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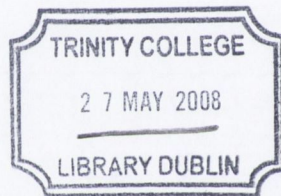
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Systemic Inflammation after Cardiac Surgery

By

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**A Thesis Submitted in Fulfillment of the Requirements for the
Degree of Doctor of Medicine (M.D)**

Faculty of Medicine

Trinity College Dublin

This work was carried out under the supervision of
Dr. Thomas Ryan and Dr Ross Mc Manus,
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Declaration

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Eddie Duggan

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Abbreviations

A	Adenine
ACCP	American College of Chest Physician
ACT	Activated Clotting Time
ANOVA	Analysis of Variance
APTT	Activated Partial Thromboplastin Time
ATA	Adenine Thymine Adenine
ATP	Adenosine triphosphate
Bp	Base pair
C	Cytosine
⁰ C	Degrees Celsius
CABG	Coronary Artery Bypass Graft
cDNA	Complementary Deoxyribonucleic acid
CI	Confidence Interval
CPB	Cardiopulmonary Bypass
Cr	Creatinine
Ct	Threshold cycle
Δ Ct	Difference in threshold cycle between the target and the reference.
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethlyenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
Fig.	Figure
FiO ₂	Fractional inspired O ₂ concentration
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gm	gram
HCL	Hydrogen chloride
HCO ₃ ⁻	Bicarbonate ion
H ₂ O	Water
hrs	hours
ICU	Intensive Care Unit
Iκβ	Inhibitor kappa beta
IL	Interleukin
INR	International Normalised Ratio
KCL	Potassium Chloride
kDa	Kilodalton
KG	Kilogram
L	Litre (s)
LPS	Lipopolysaccharide
m	metres

MAP	Mean Arterial Pressure
MGB	Minor Groove Binding
Min.	Minutes
mg	Miligrammes
MgCl	Magnesium Chloride
ml	Mililitres
mM	Milimoles
mmHg	Milimetres of Mercury
mMLV	Murine Moloney Leukaemia Virus
MODS	Multiple Organ Dysfunction Syndrome
mRNA	messenger Ribonucleic acid
MOF	Multiple Organ Failure
N	Number
NADH	Nicotinamide Adenine Dinucleotide
NFκβ	Nuclear Factor Kappa Beta
ng	nanogram
nm	nanometres
NS	Not significant
O ₂	Oxygen
p	Probability
PAI-1	Plasminogen Activator Inhibitor-1
PaO ₂ /FiO ₂ ratio	Ratio of Arterial Oxygen Tension to inspired

	Oxygen Concentration
PATS	Patient Administration and Tracking System
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PDH	Pyruvate Dehydrogenase
pH	potential of Hydrogen ion
plt	platelets
pmol	picomoles
R	Correlation coefficient (Pearson's)
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
rRNA	ribosomal Ribonucleic acid
SBP	Systolic Blood Pressure
SCCM	Society of Critical Care Medicine
SD	Standard Deviation
SE	Standard error
s	Seconds
SEM	Standard Error of the Mean
SIRS	Systemic Inflammatory Response Syndrome
SNP	Single Nucleotide Polymorphism
T	Thymine
TACE	Tumour Necrosis Factor Alpha Converting Enzyme

TNF α	Tumour Necrosis Factor Alpha
t-PA	Tissue Plasminogen Activator.
U	Units
μ l	Microlitre
μ m	Micrometres, microns
μ mol	Micromole
u-PA	Urokinase Plasminogen Activator
VLDL	Very Low Density Lipoprotein
Vs	Versus
WCC	White Cell Count

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Dedication

I wish to dedicate this thesis to my husband, Gerry Gillespie and to my parents John and Bridie Duggan. I am grateful for their continuous support and encouragement which enabled me to complete this thesis.

Summary of Thesis

Genetically determined inter-individual variation in cytokine production, influences outcome in patients with systemic inflammation and severe sepsis. However outcome in patients with sepsis is also dependant on the presence of co existing disease and the interaction between infection and inflammation. Due to this heterogeneity, the role of genetic factors in the generation of systemic inflammation is difficult to study in septic patients. Our study overcomes this by examining systemic inflammation that occurs postoperatively in cardiac surgical patients in a sterile setting. Using real time polymerase chain reaction (PCR), we measured interleukin 10 (IL 10) and tumour necrosis factor alpha (TNF α) messenger RNA in peripheral mononuclear cells at timed points after cardiopulmonary bypass. A low IL 10:TNF α mRNA ratio manifested systemically as haemodynamic instability within 24hrs post cardiac bypass. We also determined cytokine related single nucleotide polymorphisms using restriction fragment length polymorphism, and taqman technology. Haplotype analysis was performed using Phase II software for both TNF and IL 10 polymorphisms. We discovered that an IL 10 haplotype (ATA) was linked with haemodynamic instability within the first 24hrs post cardiac bypass. Previous studies have associated low IL 10 levels with this ATA IL 10 haplotype. Inflammation and coagulation activation are intimately linked. Our second study focused on the gene expression and insertion/deletion polymorphism (4G/5G) of the serine protease, Plasminogen Activator Inhibitor-1 (PAI-1). PAI mRNA decreased

in peripheral mononuclear cells after cardiac surgery and remained low 24hrs postoperatively. We demonstrated an association between PAI-1 4G/5G genotype and a propensity to receive coagulation blood products in the early post-operative period. There was a significant relation between relative change in PAI mRNA and TNF a mRNA after cardiopulmonary bypass thus confirming previous studies which have demonstrated a link between the inflammatory and fibrinolytic cascades.

Chapter 1
Introduction

1.1 Introduction

Modulation of the inflammatory cascade during sepsis has been considered as a possible means to improve survival in sepsis and prevent septic shock. However, outcome in patients with sepsis is also dependant on the presence of co existing disease and the interaction between infection and inflammation. The situation is further complicated by patient selection and presentation, with patients who acquire infection and develop systemic inflammation almost exclusively represented among the critically ill, whereas those who experience a lesser degree of systemic inflammation in response to infection remain unobtrusive and thereby difficult to identify. Thus the role of genetic factors in the generation of systemic inflammation is difficult to study in septic patients as the initial insult has already occurred and is complicated by an infective component. The ability to measure the activation of the systemic inflammatory response exists in a cardiac surgical setting. Systemic inflammation occurs after surgery in cardiac surgical patients in a sterile environment. Population studies which include all patients having cardiac surgery, would capture patients with differing propensity to develop systemic inflammation and may be an ideal environment to study the genetics of systemic inflammation.

1.2 Systemic Inflammatory Response Syndrome

1.2.1 Definition and Diagnostic Criteria

In 1991 the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) convened a “Consensus Conference”, in an attempt to provide a conceptual and practical frame-work to define systemic inflammation. The 1992 statement from the ACCP/SCCM Consensus Conference introduced into the term “systemic inflammatory response syndrome”(SIRS) (1992). The term provided a reference for systemic activation of the immune response, regardless of cause. SIRS was considered to be present when patients had more than one of the following clinical findings:

- Body temperature higher than 38⁰ C or lower than 36⁰ C
- Heart rate higher than 90/min
- Respiratory rate > 20/min or PaCO₂ < 32mmHg
- White blood cells > 12,000cells/μl or < 4,000/μl

The systemic inflammatory response can be triggered by a variety of infectious and noninfectious conditions. Signs of systemic inflammation can and do occur in the absence of infection among patients with burns, pancreatitis, and after cardiopulmonary bypass. However, in particular the 1992 criteria are unsuitable for identifying patients who develop SIRS post cardiopulmonary bypass. Patients undergoing cardiopulmonary bypass are ventilated postoperatively, may be paced and have undergoing active cooling and rewarming during the operation. This

makes the classic clinical findings of the systemic inflammatory response syndrome (i.e high temperature, fast heart rate and high respiratory rate) hard to interpret in these patients. In 2001 an International Sepsis Definitions Conference decided that the diagnostic criteria for SIRS published in 1992 were overly sensitive and non-specific (Levy et al., 2003). They suggested expanding the list of signs and symptoms in an attempt to redefine the systemic inflammatory response. This expanded version of signs and symptoms may be more useful in identifying patients who develop a systemic inflammatory response after cardiopulmonary bypass (table 1). As a result of this, we decided to use hypotension and hyperlactataemia as indicators of the systemic inflammatory response in our patient population

Table 1.1 *Expanded list of signs for diagnosis of systemic inflammation/sepsis.*

General parameters	
<i>Fever >38.3, Hypothermia <36</i>	<i>Heart rate >90 beats per minute,</i>
<i>Oedema (+ balance >20ml/kg over 24hrs)</i>	<i>Altered mental status</i>
<i>Hyperglycemia (glucose > 110mg/dl)</i>	<i>Respiratory rate >30 breaths per min</i>
Inflammatory parameters	
<i>Leukocytosis (WCC >12)</i>	<i>Leukopenia (WCC <4)</i>
<i>Plasma CRP >2SD above N</i>	<i>Procalcitonin >2D above N</i>
<i>Normal WCC with >10% immature forms</i>	
Haemodynamic parameters	
<i>Arterial Hypotension SBP <90mmHg</i>	<i>Mixed Venous O2 sat <70%</i>
<i>Cardiac Index >3.5l/min/m²</i>	<i>PaO₂/FiO₂ <300</i>
<i>Oliguria <0.5ml/kg x 2hrs</i>	<i>Cr increase >0.5mg/dl</i>
<i>Coag (INR > 1.5, Aptt >60s)</i>	<i>Ileus</i>
<i>Plt <100,000/ul</i>	<i>Hyperbilirubinemia</i>
Tissue perfusion parameters	
<i>Lactate >3mmol/l</i>	<i>Decreased cap refill</i>

1.2.2 Predisposing factors to systemic inflammation

1.2.2.1 The Systemic Inflammatory response to Cardiac Surgery

Cardiac surgery provokes a vigorous inflammatory response, which has important clinical implications. At least 12.6% of cardiac surgical patients develop systemic inflammation after cardiac surgery (Taneja et al., 2001). This pathophysiologic condition reflects a hyperdynamic circulation resulting in a high cardiac output state with reduced systemic vascular resistance, requiring treatment with fluid replacement and agents that help increase the blood pressure (Hall et al., 1997). Subsequently, lactic acidosis can occur with the risk of bleeding, respiratory distress, kidney dysfunction and ultimately multi-organ failure (Cremer et al., 1996). Although systemic inflammation after cardiac surgery is usually brief, coagulopathy with bleeding necessitating operation, and prolonged mechanical ventilation are documented sequelae. In addition, where organ dysfunction is severe, or in those with limited functional reserve, systemic inflammation after cardiac surgery can result in increased morbidity and mortality. Factors contributing to the inflammatory response after cardiac surgery are currently not well understood.

1.2.2.2 Influence of cardiopulmonary bypass on systemic inflammation

The modern era of cardiac surgery began when the technique for cardiopulmonary bypass was introduced in the early 1950's (Larmann and Theilmeier, 2004).

However, since its introduction it has become clear that cardiopulmonary bypass (CPB) is associated with an undesirable systemic inflammatory response. Many factors during CPB induce a complex inflammatory response e.g exposure of blood to nonphysiologic surfaces, surgical trauma, reperfusion to the organs after CPB, and release of endotoxin. Once initiated, the systemic inflammatory response is maintained by several factors. This consists of a broad range of host responses that include the production of protein and lipid mediators, expression of cell surface receptors and adhesion molecules, induction of enzymes and production of acute phase proteins (Hall et al., 1997). Activation of pro-coagulation proteins as well as fibrinolytic activators and inhibitors on the endothelial surface is also a feature and this represents an important link between the inflammatory, coagulation and fibrinolytic systems (Paparella et al., 2004). This complex chain of events has strong similarities with sepsis.

1.2.2.3 Effects of Anaesthetic technique on systemic inflammation

Few studies have investigated the effect of the anaesthetic technique on systemic inflammation. The assessment of the potential effects of different anaesthetics is difficult, since different factors may affect the perioperative balance between the pro and anti-inflammatory mechanisms. Conflicting data have been published on the effect of anaesthetics on the systemic inflammatory response syndrome. Some studies have shown that intravenous anaesthetics have anti-inflammatory effects in vitro. However, Brix-Christensen et al found no significant difference in cytokine production when they compared a high-dose fentanyl versus a low-dose opioid

anaesthetic technique (Brix-Christensen et al., 1998). While numerous reports have demonstrated similar changes under both intravenous and inhalational anaesthesia, other studies have observed both increases or decreases in proinflammatory cytokines using different inhalational agents (Kawamura et al., 2006, Corcoran et al., 2006). The inconsistencies between studies may be due to heterogenous patient groups with different pre-existing diseases, major differences in duration and severity of tissue injury and the small number of randomised studies.

1.3 Lactic Acidosis

Hyperlactataemia (high blood lactate levels) was included in the list of signs for diagnosing the systemic inflammatory response syndrome at the International Sepsis Definition Conference in 2001. Lactic acid was first isolated from sour milk by Scheele in 1780 (Fall and Szerlip, 2005). In 1918, Cannon made an important observation that metabolic acidosis was associated with decreased blood flow and shock. Shortly thereafter, Clausen introduced an assay for lactate, and subsequently it was demonstrated that the accumulation of lactic acid accounted for the metabolic acidosis described in diabetic patients without ketoacidosis (Fall and Szerlip, 2005). Lactic acid exists as two stereoisomers, L-lactic acid and D-lactic acid. In healthy humans, serum lactate is considered entirely L-lactate, as this is the isomer exclusively produced in the body (Uribarri et al., 1998).

1.3.1 Metabolism of Lactate

To understand the clinical syndrome of lactic acidosis requires knowledge of normal lactate metabolism. Lactate is a metabolic end product of anaerobic glycolysis and is produced by the reduction of pyruvate. Under hypoxic conditions glucose is glycolytically converted to pyruvate and lactate is used as substrate to produce ATP by the enzyme lactate dehydrogenase with NADH as a co-factor. Although lactate is produced in all tissues, skeletal muscle, brain, red blood cells and renal medulla are responsible for the majority of production (Uribarri et al.,

1998, Fall and Szerlip, 2005). Once produced, lactate can be either slowly oxidized in cells or rapidly converted back to glucose in the liver.

