West Sussex) (chapter 2.2.3). Control blood samples (n=390) were collected (chapter 2.1.1) from the Irish Blood Transfusion Service (IBTS) and the Northern Ireland Blood Transfusion Service (NIBTS) and DNA extracted (chapter 2.2.1). 1000 Guthrie card controls were collected at Temple St. Children’s Hospital (Dublin 1) (chapter 2.1.2) and DNA extracted (chapter 2.2.2).

Hypercoagulation screens were performed by staff of the coagulation laboratory at the NCHCD, St. James’s Hospital (chapter 2.3.2). The following parameters were measured: AT, FVII, PC, PS, fibrinogen, PT, APTT and LAC.

The polymerase chain reaction was performed on all samples for the FV Leiden G1691A polymorphism, the prothrombin G20210A polymorphism and the MTHFR C677T thermolabile variant (chapter 2.4.1.i-iii). Results were confirmed by an allelic discrimination approach on the ABI 7700 instrumentation (Applied Biosystems) at St. James’s Hospital by Dr. Helen Egan (chapter 2.4.1.viii).
5.3.0: RESULTS

5.3.1: Hypercoagulation screens

Two hundred and thirty eight consecutive full thrombophilia screens were carried out in the NCHCD and the Department of Genetics, Trinity College Dublin. Of the samples tested the most commonly detected risk factor for VTE was elevated circulating fibrinogen (16.59%), followed by activated protein C resistance (14.2%) and the FV Leiden polymorphism (14.9%). The discrepancy in APCR and FV Leiden prevalence is a result of APCR assays not carried out for certain individuals, for example in cases of heparin therapy. 13.67% (n=30) of individuals analysed had an elevated level of FVIII and 1.88% of individuals (n=4) were lupus anti-coagulant positive. Deficiencies in AT, PC and PS were detected in 1.79%, 5.43% and 9.94% of thrombotic patients respectively. Table 5.4 illustrates the exact number of thrombotic patients that exhibited a coagulatory defect as discovered by routine thrombophilia screening.

5.3.2: FV Leiden

The FV Leiden polymorphism was detected at 4.4% in the Irish blood donor (IBTS) population (n=389) and at 4.2% in the Irish newborn population (n=962). There was no significant variation in the heterozygosity rates for each control group (Chi-Square p=0.6521; Fisher’s Exact p=0.6566), validating the use of either population for case-control comparisons. FV Leiden was detected in 14.9% of routine thrombophilia screens (n=208) compared to 4.4% of IBTS controls and 4.2% of newborn controls, differences of statistical significance (p<0.014 and p<0.0001 respectively) (table 5.5). Comparison of patients with the FV Leiden polymorphism and APCR shows that of the 81 patients with an APCR ratio between 1.2-1.9, all were FV Leiden positive. Five patients had a ratio of 2.0, which is considered borderline. Of these, only one individual was found to be FV Leiden negative. All remaining patients had a ratio between 2.1-3.0 and were found to be FV Leiden negative (table 5.6). The APCR:FV ratio is an inexpensive, rapid screening assay for the phenotype resulting from the FV Leiden polymorphism. This study validated the use of a cut off point (APCR ratio = 2.0) using the APCR:FV ratio test in determining which patients should be screened for the more labour intensive, timely and costly FV Leiden screening.
5.3.3: FII G20210A
In an Irish control population (n=390) the prothrombin G20210A polymorphism was detected in 1.8% (A allele frequency 0.0115) and 6.34% of VTE cases (n=205, A allele frequency 0.032) (table 5.5). Both the heterozygote genotype and the A allele were associated with an increased risk of VTE (p=<0.001). Therefore, the prothrombin gene variant infers an increased risk of thrombosis in this population of VTE patients.

5.3.4: MTHFR C677T
Analysis of the t-MTHFR gene variant revealed a homozygosity prevalence of 16.9% in the Irish blood donor population compared to 14.6% in patients routinely screened for thrombophilia. This figure is higher than a previous report for an Irish control population of 6.1% homozygosity. There was no significant association with either MTHFR T allele homozygote or heterozygote genotypes and either a personal or family history of VTE. The frequency of the T allele was 0.36 in the VTE population compared to 0.41 for controls (table 5.5). There was no significant difference between allele and genotype frequencies between patients and controls (p=0.138 and p=0.289 respectively).

5.3.5: Combined thrombophilic defects
Thrombophilic defects were detected in 50% (n=119) of thrombotic patients analysed. Of these individuals, 33.2% had one thrombophilic deficiency, 10.9% had two deficiencies, 4.6% had three deficiencies and 1.3% had four thrombotic defects (table 5.6). Of the 238 screens carried out, a total of 16.8% of individuals had multiple markers considered to be of significance in VTE disease development. These results offer unequivocal evidence that the existence of one or more thrombotic defects as detected by hypercoagulable and genetic screening infers a greatly increased risk of developing thrombotic disease in this cohort of patients.
Table 5.4: Hypercoagulation results for venous thrombosis patients (n=238).

<table>
<thead>
<tr>
<th>Coagulatory Defect</th>
<th>Normal Ranges</th>
<th>Number of positive patients</th>
<th>Total Number of Tests</th>
<th>Percentage of Total Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT deficiency</td>
<td>0.76-1.20 IU/ml</td>
<td>4</td>
<td>223</td>
<td>1.79</td>
</tr>
<tr>
<td>Elevated FVIII</td>
<td>0.500-2.00 IU/ml</td>
<td>30</td>
<td>220</td>
<td>13.67</td>
</tr>
<tr>
<td>PC deficiency</td>
<td>0.70-1.61 IU/ml</td>
<td>10</td>
<td>184</td>
<td>5.43</td>
</tr>
<tr>
<td>PS deficiency</td>
<td>0.71-1.45 IU/ml</td>
<td>18</td>
<td>181</td>
<td>9.94</td>
</tr>
<tr>
<td>APCR:FV Ratio &lt;2.1</td>
<td>2.1-3.0 (Ratio)</td>
<td>30</td>
<td>211</td>
<td>14.22</td>
</tr>
<tr>
<td>Elevated Fibrinogen</td>
<td>1.5-4.0 g/L</td>
<td>35</td>
<td>211</td>
<td>16.59</td>
</tr>
<tr>
<td>Lupus Positive</td>
<td></td>
<td>4</td>
<td>213</td>
<td>1.88</td>
</tr>
</tbody>
</table>
Table 5.5a: Genetic thrombophilia screens in VTE patients and controls: Polymorphic genotype and allele frequencies

<table>
<thead>
<tr>
<th>Polymorphism (Controls)</th>
<th>Genotype</th>
<th>Raw Count (controls)</th>
<th>% of Total (controls)</th>
<th>Allele</th>
<th>Frequency (Controls)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor V</td>
<td>GA</td>
<td>31 (17)</td>
<td>14.9 (4.4)</td>
<td>G</td>
<td>0.925 (0.9781)</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>177 (372)</td>
<td>85.1 (95.6)</td>
<td>A</td>
<td>0.075 (0.0219)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>GA</td>
<td>13 (7)</td>
<td>6.34 (1.8)</td>
<td>G</td>
<td>0.968 (0.9885)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>192 (382)</td>
<td>93.6 (97.9)</td>
<td>A</td>
<td>0.032 (0.0115)</td>
<td>0.289</td>
</tr>
<tr>
<td>MTHFR</td>
<td>CC</td>
<td>83 (137)</td>
<td>41.7 (35.1)</td>
<td>C</td>
<td>0.636 (0.5910)</td>
<td>0.138</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>87 (187)</td>
<td>43.7 (48.0)</td>
<td>T</td>
<td>0.364 (0.4090)</td>
<td>0.186</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>29 (66)</td>
<td>14.6 (16.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.5b: Activated Protein C Resistance (APCR) assay (with FV deficient plasma) and relationship with the FV Leiden polymorphism.

<table>
<thead>
<tr>
<th>APCR:FV ratio</th>
<th>FV Leiden Positive patients</th>
<th>FV Leiden Negative patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2-1.9</td>
<td>81</td>
<td>0</td>
</tr>
<tr>
<td>2.0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>2.1-3.0</td>
<td>0</td>
<td>186</td>
</tr>
</tbody>
</table>
Table 5.6: Overall number of relevant hypercoagulation defects. Hypercoagulation defects included AT, PC and PS deficiencies, FVIII:C, elevated fibrinogen, lupus anticoagulant, APCR and Factor V Leiden (counted as one), Prothrombin, and MTHFR.

<table>
<thead>
<tr>
<th>Number of Defects</th>
<th>Number of individuals</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>119</td>
<td>50%</td>
</tr>
<tr>
<td>1</td>
<td>79</td>
<td>33.2%</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>10.9%</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>4.6%</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1.3%</td>
</tr>
<tr>
<td>Defect Present</td>
<td>119</td>
<td>50%</td>
</tr>
</tbody>
</table>
5.4.0: DISCUSSION

Over the last ten years our understanding of venous thromboembolic disease has increased so much that now inherited thrombophilias explain more than 50% of cases of venous thrombosis. The advantage of laboratory assessment of thrombophilia is the prediction of recurrence of thrombosis in asymptomatic individuals and the determination of the risk to relatives. Therefore, thrombophilia testing is used to identify the benefit/risk ratio of anticoagulant treatment, which can have dangerous side effects. There is a 1% risk that anticoagulation can induce major haemorrhage over a one year treatment period. The ability of thrombophilia screens to identify patients at high or low risk of thrombosis helps to optimise therapeutic strategies (Baglin, 2000). The existence of variable phenotypes between family members possessing the same defect emphasises the lack of correlation between phenotype and genotype in some cases. For this reason phenotypic analysis of thrombophilic defects requires accompaniment of genetic screening assays where possible, a process that is becoming increasingly routine in specialised coagulation laboratories. It is also becoming more apparent that the alteration of the haemostatic balance by more than one thrombophilic defect is paramount in many cases of thrombosis. Recently Swiatkiewicz et al (2002) reported 50% of DVT patients to have at least one thrombophilic defect. A familial study of VTE patients also reported that 50% of relatives were carriers of at least one inherited thrombotic defect (Siminoi et al., 1999). A single prothrombotic risk factor was also reported in 58.5% of childhood VTE patients, with 20.6% having combined defects (Nowak-Gottl et al., 2001a). The present study illustrates that 50% of VTE patients studied had an underlying thrombophilic defect of which 16.8% had more than one defect contributing to disease pathogenesis. These results are in concordance with previous investigations in European populations (Nowak-Gottl et al., 2001a; Siminoi et al., 1999; Swiatkiewicz et al., 2002).

It has been long since known that elevated fibrinogen levels are associated with arterial disease but their association with venous thrombosis is more controversial (Renner et al., 2002). The present study found that increased fibrinogen concentration was the most common risk factor for VTE in this cohort of patients with an incidence of 16.6%. These findings are similar to previous reports of significant increases in fibrinogen concentrations in DVT patients compared to controls (Koster et al., 1994; Swiatkiewicz et al., 2002). It is uncertain whether the increase in concentrations is causal or a marker
for other thrombotic risk factors as fibrinogen is an acute phase protein and may also be increased under inflammatory conditions (van der Bom, 2002). However, a recent investigation into the levels of inflammatory markers in DVT patients reported a decrease in these markers over a number of days of hospital treatment, indicating the increase to be a result rather than a consequence of venous thrombosis (Roumen-Klappe et al., 2002). A polymorphism in the fibrinogen beta gene (G455A) has been associated with increased plasma concentrations of fibrinogen but a recent study of this mutation in a group of DVT patients found no significant difference in genotype frequencies between patients and controls (Renner et al., 2002). We have also reported these findings with increased fibrinogen concentrations during pregnancy in this thesis (chapter 3). It may be of interest to investigate this polymorphism in the present cohort of patients to ascertain a causal or consequence increase in fibrinogen in this venous thromboembolic group.

The newborn control population studied here provided the first representative and accurate estimate of the frequency of FV Leiden in the Irish population. Blood donor cohorts used as control groups in most studies are a select population in that the donor must for example have survived to adulthood. These individuals must also be in good health with no history of ischaemic heart disease or recurrent thrombotic events. A further selection bias is the exclusion of individuals taking long-term medication or those who have received blood transfusion. We postulated that there may be a difference between polymorphism frequency between newborn cohorts and blood donor control populations due to this selection bias. The frequency of the FV Leiden polymorphism was determined for each of the control populations sampled and no statistically significant difference in heterozygosity was found. The archive newborn blood samples proved difficult to amplify so a nested PCR approach was undertaken. This method involved two separate PCR reactions per sample (n=1000) and was extremely time consuming and labour intensive. We determined that the use of the archive newborn DNA samples in further case-control studies was unnecessary, as the IBTS blood donor control samples were readily amplified after one PCR reaction, offering a less labour intensive method of analysis.

Increased APCR was observed in 14.2% of VTE patients accompanied by an increased prevalence of the FV Leiden polymorphism (14.9%) in this cohort. Indeed these defects represent the second most common defect in these patients. APCR is most often caused
by the presence of the FV Leiden polymorphism and this study has validated the laboratory APCR:FV ratio of 2.0. The cut off ratio of 2.0 currently in operation at St. James’s coagulation laboratory is the most labour, time and cost efficient method of determining which patients to screen for the FV Leiden polymorphism. This polymorphism is generally accepted as being the most commonly inherited risk factor for venous thrombosis and has been reported to increase the risk of thrombosis 7-fold in heterozygotes and 80-fold in homozygotes (Rosendaal et al., 1995). The current study detected the mutation in 14.9% of VTE patients compared to 4.4% of controls (p=0.01, OR=3.8), which is in agreement with previous reports (De Stefano et al., 1998; Koster et al., 1993).

We report an increase in factor VIII concentrations in this VTE cohort, with 13.7% of patients experiencing elevated plasma levels. The Leiden thrombophilia study previously reported increased FVIII concentrations in 25% of DVT patients with a relative risk of 4.8 and concluded this increase to be an independent risk factor for thrombosis (Koster et al., 1995a). A subsequent analysis of this acute phase protein, to determine the cause or consequence effect of elevated FVIII levels found an increase in 17.3% of thrombotic patients and no relationship with markers of the acute inflammatory response (O'Donnell et al., 1997). Therefore, the current findings indicate an independent risk factor for thrombosis in this VTE cohort.

Deficiency of the natural anticoagulants in VTE patients was found to occur at similar rates compared to previous reports. AT deficiency occurred in 1.8% of patients, PC deficiency in 5.4% and PS deficiency in 9.9% of patients studied. AT deficiency has been previously reported in 3.3-7% of DVT patients while the prevalence of PC and PS deficiencies has been reported as between 3-9% and 5-9% respectively (Salomon et al., 1999; Siminoi et al., 1999; Swiatkiewicz et al., 2002). The rarity and the heterogeneity of these deficiencies make it difficult to obtain equivocal frequency rates, since each gene comprises a large number of mutations.

Lupus anticoagulant along with anti-prothrombin antibodies has been associated with thrombosis in patients with SLE (Nojima et al., 2001), but its role in venous thrombotic disease has had little investigation. We describe the existence of LAC antibodies in 1.9% of venous thrombosis patients attending the NCHCD over a one year period. The detection of LAC in these patients may be indicative of the APS, which is accompanied
by recurrent thrombosis. This subset of patients may be at an increased risk of a recurrent thrombotic event. The presence of LAC has been reported in conjunction with acquired APCR (without FV Leiden) in 34.4% of thrombotic patients suffering from SLE (Nojima et al., 2002). This transient resistance to APC may be the triggering factor in thrombotic recurrence in these patients.