1.3.2 Lactic acidosis and systemic inflammation

The most common cause of lactic acidosis in the intensive care setting is the systemic inflammatory response syndrome (Stacpoole et al., 1994). Because these patients are frequently haemodynamically unstable, it has been assumed that the increase in lactate production is the result of inadequate oxygen delivery and poor tissue perfusion (Hurtado et al., 1992). However, more recently this belief had been challenged. These studies have shown that increased pyruvate production, decreased PDH activity, regional differences in lactate production, and release of lactate from lung tissue are all possible mechanisms of lactic acidosis in SIRS (Gore et al., 1996, Brown et al., 1996, Vary et al., 1998). In addition, lactate levels have been shown to correlate with the development of Multi-Organ Dysfunction Syndrome (MODS) and prolonged hyperlactataemia has been associated with increased mortality (McNelis et al., 2001).

1.3.3 Causes of Lactic acidosis after cardiac surgery

Systemic inflammation after cardiac surgery is invariably accompanied by lactic acidosis (Hall et al., 1997). Lactic acidosis in shocked cardiac surgical patients is a result of excess lactic acid production with unchanged utilisation and is not related to altered carbohydrate metabolism (Chiolero et al., 2000). Excess lactic acid accumulation after CPB has been attributed to decreased splanchnic blood flow (i.e

blood flow to the internal organs) during surgery. However Haisjackl, measuring splanchnic blood flow with indocyanine green and using gastric tonometry for mucosal pH measurement, found no evidence of reduced splanchnic blood flow (Haisjackl et al., 1998). Indeed post CPB splanchnic blood flow and lactic acid levels were both increased compared to pre CPB levels suggesting that splanchnic lactic acid production after cardiac surgery is related to systemic inflammation and not hypoperfusion (reduced blood flow).

Decreased lactate clearance has also been demonstrated with SIRS in animal models. Chrusch et al found a combination of increased splanchnic production and decreased hepatic clearance of lactate in a model of canine sepsis (Chrusch et al., 2000). Severin et al demonstrated decreased hepatic lactate clearance even in haemodynamically stable septic rats (Severin et al., 2002). Therefore, lactic acidosis in patients with SIRS not only develops in haemodynamically unstable patients but also can occur in the setting of adequate perfusion and oxygenation. This leads us to believe that lactic acidosis is not a result of hypoperfusion and hypoxia but rather that other factors contribute to the increase in lactate production.

1.3.4 Relationship between lactate production and cytokine release.

There are a few studies documenting the effects of lactate on the synthesis and release of inflammatory mediators. Lactic acid has been shown in one study to increase TNF release in mononuclear cells (Steele et al., 1998). Vary et al., using a rat model of sepsis linked TNF with excess lactic acid production by showing that

anti TNF antibody reversed both TNF mediated pyruvate dehydrogenase inhibition and excess lactate production (Vary et al., 1998). Thus their results indicate that lactic acid production in systemic inflammation appears to be a manifestation of excess TNF rather than hypoperfusion (poor blood flow). Other cytokines have been associated with lactate. Hack et al reported a direct correlation between IL6 and lactate in septic shock (Hack et al., 1989). Furthermore, IL6 has been shown to be a prognostic marker with regard to outcome in patients with SIRS.

1.4 Genetic polymorphisms

Since Watson and Crick introduced in 1953 the molecular structure for deoxyribonucleic acid, molecular biology has revolutionized medicine by increasing our understanding of the pathophysiological mechanisms of diseases. Genetic polymorphisms account for traits and varied susceptibility to complex diseases. A genetic polymorphism is an allelic variant that occurs in >1% of the population, is stable in frequency, and cannot be accounted for by new mutations.

1.4.1 Importance of Genetic Polymorphisms on Disease Susceptibility

Susceptibility to many diseases appear to have a genetic component. A study by Sorensen et al in 1988 looked at the genetic influences on the risk to die from various diseases (Sorensen et al., 1988). The authors followed 960 families that included adopted children. The risk of dying from specific groups of diseases for adoptees with a biologic or adoptive parent, who died of the same cause before, was compared. The death of a biological parent before the age of 50 resulted in relative risks of death in the adoptees of 1.71 for all causes, 1.19 for cancer, 4.52 for cardiovascular and cerebrovascular causes, and 5.81 for infections. Therefore, genetic factors appear to play an important role in the susceptibility to sepsis/systemic inflammation.

1.4.2 Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide (A, adenine; T, thymine; C, cytosine; G, guanine) in the genome sequence is altered. For a variation to be considered a SNP, it must occur in at least 1% of the population and SNPs occur approximately one in every 300 base pairs in the human genome (2003). SNPs constitute a major component to human genetic variation (Brookes, 1999). Changes to protein structure may be defined as conservative or non-conservative and may or may not alter the structure and or function of the gene product. SNPs in the promoter or other regulatory regions of a gene may change the binding affinity of nucleic acid binding proteins (an obvious example of which are transcription factors) thus altering the rate of transcription and/or translation leading ultimately to changes in protein levels. Besides influencing the rate of transcription directly, SNPs in coding regions can influence gene expression in other ways; for example by altering the stability or otherwise the availability of transcribed mRNA. Finally it should be noted that distal elements (such as enhancers and silencers) may influence gene expression from considerable distances either upstream or downstream of a gene.

1.4.3 Importance of Haplotype analysis

Single nucleotide polymorphic alleles at linked loci are often correlated (described as linkage disequilibrium (LD)) and coinherited in non-random configurations on chromosomal sections called haplotypes. Most of the genome exists in regions of

pronounced LD, called haplotype blocks, within which a limited subset of all possible haplotypes are found at appreciable frequencies for a given population (Gabriel et al., 2002). Within these blocks, a relatively small number of SNPs can mark common haplotypes and capture some of the genetic diversity in a sample (Patil et al., 2001, Daly et al., 2001). Haplotype analysis can increase the power to detect disease associations. It allows for the possibility of an ungenotyped functional variant to be in linkage disequilibrium with the genotyped polymorphism. Many common diseases in humans such as systemic inflammation/sepsis are not caused by one genetic variant within a single gene, but are determined by complex interactions among multiple genes, environmental and lifestyle factors (De Maio et al., 2005). Presumably functional genetic factors can confer either susceptibility or resistance to a particular disease and thereby influence its severity or progression of disease. By studying SNP profiles or haplotypes associated with systemic inflammation, we may begin to understand the genetic profiles of individuals who are susceptible to inflammation, sepsis and multiorgan failure after cardiac surgery. While the utilization of these SNPs and common haplotypes in genetic association studies has been recognized as an efficient and effective method to localise disease susceptibility variants (2003), because only a subset of SNPs are actually assayed a study may fail to detect a causative functional genetic variant. With the development of new genomic technologies, it has become apparent that large numbers of genomic imbalances called copy number variations exist. Copy number variations (CNV's) are defined as deletions and duplications of

DNA segments larger than 1000 bases that are present in variable copy numbers compared with a reference genome. They have recently been identified as a significant component of the human genome responsible for a considerable proportion of genetic variation. In addition, CNV's have been linked to disease susceptibility including Alzheimer's disease, coronary artery disease and diabetes mellitus. Although some disease-related CNV's may be detected via SNP analysis, many others are either not in linkage disequilibrium or are in genomic areas that have insufficient SNP's to detect these CNV's. Finally it should be noted in passing that non-linked loci may effect a considerable influence over gene expression at different sites through the action of regulatory RNA molecules such as micro-RNAs. It is becoming increasingly apparent that these molecules have important and complex regulatory roles that allow for sophisticated control of gene expression in tissue specific, temporal and developmental modes. However, these are poorly described and may well be unlinked to regulated sequences and therefore not amenable to current genetic mapping strategies.

1.5 Role of Cytokines in systemic inflammation

Cytokines are early mediators of the systemic inflammatory reaction. They serve to initiate the systemic inflammatory response and determine the magnitude and nature of the immune response. Cytokine synthesis and release are generally a brief regulated, self limiting event. The release of cytokines is triggered by many different stimuli including endotoxin exposure, tissue damage and oxidative stress (Larmann and Theilmeier, 2004). Cytokines appear to have a local effect and a systemic effect (due to the consequences of high cytokine levels circulating in the blood). The local effect involves recruitment of phagocytic cells, essential for the elimination of noxious stimuli, while the systemic effects (if prolonged or excessive) can have long lasting deleterious consequences. Excess production or activity of cytokines can lead to tissue injury, respiratory disease, shock with decreased perfusion (blood flow) to organs leading to multi-organ failure such as renal, cardiac and respiratory failure. Clinical manifestations of systemic cytokine release include fever, reduced level of consciousness, haemodynamic instability, high lactate levels and myocardial depression (Hennein et al., 1994, Dinarello, 2000). These features can also be found in the early postoperative course after CPB. In general, cytokines are not stored as preformed molecules, and their synthesis is limited to newly transcribed mRNA. Monocytes-macrophages produce $\text{TNF}\alpha$, IL1, IL6 and IL8 in response to an activating stimuli (Hagiwara et al., 1995). These peptides act on other cells and blood elements such as polymorphonuclear cells, endothelial cells, fibroblasts, platelets and even monocytes themselves. These

cytokines induce the production and release of other mediators. TNF α appears to be a key mediator in the systemic inflammatory response syndrome (Giroir, 1993). TNF α , IL1 and IL6 stimulate the synthesis of acute phase proteins by the liver. They also prime the lymphocyte response. The important biological effects of TNF α and IL1 can be modulated by IL10 (Moore et al., 2001). IL10 is an anti-inflammatory cytokine produced by macrophages/monocytes and subsets of T cells which lowers the production of TNF α and IL1 by macrophages and monocytes (Fiorentino et al., 1991). In addition, IL10 appears to enhance clotting activity (Pajkrt et al., 1997). Other anti-inflammatory cytokines which specifically inhibit TNF α and IL1 have been detected in the blood such as IL1 receptor antagonist, and soluble receptors for TNF α and IL1. The balance of pro-inflammatory and anti-inflammatory responses may be important in determining the extent of the inflammatory response and clinical outcome.

1.5.1 Cytokine production after cardiopulmonary bypass.

The cytokine response after cardiac surgery has previously been investigated at a protein level, with some studies failing to detect any TNF α and others documenting a brief but variable level of TNF α and IL10 production immediately after surgery (Roth-Isigkeit et al., 2001, McBride et al., 1995, Wei et al., 2001). These small studies failed to differentiate between patients with uncomplicated recovery and those who require inotropic support or develop hyperlactataemia after surgery.

Furthermore, measured plasma cytokine protein may not be a good index of cytokine activity after surgery, as many of these cytokines bind to cell surface receptors with such high affinity that unbound protein levels can not be measured (Munoz et al., 1991, Brix-Christensen et al., 2003, Butler et al., 1993, Misoph and Babin-Ebell, 1997). It is notable that the ligand receptor dissociation constant for both TNF α and IL10 are several orders of magnitude greater than the dissociation constant for other common biologic receptor ligand interactions, such as the interaction between antigen and immunoglobulin (Grell et al., 1998). An alternative approach to quantifying the cytokine components of inflammation by measuring cytokine protein levels would be useful. It is possible to accurately quantify inflammatory cell mRNA levels using a real-time polymerase chain reaction (PCR) assay, and this might represent an important index of cytokine mediated inflammation. Furthermore, a recent study by Fu et al discovered a positive correlation between mRNA expression and protein expression (Fu et al., 2007). Therefore, mRNA not only reflects the DNA genetic code but may also give us some insight into what is occurring at the protein level.

1.5.2 Tumour Necrosis Factor

Tumour Necrosis Factor was named over 20 years ago, on the basis of its ability to kill tumour cells in vitro and cause haemorrhagic necrosis of transplantable tumours in mice (Carswell et al., 1975). Human TNF α was purified and its complementary DNA cloned in 1984 (Pennica et al., 1984). TNF α is initially synthesized as a

preprotein of 233 amino acids, from which the N-terminal 76 amino acids are later removed (Wang et al., 1985). TNF α occurs in two forms, as a cell membrane protein (26 kd) which can be cleaved into a soluble form (17 kd) by a specific TNF α converting enzyme (TACE)(Black et al., 1997). The primary sources of TNF α synthesis include monocytes/macrophages and T cells. Although the half life of TNF α is less than 20 minutes, this brief appearance is sufficient to evoke marked metabolic and haemodynamic changes and activate mediators distally in the cytokine cascade (Tonnesen et al., 1996). TNF α is known to be a pleiotropic cytokine with many complex effects which can be viewed as both beneficial and harmful in the context of human disease (Bazzoni and Beutler, 1996). On the one hand TNF α is essential for a proper immune response to be mounted; on the other hand, it is clear that TNF α plays a key role in orchestrating the inflammatory response which contributes to numerous inflammatory diseases. With particular reference to this study, TNF α is a central endogenous mediator of endotoxic shock which is a manifestation of an acute systemic inflammatory response.

TNF α initiates signalling responses by binding to at least two receptors; TNF receptor 1(p55) and TNF receptor 2 (p75) which are expressed on most nucleated cells (Engelmann et al., 1990). However, although both receptors are expressed on cells, one is typically dominant. *In vivo* studies have suggested that p55 receptor binding is critical primarily for TNF α 's proinflammatory properties (Van Zee et al., 1994). These show that administration of p55 TNF α receptor agonists to healthy baboons produced hypotension (low blood pressure), tachycardia and leukocytic

changes simulating a systemic inflammatory response. In another study, antibodies that prevented TNF α binding to the p55 receptor and not the p75, protected mice from lethal endotoxic shock but blocked development of a protective response against infection with *Listeria monocytogenes* (Sheehan et al., 1995).

Furthermore, TNF α stimulates mononuclear phagocytes and other cell types to secrete chemokines that contribute to leukocyte recruitment and activation of neutrophils and other mononuclear phagocytes (Dinarello, 2000). TNF α , an endogenous pyrogen, stimulates prostaglandin and nitric oxide synthesis, and can induce a fever directly or through the secondary induction of IL1 and IL6. In addition, these three cytokines act on hepatocytes to increase synthesis of acute phase proteins like C-reactive protein, α_1 antitrypsin, and haptoglobin.

1.5.2.1 Genetics of Tumour Necrosis Factor Alpha

The gene encoding *TNF α* is located within the major histocompatibility complex (MHC) on chromosome 6p21.3. Multiple bi-allelic polymorphisms have been identified in the human *TNF α* promoter region. These may influence TNF α production and hence inflammatory responses. A *TNF α* polymorphism at position -308 involves replacement of guanine with adenine. Mira et al. studied six *TNF α* gene polymorphisms in patients with sepsis (Mira et al., 1999). They found an association between alleles at the G-308A position (*TNF*) and the septic group where the *TNF2* (-308A) allele was associated with a 3.75 fold increase in the probability of dying. McGuire et al in a study of malarial children found that homozygotes for the *TNF2* allele had a seven fold increase in the relative risk of

death or severe neurologic disease and that this effect was independent of HLA variation (McGuire et al., 1999). Previous studies have associated the *TNF2* (-308A) allele with increased TNF α production (Wilson et al., 1997). Nadel studied children with meningococcal disease and determined that carriage of the *TNF2* allele was associated with a significantly increased risk of severe disease and death (Nadel et al., 1996). A bi-allelic *NcoI* restriction fragment length polymorphism has also been described within the first intron of the *TNF β* gene (which is situated contiguous to *TNF α*) at position +252 with an adenine to guanine substitution (Wilson et al., 1992). The *TNFB2* (A allele) has also been associated with higher TNF α levels in patients with sepsis (Stuber et al., 1996). However, Stuber inadvertently links a polymorphism associated with lesser TNF β production with both excess TNF α production and mortality (Stuber et al., 1996). Other studies, by Westendorp and van Dissel, which examine cytokine levels suggest that infection related deaths are linked to a deficit in TNF production and excess IL10 production (Van Dissel et al., 1998, Westendorp et al., 1997). There is some discordance in the data provided by these studies with both high and low TNF α associated with mortality. This may partly be explained by the heterogenous group of patients studied and the diverse causative organism in a septic population. Therefore, the value of genotyping for TNF α as a prognostic marker for systemic inflammation requires further investigation. For example several autoimmune diseases such as rheumatoid arthritis have been successfully treated with anti-TNF agents. However, no benefit was shown in the treatment of the systemic inflammatory response

syndrome or sepsis. In addition, it must be remembered that although TNF α is an important mediator, other cytokine related polymorphisms may play a role in determining susceptibility to SIRS.

1.5.3 Interleukin 10

Interleukin 10 is an anti-inflammatory cytokine expressed and secreted by a variety of cell types, including T cells, monocytes/macrophages, dendritic cells and epithelial cells usually after an activation stimulus such as cardiopulmonary bypass (Moore et al., 2001). It circulates as a homodimer consisting of two tightly packed 160 amino acid proteins. After engaging its high-affinity 110-kd cellular receptor, IL10 inhibits monocyte/macrophage-derived TNF α , IL1 β , IL8 and nitric oxide production (Moore et al., 2001). It also inhibits the generation of cytokines by neutrophils and natural killer cells (Opal and DePalo, 2000). Prior exposure to IL10 inhibits nuclear factor kappa β (NF κ B) translocation in response to lipopolysaccharide stimulation. In addition, IL10 attenuates surface expression of TNF receptors and promotes the shedding of TNF receptors into the systemic circulation (Moore et al., 2001, Opal and DePalo, 2000). Therefore IL10 appears to inhibit non-specific inflammatory responses. In fact, inflammation is a major stimulus for the production of IL10 since previous studies have shown that both IL1 and TNF α can stimulate IL10 production directly indicating the existence of a negative feedback loop (Berg et al., 1995).

1.5.3.1 Genetics of Interleukin 10

The *IL10* gene is located on chromosome 1 (1q31-32). IL10 secretion in response to LPS shows a large interindividual variation which has a genetic component of over 70% (Westendorp et al., 1997). Polymorphisms within the *IL10* gene may plausibly account for different levels of IL10 production. The SNP's at position -1082 (A/G), -819 (T/C) and -592 (A/C) are in strong linkage disequilibrium (Fife et al., 2006). These variants have been reported to influence the transcription rate of *IL10* and therefore the production of this cytokine. Turner associated reduced IL10 secretion with the presence or absence of an 'A' at position 1082 of the human IL10 promoter region (Turner et al., 1997). The frequency of the low producing 1082 A/A genotype was shown to be increased in patients with acute myocardial infarction in a study by Lio (Lio et al., 2004). In addition, Balding showed that the frequency of the IL10 1082 A allele was higher in patients with severe meningococcal disease (2003). However, other studies have found conflicting results. To assess the genetic influence on IL10 production, Westendorp determined the capacity to produce IL10 in families of patients who had meningococcal disease. Families with high IL10 production to endotoxin stimulus had a 20 fold increased risk of fatal outcome from meningococcal disease (Westendorp et al., 1997). Gallagher associated high IL10 production and the 1082 G allele with increased mortality in patients with SIRS due to pneumonia (Westendorp et al., 1997, Gallagher et al., 2003). Lowe demonstrated that another IL10 polymorphism (the -592 A allele) was significantly associated with death in critically ill patients (Lowe et al., 2003).

The IL10 promotor SNP's -1082A, -819T, and -592A (ATA haplotype) have been associated low IL10 production. A study by Opdal associated the ATA haplotype with sudden unexpected infant death due to infection (Opdal et al., 2003). Conversely, frequency of the ATA haplotype is higher in patients who are asymptomatic carriers of hepatitis C than in patients with chronic progressive liver disease (Mangia et al., 2004). Overall, the evidence supporting a role for IL10 polymorphisms in determining outcome in inflammatory disease states is controversial and requires further investigation.

1.5.4 Cardiac surgery and cytokine related genetic polymorphisms

Genetic polymorphisms in genes coding for cytokines have been associated with poor outcome after cardiac surgery. Tomasdottir et al related a *TNF β -252* polymorphism to increased TNF α levels which was associated with prolonged ICU stay and cardiopulmonary dysfunction after cardiac surgery (Tomasdottir et al., 2003). Polymorphisms in the promoter of *IL6* promoter (-572 G-C and -174 G-C) have been associated with higher postoperative plasma IL6 levels and prolonged hospitalization after cardiac surgery with CPB. A study by Podgoreanu et al found that the -572 G IL6 polymorphism was an independent predictor of postoperative myocardial infarction. (Podgoreanu et al., 2006). Gaudino provided evidence that the IL6 -174 G allele correlated with IL6 levels (Gaudino et al., 2003) with G/G

homozygous patients having higher IL6 levels and a greater incidence of postoperative renal and pulmonary dysfunction after cardiac surgery.

1.6 Fibrinolytic system

The plasminogen activator (PA)/plasmin system, also known as the fibrinolytic system serves as one of the endogenous defense mechanisms for the prevention of intravascular thrombosis (Vaughan, 2005). Fibrin deposition occurs on activation of the coagulation cascade. This results in the ultimate conversion of prothrombin into thrombin, which then catalyzes conversion of soluble fibrinogen to fibrin. Fibrin removal occurs on activation of the fibrinolytic system. This system comprises an inactive proenzyme (plasminogen) that is converted to the active enzyme (plasmin) that degrades fibrin into soluble fibrin-degradation products. Two immunologically distinct types of physiological plasminogen activators have been identified in humans: tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (u-PA) (Lijnen and Collen, 1997). t-PA mediated plasminogen activation is mainly involved in the dissolution of fibrin in the circulation. u-PA binds to a specific cellular receptor resulting in enhanced activation of cell-bound plasminogen. Plasminogen activation is regulated by the presence of very specific and rapid acting, plasminogen activator inhibitors that are also present in plasma (Lijnen et al., 1994). When the processes of fibrin deposition and removal are

properly regulated, they perform their physiologic functions remarkably well.

However, when unbalanced, the consequences can lead to an increased tendency to bleed or conversely an increased tendency for thrombotic events.

1.6.1 Regulation of the fibrinolytic system

Regulation of the fibrinolytic system occurs via 1) inhibition of plasmin by α_2 -antiplasmin, 2) thrombin-activatable fibrinolysis inhibitor which attenuates plasmin generation and 3) PAI-1, the primary inhibitor of both plasminogen activators (Lijnen and Collen, 1995). Together these inhibitors constitute a powerful, negative regulatory system for controlling the formation and activity of plasmin.

Plasminogen activator inhibitor (PAI-1) inhibits plasminogen activators (u-PA and t-PA) by forming inert, covalent complexes. The rapid inhibition of both t-PA and u-PA involves a high affinity region of PAI-1. There appears to be no endogenous mechanism for recycling PA-PAI-1 complexes, which are cleared through the low-density lipoprotein-related (LRP) receptor and the VLDL receptor (Orth et al., 1992).

1.6.2 Plasminogen Activator inhibitor-1

PAI-1 was first identified in human endothelial cells and subsequently shown to be produced by vascular smooth muscle cells, mesangial cells, monocytes/macrophages and by stromal cells from adipose tissue (Orth et al., 1992). Except for platelets, which contain an inactive form of PAI-1, PAI-1 is not

stored within cells but is rapidly and constitutively secreted after synthesis (Lijnen, 2005). Synthesis and secretion of PAI-1 can be modulated by various agonists such as endotoxin and cytokines (Sawdey et al., 1989). PAI-1, a member of the serpin family, is a single-chain glycoprotein of about 45 kDa consisting of 379-381 amino acids (Lijnen, 2005). PAI-1 reacts very rapidly with t-PA and u-PA. In humans, increased levels of PAI activity resulting in a decreased fibrinolytic capacity have been reported in several thrombotic disease states, including thromboembolism, coronary artery disease and acute myocardial infarction (Hamsten et al., 1987, Ridker et al., 1992). Excessive fibrinolysis due to decreased PAI-1 levels has been reported in a few cases. A complete deficiency of PAI-1 has been associated with episodes of major haemorrhage, all in response to trauma or surgery (Schleef et al., 1989, Minowa et al., 1999). The effect of PAI-1 gene disruption on haemostasis, thrombosis and thrombolysis has been investigated in mice. Spontaneous bleeding or delayed bleeding was not observed after partial amputation of the tail or the caecum in PAI-1 deficient mice (Carmeliet et al., 1993). This is in contrast to the delayed rebleeding observed after trauma or surgery in patients with reduced or absent PAI-1 levels. This difference may be due to the 5-fold lower basal plasma levels of active PAI-1 in wild-type mice than in man. PAI-1 has also been shown to inhibit vascular wound healing via regulation of cell adhesion and migration (Lijnen, 2005). Elevated plasma PAI-1 levels have been associated with the progression of atherosclerosis, by inhibiting the clearance of fibrin incorporated into atherosclerotic plaques.

1.6.2.1 Genetics of Plasminogen Activator Inhibitor-1

PAI-1 expression has been observed in various cell types including mononuclear cells, and multiple regulatory factors have been identified that play a role in PAI-1 transcription. The human PAI-1 gene is mapped on chromosome 7q21.3-q22 and several polymorphisms within the PAI-1 gene have been described (Strandberg et al., 1988, Westendorp et al., 1999). While the genetic architectures of PAI-1 have not been fully elucidated, there is accumulating evidence suggesting that interindividual variation in plasma enzymes such as PAI-1 is significantly influenced by polymorphisms. A common functional polymorphism exists in the *PAI-1* promoter. A single base pair insertion (5G)/deletion (4G) polymorphism 675bp upstream from the start of transcription is functionally important in regulating the *PAI-1*. Artificial constructs containing the *PAI-1* promoter have shown that the 4G allele produces six times more RNA than the 5G allele in response to interleukin 1 β (Dawson et al., 1993). Individuals homozygous for the 4G allele have higher basal and inducible concentrations of PAI-1 than those with one or two copies of the 5G allele (Dawson et al., 1993, Hermans et al., 1999). Transcription studies of this promoter region revealed that both alleles bind a transcription activator (at position -683 to -676), whereas the 5G allele also binds a repressor protein to an overlapping binding site (at position -672 to -676). The extent to which this polymorphism influences PAI-1 protein levels has also been investigated. A study of 464 German twins estimated the heritability of PAI-1 expression levels to be 0.44 (Peetz et al., 2004). This study estimated that

approximately 56% of the PAI-1 heritability is explained by interindividual variation in the *PAI-1* 4G/5G polymorphism. In addition, Asselbergs found that the 4G/5G polymorphism is a significant predictor of plasma PAI-1 levels (Asselbergs et al., 2006).

1.6.3 Plasminogen Activator Inhibitor and Systemic Inflammation

The systemic inflammatory response is a complex clinical syndrome that involves the activation of many cells and cascade reactions. Activation of the coagulation and fibrinolytic systems are an integral part of the inflammatory response and is an important pathogenic factor in sepsis associated organ injury (Idell, 2001, Johnson et al., 1998). Endotoxin infusions in healthy individuals induces up-regulation of both tissue plasminogen activator and plasminogen activator inhibitor (PAI-1) (Suffredini et al., 1989). This is not surprising as PAI-1 is an acute phase protein, and is increased by inflammatory stimuli such as interleukin 1 β and tumour necrosis factor (Dawson et al., 1993, Ryan et al., 1996). The inflammatory process leads to the production of a great number of inflammatory mediators. These mediators induce tissue factor- mediated thrombin production and activation of the coagulation system (Binder et al., 2002). Ultimately this can result in microvascular fibrin deposition, platelet depletion and disseminated intravascular coagulopathy leading to multiorgan failure (Hermans and Hazelzet, 2005, Kidokoro et al., 1996). During this process there is rapid but transient activation of the fibrinolytic system,

which is downregulated by a sustained increase in plasma PAI-1 levels (Biemond et al., 1995, Suffredini et al., 1989). The increase in PAI-1 effectively eliminates plasma t-PA leaving undissolved fibrin deposits which is commonly seen in sepsis and multiorgan failure (Levi et al., 2003). PAI-1 levels correlate closely with the severity of disseminated intravascular coagulation and disease. Substantially increased PAI-1 levels are observed during acute lung injury, experimental sepsis, endotoxemia in volunteers, and meningococcal sepsis (Schultz et al., 2004, Hermans et al., 1999).