Heterozygosity for the prothrombin gene variant was detected in 6.3% of VTE patients compared to 1.8% of controls, a difference of statistical significance for both alleles and genotypes (p<0.001). There were no homozygote mutant individuals detected in this patient cohort. These findings indicate a similar control population prevalence to the Leiden Thrombophilia Study (1%) but the VTE prevalence of 6.3% is considerably lower than the 18% reported in the Dutch VTE group (Poort et al., 1996). However, the prevalence has been noted to be variable between populations since 3.2% of VTE patients were reported to possess the mutation compared to 1.3% of controls in a separate study (Hessner et al., 1999), which is in agreement with the present data. The variation may also be due to inter-laboratory variation but the current results were confirmed by an allelic discrimination approach on ABI 7700 instrumentation (TaqMan), giving greater strength to the confidence in this data.

The MTHFR thermolabile variant has met with controversial association in relation to VTE with what seems to be more negative associations than positive. Our data are in agreement with the theory that the t-MTHFR variant is not associated with VTE. The present study found no significant difference in the prevalence of the polymorphism between VTE patients (41.7%) and controls (35.1%). The frequency of the T allele was 0.36 in the VTE population compared to 0.41 for controls. This figure is higher than that previously reported for the general Irish population (0.29) (Whitehead et al., 1995). However previous results from a control sample from Northern Ireland revealed a T allele frequency of 0.38 and a TT genotype frequency of 14.1% (Harmon et al., 1997). Thus, the range of observed T allele frequencies in Ireland would appear to be greater than first reported, mirroring what has been found for other European countries such as the UK. Homocysteine levels were not measured in this cohort of patients, which may be an area for further research.

The findings in this study have a number of implications for selection of patient sub groups for thrombophilia screening. We have demonstrated that 50% of patients
presenting with VTE possess one or more thrombotic defect. This indicates that all patients with VTE be screened for all thrombophilic deficiencies. However, this approach would be time consuming, extremely costly and unwarranted. A closer look at the breakdown of each thrombophilia indicates that the most common defect is increased fibrinogen levels, which was detected in 16.6% of patients. Therefore, measurement of fibrinogen levels should be the first test performed on VTE patients. In addition to this, the APCR test should be performed since this phenotype occurred in 14.2% of VTE patients in this study. If an APCR of less than 2 is observed, the FV Leiden assay should also be performed to verify the mutation genotype. Prothrombin mutation analysis may prove useful at this point, but t-MTHFR genotyping does not appear to have any clinical advantages and may be an unnecessary cost. Finally, FVIII levels should be measured, as increases have been suggested as an independent risk factor for thrombophilia. The rare occurrence of defects in AT, PC and PS do not justify universal screening of all VTE patients, but suggest screening if all other factors remain normal. Such a process of elimination would prove more economical and less time consuming to laboratory personnel and clinicians alike.

It has been reported that a policy based on knowledge of diagnosis and implementation of antithrombotic treatment during risk situations modified the clinical prognosis of patients even in the absence of long-term antithrombotic therapy. The prevalence of both FV Leiden and the prothrombin polymorphism indicates the potential for vast numbers of individuals presenting for screening. For this reason it has been stated that hospitals need to outline appropriate guidelines for thrombophilia screening, targeting patients most at risk of thrombosis or recurrence (Cumming and Shiach, 1999). This strategy would ensure resources are used efficiently and expensive investigations kept to a minimum. This approach has been successfully adopted at the NCHCD at St. James’s Hospital where the experience of the nursing staff, medical team, laboratory and research scientists, and a dedicated team of social workers and counsellors has been combined, to optimise the care, management and understanding of VTE. The last three years have witnessed the progression of genetic testing from the research laboratory to the routine setting, a move that illustrates the increasing understanding and demand for thorough thrombophilia screening at the laboratory level.
CHAPTER 6

ISCHAEMIC STROKE
6.1.0: ISCHAEMIC STROKE

Cerebrovascular accident (CVA) or Stroke is the third leading cause of morbidity and mortality in Western countries with an incidence of 150-400 in 100,000 individuals and a mortality rate of 30% (Bousser, 2001; Dirnagl et al., 1999). It is a major cause of disability, dementia and depression, and is a huge financial burden to the health system. The United Kingdom’s national health services spend greater than 5% of total resource allocation on stroke patients annually (Bousser, 2001). There are two types of CVA, cerebral infarction or ischaemic stroke and intracerebral haemorrhage (ICH) (Catto and Grant, 1995). Ischaemic stroke is more common than ICH as the later only accounts for 12-15% of patients suffering acute stroke. Haemorrhagic stroke is a result of bleeding within the local environment of the brain from a ruptured artery (Brandt et al., 1996). Ischaemic stroke is a major complication of atherosclerosis, a degenerative inflammatory disease of the internal layer of arteries. It is characterised by transient or permanent reduction in cerebral blood flow as a result of atherothrombotic deposits, which are restricted to the local environment of a major brain artery, leading to accumulative thrombotic lesions and subsequent tissue damage (Dirnagl et al., 1999; Lane and Grant, 2000). Cerebral thrombosis involves the formation of a blood clot in a major artery supplying the brain, while cerebral embolism can occur if the blood clot migrates from peripheral sites. Transient ischaemic attacks (TIA’s) or ‘mini strokes’ are those where the stroke symptoms disappear within 24 hours but are warning signs of a future and more serious ischaemic episode (Catto and Grant, 1995). Due to the high incidence of ischaemic stroke, this study will be concerned with only this sub-type of the disease.

CVA is a multifactorial disease with both environmental (acquired) and genetic (inherited) influences. Environmental risk factors include smoking, inactivity, obesity, diabetes, hypertension (high blood pressure), hyperhomocysteinemia, disorders of lipid metabolism (dyslipidemia), menopause, male gender and a positive family history (Franco and Reitsma, 2001). The occurrence of Stroke at young ages and positive family histories suggests a strong genetic influence. Disordered haemostatic balance has been suggested in the pathogenesis of atherosclerosis since elevated levels of many proteins involved in haemostasis have been observed in arterial disease such as fibrinogen, FVII, vWF, PAI-1 and TPA (Lane and Grant, 2000). Many proteins of both the coagulation and fibrinolytic pathways have been investigated at the genetic level to elucidate the role haemostatic factors have on the progression of cerebrovascular accidents.
6.1.1: Stroke & Procoagulant Genetic Defects

Several studies to date have found no association between the risk of stroke and the presence of FV Leiden (Catto et al., 1995; Ridker et al., 1995; Voetsch et al., 2000), prothrombin variant (20210A allele) or thermolabile MTHFR (Voetsch et al., 2000). Conversely a recent report found 20% of childhood stroke patients to possess the FV Leiden polymorphism compared to 4% of controls increasing the risk of stroke 6-fold. This same study reported significantly increased risks of childhood stroke with the prothrombin 20210A allele (6% patients v 1.3% controls; OR=9.5), thermolabile MTHFR (23.6% v 10.4%; OR=2.4) and protein C deficiency (6% v 0.67%; OR=9.5). Co-inheritance of FV Leiden heterozygosity and MTHFR homozygosity was found in 10.8% of patients compared to 0.3% of controls (Nowak-Gottl et al., 1999). Similarly Margaglione et al (1999) reported an increased risk of stroke with the FV Leiden mutation (OR=2.56, 3.95 in subset of women) and thermolabile MTHFR (OR=1.6) but not with the FII G20210A gene defect (Margaglione et al., 1999). Further studies reported a significant increase in FV Leiden carriers in stroke patients (11.1%) compared to controls (6.6%) but no increase in the MTHFR 677TT genotype or the FII 20210A variant (Gaustadnes et al., 1999). An earlier study reported an increased prevalence of APCR in stroke patients (20%) compared to controls (2%) but did not report on the FV Leiden carrier status of these patients (Halbmayer et al., 1994). The role of these genetic defects, along with deficiencies of AT, PS and PC, in the progression of arterial thrombotic disease remains controversial and therefore requires further investigation.

6.1.1.i: Stroke & Fibrinogen

The Northwick Park Heart Study first associated increased plasma fibrinogen levels with the pathogenesis of stroke and other occlusive vascular disorders (Meade et al., 1986). Fibrinogen levels influence platelet aggregation, blood viscosity and endothelial cell injury, all important factors in the development of atherosclerosis. Homozygosity of the G→455A fibrinogen mutation has been associated with increased plasma levels of the protein (Humphries et al., 1995) and this has been the focus of many case-control studies in stroke patients. The Copenhagen City Heart Study found no association between this polymorphism and atherosclerotic disease although the disease was associated with increased fibrinogen levels and the -455A allele was independently associated with increased fibrinogen levels (Tyjaerg-Hansen et al., 1997). The G allele has been associated with a higher prevalence of coronary artery disease in patients with type II
diabetes (Carter et al., 1996). However, the A allele was implicated in the progression of atheroma in a separate study and it was suggested that the -455AA genotype promotes a stronger acute phase response in fibrinogen, influencing the progression of arterial disease (de Maat et al., 1998). Similar to other coagulatory proteins, smoking increases circulating concentrations of fibrinogen and hence the risk of arterial disease, an effect which has been reported to be reversible after cessation of smoking (Dobson et al., 1991). Once again there appears to be dispute over the role genetic polymorphisms in the gene encoding this procoagulant protein have to play in arterial disease, an issue requiring further investigation.

6.1.1.ii: Stroke & PAI-1
PAI-1, the potent inhibitor of TPA in the fibrinolytic pathway, has been implicated in arterial disease, since increased levels have been found in atherosclerotic plaques (Franco and Reitsma, 2001). High levels of PAI-1 decrease TPA activity, reducing TPA’s ability to release plasmin and therefore reducing fibrinolysis. Increased PAI-1 levels, and thus reduced ability to break down clots, have been associated with atherosclerotic progression (Lupu et al., 1993). There have been mixed reports on the association of the PAI-1 promoter polymorphism (-675) and myocardial infarction (MI), but fewer studies have investigated its effect on PAI-1 levels in relation to cerebrovascular disease progression, however the 4G allele has been inversely associated with stroke. Catto et al (1997) reported increased PAI-1 levels in stroke patients compared to controls and an association with stroke mortality but no association between genotype and stroke. A recent study of childhood and adolescent stroke reported no association between the 4G genotype and stroke either alone or when inherited along with other prothrombotic polymorphisms (FV Leiden, FII G20210A, MTHFR C677T) (Nowak-Gottl et al., 2001). Conversely a prospective cohort of women followed for 18 years found that stroke mortality was reduced in 4G homozygotes compared to 5G homozygotes with no effect on MI or other cardiac disorders (Roest et al., 2000). Similarly the 4G genotype was reported to have a significantly lower frequency in stroke patients below the age of 60 compared to patients above 60 years of age indicating a possible protective effect in younger stroke sufferers (Endler et al., 2000). Therefore further studies are necessary to elucidate the role PAI-1 plasma levels and the PAI-1 promoter polymorphism has to play in the progression of cerebrovascular disease.
6.1.1.iii: Stroke & TPA

Increased plasma concentrations of TPA have been associated with MI (Ridker et al., 1993b). The Alu repeat insertion/deletion polymorphism in the TPA gene has been associated with a 2-fold increased risk of MI (van der Bom et al., 1997). However the influence of TPA levels and polymorphisms on stroke is less clear. Genetically typed umbilical endothelial cells were found to have no difference in TPA levels between genotypes (van den Eijden-Schrauwen et al., 1995). The US Physicians Health study and a study of Italian patients with a family history of cardiovascular disease found no association between the TPA Alu repeat polymorphism and the development of thrombosis (Iacoviello et al., 1996; Ridker et al., 1997). Once again further studies are required to determine the role of the TPA polymorphism in stroke populations.

6.1.1.iv: Stroke & ACE

Due to the many functions of ACE in vascular systems, it has been the most extensively studied gene with regard to ischaemic stroke (Hassan and Markus, 2000). The DD genotype of the Alu insertion (I)/deletion (D) polymorphism in ACE gene has been shown to be a strong independent discriminator of patients with a history of stroke, an effect that is enhanced when associated with elevated levels of TPA (Margaglione et al., 1996). Sharma et al (1994) reported no association of stroke with the ACE gene polymorphism but a tendency of those with a DD genotype to experience stroke at a younger age (Sharma et al., 1994). Maeda et al (1996) reported a significantly higher D allele frequency (0.72) in hypertensive patients with a parental history of stroke than controls (0.52) and a DD genotype of 63% in patients compared to 32.6% in controls. A Japanese study of young (age of onset ≤ 60 years) and old (age of onset ≥ 60 years) patients with brain infarction revealed no significant difference in allele distribution and genotypes between all patients and controls. However, sub-group analysis uncovered a significant difference between younger patients and older patients (p=0.002), and also between young patients and controls (p=0.02) but not between sub-populations of older onset infarction patients and elderly controls (Doi et al., 1997). Subsequently a meta-analysis of a large cohort of patients (n=1918) and controls (n=722) concluded that the ACE genotype inferred only a small increased risk of disease (OR=1.31) (Sharma, 1998). A recent report revealed the frequency of the deletion polymorphism to be different between ethnic groups, with African Americans having the highest frequency of the deletion allele (0.59) compared to Indians (0.49) and Caucasians (0.44) (Mathew et
This discovery reflects population differences similar to those seen for other polymorphisms within genes affecting coagulation and inflammation. Therefore the geographical origin of the population in question is of great importance when studying polymorphic markers in any disease.

We postulate that differences in expression of genes encoding coagulation factors may be associated with an increased incidence of stroke, and that a prothrombotic phenotype may play a role in the pathophysiology of ischaemic stroke. Here we investigate the frequencies of functional polymorphisms in genes encoding coagulation factors (FV, FII, and Fibrinogen), fibrinolytic proteins (TPA and PAI-1) and ACE expression. These polymorphisms have been reported to influence protein expression and have also been associated with thrombotic disease (table 6.1).

AIMS

• To determine the prevalence of polymorphisms within genes encoding coagulatory and fibrinolytic proteins in a cohort of patients suffering ischaemic stroke.

• To determine the prevalence of such polymorphisms within the Irish population and compare to stroke victims to ascertain a relationship between these inherited markers and the incidence of stroke in the Irish population.
### Table 6.1: Thrombotic gene polymorphisms, effects on gene expression, and arterial disease associations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Effect on Expression</th>
<th>Disease Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV Leiden</td>
<td>+1691 G→A (Bertina et al., 1994)</td>
<td>A: substitutes Arg506 with Gln (R506Q), at one of three APC cleavage sites causing APCR (Bertina et al., 1994).</td>
<td>Myocardial Infarction (MI) (Holm et al., 1996)</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>+21210 G→A (Poort et al., 1996)</td>
<td>A: Elevated prothrombin levels (Poort et al., 1996)</td>
<td>Ischaemic Stroke (Biousse et al., 1998; Nowak-Gottl et al., 1999)</td>
</tr>
</tbody>
</table>
| PAI-1      | -675 insertion/deletion of G allele (Dawson et al., 1993) | 5G: transcriptional repressor binding site (Dawson et al., 1993)  
4G: Elevated plasma PAI-1 levels (Eriksson et al., 1995) (Grubic et al., 1996) | 4G: Venous & arterial thrombosis (Eriksson et al., 1995) |
| Fibrinogen | -455 G→A (Humphries et al., 1995; Scarabin et al., 1993) | A: Elevated plasma Fibrinogen levels (Koster et al., 1994) | MI (Scarabin et al., 1993) Stroke (Nishiuma et al., 1998) |
| ACE        | insertion(I)/deletion (D) of 287bp Alu repeat in intron 16 (Rigat et al., 1992) | D allele associated with increased plasma ACE levels (Rigat et al., 1992) | MI (Cambien et al., 1992) |
| TPA        | insertion(I)/deletion (D) of Alu repeat in intron 8 (Tishoff et al., 1996) | I allele conflicting reports on association with increased plasma tPA levels (Hooper et al., 2000; Jern et al., 1999; van der Bom et al., 1997) | MI (van der Bom et al., 1997) |
6.2.0: MATERIALS & METHODS

6.2.1: Subjects and DNA Isolation

EDTA anticoagulated whole blood was collected from 390 healthy blood donors and 103 patients enrolled in a stroke study. Approval was granted by the ethical committee at St Vincent’s Hospital, Dublin and consenting stroke patients were recruited. Donor bloods were collected at The Northern Ireland Blood Transfusion Service (n=60) and The Irish Blood Transfusion Service (n=330) (Chapter 2.1.1). DNA was extracted from whole blood using proteinase K (1mg/mL) cell lysis overnight at 37°C in the presence of 0.5% SDS followed by extraction with phenol/chloroform, and precipitation with ethanol (Chapter 2.2.1 and 2.2.2).

6.2.2: Polymerase Chain Reaction, Restriction Enzyme Digestion, and Agarose Gel Electrophoresis of patient and control DNA samples.

PCR amplification of all polymorphic sites was performed in a total reaction volume of 50µl. The standard PCR reaction mix (Appendix table A.3.1) consisted of Taq DNA Polymerase buffer with MgCl₂ (Promega) [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 1.5 mM MgCl₂], 0.4 units of DNA Taq polymerase, 2µl of genomic DNA, 4% dimethyl sulphoxide (DMSO, Sigma), 30µM each of dNTP (Boehringer Mannheim), and 0.2µM each of sense and antisense primers (Sigma Genosys) (chapter 2, table 2.1). The cycling parameters for each assay are listed in chapter 2, table 2.2. The FV Leiden, PAI-1, prothrombin G20210A and beta fibrinogen amplicons were digested with the appropriate enzyme (chapter 2, table 2.1) overnight at 37°C according to manufacturers instructions (New England BioLabs, NEB). The ACE and TPA PCR products were visualised directly on agarose gels (Pronadisa, Madrid). Restriction digest/PCR products were run on the appropriate percentage of agarose gel (chapter 2.4.0), containing 1.6µg/ml ethidium bromide (Sigma). Typical DNA banding patterns of PCR amplicons and restriction digest products for each polymorphism are illustrated in chapter 2, figures 2.1-2.2 and figures 2.4-2.7.
6.3.0: RESULTS

Genotype frequencies for all six polymorphisms in the control group, stroke population, and subgroups of the stroke population (previous arterial event, smoker, ex-smoker, non-smoker, and those suffering a stroke with hypertension) are listed in Table 6.2. The genotype frequencies calculated for the control population did not significantly differ from previous reports. Overall we found no significant difference in the prevalence of any polymorphism between the control and stroke populations (p>0.05). The distribution of genotypes observed in both patients and controls were found to be in Hardy-Weinberg equilibrium.

The percentage heterozygosity for the FV Leiden and prothrombin G20210A polymorphisms in the Stroke population was 1% and 0% respectively, compared to 4% and 1.8% for the controls (no statistical significance). The FII 20210 A allele was not detected in the entire stroke population. This result may simply reflect the low prevalence of this mutation in the population in general and the small sample size of the stroke cohort when looking at such an infrequent polymorphic event.

The PAI-1 4G/5G polymorphism, the Fibrinogen G-455A transition, the ACE insertion/deletion polymorphism and the TPA insertion/deletion polymorphism were not statistically different between the control population and the Stroke patient cohort. However, the frequency of the ACE D allele was almost significantly increased in stroke patients who also had hypertension, when compared to the control population (p=0.08). When the smoking sub-population were excluded from analysis, the TPA polymorphism exhibited a significant difference between controls, ex-smokers and non-smokers collectively (p=0.01).

The mean age of initial onset of stroke was calculated for smokers, and ex/non-smokers (individuals who had not smoked for >1 year). The mean age of first stroke was lower, 65.7 years (range 35-86; median 59, n=38) for smokers when compared to 70.9 years for non-smokers (range 43-99; median 70, n=67) (Prob [t]>0.036). A variation in age of onset was also noted when comparing sexes of the patients. The mean age for a first event for females was 72.0 (n=42) compared to 67.5 years for males (n=64) (Prob [t] >0.075).
The occurrence of a previous stroke with and without residual disability and with a previous MI episode was assessed. There was no significant variation between polymorphisms within these sub-populations.
Table 6.2: Genotype counts for pro-thrombotic polymorphisms in a control population and a Stroke patient cohort. Subdivisions of the Stroke population into; those with a previous event, either Myocardial Infarction or stroke, smoker, ex-smoker, non-smoker and individuals with hypertension.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Control Group</th>
<th>Stroke</th>
<th>Previous Arterial Event</th>
<th>Smoker</th>
<th>Ex-Smoker</th>
<th>Non-Smoker</th>
<th>Hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV Leiden</td>
<td>GG</td>
<td>372 (96%)</td>
<td>102 (99%)</td>
<td>33 (100%)</td>
<td>36 (100%)</td>
<td>28 (100%)</td>
<td>38 (97%)</td>
<td>49 (100%)</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>17 (4%)</td>
<td>1 (1%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>GG</td>
<td>382 (97.9%)</td>
<td>103 (100%)</td>
<td>33 (100%)</td>
<td>36 (100%)</td>
<td>28 (100%)</td>
<td>39 (100%)</td>
<td>49 (100%)</td>
</tr>
<tr>
<td>G20210A</td>
<td>GA</td>
<td>7 (1.8%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>1 (0.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>PAI-1</td>
<td>4/4</td>
<td>128 (33%)</td>
<td>35 (35%)</td>
<td>11 (36%)</td>
<td>11 (32%)</td>
<td>12 (44%)</td>
<td>12 (31%)</td>
<td>20 (43%)</td>
</tr>
<tr>
<td></td>
<td>4/5</td>
<td>183 (47%)</td>
<td>52 (53%)</td>
<td>14 (45%)</td>
<td>18 (53%)</td>
<td>14 (52%)</td>
<td>20 (53%)</td>
<td>22 (48%)</td>
</tr>
<tr>
<td></td>
<td>5/5</td>
<td>75 (19%)</td>
<td>12 (12%)</td>
<td>6 (19%)</td>
<td>5 (15%)</td>
<td>1 (4%)</td>
<td>6 (16%)</td>
<td>4 (9%)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>GG</td>
<td>274 (70%)</td>
<td>71 (69%)</td>
<td>20 (61%)</td>
<td>25 (69%)</td>
<td>17 (61%)</td>
<td>29 (74%)</td>
<td>32 (65%)</td>
</tr>
<tr>
<td>G-455A</td>
<td>GA</td>
<td>103 (27%)</td>
<td>29 (28%)</td>
<td>10 (30%)</td>
<td>11 (31%)</td>
<td>9 (32%)</td>
<td>9 (23%)</td>
<td>15 (31%)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>13 (3%)</td>
<td>33 (3%)</td>
<td>3 (9%)</td>
<td>0 (0%)</td>
<td>2 (7%)</td>
<td>1 (3%)</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>ACE</td>
<td>DD</td>
<td>94 (24%)</td>
<td>30 (29%)</td>
<td>7 (21%)</td>
<td>7 (19%)</td>
<td>5 (18%)</td>
<td>12 (31%)</td>
<td>14 (30%)</td>
</tr>
<tr>
<td></td>
<td>ID</td>
<td>200 (51%)</td>
<td>54 (53%)</td>
<td>19 (58%)</td>
<td>22 (62%)</td>
<td>11 (41%)</td>
<td>21 (54%)</td>
<td>29 (60%)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>96 (25%)</td>
<td>18 (18%)</td>
<td>7 (21%)</td>
<td>7 (19%)</td>
<td>11 (41%)</td>
<td>6 (15%)</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>TPA</td>
<td>DD</td>
<td>81 (21%)</td>
<td>21 (21%)</td>
<td>6 (18%)</td>
<td>7 (20%)</td>
<td>6 (22%)</td>
<td>8 (20%)</td>
<td>8 (17%)</td>
</tr>
<tr>
<td></td>
<td>ID</td>
<td>182 (47%)</td>
<td>52 (51%)</td>
<td>17 (52%)</td>
<td>21 (58%)</td>
<td>12 (44%)</td>
<td>19 (49%)</td>
<td>24 (50%)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>126 (32%)</td>
<td>29 (28%)</td>
<td>10 (30%)</td>
<td>8 (22%)</td>
<td>9 (33%)</td>
<td>12 (31%)</td>
<td>16 (33%)</td>
</tr>
</tbody>
</table>
6.4.0: DISCUSSION

Stroke is a complex disease and many factors, both inherited and environmental, are known risk indicators. Generally accepted risk factors include smoking status, physical inactivity, dyslipidaemia, hypertension, diabetes, obesity, metabolic syndromes, menopause, hyperhomocysteinaemia, male gender and a positive family history of arterial disease. In addition, blood coagulation defects may be a risk factor for arterial thrombosis. Small blood clots that occlude major arteries represent a leading complication of atherosclerosis resulting in MI or ischaemic stroke (Fuster et al., 1996; Rentrop, 2000). The search for candidate genes should therefore include those encoding haemostatic factors in order to improve our understanding of the pathophysiology of arterial thrombotic disease. In this study we investigated the prevalence of such gene polymorphisms in control and stroke populations.

The Factor V Leiden polymorphism is the most prevalent genetic defect associated with venous thrombosis (Bertina et al., 1994; Zoller and Dahlback, 1994). This study confirms findings in previous reports that have failed to demonstrate an association between APCR or FV Leiden and arterial disease (Franco and Reitsma, 2001). However, the discovery of an association between FV Leiden and childhood stroke has not ruled out this polymorphism in very young victims of arterial disease (Nowak-Gottl et al., 1999). It appears that FV Leiden is not a major risk factor of arterial thrombosis but it may be possible that the mutation increases the risk conferred by classical risk factors such as smoking (Franco and Reitsma, 2001).

The G to A transition at nucleotide 20210 in the 3' untranslated region of the FII gene is associated with hyperprothrombinaemia (Poort et al., 1996) and an increased risk of venous thrombosis (Makris et al., 1997; Poort et al., 1996; Souto et al., 1998). Conflicting reports have been published concerning the role of this mutation in arterial thrombosis with mainly negative associations (Franco and Reitsma, 2001). A recent analysis of childhood stroke identified 6% of patients having the mutation compared to 1.3% of controls, figures similar to those reported here (Nowak-Gottl et al., 1999). We did not detect the prothrombin G20210A polymorphism in the Stroke population. However, this may be a statistical error due to the numbers recruited in the study (n=103) and the low frequency of the FII 20210A allele in the Irish population (Keenan et al., 2000). Similar to FV Leiden, the FII G20210A mutation may contribute to
thrombosis in the presence of other risk factors such as oral contraceptive use, as could be found in younger stroke victims (<45 years) (Martinelli et al., 1998).

Fibrinogen is the precursor of fibrin, its levels influencing platelet aggregation, blood viscosity and endothelial cell injury. It has been observed that elevated fibrinogen levels are associated with a 2-fold increased risk of cardiovascular disease in both high-risk subjects and controls (Maresca et al., 1999; Scarabin et al., 1993). A G to A substitution at position -455 in the promoter region of the fibrinogen gene has been associated with variations in levels of circulating fibrinogen (Koster et al., 1994) and has been linked to an increased risk of stroke (Nishiuma et al., 1998) and MI (Scarabin et al., 1993). However, we found no association with fibrinogen G-455A genotype and risk of stroke in this study. Fibrinogen is an acute phase protein that responds to cytokines in the local environment as a result of inflammatory processes. The association of increased fibrinogen concentrations and cardiovascular disease may reflect the cytokine release during inflammation of the damaged arteries. Further studies of the G-455A transition in association with inflammatory markers in stroke patients may reveal if fibrinogen increases are a cause or consequence of arterial damage.

We also investigated the prevalence of gene mutations in the fibrinolytic factors, TPA and PAI-1, and detected no significant variation in genotypes or allele frequencies between the stroke and control populations. PAI-1 functions as an inhibitor of plasminogen activation, thereby regulating fibrinolysis. The presence of the 4G allele of the promoter polymorphism has been associated with an increased plasma concentration of PAI-1 and controversial results regarding its role in arterial thrombosis have been reported (Franco and Reitsma, 2001). Contrary to its role in venous thrombosis, the 4G allele appears to have a protective effect in arterial thrombosis with homozygotes experiencing stroke at a later age and having reduced mortality (Endler et al., 2000; Roest et al., 2000). Similarly, the insertion deletion polymorphism in the TPA gene influences the release rates of the TPA protein. The homozygous state for the Alu insertion polymorphism has been associated with an increased risk of MI (van der Bom et al., 1997). Our results are similar to the US Physicians Health study that reported no association with the TPA Alu repeat polymorphism and ischaemic stroke (Ridker et al., 1997).
The insertion/deletion polymorphism in the ACE gene is associated with a marked variation in serum and cellular ACE levels, with higher levels associated with the DD genotype (Costerousse et al., 1993; Mathew et al., 2001; Rigat et al., 1990). The DD genotype has also been reported to be a risk factor for MI (Cambien et al., 1992) and an independent discriminator of patients with a history of stroke (Margaglione et al., 1996). In this study we identified a small increase in the DD genotype in individuals suffering stroke with hypertension (24-30% \( p=0.08 \)). Whilst only approaching statistical significance, this result suggests that the DD genotype may exert an effect through hypertension via an increase in circulating angiotensin II (either via the renin-angiotensin system or its inhibitory action on the fibrinolytic pathway) (Brown et al., 1998; Ridker et al., 1993a). The present results are in agreement with a previous report of an increase in the frequency of D allele in stroke patients with hypertension (Maeda et al., 1996). A larger patient cohort may be needed to verify this association with greater significance.

The current study found the mean age of first incidence of stroke to be significantly lower for smokers at 65.7 years than for non- or ex-smokers (individuals who had not smoked for > 1 year) at 70.9 years (\( p=0.036 \)). There was also a variation in age of onset between male and female subjects, with females experiencing their first stroke at a mean age of 72 years compared to 67.5 years in males (\( p=0.075 \)). These classic environmental risk factors may influence the inherited risk factors mentioned previously in this multifatorial disease.

Clearly, Stroke is a multifactorial disorder without one isolated cause. Characterising both phenotypic and genetic markers for stroke will be an important step forward in elucidating the pathogenesis of the disease. However, results published to date comparing the prevalence of genetic polymorphisms in genes encoding haemostatic factors in control and study populations have been conflicting. The problems are that sources of data are heterogenous both in ethnicity and clinical criteria, sample and control population sizes differ between studies, study design and methods of data analysis are also variable, and finally candidate polymorphisms may be in linkage disequilibrium with actual disease-causing polymorphisms. The degree of disequilibrium may vary from population to population, making detection of a disease causing mutation more complex (Franco and Reitsma, 2001). It is essential however, to continue in our endeavors to investigate the molecular basis of stroke as a clearer
understanding may allow preventative measures to be taken and more effective therapeutics for individual cases. More information would be available to aid in the management, for example, of unaffected family members with the presence of a strong familial history of stroke.
CHAPTER 7

INFLAMMATORY BOWEL DISEASE
7.1.0: INFLAMMATORY BOWEL DISEASE

Inflammatory Bowel Disease (IBD) is characterised by inappropriate activation of the mucosal immune system and comprises two chronic relapsing and remitting inflammatory disorders of the gastrointestinal tract, ulcerative colitis (UC) and Crohn’s disease (CD) (Podolsky, 2002). The excessive activation of the mucosal immune system is driven by the presence of normal luminal flora, a response facilitated by defects in the barrier function of the intestinal epithelium and the mucosal immune system (Podolsky, 2002). UC affects only the colon and rectum while CD may affect any part of the digestive tract. In the acute and moderately severe forms of the disease, UC presents with symptoms of frequent bouts of diarrhoea with blood and mucus, abdominal pain, fever and anorexia. These conditions, especially if they are severe or untreated, can lead to anaemia, sepsis and shock. A third but much less severe form of the IBD termed active proctitis, generally causes some rectal bleeding but the general health of the patient is maintained. The symptoms of Crohn’s disease tend to be more varied and depend on its site in the digestive tract. Patients suffering from inflammation of the ileum may or may not present with diarrhoea but generally suffer from obstruction of the small bowel resulting in constipation and vomiting. Patients with extensive small bowel disease have the above symptoms but in addition may have features of malabsorption and anaemia (Hendrickson et al., 2002). Extra-intestinal manifestations are also quite common with CD, especially if it affects the large bowel. Examples of such are inflammation of the eye, skin, joints and mouth, arterial and venous thrombosis, chronic hepatitis and other complications involving the liver, biliary tract and kidneys (Bernstein, 2001). There is considerable overlap in the clinical features of both UC and CD and in 10% of patients presenting with IBD it is not possible to determine whether the individual is suffering from UC or CD (Podolsky, 2002).