1.6.4 Influence of Cardiac Surgery on PAI-1 levels

Previous studies measuring PAI-1 after cardiac surgery have shown inconsistent results, with most but not all studies noting an increase in PAI-1 activity (Valen et al., 1994, Dixon et al., 2005, Chandler and Velan, 2003). PAI-1 protein levels in plasma can be measured as either PAI-1 antigen or PAI-1 activity. PAI-1 antigen comprises both active and inactive PAI-1 and methods of measurement vary considerably (Declerck et al., 1993, Hoekstra et al., 2004). PAI-1 activity can also be used as a marker of plasma PAI-1, however endogenous plasma constituents, can interfere with accurate determination of this assay (Chiu et al., 2000). Furthermore PAI-1 is a labile protein with an *in vivo* half-life of 30 minutes, thus requiring prompt measurement (Kooistra et al., 1986). Valen et al found that cardiopulmonary bypass was associated with an increase in tPA and a decrease in PAI-1 levels (Valen et al., 1994). In contrast, Chandler et al noted a heterogeneous fibrinolytic response

after cardiac bypass with either an increase or no change in active PAI-1 (Chandler and Velan, 2003). Inhibition of gene expression may result in undetectable protein levels which can be difficult to quantify with the current assays available. The difference in measurement technique may account for discordance between the results of these studies.

1.6.5 Functional Relevance of 4G/5G PAI-1 polymorphism

Concentrations of PAI-1 in plasma are very high in children with meningococcal sepsis, with the highest concentrations being found in severe and fatal disease (Kornelisse et al., 1996). Carriage of the 4G deletion polymorphism in the PAI-1 gene has been associated with increased mortality from meningococcal sepsis (Hermans et al., 1999). In a similar study, Westendorp analysed 50 patients who survived meningococcal infection and 131 control subjects from the same geographic region, as well as 183 first-degree relatives of patients with meningococcal infection for the 4G/5G polymorphism (Westendorp et al., 1999). Because Westendorp had no information on genotypes of patients who died, he included first-degree relatives of patients with meningococcal infection to avoid under-representation of the patients who did not survive. The 5G/5G genotype was more common among relatives of patients with meningitis (31% vs 11%, $p=0.001$). However, patients whose relatives were carriers of the 4G/4G genotype had a 6 fold higher risk of developing septic shock as opposed to meningitis.

In a study of severely traumatised patients, Menges et al demonstrated that the 4G/4G PAI polymorphism is associated with poor prognosis and outcome after severe trauma (Menges et al., 2001) The PAI-1 4G allele is associated with high PAI-1 plasma concentrations and a poor survival rate in these patients. Conversely, as previously mentioned a few studies have implicated deficiencies in PAI-1 activity with an increased likelihood of haemorrhagic events (Schleef et al., 1989, Fay et al., 1997).

1.7 Hypothesis and Aims

1.7.1 Aim #1

The hypothesis that interindividual variability in cytokine gene expression after cardiac surgery is associated with clinically important events, can be investigated by measuring change in cytokine mRNA before and after cardiac surgery using real-time PCR. With this approach, the interrelation of pro-inflammatory and anti-inflammatory cytokine gene expression in the pathophysiology of systemic inflammation could be examined in vivo. Hence, the aim of the study was to investigate the relation between cytokine gene expression in peripheral blood mononuclear cells, genotype and clinical events after cardiac surgery.

1.7.2 Aim #2

The purpose of this study was to examine the temporal pattern of changes in PAI-1 messenger RNA (mRNA) levels in peripheral blood mononuclear cells in patients undergoing cardiac surgery, to determine whether PAI 4G/5G gene polymorphism influences PAI-1 mRNA expression after cardiac surgery, and to obtain preliminary information on the relation between PAI-1 mRNA levels and clinical outcomes. We hypothesized that PAI-1 gene expression and indices of systemic inflammation in cardiac surgical patients might be linked. Furthermore the pattern of change in PAI-1 gene expression after cardiac surgery might be modulated by carriage of functional polymorphic PAI-1 alleles.

1.8 Summary

Systemic inflammation may occur after cardiopulmonary bypass, when it frequently presents as arterial vasodilation and lactic acidosis. Inflammation is cytokine mediated with tumour necrosis factor a recognized as a critically important molecule involved in the initiation, propagation and regulation of the systemic inflammataory response while interleukin 10 is a potent anti-inflammatory cytokine that down-regulates the proinflammatory response. The balance between the proinflammatory and anti-inflammatory response may significantly influence outcome after cardiac surgery. In addition, the inflammatory cascade is linked to the fibrinolytic system with both TNFa and IL1 influencing the release of PAI- I. Indeed, susceptibility to thrombosis has been confirmed in the setting of meningococcal septicemia, trauma, and deep vein thrombosis. Additionally, there are clinical data associating deficiencies in PAI-1 production with an increased likelihood of haemorrhagic events. Analyzing SNPs and haplotypes may help identify genetic predisposition to complications after cardiac surgery.

Chapter 2

Study Design and Methodology

2.1 Subject Recruitment

Following approval of the study protocol by the institutional ethics committee, a written informed consent was obtained for each patient before inclusion in to the study. Eighty-two patients scheduled for routine cardiac surgery were recruited over a 2 year period. Exclusion criteria included: renal impairment as defined by a creatinine concentration of $> 150\mu\text{mol/L}$, history of liver disease, coagulation disorders as defined by International Normalised Ratio (INR) > 1.5 , haematological malignancies, coronary artery bypass graft (CABG) surgery without cardiopulmonary bypass (CPB), emergency surgery, and use of corticosteroids or anticoagulant drugs before surgery.

2.2 Cardiac Surgery and Anaesthesia

Perioperative clinical care included anaesthesia with propofol, fentanyl, pancuronium, and isoflurane. Non-pulsatile CPB was performed using a membrane oxygenator (Cobe Inc, Denver, CO, USA) with flow maintained between 2.0 and 2.4 L/min/m². Suction systems were controlled. Body temperature was kept between 32 and 35⁰C during CPB. Heparin 3mg/kg was given to obtain an ACT of > 480 seconds prior to CPB and reversed after CPB with protamine (1:1 ratio of protamine with heparin). All patients were transferred to the ICU and controlled

mechanical ventilation was continued until the patient was ready for extubation.

Extubation was determined by a consultant anaesthetist.

2.3 Randomisation

Post operatively patients were divided into two groups;

- a) the uncomplicated group and
- b) the complicated group i.e those that developed a systemic inflammatory response within 24hrs.

The complicated group fulfilled the following criteria:

- 1) Hypotension including those requiring the infusion of a potent vasoconstrictor or an intra-aortic balloon pump to maintain mean blood pressure $> 70\text{mmHg}$, or
- 2) Hyperlactataemia as defined by lactate levels $\geq 4.0\text{mmol/L}$ within 24hrs post surgery.

2.4 Physiological Variables

Demographics such as age, gender, weight, preoperative chronic disease state and operative details were recorded on a patient information database (PATS system).

Relevant data was retrieved from a patient clinical information system (Care Vue,

Agilent Technology) including hourly haemodynamics, inotropic requirement, requirement for mechanical assist devices such as intra-aortic balloon counterpulsation, duration of mechanical ventilation and indices of oxygenation, coagulation parameters and blood loss in the first 24 hours after surgery, and duration of intensive stay.

2.5 Blood sampling protocol

2.5.1 RNA Extraction

Blood samples for RNA analysis were obtained at three time points: a) prior to cardiac surgery, b) 1 hour post cardiopulmonary pump, and c) 6 hours post cardiopulmonary pump. A 24hr sample was taken on a subgroup of patients who remained in ICU after 24hrs. Blood was collected in EDTA containing tubes and processed immediately. PBMC's (peripheral blood mononuclear cells) were obtained by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) using a standard protocol (Woollard et al., 2002, Bugalho et al., 2001). Briefly, this involved diluting 10ml blood samples in Hanks' buffered saline (1:1 dilution) (Life Technologies Inc.), and layering it on 5 ml of Lymphoprep. Each sample was spun at 1,200 rpm for 30 minutes, and the mononuclear cell interphase was collected into a fresh tube and washed with Hanks' medium for 10 minutes at 1,200rpm. The resultant cell pellet was reconstituted with Hanks Medium at 4⁰C and a cell count was performed. The cells were then spun for 10 minutes at 1,200

rpm thus producing a cell pellet. The cell pellets were stored in a lysis buffer (RLT buffer, RNeasy mini Kit, Qiagen, Sussex, England) at -80°C . Total RNA was extracted using RNeasy mini kit (Qiagen, Sussex, England). The RNA was treated with 10 units of RNase-free DNase for 15min during the extraction procedure in order to avoid amplification of contaminating genomic DNA. Concentration and purity of RNA was determined by measuring the absorbance at 260nm and 280nm on a spectrophotometer (Eppendorf, Biophotometer). Ribosomal RNA (28S rRNA and 18S rRNA) was visualized on a 1.2% agarose/formaldehyde gel following electrophoresis to demonstrate the integrity of the RNA.

2.5.2 DNA extraction

Blood was collected in ethylenediamine-tetraacetic acid (EDTA) tubes pre-operatively. DNA was extracted using the QIAamp DNA Blood Midi Kit (Qiagen, Sussex, England) according to manufacturers instructions, and DNA was stored at -20°C until amplification. Concentration of DNA was determined by measuring the absorbance at 260nm and the purity was assessed by calculating the ratio of absorbances at 260nm and 280nm on a spectrophotometer (Eppendorf, Biophotometer).

2.6 RNA processing

2.6.1 Reverse Transcriptase

Total RNA was reversed transcribed to produce complementary DNA (cDNA) using a murine Moloney Leukaemia Virus (mMLV) reverse transcriptase. Reverse

transcription was performed at 37⁰ C for a duration of 60 min in a final volume of 30µl containing: 500ng sample RNA, 1.25µl (250U) mMLV reverse transcriptase (Promega,) with 2µl random hexamers (pd (N)₆), 0.2mM dNTP's, 0.6µl RNAsin 1U/µl, 10mM dithiothreitol, 4.5µl 11% DMSO and 6µl 5 X RT buffer (250mM Tris-HCL, pH 8.3, 375 mM KCl, 15mM MgCl₂). The cDNA was analysed immediately or stored at -20⁰C for later analysis.

2.6.2 Real time PCR

The extent of cytokine gene expression after cardiopulmonary bypass was determined using quantitative real time RT-PCR. Messenger RNA for interleukin 10 (IL10), plasminogen activator inhibitor 1 (PAI-1) and tumour necrosis Factor alpha (TNF α) were measured. Complimentary DNA (cDNA) was used as a template for the polymerase chain reaction. All samples and controls were assayed in triplicate in 25µl reactions. Quantification of mRNA using group-specific primer pairs and FAM probes (Assay by Demand, Applied Biosystems Perkin Elmer, Foster City, CA) for the detection of IL 10, PAI-1 and TNF α was carried out on an ABI PRISM GeneAmp 7000 Sequence Detection System (Applied Biosystems Perkin Elmer, Foster City, CA) with real-time detection and quantification taking advantage of the fluorescence TaqMan technology.

The reaction conditions were: Taqman Universal Mastermix (12.5µl), template cDNA (50ng), Assay on demand (1.25µl). The balance of the 25µl reaction was made up with RNase free H₂O. Real time quantification was carried out under the

following conditions: 50⁰ C for 2minutes, 95⁰ C for 10minutes and then 40 cycles of 95⁰ C for 15s and 60⁰ C for 1minute. Normalisation of cDNA templates was achieved by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) quantification. All probes used in the Taqman reaction were designed to have nonfluorescent quenchers and minor groove-binding (MGB) modifications. Negative template controls and no amplification controls were included in each Taqman run. The comparative C_T method of quantification was used comparing the timed and baseline samples (ABI Prism 7700 Sequence Detection System User Bulletin 2, PE Applied Biosystems).

2.6.3 Relative Quantification of gene expression

Relative quantification can be performed using the standard curve method or the comparative method using ABI prism 7000. The comparative Ct method is similar to the standard curve method, except it uses arithmetic formulas to achieve the same result for relative quantification (Livak and Schmittgen, 2001). The comparative method can be used provided a validation experiment is performed.

2.6.3.1 Validation Experiment

Before using the $\Delta\Delta C_t$ method for quantification, a validation experiment was performed to demonstrate that the efficiencies of the target and reference are approximately equal. A sensitive method for assessing if two amplications have the same efficiency is to look at how ΔC_t varies with template dilution.

Therefore a dilution series was prepared for each target. A dilution series of cDNA in triplicate was amplified using ABI PRISM 7000 (see fig 3.1). The average Ct values for the target were subtracted from the average Ct values for the endogenous reference at each dilution and the differences in Ct (Δ Ct) values were plotted against the log of the template amount (i.e.100ng, 50ng, 25ng, 10ng). The slope was <0.1 and therefore the amplification efficiencies were comparable.

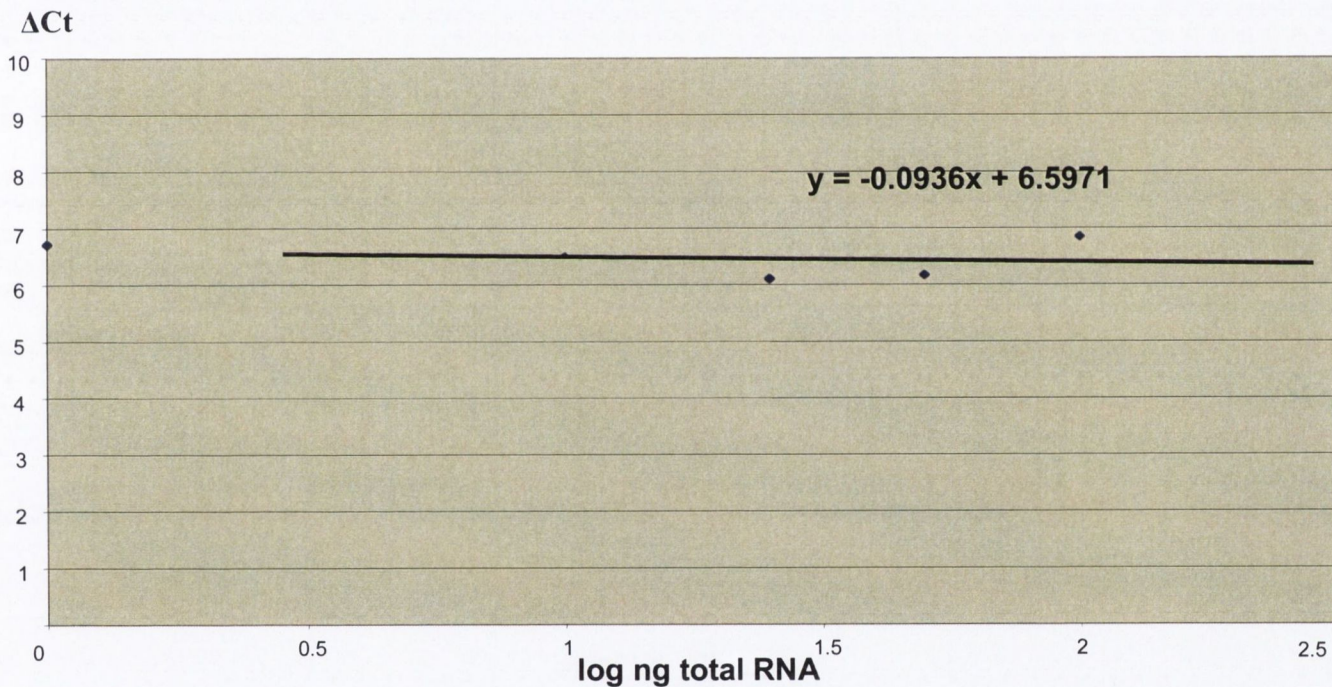


Fig 2.1 PCR Efficiency curve

The threshold cycle (Ct) indicates the fractional cycle number at which the amount of the amplified target reaches a fixed threshold. The difference in Ct values (between the reference and the target) were plotted against the log of the RNA amount. Slope = 0.0936

Ct; threshold cycle, Δ Ct; delta Ct (difference in Ct values), ng; nanogrammes.