The exact aetiology of IBD is unknown although it is widely accepted that a number of genetic and environmental factors are involved, giving IBD a multifactorial status. Smoking has been reported to have a protective effect in UC and a detrimental effect in CD, with CD patients who smoke experiencing significantly more relapses, hospital admissions, surgery, pain and diarrhoea than non-smokers with CD (Farrell and Peppercorn, 2002; Wakefield et al., 1991). The use of the oral contraceptive pill has been implicated in IBD disease progression, namely, improvement in disease activity is seen after discontinuation of the pill (Wakefield et al., 1991). The theory of a genetic basis to disease susceptibility comes from a number of observations. Wide variations in the
incidence and prevalence of IBD among different populations and the familial aggregation of the disease have suggested a genetic predisposition to disease development (Podolsky, 2002). Twin studies have demonstrated the heritability of CD (1.0, 95% CI 0.34-1.0) and UC (0.53, 95% CI 0.24-1.0) which was found to be higher in monzygotic than dizygotic twins, especially in those with Crohn’s disease (Tysk et al., 1988). First degree relatives of an affected patient have a 4-20-fold increased risk of developing the disease, with offspring having an 8.9% risk, siblings an 8.8% risk and parents of an affected child having a 3.5% risk of disease (Podolsky, 2002; Tysk et al., 1988).

### 7.1.1: Role of coagulation in IBD pathophysiology

IBD has traditionally been regarded as an immune mediated disorder since there is an increase in pro-inflammatory cytokines, chemokines and adhesion molecules (Ang et al., 2000). The occurrence of microthrombi and inflammatory vasculitis on the intestinal mucosa indicate the importance of a hypercoagulable state in disease pathogenesis (Ang et al., 2000). A UK study found the incidence of CD in patients with the bleeding disorders Haemophilia or von Willebrands disease to be much lower than expected, indicating the protective effect of a hypercoagulable state (Thompson et al., 1995). Also, IBD patients were found to have a 3-fold increased risk of developing DVT or PE in a recent study (Bernstein et al., 2001), and these venous thrombotic events occur at an earlier age than non-IBD thrombotic patients (53 vs. 64 years, p=0.0225) (Grip et al., 2000).

A number of haemostatic parameters have been reported to be abnormal in IBD patients by several groups. An Italian study of 41 CD patients, 19 UC patients and 40 controls found a significant increase in platelet number (p<0.001), fibrinogen concentration (p<0.001), prothrombin fragments (F1 +2) (p=0.005 CD, p<0.001 UC), and spontaneous platelet aggregation (p<0.01) in both sub-populations of IBD compared to controls, while FXIII concentrations were however significantly reduced (p<0.005), whilst fibrinolytic activity was not found to be decreased (Chiarantini et al., 1996). Conversely, fibrinolytic factors were found to be significantly altered in IBD patients in an earlier Dutch study of 28 IBD patients (12 CD, 16 UC) and 28 controls. Significantly decreased TPA (p<0.02), increased PAI-1 (p<0.01), and increased uPA (p<0.01) were reported along with prolonged clotting times (p<0.001) (de Jong et al., 1989). This has been supported by the observation that significantly increased fibrinogen degradation products.
(FDP's) was reported in two separate studies of IBD patients (Kjeldsen et al., 1998; van Bodegraven et al., 2002). Increased fibrinogen and FVII concentrations have also been reported in a UK cohort of 110 IBD patients (CD n=75, UC n=35) compared to 85 controls (p<0.001) (Hudson et al., 1996). A recent investigation in an Irish cohort of paediatric IBD patients (n=35) revealed increased FVIII levels in 50% of patients compared to 10% in the normal population (p<0.01), with no deficiencies of the anticoagulants AT, PC, PS and no increased prevalence of the FV Leiden polymorphism (personal communication, Dr. Barry White).

Anti-inflammatory therapy with corticosteroids and 5-aminosalicylic acid have been the standard traditional treatments for IBD. Although these therapies have been successful in the majority of cases, significant numbers of patients have shown therapy resistance and some have experienced serious side effects (Jamerot et al., 1985; Podolsky, 2002). As mentioned earlier, heparin enhances the action of AT, promoting inhibition of FXa and thrombin. Elevated thrombin production has been reported in patients with IBD, promoting both coagulatory and pro-inflammatory processes such as stimulation of cytokine production on endothelial cells and monocytes, inducing cell-mediated immunity and further coagulation activation (Kjeldsen et al., 1998; White et al., 1999). A recent study of heparin therapy versus corticosteroid therapy in patients with active IBD revealed clinical, biochemical and endoscopic improvement to be similar in both treatment groups, suggesting heparin therapy to be an effective first line of treatment in IBD (Ang et al., 2000). The success of heparin treatment in IBD further emphasises the importance of coagulation abnormalities in the pathogenesis of the disease. These observations strongly suggest that the pathways of procoagulation, anticoagulation and fibrinolysis are perturbed in patients with active IBD, implying they may be involved in the pathobiology of this disease. A number of genes encoding coagulation and fibrinolytic proteins are reported to have functional polymorphisms, altering expression and production of their protein products in the circulation. Some of these polymorphisms have been investigated for associations with IBD pathogenesis.
7.1.2: Procoagulant gene mutations

7.1.2.i: FV Leiden

The FV Leiden G1691A polymorphism (see Chapters 6 and 8 for review of all polymorphisms) has been studied for its role in IBD pathophysiology with contradictory results (table 7.1). In a recent Hungarian study, 14.28% of CD patients (n=49) and 27.58% of UC patients (n=29) were found to be carriers of the FVL polymorphism compared to 5.26% of controls (n=57) (p<0.05 for both patient groups) (Nagy et al., 2001). In a French IBD study, FVL was looked for in patients with thrombosis (n=15), without thrombosis (n=58), thrombotic patients without IBD (n=110) and a healthy control population (n=84). The results indicated an increased prevalence of FVL in patients with thrombosis as a complication of IBD but no significant difference from the thrombotic population in general (Guedon et al., 2001). Furthermore, several studies have found no association between FVL and IBD. Similar frequencies of heterozygosity for FVL compared to controls were reported in an Irish study (Shanahan, 1999) and by three independent Italian studies, with a frequency range from 1.5-5% in patients between studies (Papa et al., 2000; Turri et al., 2001; Vecchi et al., 2000).

7.1.2.ii: Prothrombin G20210A

Three independent Italian studies reported similar frequencies of heterozygosity for the prothrombin G20210A polymorphism in IBD patient cohorts compared to controls, with a frequency range from 1.1-1.9% in patients between studies (Papa et al., 2000; Turri et al., 2001; Vecchi et al., 2000) (table 7.1). A recent UK investigation of 39 IBD patients and 100 controls prospectively evaluated the presence of the polymorphism, with no patients possessing the gene variant compared to 4% of controls (Haslam et al., 2001). This study highlights how small population studies may misrepresent the actual frequency of a polymorphism. Where the frequency of the GA genotype is 1-2% in the general population, 39 patients is clearly insufficient to accurately determine the prevalence in a study population and hence a bigger patient cohort needs to be studied to include or exclude a true association.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Effect on Expression</th>
<th>Association with IBD</th>
<th>No Association with IBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV Leiden</td>
<td>+1691 G→A</td>
<td>A: substitutes Arg506 with Gln (R506Q), at one of three APC cleavage sites causing APCR (Bertina et al., 1994).</td>
<td>Liebman et al., 1998</td>
<td>Shanahan, 1999</td>
</tr>
<tr>
<td></td>
<td>(Bertina et al., 1994)</td>
<td></td>
<td>Nagy et al., 2001</td>
<td>Papa et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Guedon et al., 2001</td>
<td>Vecchi et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Turri et al., 2001</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>+21210 G→A</td>
<td>A: Elevated prothrombin levels</td>
<td>Guedon et al., 2001</td>
<td>Papa et al., 2000</td>
</tr>
<tr>
<td></td>
<td>(Poort et al., 1996)</td>
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<td>Papa et al., 2000</td>
<td>Vecchi et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vecchi et al., 2000</td>
<td>Turri et al., 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Haslam et al., 2001</td>
</tr>
<tr>
<td>MTHFR</td>
<td>+677 C→T</td>
<td>T: allele creates a thermolabile enzyme with reduced activity for metabolising homocysteine (tHcy), increasing plasma homocysteine concentrations (Froost et al., 1995)</td>
<td>tHcy (Cattaneo et al., 1998; Papa et al., 2001; Romagnuolo et al., 2002)</td>
<td>Vecchi et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TT genotype (Mahmud et al., 1999)</td>
<td>Papa et al., 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Guedon et al., 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vecchi et al., 2000</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>-455 G→A</td>
<td>A: Elevated plasma Fibrinogen levels</td>
<td>No studies to date in IBD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Humphries et al., 1995; Scarabin et al., 1993)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>-675 insertion/deletion of G allele (Dawson et al., 1993)</td>
<td>5G:transcriptional repressor binding site (Dawson et al., 1993)</td>
<td>No studies to date in IBD</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4G:Elevated plasma PAI-1 levels (Eriksson et al., 1995; Grubic et al., 1996)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>insertion(I)/deletion (D) of Alu repeat in intron 8 (Tishoff et al., 1996)</td>
<td>I allele conflicting reports on association with increased plasma tPA levels (Hooper et al., 2000; Jern et al., 1999; van der Bom et al., 1997)</td>
<td>No studies to date in IBD</td>
<td></td>
</tr>
<tr>
<td>ACE</td>
<td>insertion(I)/deletion (D) of 287bp Alu repeat in intron 16 (Rigat et al., 1992)</td>
<td>D allele associated with increased plasma ACE levels (Rigat et al., 1992)</td>
<td>↓ serum ACE (Sommer et al., 1986; Takeuchi et al., 1992)</td>
<td>No ↓ serum ACE (Nunez-Gornes and Tewksbury, 1981)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>All genotypes ↓ serum ACE (Matsuda et al., 2001)</td>
<td></td>
</tr>
</tbody>
</table>
Hyperhomocysteinemia has been reported as a risk factor for both venous and arterial thrombosis (Cattaneo et al., 1998). The MTHFR C677T thermolabile variant in homozygous form, is the commonest cause of increased homocysteine. Therefore, folate deficiencies can exacerbate the effect of homozygous MTHFR in elevating the circulating plasma homocysteine levels (Cattaneo et al., 1998). The incidence of MTHFR C677T, hyperhomocysteinemia and folate deficiency has been investigated in IBD patients in several studies (table 7.1). Increased homocysteine concentrations were reported in an Italian cohort of 61 IBD patients (13%) compared to 183 age and sex matched healthy controls (5%) (p=0.04), with folate deficiency found in 15% of patients and 5% of controls (p=0.02) (Cattaneo et al., 1998). Similarly increased homocysteine levels were found in a cohort of 65 Canadian IBD patients (CD=56, UC=9) compared to 138 controls (15.4% vs. 2.2%, p<0.05) (Romagnuolo et al., 2002). In contrast, increased homocysteine concentrations were not associated with the TT genotype of the MTHFR gene variant in 106 IBD patients in a recent Danish study with increased coagulation activation in active disease (Bjerregaard et al., 2002). Moreover, the MTHFR thermolabile variant has also been reported at similar frequencies between IBD patients with thrombosis and all control groups in a French study (TT: IBD & thrombosis, 7.1%; IBD alone, 10.3%; thrombosis alone, 11.8%; healthy controls, 10.7%) (Guedon et al., 2001) and in an Italian study (IBD n=102, controls n=204) (Vecchi et al., 2000). However, in a recent Irish study, IBD was significantly associated with TT homozygosity (IBD n=174, controls n=273) (p<0.02). Hyperhomocysteinemia along with folate deficiency was found in both patients and controls with the TT genotype, and further analysis excluding these individuals found homocysteine concentrations remained higher in IBD patients than controls (p<0.05). Folate supplementation for all genotypes revealed a decrease in plasma homocysteine concentrations after six weeks of treatment (p=0.0001) (Mahmud et al., 1999). In summary, controversy still surrounds the role of MTHFR in the development of IBD, and also in the aetiology of VTE.
7.1.2.iv: Fibrinogen β chain G–455A

The fibrinogen β gene promoter polymorphism (-455) has been associated with increased plasma fibrinogen concentrations (Humphries et al., 1995). Elevated fibrinogen concentrations and increased fibrinogen degradation products have been reported in patients with IBD in several studies (Chiarantini et al., 1996; Hudson et al., 1996; Kjeldsen et al., 1998; van Bodegraven et al., 2002). Surprisingly, the fibrinogen β gene polymorphism has not been investigated in IBD patients, given its important biological role in the promotion of clot formation and thrombotic disease.

7.1.2.v: TPA & PAI-1 polymorphisms

The concept of altered global fibrinolytic activity in IBD patients is still in dispute. However, as discussed previously, TPA concentrations have been reported to be significantly decreased along with significantly increased PAI-1 concentrations in IBD (de Jong et al., 1989). The TPA Alu insertion/deletion polymorphism has been associated with altered TPA release, a possible cause of decreased TPA concentrations in IBD patients and subsequent promotion of thrombotic persistence. To date, the role of this polymorphism has however not yet been investigated in IBD. The PAI-1 promoter polymorphism (–675) has been associated with plasma PAI-1 levels, with the 4G allele having the highest levels (Eriksson et al., 1995; Grubic et al., 1996). Increased PAI-1 concentrations promote the inhibition of TPA, preventing the activation of the fibrinolytic pathway and subsequently promoting a prothrombotic state. To date, the role of the PAI-1 promoter polymorphism has not yet been elucidated in IBD pathogenesis or disease susceptibility.

7.1.2.vi: ACE

Angiotensin converting enzyme (ACE) has many functions including vasoconstriction, regulation of endothelial function and smooth muscle cell proliferation along with a role in the absorption of minerals, proteins and water (Hassan and Markus, 2000) (Matsuda et al., 2001). ACE downregulates TPA production via the catabolism of bradykinin, a potent stimulator of TPA (Dzau, 2001). It also includes the release of PAI-1 inhibiting fibrinolysis and promoting a procoagulant state (Vaughan, 2002). Serum ACE concentrations have been reported to be both decreased and unchanged in patients with IBD, with decreases observed more so in CD patients than UC (D’Onofrio et al., 1984; Nunez-Gornés and Tewksbury, 1981; Silverstein et al., 1981; Sommer et al., 1986;
Takeuchi et al., 1992). The Alu insertion (I)/deletion (D) polymorphism in the ACE gene is associated with marked differences in serum and cellular ACE levels, higher levels being associated with homozygosity for the ACE DD genotype (Costerousse et al., 1993; Mathew et al., 2001; Rigat et al., 1990). Until recently, there have been no reports of this genetic variant in the pathophysiology of IBD. The production of proinflammatory cytokines by mucosal macrophages and lymphocytes in IBD, may downregulate the production of ACE preventing it from regulating endothelial function, exacerbating the inflammatory reaction and promoting a procoagulant environment at the mucosal level (Matsuda et al., 2001). The role of the ACE gene polymorphism requires further investigation in cohorts of IBD patients.

We postulate that differences in expression of genes encoding coagulation and fibrinolytic proteins may be associated with an increased incidence of IBD, and that a prothrombotic phenotype plays a role in the pathophysiology of IBD. Here we investigate the frequencies of functional polymorphisms in genes encoding coagulation factors (FV, FII, and Fibrinogen), fibrinolytic proteins (TPA and PAI-1) and ACE expression. Table 7.1 lists the associations of each gene mutation with IBD.

AIMS

• To determine the prevalence of polymorphisms within genes encoding coagulation and fibrinolytic proteins in patients suffering inflammatory bowel disease.
• To compare these frequencies to those within the Irish population to determine a relationship between the presence of such polymorphisms and the incidence of IBD in the Irish population.
7.2.0: MATERIALS & METHODS

7.2.1: Subjects and DNA Isolation

IBD patients were recruited from St. James’s Hospital, Dublin 8, Ireland. 174 patients with Crohn’s Disease (n=64), Ulcerative Colitis (n=108) and undetermined IBD (n=2) were collected and homocysteine, folate and vitamin B₁₂ levels were assessed (chapter 2.1.5). Donor bloods were collected at The Northern Ireland Blood Transfusion Service (n=60) and The Irish Blood Transfusion Service (n=330) (chapter 2.1.1). DNA was extracted from whole blood using proteinase K (1mg/mL) cell lysis overnight at 37°C in the presence of 0.5% sodium dodecyl sulphate followed by extraction with phenol/chloroform, and precipitation with ethanol (chapter 2.2.1 and 2.2.2).

7.2.2: Polymerase Chain Reaction, Restriction Enzyme Digestion, and Agarose Gel Electrophoresis of patient and control DNA samples.

PCR amplification of all polymorphic sites was performed in a total reaction volume of 50µl. The standard PCR reaction mix (Appendix table A.3.1) consisted of Taq DNA Polymerase buffer with MgCl₂ (Promega) [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 1.5 mM MgCl₂], 0.4 units of DNA Taq polymerase, 2µl of genomic DNA, 4% dimethyl sulphoxide (DMSO, Sigma), 30µM each of deoxyribonucleoside triphosphates (Boehringer Mannheim), and 0.2µM each of sense and antisense primer (Sigma Genosys) (chapter 2, table 2.1). The cycling parameters for each assay are listed in chapter 2, table 2.2. The FV Leiden, prothrombin G20210A, beta fibrinogen and PAI-1 amplicons were digested with the appropriate enzyme (chapter 2, table 2.1) overnight at 37°C according to manufacturers instructions (New England BioLabs, NEB). The ACE and TPA PCR products were visualised directly on agarose gels (Pronadisa, Madrid). Restriction digest/PCR products were run on the appropriate percentage of agarose gel (chapter 2.4.0) containing 1.6µg/ml ethidium bromide (Sigma). Typical DNA banding patterns of PCR amplicons and restriction digest products for each polymorphism are illustrated in chapter 2, figures 2.1-2.7.
7.3.0: RESULTS

7.3.1.i: Genetic analysis

Table 7.2 lists raw counts, genotype and allele frequencies for all IBD patients, subgroups of UC and CD patients and the controls population for all polymorphic markers studied. The FV Leiden polymorphism, the prothrombin G20210A polymorphism and the MTHFR C677T gene variant were not detected at significantly different frequencies between IBD patients and controls for both genotypes and alleles (p>0.05). No homozygotes were identified for both FV Leiden and prothrombin polymorphism. Heterozygotes were detected at similar frequencies to controls for all three polymorphisms (FVL 3.5% vs. 4.4%; FII 1.7% vs. 1.8%; MTHFR 16.9% vs. 16.9%). Analysis of UC and CD patients separately did not achieve statistically significant differences for FVL and prothrombin polymorphisms. The frequency of MTHFR heterozygotes in CD patients appeared to be increased (20.3%) compared to controls (16.9%), however this difference did not reach statistical significance.

The fibrinogen G-455A polymorphism was detected at a statistically significantly different frequency in IBD patients compared to controls. Both genotype and allele frequencies were significantly different from blood donor controls (p=0.02, 0.01 respectively). Homozygotes for the AA genotype were detected in 4% of IBD patients. UC patients exhibited statistically significant variations in allele frequencies compared to controls (p=0.03) but not genotype frequencies (p=0.86). CD patients demonstrated significant variations in genotype frequencies compared to controls (p=0.057) but not allele frequencies (p=0.14).

The PAI-1 4G/4G genotype was detected in 38.1% of all IBD patients, 43% of UC patients and 31.2% of CD patients compared to 33.2% in controls. There was no significant difference in both genotype and allele frequencies between all IBD patients and controls (p=0.45, 0.20). Both genotype and allele frequencies in CD patients were detected at similar frequencies to controls (p=0.89, 0.65). There was no significant difference in genotype frequencies in UC patients (p=0.11), however allele frequencies were detected at a significantly different frequency than controls (p=0.03).
The frequency of the TPA insertion/deletion polymorphism was statistically significantly different between all IBD patients and controls for both genotypes and alleles ($p=0.0004$, 0.006). Analysis of UC patients revealed both genotype and allele frequencies to be significantly different ($p<0.001$, $<0.001$), with the DD genotype detected at a much higher frequency than controls (40.7% vs. 20.8%). Similar analysis of CD patients revealed no statistical significance in genotype and allele frequencies compared to controls ($p=0.48$, 0.97).

Frequency of the ACE insertion/deletion polymorphism was significantly different between all IBD patients and controls for both genotypes and alleles ($p=0.006$, 0.003). The DD genotype was detected in 36.8% of all IBD patients, 38% of UC patients and 34.4% of CD patients compared to 24.1% of controls (UC $p=0.01$, CD $p=0.09$).
Table 7.2: Genotype and allele frequencies of polymorphisms in control and inflammatory bowel disease populations. IBD patients are subdivided into ulcerative colitis (UC) and Crohn’s disease (CD). Raw data is shown as counts (%).

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Controls</th>
<th>IBD</th>
<th>UC</th>
<th>CD</th>
<th>Allele Frequency</th>
<th>Controls</th>
<th>IBD</th>
<th>UC</th>
<th>CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV Leiden</td>
<td>AA</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>A</td>
<td>0.02</td>
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<tr>
<td></td>
<td>GA</td>
<td>17 (4.4)</td>
<td>6 (3.5)</td>
<td>4 (3.7)</td>
<td>2</td>
<td>3.1</td>
<td>G</td>
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</tr>
<tr>
<td></td>
<td>GG</td>
<td>372 (95.6)</td>
<td>168 (96.5)</td>
<td>104 (96.3)</td>
<td>62</td>
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<td></td>
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<td>FII G20210A</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>0.02</td>
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<tr>
<td></td>
<td>GA</td>
<td>7 (1.8)</td>
<td>3 (1.7)</td>
<td>2 (1.9)</td>
<td>1</td>
<td>1.6</td>
<td>G</td>
<td>0.98</td>
<td>0.99</td>
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</tr>
<tr>
<td></td>
<td>GG</td>
<td>382 (97.9)</td>
<td>171 (98.3)</td>
<td>106 (98.1)</td>
<td>63</td>
<td>98.4</td>
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<td>MTHFR C677T</td>
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<td>69 (40.1)</td>
<td>49 (46.2)</td>
<td>19</td>
<td>29.7</td>
<td>C</td>
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<tr>
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<td>CT</td>
<td>187 (48.0)</td>
<td>74 (43.0)</td>
<td>42 (39.6)</td>
<td>32</td>
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<td>G-455A</td>
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<td>65 (37.4)</td>
<td>38 (35.2)</td>
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<td>37</td>
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<td>44</td>
<td>128 (33.2)</td>
<td>66 (38.1)</td>
<td>46 (43.0)</td>
<td>20</td>
<td>31.2</td>
<td>4</td>
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<tr>
<td>4G:5G</td>
<td>45</td>
<td>183 (47.4)</td>
<td>79 (45.7)</td>
<td>47 (43.9)</td>
<td>30</td>
<td>46.9</td>
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<td>55</td>
<td>75 (19.4)</td>
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<td>41 (38.0)</td>
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</table>
7.3.1.ii: Sex distribution & age of onset

The male to female ratio was 42% versus 58% respectively. CD patients consisted of 72% females and 28% males while UC patients consisted of 51% females and 49% males. This difference in sex distribution was significantly different between CD and UC patients (p=0.008). The mean age of onset in women was 29.5 years compared to 32.7 years in men (figure 7.1), a difference approaching statistical significance (p=0.06). The mean age of onset between CD and UC patients was 30 years and 31.5 years respectively, reflecting the female to male age of onset distribution above. This gives strength to the finding that CD patients consisted more predominantly of women.

7.3.1.iii: Homocysteine, Folate & Vitamin B12

No significant difference in the concentration of folate and vitamin B12 was detected between UC and CD patients (p=0.3, 0.5). The overall mean concentration of homocysteine was 12.44 μmol/L in all IBD patients, with a mean of 11.43 μmol/L in CD patients and 13.1 μmol/L in UC patients, however this difference was not statistically different between patient groups (p=0.1). The mean homocysteine concentration exhibited some variance between MTHFR genotypes with the greatest increase in homozygotes for the mutation (CC: 11.7; CT: 12.2; TT: 14.8) (figure 7.2). This variance was found approaching statistical significance (p=0.07).
Figure 7.1: Age of onset of inflammatory bowel disease in male and female patients.
Figure 7.2: Homocysteine concentration variance between MTHFR genotypes in IBD patients.
7.4.0: DISCUSSION

Inflammatory Bowel Disease involves excessive activation of the mucosal immune system in the form of two chronic inflammatory disorders, ulcerative colitis (UC) and Crohn's disease (CD). Considerable overlap in the clinical features of both UC and CD exists and 10% of patients cannot be sub-classified into either disease group (Podolsky, 2002). The aetiology of IBD is unknown although it is widely accepted that it is a multifactorial disease. Wide variations in the incidence and prevalence of IBD among different populations and the familial aggregation of the disease have suggested a genetic predisposition to disease development (Podolsky, 2002). The emerging role of thrombotic complications in the progression of disease has led to studies of coagulation factors in IBD, showing changes in these parameters during active disease, and successful treatment with heparin therapy. These findings led us to initiate genetic investigations into functional polymorphisms in genes encoding coagulation factors.

The Factor V Leiden polymorphism is the most prevalent genetic defect associated with venous thrombosis and predicts FVα's resistance to inhibition by activated protein C, promoting a procoagulant environment (Bertina et al., 1994; Zoller and Dahlback, 1994). A small number of studies have reported an increased incidence of FVL in IBD patients compared to controls in patients from the US, Hungary and France (GA range 14.3%-36% IBD vs. 0%-5.26% controls). The present study found no increased frequency in FVL between a large cohort of IBD patients (n=174) and controls (n=390). Sub-division of IBD patients into CD (n=64) and UC (n=108) also revealed similar genotype and allele frequencies between patients and controls. These results confirm findings in several other investigations failing to demonstrate an association with FVL and inflammatory bowel disease (Papa et al., 2000; Shanahan, 1999; Turri et al., 2001; Vecchi et al., 2000). Therefore, it is unlikely that the FVL polymorphism contributes significantly to susceptibility to inflammatory bowel disease. Nonetheless, it may increase the risk of thrombotic complications in IBD.

The prothrombin G20210A polymorphism is the second commonest identified genetic risk factor for venous thrombosis predicting increased plasma concentrations of the protein (Poort et al., 1996). No increase in prevalence of the prothrombin G20210A polymorphism was observed in this Irish cohort of IBD patients (n=174) compared to controls (n=390). Hetereozygotes were detected in 1.7% of all IBD patients, 1.9% of UC
patients, 1.6% of CD patients and 1.8% of controls. Similarly, several other groups have failed to demonstrate any significant difference in frequency of this mutation between IBD patients and controls (Italian and UK populations) (Haslam et al., 2001; Papa et al., 2000; Turri et al., 2001; Vecchi et al., 2000). Increased prothrombin fragments and fibrinogen concentrations, previously reported in IBD patients by several investigators (Chiarantini et al., 1996; Hudson et al., 1996), are most likely not a result of increased prothrombin generation but a result of global coagulation activation via the proinflammatory cytokine stimulation of the tissue factor pathway of coagulation activation.

Hyperhomocysteinemia may be a risk factor for both venous and arterial thrombosis (Cattaneo, 1999; Cattaneo et al., 1998) and the MTHFR C677T thermolabile gene variant has been associated with increased homocysteine concentrations due to the reduced activity of the enzyme (Salomon et al., 1999). Increased homocysteine concentrations have been reported in two Italian and one Canadian population of IBD patients. Homocysteine concentrations ranged from 8.7-12.2-μmol/L for IBD patients compared to 6.6-10.5-μmol/L between studies (p=0.3, 0.001 and <0.05). The present study found mean concentrations of homocysteine in all IBD patients to be 12.44μmol/L, 13.1μmol/L in UC patients and 11.43μmol/L in CD patients. The MTHFR thermolabile variant was detected in 16.9% of all IBD patients, 14.2% of UC patients and 20.3% of CD patients compared to controls (16.9%). The prevalence of the TT genotype and the T allele was not statistically significantly different between these sub-populations and the control cohort, as in previous studies (Bjerregaard et al., 2002; Guedon et al., 2001; Papa et al., 2001; Vecchi et al., 2000). A tendency toward hyperhomocysteinemia was observed in patients with the TT genotype, however this did not reach statistical significance (p=0.07). These results support previous findings of an increase in homocysteine with the TT genotype (Salomon et al., 1999) and suggest a possible role of hyperhomocysteinemia in thrombotic development in IBD.

The fibrinogen protein is a precursor of fibrin, the basis of a thrombotic clot. Its levels influence platelet aggregation, blood viscosity and endothelial cell injury (Maresca et al., 1999). Fibrinogen concentrations have been reported to be increased in patients with IBD compared to controls in several studies (Chiarantini et al., 1996; Hudson et al., 1996; Kjeldsen et al., 1998; van Bodegraven et al., 2002). Homozygosity for the fibrinogen β
gene promoter polymorphism (−455) has been associated with increased plasma fibrinogen concentrations (Humphries et al., 1995). To our knowledge, this is the first report of an investigation of this polymorphism in a cohort of IBD patients. We detected homozygosity for the AA genotype in 4% of all IBD patients, 5.6% in UC patients and 1.6% in CD patients compared to 3.3% in controls (p=0.03). All genotype and allele frequencies were significantly different in IBD populations than controls (p=0.02, 0.01). Analysis of UC patients revealed significantly different genotype and allele frequencies compared to controls (p<0.001, <0.001). Fibrinogen concentrations were not measured in this study but the increases observed by several others may be due to homozygosity for the AA genotype and increased prevalence of the A allele in IBD populations. The relevance of the A allele in IBD patients requires further investigation along with fibrinogen concentrations to elucidate its role in IBD pathogenesis. Glucocorticoid treatment of IBD has been shown to reduce fibrinogen concentrations over time, but non-responsive patients to treatment did not achieve a reduction in fibrinogen levels (Kjeldsen et al., 1998; van Bodegraven et al., 2002). The presence of the A allele in patients may be a factor in those non-responsive to treatment and requires further investigation. Fibrinogen is an acute phase protein that is influenced by the presence of inflammatory cytokines and hence is a marker of inflammation. Increased fibrinogen levels in IBD may therefore be either a cause or consequence of inflammation occurring in these patients (van der Bom, 2002).