2.6.3.2 The Comparative Method of Relative Quantification

The comparative ($\Delta\Delta C_T$) method of relative quantification was used to determine the amount of mRNA at each time point relative to the baseline sample. A correlation between PCR efficiency of the endogenous reference (GAPDH) compared to targets (IL-10, TNF α , PAI) allowed the use of the $\Delta\Delta C_T$ method for quantification (see above). The comparative method uses an arithmetic formula for relative quantification. The relative quantification of the target, normalized to the endogenous reference (GAPDH) and relative to a baseline sample is given by:

$$\text{Relative quantification} = 2^{-\Delta\Delta C_T}$$

Where $\Delta\Delta C_T$ is defined as the difference of mean ΔC_T (timed sample) and the mean $\Delta\Delta C_T$ (baseline sample)

$$\Delta\Delta C_T = \Delta C_T (\text{timed sample}) - \Delta\Delta C_T (\text{baseline sample})$$

and the ΔC_T is defined as the difference in mean C_T (IL-10, TNF α , PAI) and mean $C_{T(\text{GAPDH})}$ as endogenous control,

$$C_T (\text{IL-10, TNF}\alpha, \text{PAI}) - C_{T(\text{GAPDH})}$$

Derivation of the formula:

The equation that describes the exponential amplification of PCR is:

$$X_n = X_0 \times (1 + E_x)^n$$

Where:

X_n	=	Number of target molecules at cycle n
X_0	=	Initial number of target molecules
E_x	=	Efficiency of target amplification
n	=	Number of cycles

The threshold cycle (C_t) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Thus,

$$X_T = X_0 \times (1 + E_x)^{C_{T,X}} = K_x$$

Where:

X_T	=	Threshold number of target molecules
$C_{T,X}$	=	Threshold cycle for target amplification
1.8.1.1.1.1.1.1.1 K_x	=	Constant

A similar equation for the endogenous reference reaction is:

$$R_T = R_0 \times (1 + E_R)^{C_{T,R}} = K_R$$

Where:

R_T	=	Threshold number of reference molecules
R_0	=	Threshold number of reference molecules
E_R	=	Efficiency of reference amplification
$C_{T, R}$	=	Threshold cycle for reference amplification
K_R	=	Constant

Dividing X_T by R_T gives the following expression:

$$\frac{X_T = X_0 \times (1 + E_X)^{C_T}, X = KX}{R_T = R_0 \times (1 + E_R)^{C_T}, R = KR} = K$$

The exact values of X_T and R_T depend on a number of factors, including:

- Reporter dye used in the probe
- Sequence context effects on the fluorescence properties of the probe
- Efficiency of probe cleavage
- Purity of probe
- Setting of the fluorescence threshold.

Therefore, the constant K does not have to be equal to one.

Assuming efficiencies of the target and the reference are the same:

$$E_X = E_R = E,$$

$$X_0 \times (1 + E)^{C_T}, X - C_T, R = K$$

$$\text{Or } X_N \times (1 + E)^{\Delta C_T} = K$$

Where:

X_N	=	X_0 / R_0 , the normalized amount of target
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ΔC_T	=	$C_{T,X} - C_{T,R}$, the difference in threshold cycles for target and reference
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Rearranging gives the following expression:

$$X_N = K \times (1+E)^{-\Delta C_T}$$

The final step is to divide the X_N for any sample q by the X_N for the calibrator (cb):

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K \times (1+E)^{-\Delta C_{T,q}}}{K \times (1+E)^{-\Delta C_{T,cb}}} = (1+E)^{-\Delta \Delta C_T}$$

Where:

$\Delta \Delta C_T$	=	$\Delta C_{T,q} - \Delta C_{T,cb}$
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Therefore, the amount of target, normalized to an endogenous reference and relative to a calibrator is given by:

$$2^{-\Delta \Delta C_T}$$

In summary, gene expression is measured as increased fluorescence corresponding to amplification of the target mRNA. The cycle in which fluorescence exceeds the background signal is termed the threshold cycle (Ct) (see fig 3.2). The Ct will always occur during the exponential phase of amplification. The higher the starting quantity of the target mRNA, the earlier a significant increase in fluorescence, and the smaller the Ct value obtained. The Ct values of the timed samples (i.e 1hr, 6hr, and 24hr) were compared to the preoperative baseline sample to determine the proportional change in mRNA after cardiopulmonary bypass. The baseline levels

are taken as 1 and subsequent samples are taken as fractional/proportional change to this. This methodology does not provide absolute quantification of messenger RNA at any time point. This method has been previously described (Girault et al., 2002, Peirce et al., 2001)

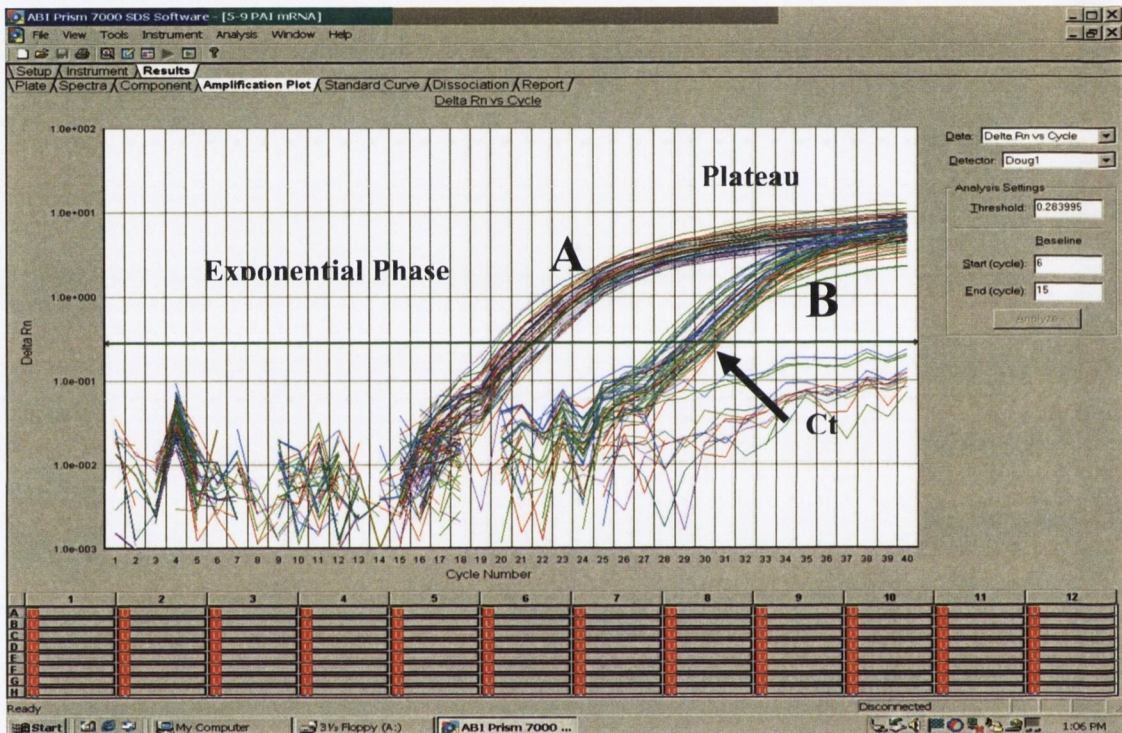


Figure 2.2. *RT- PCR amplification curve for PAI-1 mRNA.*

Real time PCR detects the amplified products in the exponential phase. Changes in the fluorescent reporter signal (delta Rn) is plotted against cycle number. Ct (Threshold cycle) represents the cycle at which a significant increase in the delta Rn above a baseline signal is first detected. Graph A represents the endogenous reference GAPDH for each sample; Graph B represents the target reference PAI-1.

2.7 DNA processing

2.7.1 Restriction fragment length polymorphisms (RFLP's)

2.7.1.1 PCR of TNF β /LTA Biallelic polymorphic site.

The -252 SNP in the *TNF β /LTA* proximal promoter contains a *NcoI* restriction site and thus may be genotyped by digestion with this enzyme. A 782bp fragment of extracted genomic DNA containing the polymorphic *NcoI* site was amplified using polymerase chain reaction. This fragment was then digested with *NcoI* restriction enzyme. 100ng of DNA served as the template in a 25 μ l reaction. Amplification involved the use of 10pmol of the following primers.

TNF β 1 - 5'- CCGTGCTTCGTGCTTTGGACTA-3'

TNF β 2 – 5'- AGAGGGGTGGATGCTTGGGTTC-3'

A concentration of 2mM MgCl₂ and 0.15mM dNTP's was used in the reaction. A 'hot start' procedure with *Taq* polymerase at a concentration of 0.2 units per reaction was employed. The cycling conditions for the PCR amplification were as follows: 95⁰ C x 3mins, 80⁰ C x 5min, 37 cycles at 95⁰ C, 68⁰ C and 74⁰ C each for 1min, and then 72⁰C for 6 mins and finally 4⁰C.

10 μ l of the amplified product was then run on a 1.5% agarose gel containing ethidium bromide. A 100bp DNA ladder was also run along with the samples. The gel was then placed on an ultra-violet light box. Positive samples with a product of 782bp were then analysed by *NcoI* restriction digest.

2.7.1.2 *NcoI* digestion of PCR product

5 μ l of PCR product was mixed with 1 μ l of Buffer H (Roche) and 0.2 μ l of *NcoI* restriction enzyme (10units/ μ l, Boehringer Mannheim) in a final volume of 10 μ l. This mixture was incubated overnight at 37⁰C overnight. The digested sample (10 μ l) of amplified DNA was subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. Restriction with *NcoI* produced fragments of 586bp and 196bp for TNF β 1 (G allele) and 782bp for TNF β 2 (A allele) (see figure 3.3).

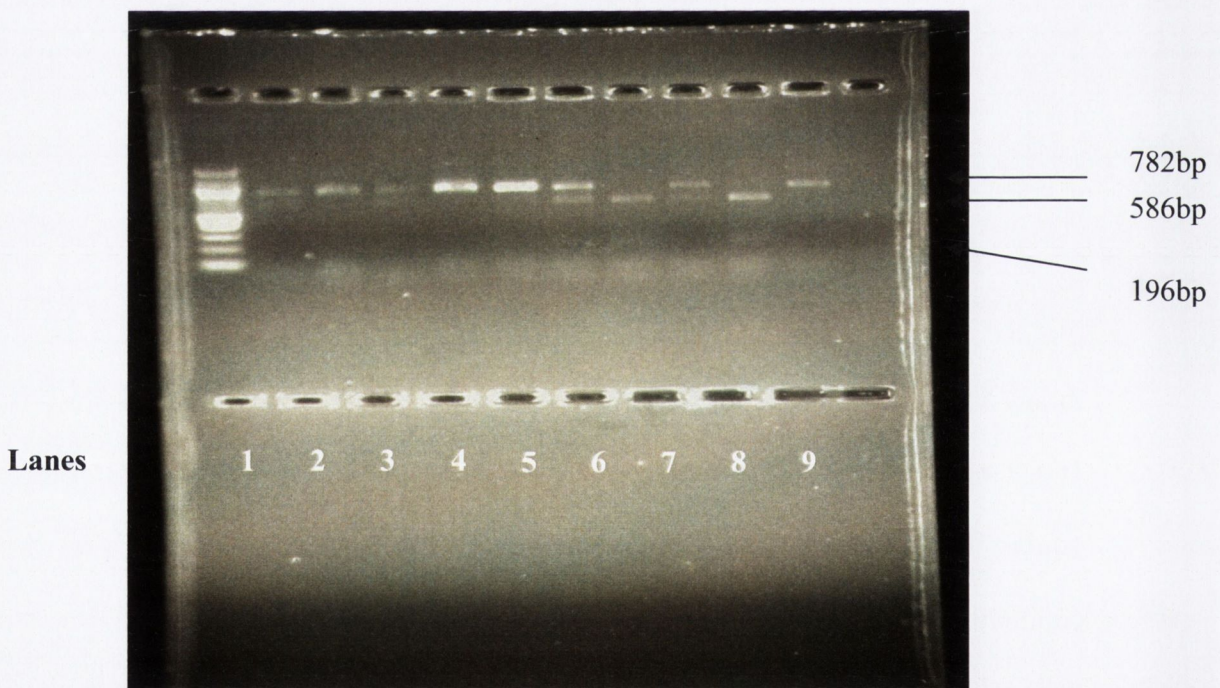


Figure 2.3. *TNF β +252 G/A genotyping using Restriction Fragment Length Polymorphisms with *NcoI*.* Restriction fragments of *NcoI*. Lane 1; 100bp DNA

ladder, Lanes 2,3,6,8 patients with TNF B1/B1 genotype, – Lanes 2,4,5,9 patients with TNF B1/B2 genotype- Lanes 7,8 patients with TNF B2/B2 genotype. Allelic determination for *TNFβ* was also confirmed using the amplifluor method. Bp; base pairs

2.7.2 Taqman™ SNP Allelic discrimination

2.7.2.1 IL10 Single nucleotide polymorphisms

Specific primers and fluorescent dye-labelled Taqman™ MGB probes for IL10-1082 and IL10-592 were designed using Primer Express Version 1.5 (Applied Biosystems/Perkin Elmer, California 94402, USA). The following primers and probes were used for amplification and allelic discrimination of IL10-1082 and IL10-592.

IL10-1082

Forward Primer 5'-GGAGGCTGGATAGGAGGTCC-3'

Reverse Primer 5'-CACACAAATCCAAGACAACACTACTAAG-3'

Allele 1 Probe 5'-CTACTTCCCCTTCCCA-3' 5'Fluor Label,6-FAM

Allele 2 Probe 5' –CTACTTCCCCCTCCCA-3' 5'Fluro Label, VIC

IL10-592

Forward Primer 5' –AGCAGCCCTTCCATTTTACTTTC-3'

Reverse Primer 5' – GCCTGGAACACATCCTGTGA-3'

Allele 1 Probe 5' – CCTACAGGACAGGCG –3' 5'Fluro Label,6-FAM

Allele 2 Probe 5' – TCCTACAGTACAGGCGG-3' 5'Fluro Label, VIC

The following mixture was used for PCR amplification in 25µl volume: 12.5µl of Taqman universal PCR master mix (Applied Biosystems/Perkin Elmer), 5µM of FAM probe, 1µM of VIC Probe, 10µM of forward primer, 10µM of reverse primer and 2.65µl of H₂O. The following conditions were used for PCR amplification: denaturation step at 95⁰C for 10 mins, 40 cycles at 95⁰C for 15 secs (denaturing step), a 60 second annealing step at 62⁰C and finally a holding temperature of 15⁰C.

2.7.2.2 Plasminogen Activator Inhibitor-1 4G/5G SNP

Specific primers and fluorescent dye-labelled TaqmanTM MGB probes for Plasminogen Activator Inhibitor –4G/5G were designed using Assay by Design (Applied Biosystems/Perkin Elmer, California 94402, USA); see Fig 3.4. The following primers and probes were used for amplification and allelic discrimination of PAI 4G/5G.

PAI 4G/5G

Forward Primer 5'-AGCCAGACAAGGTTGTTGACA-3'

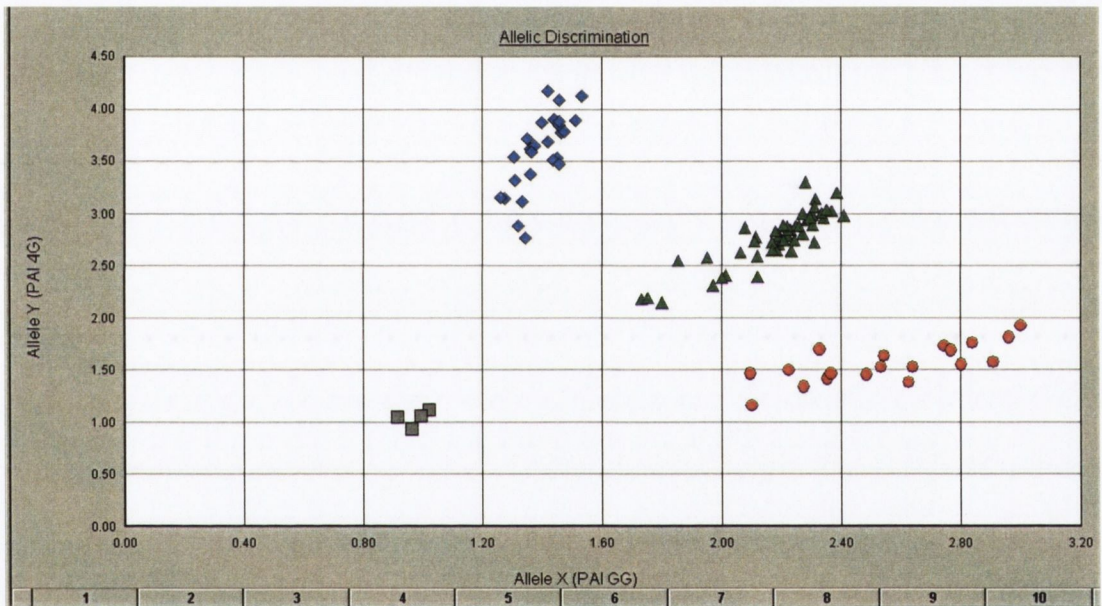
Reverse Primer 5'- GCCGCCTCCGATGATACAC-3'

Allele 1 Probe 5'- CTGACTCCCCACGTGT-3', 5'Fluor Label,6-FAM

Allele 2 Probe 5' – CTGACTCCCCCACGTGT-3' 5'Fluro Label, VIC

The following mixture was used for PCR amplification in 25µl volume: 12.5µl of Taqman universal PCR master mix (Applied Biosystems/Perkin Elmer), 0.2µM of

FAM probe, 0.2 μ M of VIC Probe, 0.9 μ M of forward primer, 0.9 μ M of reverse primer, 2 μ l DNA (1-20ng) and 9.875 μ l of H₂O. The conditions used for PCR amplification included an initial step at 95⁰C for 10 mins, a denaturing step of 40 cycles at 92⁰C for 15 secs, an annealing step of 40 cycles at 60⁰C for 1 minute and finally a holding temperature of 15⁰C.



- ▲ PAI 4G/4G Genotype
- ▲ PAI 4G/5G Genotype
- PAI 5G/5G Genotype
- Non Template Control (contains no DNA)

Figure 2.4. Allelic Discrimination using Taqman Technology

Genotyping results for PAI-1 4G/5G polymorphism: y axis indicates the 4G allele and x axis the 5G.

2.7.2.3 Amplifluor Technology – remaining polymorphisms

The remaining polymorphisms TNF -308, TNF -863, TNF -857, and TNF -238 were determined using amplifluor technology. TNF 252 was also genotyped with amplifluor technology to demonstrate reproducibility with the RFLP results.

Primers and probes are listed in appendix 3.

2.8 Outcome measures

Lactate levels were measured routinely on every arterial blood gas every four to six hours. Hyperlactatemia was considered significant when lactate levels exceeded 4 mmol/L on any sample within 24hrs after CPB. Need for vasoconstrictor drugs (noradrenaline/adrenaline) to maintain the systemic mean blood pressure greater than 70mmHg within 24hrs was also recorded. There was no algorithm for administration of vasoactive drugs. Hemoglobin levels and platelet levels were measured immediately postoperatively and at 12hrs post cardiopulmonary bypass. Additional levels were obtained within 24hrs post cardiopulmonary when considered necessary by the physician caring for the patient. Administration of packed red blood cells, hemostatic blood products, and antifibrinolytic agents were determined by the clinician caring for the patient independent of the investigators and blinded to cytokine and PAI genotype or gene expression.

2.9 *Statistical analysis*

Differences in cytokine and PAI mRNA levels between the two groups were examined using Wilcoxon rank sum test. The relative change in cytokine and PAI mRNA from baseline is expressed as the median with interquartile range. A $p < 0.05$ was considered statistically significant. A multivariate logistic regression analysis to assess the predictors for hyperlactataemia, requirement for inotropes and composite endpoints was performed. In this model, the duration of CPB was dichotomized around a median value of 95mins and patients were dichotomized above and below the lower quartile of the IL10/TNF α ratio. The relation between postoperative blood loss and requirement for transfusion of coagulation products, the carriage of specific PAI-1 alleles, and changes in PAI-1 mRNA were analysed by Wilcoxon rank sum test and Spearman rank correlation coefficient. A multivariate logistic regression to assess the predictors for transfusion of coagulation products was performed. DNA was extracted and analysed on all patients. The distribution of alleles between the complicated and uncomplicated group was analysed by Chi square testing. The relationship between cytokine gene expression and PAI gene expression was analysed using Spearman rank correlation. Statistical analysis was performed using the JMP statistical package (SAS institute, Cary, North Carolina).

2.9.1 Haplotype Analysis

Haplotypes were inferred by the statistical software packages PHASE, version 2.0 (Stephens et al., 2001, Stephens and Donnelly, 2003). The PHASE program uses coalescent models and Markov Chain Monte Carlo (MCMC) methods to assign phases in each individual and estimate haplotype frequencies. These algorithms use population genetic models to relate different haplotype patterns such that a haplotype that is more similar to the commonly observed haplotype patterns is more likely to be inferred to be present than less similar haplotypes.

Chapter 3

Tumour necrosis factor alpha and Interleukin 10 gene expression in peripheral blood mononuclear cells after cardiac surgery – Experimental Series I

3.1 Abstract

Objective Cytokine response after cardiac surgery may be genetically influenced. A study was carried out to investigate the relation between cytokine gene expression in peripheral blood mononuclear cells, genotype and clinical events after cardiac surgery.

Design A case-control study was performed.

Setting Cardiac intensive care unit in a university hospital.

Subjects A total of 82 patients having elective cardiac surgery were divided into those having uncomplicated recovery (n=48) or recovery complicated by hyperlactataemia and or requirement for inotropic support (n = 34).

Interventions The relative change in peripheral blood mononuclear cell (PBMC) TNF α and IL10 messenger RNA, 1 and 6 hours after cardiopulmonary bypass, was compared with a baseline preoperative level using quantitative reverse transcriptase polymerase chain reaction. DNA was analyzed for carriage of TNF α and IL10 polymorphic alleles.

Measurements and main results Cardiopulmonary bypass was longer in the complicated group. TNF α gene expression decreased and IL10 gene expression increased in peripheral blood mononuclear cells after surgery when compared with preoperative levels. One hour after cardiopulmonary bypass the complicated group had greater TNF α and lesser IL10 mRNA production than the uncomplicated group. The IL10/TNF α ratio was greater in uncomplicated than in complicated recovery patients. An IL10 haplotype was identified which was less frequent in the

complicated group. There was no difference between groups in TNF α genotype. On multivariate analysis, cardiopulmonary bypass time and the ratio IL10/TNF α mRNA were independent predictors of outcome.

Conclusions There is a predominant anti-inflammatory cytokine response after uneventful cardiac surgery. IL10 may have a protective role after cardiac surgery.

3.2 Introduction

Systemic inflammation may occur after cardiopulmonary bypass, when it frequently presents as arterial vasodilatation and lactic acidosis (Cremer et al., 1996).

Inflammation is cytokine mediated with tumor necrosis factor alpha (TNF α) recognised as a critically important molecule involved in the initiation, propagation and regulation of the systemic inflammatory response (Goldie et al., 1995, Hall et al., 1997) and IL10 a potent anti-inflammatory cytokine that downregulates the pro-inflammatory response. The balance between the pro- and anti-inflammatory response may significantly influence outcome after cardiac surgery. The acute phase levels of cytokines are characterized by wide inter-individual variations which may be explained, in part, by genetic factors, with carriage of specific single nucleotide polymorphic alleles associated with inter-individual variation in protein levels (Turner et al., 1997, Heesen et al., 2003). Numerous TNF α and IL10 polymorphisms have been linked to adverse outcome in sepsis (Tang et al., 2000, Mira et al., 1999). Data from a prior study suggested an association between lactic acidosis after cardiac surgery and IL10 genotype, but in the absence of any direct index of gene expression, that study could not confirm the relation between genotype and variation in gene expression (Ryan et al., 2002).

3.3 Aims and Hypotheses

To determine the relationship between cytokine gene expression, genetic polymorphisms and clinical outcome after cardiac surgery. The hypothesis that inter-individual variability in cytokine gene expression after cardiac surgery is associated with clinically important events, can be investigated by measuring change in cytokine mRNA before and after cardiac surgery using real time PCR. With this approach, the inter-relation of pro- and anti-inflammatory cytokine gene expression in the patho-physiology of systemic inflammation could be examined *in vivo*. Hence, we performed a prospective study of cytokine mRNA levels in peripheral blood mononuclear cells of patients undergoing cardiac surgery and investigated this relationship with cytokine genetic polymorphisms and clinical outcome.

3.4 Methods

3.4.1 Patient population

Having obtained ethical approval and informed consent, eighty-two patients undergoing elective cardiac surgery were enrolled in this study over a two year period. Patients were anaesthetised in a standardized manner with propofol, fentanyl and pancuronium. Cardiopulmonary bypass was performed using an open system primed with 1,500 ml of Hartmann's solution with heparin-coated circuits and roller pumps. Suction systems were controlled. All patients were transferred to the ICU and controlled mechanical ventilation was continued until the patient was ready for extubation as determined by a consultant anaesthetist. Thirty-four patients met the criteria for inclusion into the complicated group as defined by the presence of hyperlactataemia or inotrope requirement. Forty-eight patients were recruited into the uncomplicated group.

3.4.2 Measurements

Demographics such as age, gender, weight, preoperative chronic disease state and operative details were recorded on Patient Analysis Tracking System (PATS) database. In addition, hourly haemodynamics, inotropic requirement, requirement for mechanical assist devices such as intra-aortic balloon counter-pulsation, duration of mechanical ventilation and indices of oxygenation, duration of intensive care and hospital stay were recorded.

Blood samples for RNA analysis were obtained from each patient at three time points: a) prior to cardiac surgery, b) 1 hour post cardiopulmonary pump, and c) 6 hours post cardiopulmonary pump. RNA was extracted, reverse transcribed and then quantified using real time PCR. In addition, blood samples for DNA analysis were obtained preoperatively. The DNA was extracted and genotyped using either taqman technology, restriction fragment length polymorphism analysis or amplifluor technology.

3.4.3 Statistical Analysis

Differences in cytokine gene expression between the two groups were examined using Wilcoxon rank sum test. IL 10 and TNF α mRNA quantification was unsuccessful for 1 patient and 3 patients respectively at the 1hr time point. DNA was extracted and analysed on all patients. Haplotypes were inferred by the statistical software packages PHASE, version 2.0 (Stephens et al., 2001, Stephens and Donnelly, 2003).

3.5 Results

3.5.1 Patient Demographics

Eighty-two patients undergoing elective cardiac surgery were enrolled in this study. Thirty-four patients met the criteria for inclusion into the complicated group as defined by the presence of hyperlactataemia or inotrope requirement. Forty-eight patients were recruited into the uncomplicated group. Demographic parameters for both groups of patients are presented in table 4.1. Age, gender distribution, weight, body surface area and nature of the surgical procedure were similar in both groups. The prevalence of diabetes and hypertension were similar in both groups. Cardiopulmonary bypass time was significantly longer in the complicated patients ($p=0.034$) as was cross clamp time ($p=0.0049$).