It is widely accepted that coagulation markers are increased in IBD but controversy surrounds the role of fibrinolysis in the pathophysiology of this disorder. TPA concentrations have been reported to be significantly decreased along with significant increases in PAI-1 concentrations in IBD (de Jong et al., 1989). Here we report similar frequencies of heterozygosity for the PAI-1 insertion/deletion polymorphism in patients with IBD (45.7% vs. 47.4%, p=0.45). The 4G/4G genotype (which is associated with increased PAI-1) was detected in 38.1% of all IBD patients, 43% of UC patients and 31.2% of CD patients compared to 33.2% of controls (p>0.05). Subsequently, allele frequencies were significantly different in UC patients compared to controls (p=0.03). The TPA insertion/deletion polymorphism was found to occur at a significantly different frequency in IBD patients and sub-groups compared to controls for both genotypes and alleles (p= 0.0004, 0.006). The I allele was found at a slightly higher frequency in CD patients, however this did not differ significantly from controls (p=0.97). The I allele of
the TPA polymorphism has been associated with regulation of the release rates of TPA and conflicting reports exist on whether this regulation results in increased or decreased plasma TPA levels (Hooper et al., 2000; Jern et al., 1999; van der Bom et al., 1997). If TPA concentrations are truly decreased in IBD, then the present results would indicate that the I allele is associated with reduced expression of TPA in plasma. Decreased TPA would have a knock on effect on PAI-1 levels, increasing free circulating PAI-1 by reducing TPA:PAI-1 complexes. It is possible that both mutations inherited together lead to exacerbation of this effect. Under these conditions, the fibrinolytic pathway in IBD patients would not become activated and a procoagulant state would result. Plasma concentrations of these proteins must be investigated along with these gene mutations to determine the role they play in the pathophysiology of IBD. This study suggests that both the PAI-1 and TPA polymorphisms are potential risk factors for decreased fibrinolysis in IBD patients.

The many functions of ACE include downregulation of TPA production via the catabolism of bradykinin, a potent stimulator of TPA, and the promotion of PAI-1 production (Dzau, 2001; Vaughan, 2002). All of these processes promote a procoagulant state. Serum ACE concentrations have been investigated in IBD patients with reports of both decreased and unchanged levels (D'Onofrio et al., 1984; Nunez-Gornes and Tewksbury, 1981; Silverstein et al., 1981; Sommer et al., 1986; Takeuchi et al., 1992). The ACE Alu insertion/deletion polymorphism has been reported to affect concentrations of serum ACE, higher levels being associated with the DD genotype (Costerousse et al., 1993; Mathew et al., 2001; Rigat et al., 1990). The ACE DD genotype was detected in 36.8% of all IBD patients, 38% of UC patients and 34.4% of CD patients compared to 24.1% of controls. The DD genotype and the D allele were significantly associated with susceptibility to IBD (p=0.0006, 0.003). The frequencies in UC patients were significantly different from controls for both genotypes and alleles (p=0.01, 0.007). CD patients were not found to have significantly different genotype or allele frequencies compared to controls, probably due to the smaller sample size in this group. The observed increased frequency of the DD genotype in IBD patients suggests a possible increase in serum ACE concentrations in this disorder. Increased serum ACE has the potential to decrease TPA concentrations and increase PAI-1 concentrations, as has been observed in IBD patients previously. Assessment of these levels would aid in determining the role the ACE polymorphism has in IBD pathophysiology.
It is clear that the pathophysiology of inflammatory bowel disease is governed by many factors, both environmental and genetic. The occurrence of thrombotic episodes in patients with IBD does not appear to be a result of impaired regulation of coagulation factors at the genetic level. However the interaction of these coagulation gene polymorphisms and environmental risk factors such as smoking and the oral contraceptive pill cannot be ruled out. In contrast fibrinogen concentrations regulated by the β fibrinogen gene may influence the progression of disease, the development of thrombosis and the response to standard therapies in IBD. To our knowledge, the present study is the first of its kind, assessing both coagulant and fibrinolytic polymorphisms in IBD. The association of fibrinolytic gene mutations with IBD has not been previously reported. Contrary to current opinions, regulation of the fibrinolytic pathway appears to be an important parameter in the pathophysiology of IBD and TPA and ACE may both play key roles.
CHAPTER 8

DISCUSSION & CONCLUSIONS
8.1.0: DISCUSSION & CONCLUSIONS

This study investigated several disease states associated with or complicated by both coagulatory and inflammatory pathologies. These diseases are also multifactorial, being influenced by both environmental and genetic factors. Such diseases include ischaemic stroke and inflammatory bowel disease, disorders of thrombosis and inflammation respectively, and venous thromboembolism and recurrent foetal loss, disorders of both thrombosis and inflammation. The existence of diseases influenced by both coagulatory and inflammatory dysregulation suggests those traditionally associated with only one mechanism may also be dictated by the other. The emergence of an interaction between these pathways warrants their investigation in tandem in diseases associated with either mechanism. In order to gain a greater understanding of these complex systems in disease pathogenesis, we initiated our investigations in pregnant patients since the cause of many pathological pregnancies has been attributed to dysregulation of inflammatory processes and upregulation of coagulatory mechanisms. Furthermore, normal pregnancy is associated with a hypercoaguable state and changes in the inflammatory response, mechanisms that against all odds, result in a successful outcome. This phenomenon makes the pregnant state a good model for assessing the relationship between coagulatory and inflammatory mechanisms, as a preliminary step to investigating the disturbances occurring in recurrent foetal loss and other diseases. Moreover, such processes in the pregnant state are still undetermined and knowledge of such may be beneficial for aiding human reproduction and for the treatment of unrelated complications throughout gestation.

We identified increased production of coagulatory proteins and decreased activation of anticoagulant processes during pregnancy, allowing for potential thrombosis formation in the absence of other regulatory mechanisms. These findings suggest that the increase in coagulation in normal pregnancies is controlled by mechanisms other than the natural anticoagulants, since both PC and AT remained unchanged throughout gestation. However, detection of decreased PS concentrations suggests inactivation of the PC pathway during pregnancy. Furthermore, the significant increase in PS in patients with a history of RFL at 26 weeks gestation may be indicative of saturated C4bBP that cannot complex with excessive PS that is free in circulation.

The decrease in inflammatory cytokines noted here may be a method of regulating the coagulatory response by preventing the induction of the tissue factor pathway in
response to inflammatory mediators such as TNFα. A reduction in TNFα will prevent
the inducement of other cytokines since TNFα is the primary cytokine produced by cells
of the immune system in response to tissue injury. Therefore, this would indicate that the
increase in coagulatory factors such as FVIII and fibrinogen is initiated by proteins of the
contact pathway of activation. However, the contact pathway was demonstrated as
unaffected in pregnancy by measurement of the APTT. Therefore, it is possible that
increased coagulatory factors during pregnancy are a result of mechanisms involved in
the binding of phospholipids on the surface of endothelial cells. β2GP1 is a suspected
anticoagulant thought to bind these phospholipids, preventing their availability for
coaulation activation. Analysis of this protein revealed a reduction in levels until 36
weeks gestation, indicating that this protective process may be hampered during
pregnancy by some unknown mechanism. In fact RFL patients exhibited consistently
lower levels of β2GP1 than normal pregnant controls, giving support to this hypothesis
since placental thrombosis is thought to be one cause of RFL. Further understanding of
the mechanism of action of β2GP1 may aid in the understanding of increased
coaulation during pregnancy and RFL.

This study reports a decrease from non-pregnant ranges in all cytokines investigated
including the anti-inflammatory mediator IL-10, which has been reported as beneficial to
human reproduction (Wegman et al., 1993). The significantly increased concentrations
of IL-10 in RFL patients suggests that IL-10 is important for the maintenance of
pregnancy and may be the key mediator in the successful pregnancies experienced by
such individuals in this study. This may also be true for IL-6, which has also been shown
as beneficial in the maintenance of pregnancy and was found here to increase in RFL
pregnancies. Therefore, if IL-6 has beneficial roles in the maintenance of pregnancy,
increased levels in RFL patients indicate that a threshold exists, above which the effect
of IL-6 could be harmful. Furthermore, the increased IL-6 concentrations observed with
the G allele of the IL-6 promoter polymorphism in RFL patients may aid in increasing
IL-6 levels above this threshold. However, the finding that a larger cohort of patients
with a history of RFL had an increased frequency of the C allele indicates that the small
sample size in patients with IL-6 protein measurements was not sufficient to determine
the exact genotype-phenotype relationship. In addition, IL-6 is a known inducer of
fibrinogen transcription, increasing its production during the acute phase response.
Therefore, the increased fibrinogen observed in normal pregnancies suggests that
increased IL-6 may not be harmful to successful pregnancies. These conflicting results may be due to the small sample number again, since comparisons between genotype and phenotype in RFL patients were performed with only eleven patients. In conclusion, it is our belief that TNFα is probably the key inflammatory mediator during pregnancy, dictating the expression of all other cytokines. However, increased IL-10 in RFL patients may be the result of as yet unknown mechanisms.

We have demonstrated that endothelial cells in culture exhibit pro-inflammatory responses to both estrogen and progesterone, as do monocytes. Although these results are contrary to previous reports, these hormones have both been suggested as having similar effects on PAI-1 levels in the same endothelial cell line (Fujimoto et al., 1996). Therefore, maintenance of pregnancy may be achieved by overall downregulation of cytokine production, but a certain degree of pro-inflammatory action may be necessary to maintain support of the feto-allograft. From these studies, we believe that the action of these steroid hormones in the endometrium is not directed towards T cells but towards monocytes or possibly endothelial cells. This may explain the absence of a cell shift in T helper cell types observed in in vivo studies. However, the multifaceted nature of T helper cells may prevent this theory from being categorically proven, since T cells do not always fall into defined categories (Gleicher, 2002). Alternatively, in vitro environments may not adequately mimic the pregnant uterus, requiring further animal studies to elucidate the T helper cell role in the maintenance of pregnancy.

Increased fibrinogen and FVIII levels have been demonstrated as normal occurrences throughout pregnancy in this study. In venous thrombosis patients, both of these coagulatory proteins have been implicated as risk factors for thrombotic disease (Koster et al., 1995; Renner et al., 2002). Our findings support previous observations, since we detected fibrinogen as the most common risk factor for thrombotic disease in an Irish cohort of VTE patients. Increased FVIII levels were also found to be an independent risk factor for VTE in this cohort. If elevations in these proteins are such high risk factors for thrombotic disease then why do they not cause thrombosis in normal pregnancy. It may be that the concomitant decrease in inflammatory processes during pregnancy is not regulated in thrombotic disease, creating a greater potential for thrombogenicity. We have demonstrated that the polymorphism within the fibrinogen gene does not appear to influence circulating levels of fibrinogen in affected individuals during pregnancy. Similarly, the increased fibrinogen concentrations inferring a risk of thrombosis in VTE
patients may be due to environmental factors such as smoking, hypertension, surgery or major trauma, oral contraceptive use, immobilisation or hormone replacement therapy (Martinelli, 2001). The same may be true for FVIII concentrations although a genetic assay is not available as yet to determine the association between genotype and phenotype, however, these methods do not always correspond as expected, as demonstrated with the fibrinogen polymorphism.

We have detected the FV Leiden mutation as a major risk factor for venous thromboembolism in VTE patients, along with verifying a sufficient laboratory cut off point in the APCR assay to warrant FV Leiden determination. The FV Leiden polymorphism was also found at an increased frequency in the RFL cohort, further supporting its role in thrombotic disease development. Our studies support the general acceptance of FV Leiden as being the most commonly inherited risk factor for venous thrombosis (Bertina et al., 1994; Rosendaal et al., 1995; Zoller and Dahlback, 1994). Investigation of stroke patients and individuals suffering from inflammatory bowel disease revealed no increased frequency of the FV Leiden mutation in these cohorts, therefore indicating no role for this mutation in the development of disease pathophysiology. These findings have been reported previously by others in stroke and IBD cohorts (Franco and Reitsma, 2001; Papa et al., 2000; Shanahan, 1999; Turri et al., 2001; Vecchi et al., 2000). The current investigation of VTE patients also supports the previous associations of the prothrombin G20210A polymorphism with venous thrombosis (Alhenc-Gelas et al., 1999; Poort et al., 1996; Salomon et al., 1999), although we did not find this mutation to be a risk factor for RFL, Stroke or IBD disease pathogenesis.

Characterising both phenotypic and genetic markers for stroke is an important step in understanding the pathogenesis of this disorder. Coagulatory and fibrinolytic gene mutations have previously been investigated in stroke patients but with conflicting reports. We have determined no increased prevalence of any coagulatory or fibrinolytic polymorphism in this cohort of Irish stroke victims, supporting previous observations (Nowak-Gottl et al., 2001; Ridker et al., 1997; Sharma et al., 1994; Tyjaerg-Hansen et al., 1997; Voetsch et al., 2000). However, patients with hypertension were found to have an increased frequency of the D allele of the ACE gene polymorphism, which has been associated with increased angiotensin II, a potent vasoconstrictor, supporting previous findings (Sharma et al., 1994). This emphasises the multifactorial basis of this disease,
suggesting control of environmental factors in the treatment of disease progression. We have shown that male subjects and smokers are at an increased risk of developing an ischaemic event, re-emphasising the environmental impact on this disease. Further studies of stroke victims are required to elucidate the cause of this condition by continuation of sample collection from individuals with an ischaemic episode. By increasing the sample size, greater confidence may be gained in the reported prevalence of such polymorphisms in the stroke population. For example the lack of detection of the prothrombin G20210A variant suggests that a greater number of patients is required to detect this mutation in this population.

Similar to venous thrombosis and ischaemic stroke, IBD is a multifactorial disease, whose symptoms may be induced or exacerbated by environmental stimuli mentioned previously. Moreover, the familial aggregation of the disease and the wide variation in incidence among different populations suggests a genetic predisposition to disease progression (Podolsky, 2002). The recent finding that heparin therapy is an effective mode of treatment for IBD patients suggested that the coagulation process is upregulated in IBD patients. We were unable to detect an increased incidence of coagulatory gene defects in these patients. However, fibrinolytic polymorphisms may have a role to play in regulating the fibrinolytic process in these patients in particular in UC patients where increased frequency of the fibrinogen gene polymorphism was detected compared to CD patients.

The current study involved many diseases that are associated with a thrombotic phenotype and are themselves considered to be multifactorial by nature. The association of several polymorphic markers with these diseases and the association of environmental influences further emphasise the multifactorial nature of these diseases. Although thrombosis has not been detected in many of these disease groups, the association of coagulatory and fibrinolytic markers with inflammatory diseases indicates a link between the inflammatory and coagulatory pathways, mechanisms that may influence the progression of disease pathogenesis. Due to the complex nature of both systems, the exact mechanisms of interaction remain to be determined for individual diseases. However, it can be concluded that coagulation and inflammatory pathways influence the development of thrombotic disease. Continuation of patient sample collection to increase study sample sizes may aid in further elucidating the roles of these markers in disease progression.
This study is the first of its kind in an Irish population, to study longitudinal cytokine responses throughout pregnancy. In doing so, we have demonstrated that the immune response is downregulated during pregnancy to allow the maternal acceptance of the foetal allograft. In particular, we have shown that IL-10 may be an important regulatory protein in the maintenance of foetal survival, with increased concentrations acting in a protective manner. Furthermore, the pro-inflammatory cytokine TNFα may have a deleterious effect on the developing foetus, if allowed to increase above certain levels. This thesis demonstrates that the maternal immune system is kept under tight control to prevent the increase of pro-inflammatory cytokines above the safe threshold. Such findings may be useful in a clinical practice where RFL patients have been screened for all other known causative factors with no cause identified. The monitoring of serum cytokine levels throughout pregnancy may be useful, to ensure the maternal immune system is not acting in an autoimmune fashion, and that all cytokines are within the normal pregnant range.