Table 3.1. Patient Demographics and Operative Details –Series I

	Uncomplicated Recovery	Complicated Recovery	p value
Number	48	34	
Age (years)	62.5 (1.5)	64.3 (1.5)	0.43
Sex (M:F)	40:8	24:10	0.17
Body Surface Area (m ²)	1.91 (0.03)	1.90 (0.03)	0.81
Weight (Kg)	80.5 (1.9)	79.5 (2.5)	0.70
Hypertension	20 (42)	17 (50)	0.45
Myocardial Infarction	20 (40)	7 (21)	0.045
Diabetes Mellitus	6 (12.5)	7 (20.5)	0.45
CABG	35 (73)	22 (65)	
Valve	11 (23)	9 (26)	
CABG/Valve	2 (4)	3 (9)	0.45
CPB time (min)	95.5 (5.1)	111.5 (4.9)	0.034
Cross Clamp time (min)	55.8 (3.4)	71.8 (4.4)	0.0049
Deaths	1	0	NS

M:F, male:female; CABG, Cardiopulmonary bypass graft; CPB, cardiopulmonary bypass; NS, non significant. Values are provided as mean (SE) for continuous variables; percentages for categorical data in italics

3.5.2 Predictors of Outcome after cardiac surgery

Lactate levels were significantly higher in the complicated recovery group at all times, with the greatest difference seen 6 hours after intensive care admission (table 4.2). There was a significant association between lactate levels and proportional change from baseline in TNF α mRNA at 6 hours, (Lactate level = 0.6 Δ TNF α + 1.9, p = 0.006, and R² = 0.11). There was no significant association between proportional change in IL10 mRNA and lactate levels.

Table 3.2. Lactic acid levels and inotrope use

	Uncomplicated group	Complicated group	p value
Number	48	34	
1hr lactate, mmol/l	1.9 (0.2)	2.5 (0.2)	0.01
Inotrope at 1 hr (%)	0	5 (<i>15%</i>)	0.01
1-hr pH	7.37 (0.01)	7.35 (0.01)	NS
1-hr MAP, mmHg	78 (1.4)	75 (1.68)	NS
6hr lactate, mmol/l	1.7 (0.2)	3.0 (0.3)	0.0002
Inotrope at 6hrs (%)	0	13 (<i>40%</i>)	0.0001
1-hr pH	7.37 (0.01)	7.35(0.01)	NS
6-hr MAP	75 (1.4)	74 (1.6)	NS

Values quoted as mean (SE) for continuous variables. Percentages are in Italics
MAP,mean arterial pressure mmHg; NS, not significant.

3.5.3 Cytokine gene expression after cardiac surgery

Overall TNF α gene expression decreased and IL10 gene expression increased over time in both groups. However, in patients who did not receive inotropes, there was a greater decrease in TNF α mRNA and a greater increase in IL10 mRNA at both 1 and 6 hrs post CPB (see table 4.3). Consequently, IL10:TNF α ratios were higher in patients who did not receive inotropes after bypass. In patients who did not develop hyperlactataemia, only the ratio of IL10:TNF α mRNA was greater 1hr after cardiopulmonary bypass (table 4.4).

Table 3.3 *Relationship between requirement for inotrope and changes in cytokine messenger ribonucleic acid after cardiopulmonary bypass.*

	Inotrope	No Inotrope	p value
TNF α mRNA at 1hr	0.87 (0.52-1.9) N=25	0.48 (0.3-0.8) N=54	0.01
TNF α mRNA at 6hr	0.49 (0.34-1.24) N=26	0.4 (0.21-0.57) N=51	0.04
IL10 mRNA at 1hr	1.7 (0.74-3.4) N=26	3.7 (1.9-6.1) N=55	0.0046
IL10 mRNA @ 6hr	2.0 (1.5-3.8) N=26	3.0 (1.86-7.1) N=51	0.04
Ratio IL10:TNF α mRNA @ 1hr	2.8 (0.67-2.8) N=25	6.56 (2.4-17.9) N=54	0.009
Ratio IL10:TNF α mRNA @ 6hr	3.4 (1.3-9.6) N=26	9.4 (4.3-18.4) N=49	0.002

Cytokine mRNA levels are expressed as N fold change from baseline (preoperative levels). Values are given as median (interquartile range). Comparisons are by Wilcoxon rank sum test. N, number of patients; TNF α , Tumour necrosis factor α ; IL10, interleukin 10.

Table 3.4 Relationship between hyperlactataemia and changes in cytokine mRNA after cardiopulmonary bypass.

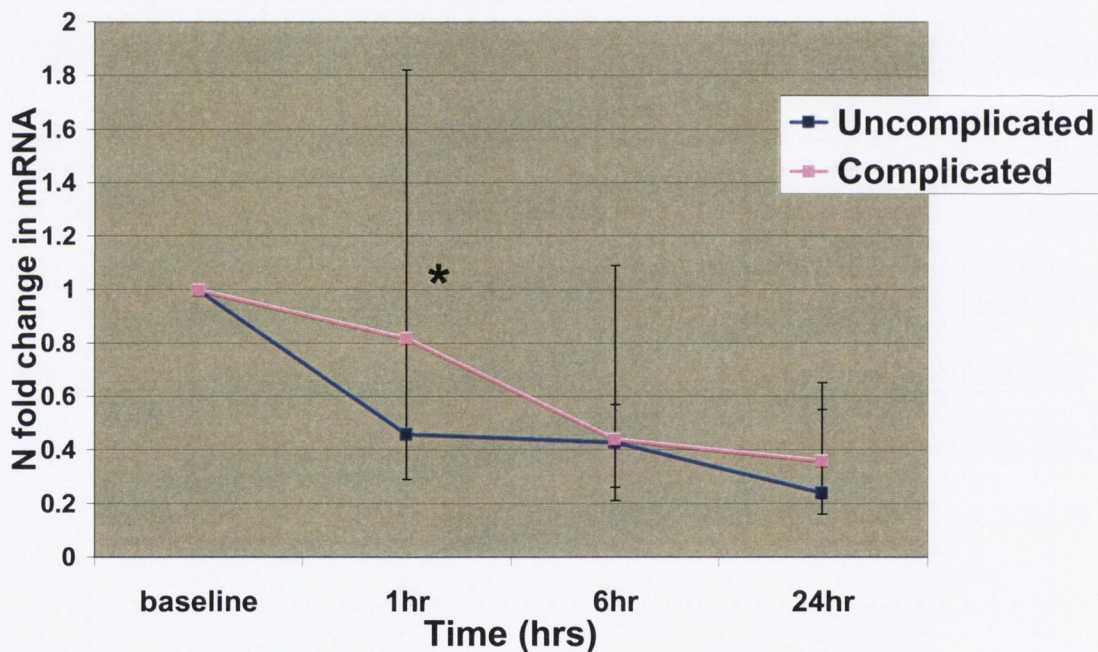
	Lactate >4mmol/L	Lactate <4mmol/L	P value
TNF α mRNA @ 1hr	0.67 (0.44-2.03)N=20	0.57 (0.29-0.93) N=59	0.06
TNF α mRNA @ 6hr	0.43 (0.27-1.4) N=20	0.43 (0.22-0.62) N=57	0.41
IL10 mRNA @ 1hr	2.0 (0.78-4.3) N=20	2.9 (1.8-6.0) N=61	0.08
IL10 mRNA @ 6hr	2.7 (1.5-4.7) N=20	2.7 (1.6-5.3) N=57	0.85
Ratio IL10:TNF α mRNA @ 1hr	2.2 (0.98-5.82) N=20	5.8 (2.2-13.3) N=59	0.009
Ratio IL10:TNF α mRNA @ 6hr	5.0 (2.07-13.8) N=20	7.25 (3.0-16.2) N=55	0.52

Values expressed as median and interquartile range. Comparison are by Wilcoxon rank sum test. N= number of patients, TNF α = Tumour necrosis factor alpha, IL10 = interleukin 10

When the two groups were analysed as a composite group (ie patients requiring inotropes and those with hyperlactataemia), there was a significant difference in IL10 and TNF α mRNA (p=0.005 and 0.005 respectively) at 1hour after cardiopulmonary bypass with relatively lower TNF α (Fig 3.1) and higher IL10 (Fig

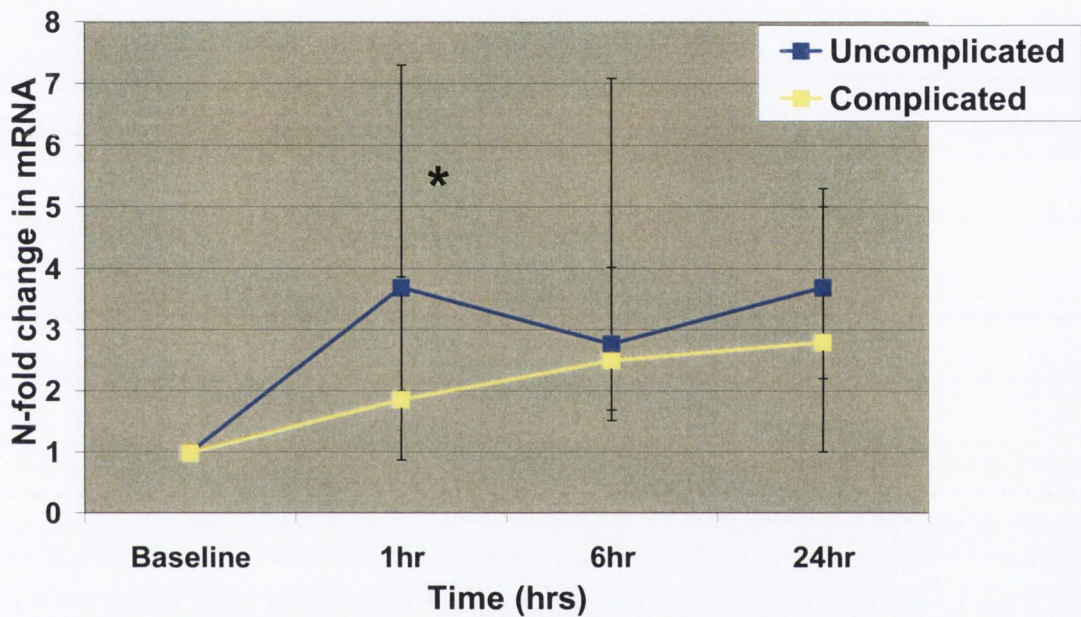
3.2) seen in the uncomplicated group. This effect was not apparent at 6 hours after cardiopulmonary bypass. The TNF α :IL10 mRNA was significantly greater in the group with complications at 1 and 6 hours after cardiopulmonary bypass (Fig 3.3).

Figure 3.1 N-fold change in TNF α mRNA after cardiac surgery in patients with uncomplicated versus complicated postoperative recovery.



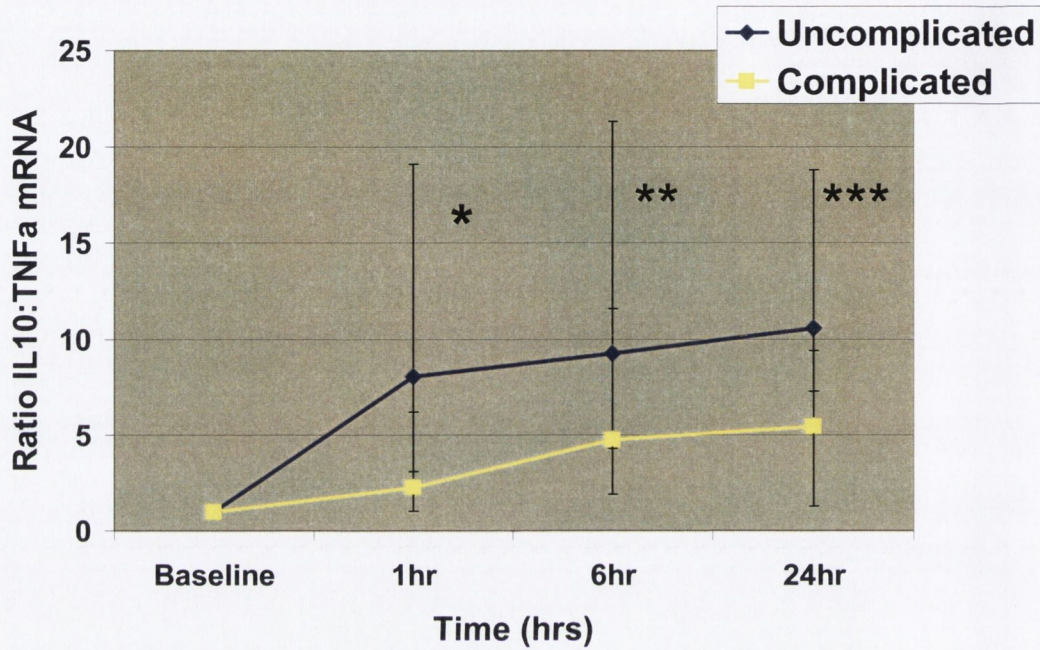
Values expressed as median and interquartile range. TNF α = Tumour Necrosis Factor alpha, mRNA = messenger RNA, baseline = preoperative sample. N-fold is the proportional change in TNF α mRNA gene expression in the timed samples after cardiopulmonary bypass compared to the baseline in patients with complicated versus uncomplicated postoperative recovery. Baseline levels are taken as 1. Comparisons are by Wilcoxon rank sum test. * p = 0.005.

Figure 3.2 IL10 mRNA after Cardiac Surgery in patients with Uncomplicated versus complicated postoperative recovery.



Values expressed as median and interquartile range. IL10 = Interleukin 10, mRNA = messenger RNA, baseline = preoperative sample. N-fold is the proportional change in IL10 mRNA gene expression in the timed samples after cardiopulmonary bypass compared to the baseline in patients with complicated versus uncomplicated postoperative recovery. Baseline levels are taken as 1. Comparisons are by Wilcoxon rank sum test. * $p = 0.005$.