The finding that endothelial cells in culture exhibit a pro-inflammatory response to steroid hormones (both estrogen and progesterone) has not been demonstrated previously and may have great importance in the mystery of RFL. As we have demonstrated previously, a pro-inflammatory environment in the maternal immune system must remain within the safe threshold for survival of the foetus. However, this new observation that steroid hormones may disturb this balance may have further therapeutic ramifications. The measurement of hormone levels in women at risk of RFL throughout pregnancy may be another method of monitoring the change in risk to a particular individual. It may be that the concentration of both estrogen and progesterone must remain within certain levels at certain stages throughout pregnancy, in order that the correct cytokine profile ensues. Hormonal monitoring in conjunction with the measurement of cytokine concentrations throughout pregnancy may prove invaluable to the care and therapeutic strategy toward at risk patients in the clinical practice.
8.2.0: FUTURE PROSPECTS

Findings in this study indicate that a greater number of patients are required to elucidate the true pathophysiology of RFL in relation to dysregulation of coagulatory and inflammatory mechanisms and also to determine the relationship between genotype and phenotype. Therefore, future work should involve further collection of patient samples longitudinally, in both normal pregnancies and in pregnancies of individuals with a history of RFL. To increase sample numbers sufficiently we suggest that a multi-centre study be performed in an Irish cohort within a shorter time period, giving greater power to data. The present study examined patients from the Rotunda Maternity Hospital longitudinally and those attending St. James’s Hospital for genetic analysis. A multi-centre study could be achieved through collaboration of the above hospitals with the National Maternity Hospital (Holles, St, Dublin 2) and the Coombe Women’s Hospital (Crumlin, Dublin 12).

Expression of TF on the surface of monocytes has been shown to be reduced during pregnancy in a recent study (Holmes et al., 2002). The decrease in TNFα demonstrated here during pregnancy, suggests that it may be responsible for the reduction in TF. Therefore, measurement of TF in the present patients by whole blood cytometric analysis would yield interesting results if found to be decreased in conjunction with TNFα. Such a finding would suggest that the decrease in TF exhibited during pregnancy is directly related to the decreased TNFα expression.

Further assessment of the acute phase proteins fibrinogen and FVIII during pregnancy may be achieved in vitro by determining their response to steroid hormone stimulation in endometrial cells. Since we have demonstrated an increase in IL-6 in response to both estrogen and progesterone, a similar increase in fibrinogen and FVIII may be achieved, as a direct result of IL-6 induction by these hormones.

Analysis of circulating concentrations of fibrinolytic proteins should be performed to enable determination of a genotype-phenotype relationship, in particular in patients with active IBD, since we have demonstrated statistically significant differences between fibrinolytic polymorphisms in IBD compared to the control population. Furthermore, the role of inflammatory markers on the development of thrombotic events in VTE, IBD and ischaemic stroke could be investigated by performing cytokine measurements in such patients at the time of the thrombotic event.
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A.1.1.0: PATIENT QUESTIONNAIRES FOR PREGNANCY STUDY

Table A.1.1: First visit questionnaire completed by medical staff on behalf of patients participating in the pregnancy study.

<table>
<thead>
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<th>Lab Number</th>
<th>Control / Study patient</th>
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<tbody>
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</tbody>
</table>

1<sup>st</sup> VISIT

**Date** / /  
**Weeks Gestation**  

**Personal History**

Venous Thrombosis Y / N  
If Y how many  

Arterial Thrombosis Y / N  
Stroke/MI /Other  
How Many  

How Many Full Term Normal Pregnancies  

How Many Foetal Loss/at what gestation /  

**Previous Treatment**: Steroids/Aspirin/UF Heparin/LMW Heparin/Other  

**Present Treatment**: Steroids/Aspirin/UF Heparin/LMWH/Other  

Folic Acid Supplements Y / N  

Smoker / Non-smoker  

**Family History**

Venous Thrombosis/ How many  

Arterial Thrombosis Stroke/MI/Other  

Foetal Loss/at what gestation  


Table A.1.2: Questionnaire completed on each return visit by medical staff on behalf of patients participating in the pregnancy study.

<table>
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<th>Control / Study patient</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Place addressograph label here

Date ____/____/____  Weeks Gestation ________

Visit___________

**Current Medical Details**

Venous Thrombosis Y / N ___________________________

Arterial Thrombosis Y / N : Stroke/MI/Other ___________________________

Treatment: Steroids/Aspirin/UF Heparin/LMWH/Other ___________________________

Folic Acid Supplements Y / N

Weeks Gestation at time of loss (where applicable) ___________________________

Smoker / Non-smoker ___________________________

Pre-eclampsia : HBP / Swelling / Proteinuria
A.2.1.0: DNA EXTRACTION

A.2.1.1: Crude Extracts for IBTS controls

**Buffer A:** (1 Litre)
- 1M TRIS (pH 7.5) 10ml
- 1M MgCl₂ 5ml
- 100% Triton-X100 10ml
- 0.32M Sucrose 100.5g
- dH₂O up to 1L

**Buffer B:** (200ml)
- 0.5M NaCl 30ml
- 0.5M EDTA 7.5ml
- dH₂O up to 200ml

5ml of blood was added to 20ml of Buffer A and vortexed. Samples were spun at 3.5K for 20 minutes in a Sorvall RC-5B refrigerated centrifuge. Pellets were resuspended in 1.25ml of Buffer B (Appendix A.2.1.1). 25μl of proteinase K (10mg/ml) was added, followed by 62.5 μl of 10% SDS. The contents were mixed thoroughly by inversion and incubated overnight at 37°C. An equal volume of saturated phenol was added to each of the samples, which were then mixed thoroughly and spun for 30 minutes at 750g in a bench top centrifuge. The aqueous layer of clear fluid was removed and extracted twice more. Two volumes of ice-cold ethanol (100% v/v) were added to each of the samples, which were then placed in the freezer at -20°C for 30 minutes, allowing the DNA to precipitate out. Following removal using a glass ‘hook’, made using a pasteur pipette, the DNA was washed 3 times in 70% ethanol. The DNA was dissolved in 200 μl of sterile water, labelled accordingly and stored at -20°C.

A.2.1.2: Phenol/Chloroform Extracts for Guthrie cards and patient/control whole blood samples

Reagents

**4M NaCl**
- 1M NaCl = 58.44g/L
- 4M NaCl = 233.76g/L

**1M Tris**
- 1M Tris = 121.1g/L

**3M NaAc**
- 1M NaAc = 82.03g/L
- 3M NaAc = 246.09g/L
**TNE Buffer 0.5% SDS:**

<table>
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<tr>
<th>Stocks</th>
<th>500ml Buffer</th>
<th>250ml Buffer</th>
<th>100ml Buffer</th>
<th>Final Conc</th>
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</thead>
<tbody>
<tr>
<td>Distilled H₂O</td>
<td>430.1ml</td>
<td>215.05ml</td>
<td>86.02ml</td>
<td></td>
</tr>
<tr>
<td>4M NaCl</td>
<td>13.8ml</td>
<td>6.9ml</td>
<td>2.76ml</td>
<td>0.11M</td>
</tr>
<tr>
<td>1M Tris pH 8.0</td>
<td>27.5ml</td>
<td>13.8ml</td>
<td>5.5ml</td>
<td>55mM</td>
</tr>
<tr>
<td>0.5M EDTA pH 8.0</td>
<td>1.1ml</td>
<td>550µl</td>
<td>220µl</td>
<td>1.1mM</td>
</tr>
<tr>
<td>10% SDS</td>
<td>27.5ml</td>
<td>13.8ml</td>
<td>5.5ml</td>
<td>0.55%</td>
</tr>
</tbody>
</table>

Phenol H₂O Saturated (Sigma)
Chloroform : Iso-amyl Alcohol (50:1) – Make fresh each day
3M Na-acetate pH 5.2
Proteinase K (20mg/ml) (Boehringer Mannheim)

Prepare TNE Lysis buffer as follows (fresh)

**Table A.2.2:** Lysis buffer preparation

<table>
<thead>
<tr>
<th>Reagent</th>
<th>6 reactions</th>
<th>12 reactions</th>
<th>24 reactions</th>
<th>48 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNE Buffer</td>
<td>2.25ml</td>
<td>4.5ml</td>
<td>9ml</td>
<td>18ml</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>250µl</td>
<td>500µl</td>
<td>1ml</td>
<td>2ml</td>
</tr>
</tbody>
</table>

**A.2.1.2.i: Guthrie card DNA extraction**

One punch from a Guthrie card or 200µl of EDTA anticoagulated whole blood was placed in a 1.5ml eppendorf tube (Treff Labs) while contained in a Category II safety cabinet. 400µl of 0.5% SDS TNE lysis buffer (Appendix table A.2.2) and proteinase K (20mg/ml) were added and mixed, and samples were incubated overnight in a 37°C water bath (Grant Instruments Ltd., Cambridge). 450µl of saturated phenol (Sigma Aldrich, USA) was added and vortexed for 10 minutes to ensure thorough mixing. Samples were centrifuged (IEC Micromax RF refrigerated centrifuge, International Equipment Company) at 15,000rpm for 5 minutes to separate layers. The aqueous layer was transferred to a new 1.5ml eppendorf, 450µl of Chloroform:Isoamyl alcohol added, and mixed and centrifuged as before. The aqueous layer was transferred to a new 1.5ml eppendorf containing 40µl of NaAc (pH 5.2). 800µl of ice-cold 100% ethanol was added, samples mixed thoroughly and incubated at -20°C overnight or -70°C for 30 minutes. Nucleic acid extract was collected by centrifuging samples at 15,000rpm at 0°C (refrigerated centrifuge) for 10 minutes. Supernatants were removed and pellets washed twice with 80% ethanol and centrifuged as before for 5 minutes. After removal of ethanol, pellets were dried using a vacuum drying centrifuge (DNA SpeedVac ©, DNA 110, Savant).
A.2.1.2.H: DNA extraction using the QIAmp™DNA Mini kit

EDTA anticoagulated blood samples were collected from patients and stored at 4°C until DNA was extracted. 200μl of whole blood was transferred into a 1.5ml microfuge tube (Treff Labs) containing 25μl of proteinase K (Qiagen). 200μl of lysis buffer (Buffer AL) was added to this and samples vortexed for 15 seconds. Manufacturers recommended that the proteinase K not be added directly to the Buffer AL. Samples were incubated at 70°C for 10 minutes after which 210μl of ethanol was added to each samples and vortexed to ensure thorough mixing. A spin column was inserted into a collection tube for each sample and the contents of the microfuge tube transferred to this. Samples were centrifuged at 8000rpm for 1 minute and collection tubes discarded. Spin columns were placed in fresh collection tubes to which 500μl of wash buffer (Buffer AW) was added. Samples were centrifuged as before and placed in clean collection tubes. Another 500μl of wash buffer was added to each sample and centrifuged at 12,000rpm for 3 minutes. The Spin column was subsequently placed in a clean labelled1.5ml microfuge tube. Elution buffer (Buffer AE) was preheated to 70°C and 50μl was added to each sample. Samples were incubated at 70°C for 5 minutes to allow elution of the DNA from the spin column into the microfuge tube. Samples were centrifuged at 8000rpm for 1 minute and the spin column discarded.

A.3.1.0: POLYMERASE CHAIN REACTION (PCR) STANDARD REACTION SOLUTION

Table A.3.1: Standard PCR reactions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Conc.</th>
<th>1X Volume (μl)</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq DNA Buffer w/ MgCl₂</td>
<td>15mM MgCl₂</td>
<td>5.0</td>
<td>1.5mM MgCl₂</td>
</tr>
<tr>
<td>(Promega)</td>
<td>10X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO (Sigma)</td>
<td>100%</td>
<td>2.0</td>
<td>4%</td>
</tr>
<tr>
<td>dNTP's (Boehringer Mannheim)</td>
<td>3mM</td>
<td>0.5</td>
<td>30μM</td>
</tr>
<tr>
<td>Sense primer</td>
<td>100pmol/μl</td>
<td>0.5</td>
<td>1pmol/μl</td>
</tr>
<tr>
<td></td>
<td>100X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense primer</td>
<td>100pmol/μl</td>
<td>0.5</td>
<td>1pmol/μl</td>
</tr>
<tr>
<td></td>
<td>100X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taq DNA Polymerase (Promega)</td>
<td>5units/μl</td>
<td>0.2</td>
<td>1unit</td>
</tr>
<tr>
<td>Nuclease Free H₂O</td>
<td></td>
<td>Up to 50μl</td>
<td></td>
</tr>
</tbody>
</table>
A.4.1.0: ELISA REAGENTS

A.4.1.1: PBS (1L)
PBS tablets (Sigma Aldrich) = 5 tablets + 1L dH₂O

A.4.1.2: Wash Buffer (1L)
1L PBS + 0.05% Tween 20 (500μl) (Sigma Aldrich)
pH 7.2-7.4

A.4.1.3: Block Buffer (500ml)
1% BSA (5g) (Sigma Aldrich)
5% Sucrose (25g)
0.05% Sodium Azide (0.25g/pinch)(NaN₃)
500ml PBS

A.4.1.4: Reagent Diluent (RD) (500ml)
0.1% BSA (0.5g)
0.05% Tween 20 (250μl)
500ml TBS

A.4.1.5: TBS pH 7.2-7.4 (500ml)
20mM Trizma Base (1.21g)(Sigma Aldrich)
150mM NaCl (4.38g)

1M Tris = 121.1g in 1L
1mM Tris = 121.1mg in 1L
1mM Tris = 60.55mg in 500ml
20mM = 1211mg in 500ml
20mM = 1.211g in 500ml

A.4.1.6: HRP-Streptavidin
1/200 dilution of stock with a total of 10ml per plate (R & D Systems)
10ml/200 = 50μl HRP in 10ml RD

A.4.1.7: Substrate Solution

A.4.1.7.(i): IFNγ, TNFα and IL-6
1:2 mixture of H₂O₂ (reagent A) and Tetramethylbenzidine (TMB) (reagent B) (supplier)
6ml dH₂O + 3ml Reagent A + 3ml Reagent B = 12ml TMB substrate per plate

A.4.1.7. (ii): IL-10

1:1 mixture of H₂O₂ (reagent A) and Tetramethylbenzidine (TMB) (reagent B) (supplier)

6ml Reagent A + 6ml Reagent B = 12ml TMB substrate per plate

A.4.1.8: Stop Solution

\[ 2N \text{H}_2\text{SO}_4 \ (1N = 2M) \]

Concentrated \( \text{H}_2\text{SO}_4 \) = 18M = 9N

\[ 1/4.5 \text{ dilution} = 4M = 2N \]

100ml 2N H₂SO₄ = 22.2ml H₂SO₄ + 77.8ml dH₂O

A.5.1.0: R & D SYSTEMS DUOSET IMMUNOASSAY KITS

A.5.1.1: Capture Antibody (cAb)

A.5.1.1.(i): IFNγ, TNFα and IL-10

antibody calculations are identical:

Need 10ml of cAb at a concentration of 4µg/ml = 40µg/10ml

\[ 1/18 \text{ dilution} \]

Stock = 720µg/ml (in 1000µl PBS) → 40µg/10ml

1000µl / 18 = 55.6µl

:: One ELISA plate was coated with 55.6µl cAb in 10ml PBS = 4µg/ml cAb per well for both IFNγ and TNFα.

IL-10 optimisation proved that double this concentration of cAb achieved the best results

:: One IL-10 ELISA plate was coated with 111.20µl cAb in 10ml PBS = 8µg/ml cAb per well.

A.5.1.1.(ii): IL-6

Stock = 360µg/ml (in 1000µl PBS)

Add Acid to Water
Store in 200ml glass Duran
Keep solution in dark (wrap bottle in aluminium foil)
Appendix

Need 10ml of cAb at a concentration of 2μg/ml = 20μg/10ml

1/18 dilution

Stock = 720μg/ml (in 1000μl PBS) → 20μg/10ml

1000μl / 18 = 55.6μl

.: One IL-6 ELISA plate was coated with 55.6μl cAb in 10ml PBS = 2μg/ml cAb per well.

A.5.1.2: Detection Antibody (dAb)

A.5.1.2.(i): IFNγ

Need 10ml of dAb at a concentration of 100ng/ml (0.1μg/ml) = 1μg/10ml

1/18 dilution

Stock = 18μg/ml (in 1000μl RD) → 1μg/10ml

1000μl / 18 = 55.6μl

.: One IFNγ ELISA plate was incubated with 55.6μl dAb in 10ml RD =100ng/ml dAb per well.

A.5.1.2.(ii): TNFα

Need 10ml of dAb at a concentration of 300ng/ml (0.3μg/ml) = 3μg/10ml

1/18 dilution

Stock = 54μg/ml (in 1000μl RD) → 3μg/10ml

1000μl / 18 = 55.6μl

.: One TNFα ELISA plate was incubated with 55.6μl dAb in 10ml RD=300ng/ml dAb per well.

A.5.1.2.(iii): IL-10

Need 10ml of dAb at a concentration of 500ng/ml (0.5μg/ml) = 5μg/10ml

1/18 dilution

Stock = 90μg/ml (in 1000μl RD) → 5μg/10ml

1000μl / 18 = 55.6μl
A.5.1.2.(iv): IL-6

Need 10ml of dAb at a concentration of 200ng/ml (0.2μg/ml) = 2μg/10ml

\[
\text{Stock} = 36\mu g/ml \text{ (in 1000μl RD)} \quad \rightarrow \quad 2\mu g/10ml
\]

\[
1000\mu l / 18 = 55.6\mu l
\]

\[\therefore \text{One IL-6 ELISA plate was incubated with 55.6μl dAb in 10ml RD=200ng/ml dAb per well.}\]

A.6.1.0: Antibody Standards

A.6.1.1: IFNγ and TNFα

Need 600μl of standards to make serial dilutions starting at a concentration of 1ng/ml (1000pg/ml)

\[
\text{Stock} = 70\mu g/ml \text{ (in 500μl RD 0.2μm filtered)} \quad \rightarrow \quad 1\text{ng/ml}
\]

\[
600\mu l / 70 = 8.6\mu l \text{ Stock in 600μl RD}
\]

\[\therefore 8.6\mu l \text{ Stock in 600μl RD = 1000pg/ml}\]

1000pg/ml

\[
\begin{align*}
\downarrow \\
500pg/ml \\
\downarrow \\
250pg/ml \\
\downarrow \\
125pg/ml \\
\downarrow \\
62.5pg/ml \\
\downarrow \\
31.25pg/ml \\
\downarrow \\
15.625pg/ml \\
\downarrow \\
7.8pg/ml
\end{align*}
\]

0pg/ml (RD alone)

1/2 serial dilutions of 1000pg/ml with Reagent Diluent (filtered)
Appendix

A.6.1.2: IL-10

Need 600μl of standard to make serial dilutions starting at a concentration of 0.125ng/ml (125pg/ml)

$\frac{1}{560}$ dilution

Stock = 70ng/ml (in 500μl RD 0.2μm filtered) $\rightarrow$ 0.125ng/ml

$600\mu l / 560 = 1\mu l$ Stock in 600μl RD

$\therefore 1\mu l$ Stock in 600μl RD = 125pg/ml

125pg/ml

↓

62.5pg/ml

↓

31.25pg/ml

↓

15.625pg/ml

↓

7.8pg/ml

↓

3.9pg/ml

↓

1.95pg/ml

0pg/ml (RD alone)

A.6.1.3: IL-6

Need 600μl of standard to make serial dilutions starting at a concentration of 0.3ng/ml (300pg/ml)

$\frac{1}{100}$ dilution

Stock = 30ng/ml (in 500μl RD 0.2μm filtered) $\rightarrow$ 0.3ng/ml

$600\mu l / 100 = 6\mu l$ Stock in 600μl RD

$\therefore 6\mu l$ Stock in 600μl RD = 300pg/ml
Appendix

300pg/ml
↓
150pg/ml
↓
75pg/ml
↓
37.5pg/ml
↓
18.75pg/ml
↓
9.4pg/ml
↓
4.7pg/ml
↓
0pg/ml (RD alone)

1/2 serial dilutions of 300pg/ml with Reagent Diluent (filtered)

A.7.1.0: BUFFERS FOR dHPLC WAVE® INSTRUMENTATION

Buffers should be made up in a fume hood to prevent inhalation of toxic fumes. All flasks/bottles used for making buffers for the WAVE® machine must be washed several times with 80mΩ distilled water (HPLC grade, Millipore). All dilutions must use this high grade water also to protect the integrity of the capillary column.

A.7.1.1: Buffer A

0.1M Triethylammonium acetate (TEAA) (Transgenomics)

Stock TEAA = 2M

Measure 50ml TEAA into a designated 50ml volumetric flask until the bottom of the meniscus touches the measure line. Transfer into a designated 1L volumetric flask. Rinse out the 50ml flask using HPLC grade water 2/3 times, pouring the washings into the 1L flask to ensure the proper volume of TEAA is transferred. Add 250µl of HPLC grade acetonitrile to prevent bacterial growth. Add HPLC grade water, mixing thoroughly at the 500ml stage and the 800ml stage until the bottom of the meniscus reaches the 1L measure line. This buffer must be made weekly if not used within this time period.

A.7.1.2: Buffer B

0.1M TEAA and 25% Acetonitrile (250ml)

Measure 50ml TEAA as for buffer A above, pouring the washings into a designated 1L volumetric flask for buffer B. Measure 250ml acetonitrile into a designated 250ml volumetric flask until the bottom of the meniscus touches the measure line. Pour acetonitrile into the 1L flask containing TEAA. Rinse out the 250ml flask using HPLC grade water 2/3 times, pouring the washings into the 1L flask to ensure the proper volume of TEAA is transferred. Add 250µl of HPLC grade water, and then add HPLC grade water, mixing thoroughly at the 500ml stage and the 800ml stage until the bottom of the meniscus reaches the 1L measure line. This buffer must be made weekly if not used within this time period.
grade water 2/3 times, pouring the washings into the 1L flask to ensure the proper volume of acetonitrile is transferred. Add HPLC grade water, mixing thoroughly at the 500ml stage and the 800ml stage until there is approximately 900ml of solution in the flask. Cap and invert several times to mix contents thoroughly. Upon mixing the solution turns cold (endothermic reaction) and gas is produced (dissolved oxygen). Release the gas by removing the cap and replacing it loosely. Due to the temperature change, the solution needs to be equilibrated to room temperature by heating in a water bath for 10-20 minutes. After temperature equilibration has occurred, adjust the final volume to 1L (until the bottom of the meniscus touches the measure line). This buffer must be made weekly if not used within this time period.

A.7.1.3: Buffer C

75% Acetonitrile cleaning solution

Measure 250ml acetonitrile using the 250ml volumetric flask as indicated above and add to the designated 1L volumetric flask for buffer C. Repeat this a further two times to obtain 750ml acetonitrile in the 1L flask. Add HPLC grade water (mixing intermittently) to approximately 900ml and release the dissolved oxygen as per instructions for buffer B. Add HPLC grade water until the bottom of the meniscus reaches the 1L measure line. This buffer should be made up every three weeks if not used within this time period.

A.7.1.4: Buffer D

8% Acetonitrile syringe washing solution

Measure 80ml of HPLC grade acetonitrile using a glass graduated cylinder, until the bottom of the meniscus touches the 80ml line. Transfer this into a designated 1L volumetric flask for buffer D. Rinse out the graduated cylinder using HPLC grade water 2/3 times, pouring the washings into the 1L flask to ensure the proper volume of acetonitrile is transferred. Add HPLC grade water, mixing thoroughly at the 500ml stage and the 800ml stage until the bottom of the meniscus reaches the 1L measure line. This buffer must be made weekly if not used within this time period.

Transfer all buffers into labelled 1L Duran bottles that have been rinsed several times with HPLC grade water.

A.7.1.5: Use of WAVE® machine

The initial step in setting up a mutation detection run on the WAVE® instrument involved choosing the ‘mutation detection’ option in the command window. Then each gene sequence to be used was saved to the hard drive of the designated computer, where the chromatographic output will be displayed and from where the instrument received its commands. Once this was achieved, the column temperature was chosen. Upon opening a particular gene sequence using the instruments software, a particular melting temperature was chosen based on the G:C content of the sequence and the length of the strand to be analysed. The ideal temperature would provide a graphical
Appendix

suggested, with graphical outputs displayed on the one graph (Figure 2.13). By drawing a line from 75% on the Y axis to the line of a particular temperature (designated by colours), and down to the X axis (representing the base pairs of the sequence), the area of the sample to be analysed by that temperature was determined (see Figure 2.13 for demonstration). This method illustrated how more than one temperature was often required to ensure the whole strand would be analysed. The first temperature the software chose was usually the most appropriate for analysing the largest possible area of the DNA strand. The melting temperature was also determined for individual target sequences using a melting profile program available from the Stanford University website (http://insertion.stanford.edu/melt.html). This program gave a list of appropriate temperatures for each block of base pairs and an overall recommended melting temperature(s) for the whole strand, including the appropriate percentage of buffers required for optimal column performance at each temperature. After accepting a particular temperature, the ‘apply’ button was clicked to confirm its acceptance. This opened the next window illustrating the predicted separation pattern for that gene sequence at that particular temperature (Figure 2.14). It was important to ensure that the elution peak would occur between 3 and 4 minutes of run time. This ensured that the DNA peak would not be masked by the large injection peak early in the run (~ 1 min, present on all chromatograms illustrating sufficient sample injection onto the column), and that it would not occur at the end of the run and be masked by the wide buffer peak at 6-7 minutes. It was possible to manipulate the position of the elution peak in this window by changing the percentage of buffer B for each temperature. Once these parameters were set and accepted, the sample table was prepared. A mutation standard was obtained from Transgenomics, which was included in every run with new buffers to ensure correct elution of samples. A dummy or no DNA sample was always used as the first sample for every change in temperature to allow the column sufficient time to equilibrate to the correct temperature. The steps described above were performed for each temperature chosen for a particular gene sequence. It was possible to run more than one gene sequence in each run for a large number of samples. Once the oven temperature was equilibrated for one hour, the buffer inlet lines were purged with new buffers, the sample table was made and saved, and the DNA samples were placed in the correct position on the sample tray, the run was started.

A.8.1.0: TISSUE CULTURE REAGENTS

Cell Counting

The surface of the haemacytometer was cleaned with 70% ethanol before and after use. The sides of the chambers were moistened with a drop of water and the coverslip placed on top, directly above the counting chambers. Cells were diluted 1/100 with 0.4% Trypan blue (Sigma) (10μl of cells + 90μl dye) and mixed thoroughly. 10μl of this cell suspension was added to both sides of the haemacytometer by placing the pipette at the edge of the coverslip, cells filled the chambers completely via capillary action. Cells were counted under the microscope (Olympus) using a 10X objective lens and a cell counter. The following formula was used to calculate the number of cells per ml of media.
C = N \times 10^4 \\
Where: \\
C = \text{cells per ml} \\
N = \text{average of cells counted} \\
10^4 = \text{volume conversion factor for 1mm}^2 \text{ surface of haemacytometer} \\

Total yield = C \times V \\
Where: \\
V = \text{total volume of cells (ml)}

Table A.8.1: Media supplementation for each cell line

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Medium (500ml)</th>
<th>10% FCS</th>
<th>1% P/S</th>
<th>1% L-Glutamine</th>
<th>1% NEAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ishikawa</td>
<td>MEM</td>
<td>50ml</td>
<td>5ml</td>
<td>5ml</td>
<td>5ml</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>RPMI 1640</td>
<td>50ml</td>
<td>5ml</td>
<td>5ml</td>
<td>-</td>
</tr>
<tr>
<td>Jurkat</td>
<td>RPMI 1640</td>
<td>50ml</td>
<td>5ml</td>
<td>5ml</td>
<td>-</td>
</tr>
<tr>
<td>THP-1</td>
<td>RPMI 1640</td>
<td>50ml</td>
<td>5ml</td>
<td>5ml</td>
<td>-</td>
</tr>
</tbody>
</table>

A.8.1.1: Estrogen & Progesterone Reconstitution

Lyophilised hormones reconstituted with 1ml absolute ethanol and 49ml sterile medium (RPMI 1640).

Stock each hormone = 63.59 \times 10^{-6} \text{M}

Cells were growing in 1ml of media therefore 1ml divided by the dilution factor = the volume of stock hormone required to give a final concentration of 1 \times 10^{-6} \text{M}.

1000\mu l / 63.59 = 15.72\mu l

\therefore 15.72\mu l each hormone was added directly to 1ml of cells to obtain a final concentration of 1 \times 10^{-6} \text{M}.

1/100 serial dilutions of stock hormones (10\mu l hormone + 990\mu l media) were made to achieve the remaining hormone concentrations and 15.72\mu l of these added to 1ml of cells.
A.8.1.2: Low Molecular Weight Heparin (LMWH) dilution

500 units of LMWH to be used in 1000μl of cells and media from stock of 20,000 IU/ml

\[
\begin{align*}
20,000 \text{ units} & \quad \rightarrow \quad 500 \text{ units} \\
1000 \mu\text{l} & \quad = \quad 25 \mu\text{l}
\end{align*}
\]

\[\begin{array}{c}
10 \mu\text{l of stock} + \\
990 \mu\text{l media}
\end{array}\]
\[63.59 \times 10^{-6} \text{M} \quad \downarrow \quad \frac{1}{63.59} \text{ dilution}\]
\[10 \mu\text{l of } 1 \times 10^{-6} \text{M} \quad + \\
990 \mu\text{l media}
\]
\[1 \times 10^{-6} \text{M} \quad \downarrow \quad \frac{1}{100} \text{ dilution}\]
\[10 \mu\text{l of } 1 \times 10^{-8} \text{M} \quad + \\
990 \mu\text{l media}
\]
\[1 \times 10^{-8} \text{M} \quad \downarrow \quad \frac{1}{100} \text{ dilution}\]
\[10 \mu\text{l of } 1 \times 10^{-10} \text{M} \quad + \\
990 \mu\text{l media}
\]
\[1 \times 10^{-10} \text{M} \quad \downarrow \quad \frac{1}{100} \text{ dilution}\]
\[10 \mu\text{l of } 1 \times 10^{-12} \text{M} \]

\[= \text{Add 25μl of LMWH to 1000μl of cells in media + hormones (15.72μl) and bring to a final volume of 2000μl with ice-cold PBS to stop the reaction}\]
### A.9.1.0: IRISH BLOOD TRANSFUSION SERVICE POLYMORPHIC FREQUENCIES

Table A.9.1: IBTS Control Genotype & Allele Counts (n=390)

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Raw Values</th>
<th>%</th>
<th>Allele</th>
<th>Raw Values</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV</td>
<td>AA</td>
<td>0</td>
<td>0</td>
<td>A</td>
<td>17</td>
<td>0.0219</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>17</td>
<td>4.4</td>
<td>G</td>
<td>761</td>
<td>0.9781</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>372</td>
<td>95.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FII</td>
<td>AA</td>
<td>1</td>
<td>0.3</td>
<td>A</td>
<td>9</td>
<td>0.0115</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>7</td>
<td>1.8</td>
<td>G</td>
<td>771</td>
<td>0.9885</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>382</td>
<td>97.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTHFR</td>
<td>CC</td>
<td>137</td>
<td>35.1</td>
<td>C</td>
<td>461</td>
<td>0.5910</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>187</td>
<td>48.0</td>
<td>T</td>
<td>319</td>
<td>0.4090</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>66</td>
<td>16.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>AA</td>
<td>13</td>
<td>3.3</td>
<td>A</td>
<td>129</td>
<td>0.1654</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>103</td>
<td>26.4</td>
<td>G</td>
<td>651</td>
<td>0.8346</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>274</td>
<td>70.3</td>
<td></td>
<td></td>
<td></td>
</tr>
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