Figure 3.3 Ratio IL10:TNF α mRNA after cardiac surgery



Values expressed as median and interquartile range. TNF α = Tumour Necrosis Factor alpha, IL10 = Interleukin 10, mRNA = messenger RNA, baseline = preoperative sample. The ratio of IL10:TNF α mRNA gene expression in timed samples after cardiopulmonary bypass in patients with complicated postoperative recovery is compared to those with an uncomplicated recovery. Baseline levels are taken as 1. Comparisons are by Wilcoxon rank sum test. * p = 0.0009. ** p= 0.05, *** p= 0.02

There was no association between duration of cardiopulmonary bypass or aortic cross clamping and the IL10/TNF α mRNA ratio after one and six hours. In a nominal logistic regression model, duration of cardiopulmonary bypass was

dichotomised around a median value of 95 mins, and patients were dichotomised above and below the lower quartile of IL10:TNF α ratio. The lower quartile was chosen to categorise those patients who have a predominantly pro-inflammatory response post cardiac surgery. Using this model, the factors which were involved in the occurrence of hyperlactataemia, requirement for inotropic support and a composite of these outcomes were analysed (see table 4.6). In this model both duration of bypass and low IL10:TNF α ratio were independently associated with occurrence of hyperlactataemia, requirement of inotropes and the composite outcome.

Table 3.6

Multivariate logistic regression analysis of cardiopulmonary bypass and IL10:TNF α mRNA ratio with lactate, inotrope and composite endpoints.

Endpoint	R ²	Chi	p value	Odds Ratio	CI
Lactate:					
Model	0.12	11	0.004		
Low IL10:TNF α mRNA		3.76	0.053	3.28	(0.99-11.4)
Prolonged CPB		7.1	0.008	5.1	(1.6-18.6)
Inotrope:					
Model	0.13	12.9	0.002		
Low IL10:TNF α mRNA		5	0.03	3.8	(1.2-12.9)
Prolonged CPB		7.86	0.005	4.8	(1.7-15.8)
Composite:					
Model	0.13	14.2	0.0008		
Low IL10: TNF α mRNA		6.71	0.01	4.67	(1.5-16)
CBP prolonged		7.59	0.006	4.22	(1.6-12.4)

Cardiopulmonary bypass (CPB) was dichotomized around the median, IL10:TNF α mRNA ratio was dichotomized around the lower quartile, composite endpoint i.e. patients with and without complications, CI = confidence intervals

3.5.4 Cytokine gene polymorphisms associated with outcome

The IL10 -592 polymorphism is in linkage disequilibrium with IL10 -819 (see table 4.7). The distributions of alleles in the two groups were in Hardy-Weinberg equilibrium for all loci ($p > 0.05$). There were no association between patient grouping and TNF α haplotypes. However, there was a trend towards significance

for one TNF haplotype, $p=0.08$ (see table 4.8). The frequency of the minor alleles, IL10 -819T and IL10 -592A, were significantly higher in the group who developed complications postoperatively. Haplotype analysis identified an IL10 haplotype, which was observed with greater frequency in the study group expression (table 4.9).

Table 3.7 IL10 allele frequencies in 82 cardiac surgical patients

SNP	Population	allele G freq	allele A freq	p value
IL10 1082 G→A	Uncomplicated	0.58	0.42	0.12
	Complicated	0.47	0.53	
IL10 819 C→T	Uncomplicated	0.80	0.2	0.02
	Complicated	0.66	0.34	

SNP = single nucleotide polymorphism , C = Cytosine, T = Thymine, A = Adenine, G= Guanine

Table 3.8 HAPLOTYPE ANALYSIS for IL10 for 82 patients Uncomplicated (*U*) vs. Complicated (*C*)

Haplotype	SNP			pop freq	p value
	1082	819	592		
	G→A	C→T	C→A		
GCC				<i>U</i> 0.61	0.15
				<i>C</i> 0.45	
ACC				<i>U</i> 0.22	0.66
				<i>C</i> 0.18	
ATA				<i>U</i> 0.17	0.04*
				<i>C</i> 0.37	

SNP = single nucleotide polymorphism, *U*=Uncomplicated recovery, *C*= Complicated recovery, A= Adenine, T= Thymine, C = Cytosine, G= Guanine

Table 3.9
HAPLOTYPE ANALYSIS FOR TNF for 82 patients – Uncomplicated (U) vs. Complicated (C)

Haplotype	SNP					Pop	Freq	p value
Analysis 308	252	863	857	238				
Description	G→A	G→A	C→A	C→T	G→A			
GACCG						<i>U</i>	0.33	0.63
						<i>C</i>	0.33	
AGCCG						<i>U</i>	0.28	0.17
						<i>C</i>	0.18	
GAACG						<i>U</i>	0.15	0.53
						<i>C</i>	0.15	
GGCCG						<i>U</i>	0.11	0.08
						<i>C</i>	0.23	
GACCA						<i>U</i>	0.1	0.3
						<i>C</i>	0.05	
GACTG						<i>U</i>	0.03	0.2
						<i>C</i>	0.06	

SNP = single nucleotide polymorphism, *U*=Uncomplicated recovery, *C* =

Complicated recovery, A= Adenine, T= Thymine, C = Cytosine, G= Guanine

3.6 Discussion

In this study of elective cardiac surgical patients, TNF α gene expression decreased and IL10 gene expression increased after cardiac surgery at all time points. A lower IL10:TNF α mRNA ratio was associated with inotrope requirements and the occurrence of hyperlactataemia. This cytokine mRNA imbalance was evident at both 1hr and 6hrs post cardiopulmonary bypass. These findings are consistent with an inflammation reaction in response to the surgical insult and cardiopulmonary bypass.

Our study also identified an IL10 haplotype that was seen with greater frequency in the group of patients whose recovery was complicated by inotrope requirements and hyperlactataemia. Although we were unable to link gene expression with the IL10 haplotype, the ATA haplotype is considered to be a low IL10 producer, based on the findings of earlier studies. There was no association between patient groupings and TNF α haplotypes.

Previous studies have shown an increase in TNF α levels in peripheral blood after cardiopulmonary bypass. This discrepancy may be due to the timing of the samples. Our study was on mRNA levels instead of protein levels. Although we measured 1 hr post cardiopulmonary bypass we may have missed the rise in TNF α mRNA levels which would be expected to precede any increase in plasma protein levels. In contrast, a study by Mitchell demonstrated a peak in plasma TNF α levels 2.5 hrs after CPB. McBride noted a small increase in TNF α levels after induction of anaesthesia which decreased to baseline after initiation of CPB. The anti-

inflammatory response began during CPB with an increase in IL10 levels which peaked 2hrs post CPB. Unfortunately, by limiting our time points we may have missed the rise in TNF α mRNA although we did capture the increase in IL10. In addition, by only measuring TNF α gene expression in peripheral blood monocytes, we have not accounted for TNF α released from other sources e.g myocardium, liver and lungs. The heart has been reported to produce TNF α in response to ischaemia. Meldrum noted an increase in myocardical TNF α levels after CPB (Meldrum et al., 2003). Massoudy demonstrated significant TNF α release from the lungs during CPB (Massoudy et al., 2001). The contribution of each organ to systemic levels remains unknown at present. However, a CPB study in pigs demonstrated that increased expression of porcine mRNA IL10 and TNF α correlated with an increased production of IL10 and TNF α in the myocardium (Vazquez-Jimenex et al., 2001). The IL10 haplotype ATA identified in this study predisposed patients to haemodynamic instability post cardiac surgery. Other haplotypes within the *IL10* gene have been associated with increased mortality and organ dysfunction from pulmonary sepsis (Wattanathum et al., 2007). Lowe demonstrated that the IL10-592 A allele was associated with lower IL10 levels and increased mortality from sepsis (Lowe et al., 2003). However, in contrast to this, Stanilova demonstrated that the IL10 1082 G allele was associated with higher IL10 production and increased mortality from severe sepsis. The discrepancies between these studies are difficult to explain and may in part be due to the timing of the IL10 levels in the septic

patients. Unfortunately, we did not investigate the association between this IL10 haplotype and late complications in our cohort of patients.

There are several potential implications from the current study. Understanding the molecular milieu associated with an inflammatory state post cardiac surgery may help to advance the quest for finding an effective treatment/prevention of SIRS and its deleterious sequelae. Furthermore, identifying an IL10 haplotype that is associated with a complicated recovery post bypass may help to further predict those patients who are genetically susceptible to developing SIRS. Whether this knowledge will be relevant for the treatment/prevention of sepsis remains to be seen.

Chapter 4

Coagulopathy following cardiac surgery: Influence of Plasminogen Activator Inhibitor polymorphism.

Experimental Series II

4.1 Abstract

Background: Cytokine mediated inflammation and coagulopathy may occur after cardiac surgery. The aim of this study was to investigate the temporal pattern of plasminogen activator-1 (PAI-1) gene expression after cardiac surgery, its relation with PAI genotype and to obtain preliminary data regarding its relation to perioperative morbidity.

Methods: The relative change in PAI-1 mRNA 1, 6, and 24 hours after cardiopulmonary bypass (CPB) was measured from mononuclear cells in eighty-two patients undergoing elective cardiac surgery. DNA was analysed for carriage of the 4G/5G PAI-1 polymorphism.

Results: PAI-1 gene expression decreased after CPB in all patients. A greater reduction in PAI-1 gene expression was observed in homozygous carriers of the 5G allele. Homozygous carriers of the 5G allele were also more likely to receive transfusion of coagulation blood products. There was no relation between change in PAI-1 gene expression and duration of cardiopulmonary bypass.

Conclusions: PAI-1 gene expression decreased over time after CPB. This study found a link between PAI-1 genotype, PAI-1 gene expression and transfusion of coagulation products after cardiac surgery.

4.2 Introduction

Cardiac surgery is known to trigger a marked systemic inflammatory response, which is usually quite brief, but may induce a coagulopathy with excessive bleeding which necessitates re-operation and subsequent prolonged mechanical ventilation. This inflammatory response is likely related to exposure of heparinised blood to non biologic surfaces during cardiopulmonary bypass and is cytokine mediated. The link between activation of the coagulation and inflammatory systems during CPB is complex. Tumour Necrosis Factor α (TNF α) is an early mediator of the inflammatory cascade and stimulates the release of other cytokines (IL1, IL6 and IL8). These mediators have broadly pro-coagulant actions which are generated by activation of the extrinsic pathway of the coagulation cascade by increased tissue factor expression, and by increase in plasminogen activator inhibitor type 1 activity (PAI-1) with subsequent inhibition of fibrinolysis.

Originally isolated from human endothelial cells, plasminogen activator inhibitor-1 (PAI-1) functions primarily as an inhibitor of endogenous tissue plasminogen activator (tPA). A variety of cell types, including mononuclear and vascular smooth muscle cells, are known to contribute to PAI-1 production. It is plausible that increased production of PAI-1 induces a pro-thrombotic state by inhibiting tPA-dependant plasmin production and, thus, fibrinolysis. Indeed, susceptibility to thrombosis has been confirmed in the setting of meningococcal septicaemia, trauma, and deep vein thrombosis (Hermans et al., 1999, Sartori et al., 2003, Menges T, 2001). Additionally, there are clinical data associating deficiencies in PAI-1

production with an increased likelihood of hemorrhagic events (Schleef et al., 1989, Lee et al., 1993, Fay et al., 1997).

One explanation for inter-individual variability in PAI-1 levels might be PAI-1 genotype. A common functional insertion/deletion (4G/5G) polymorphism has been described in the promoter region of the PAI-1 gene with homozygosity for the 4 G variant associated with greater basal PAI-1 transcription and the 5G allele with lower levels of PAI-1 (Eriksson et al., 1995). The clinical relevance of PAI-1 allelic variability is further underscored by studies in meningococcal disease demonstrating increased mortality rates in patients homozygous for the PAI-1 4G variant (Westendorp et al., 1999). There is additional evidence linking this variant to increased PAI-1 protein levels which themselves independently predict mortality (Hermans et al., 1999). There is a paucity of data, however, on the implications of PAI-1 gene expression and PAI-1 genetic variants in clinical situations involving significant haemodynamic perturbations, blood loss, and vigorous activation of coagulation and inflammatory pathways. This scenario is typical of cardiac surgery utilizing cardiopulmonary bypass (CPB).

4.3 Aims and Hypotheses

The aim of this study was to determine the relation between PAI-1 gene expression, PAI 4G/5G genotype and clinical outcome after cardiac surgery. Previous studies measuring PAI-1 protein levels after cardiac surgery have not shown consistent results with most studies showing an increase in PAI levels (Chandler and Velan, 2003, Valen et al., 1994, Dixon et al., 2005). However these studies did not differentiate between patients who had uneventful recovery after cardiac surgery and patients with clinical manifestations of systemic inflammation after cardiac surgery. The purpose of this study was to examine the temporal pattern of changes in PAI-1 messenger RNA (mRNA) in mononuclear cells in patients undergoing cardiac surgery with CPB, to determine whether PAI 4G/5G gene polymorphism influences PAI-1 mRNA expression, and to obtain preliminary information on the relationship between PAI-1 mRNA and clinical outcomes.

4.4 Methods

4.4.1 Patient population

Eighty two patients undergoing elective cardiac surgery were enrolled in this study as previously described. Thirty-four patients met the criteria for inclusion into the complicated recovery group as defined by the presence of hyperlactataemia or vasopressor requirement. Forty-eight patients were recruited into the uncomplicated recovery group.

4.4.2 Measurements

Blood samples for RNA analysis were obtained from each patient at three time points: a) prior to cardiac surgery, b) 1 hour post cardiopulmonary pump, and c) 6 hours post cardiopulmonary pump. RNA was quantified using RT-PCR. In addition, blood samples for DNA analysis were obtained preoperatively. The DNA was extracted and genotyped for the PAI-1 4G/5G polymorphism using Taqman technology.

Lactate levels were measured routinely from arterial blood specimens every 4-6hrs after surgery. Need of vasoconstrictor drugs (noradrenaline/adrenaline) to maintain systemic mean blood pressure >70mmHg within 24hrs was also recorded. There was no algorithm for administration of vasoactive drugs. Haemoglobin levels and platelet levels were measured immediately postoperatively and at 12hrs after CPB. Additional levels were obtained within 24hrs when considered necessary by the

physician caring for the patient. Chest tube blood losses were measured at 12hrs and 24hrs post insertion. Administration of packed red blood cells, haemostatic blood products, and antifibrinolytic drugs were determined by the clinician caring for the patient independent of the investigators and blinded to PAI-1 genotype or other data.

4.4.3 Statistical Analysis

The relative change in PAI mRNA at each time point compared with baseline is expressed as median with interquartile range. The difference in comparative changes in PAI-1 mRNA was analysed by Wilcoxon's signed rank test for comparison of matched pairs. Patients were categorized according to requirement for vasoconstrictor infusion, the occurrence of hyperlactataemia and the association between PAI-1 gene expression. These patient groups were compared using Wilcoxon's ranked sum test. The distribution of PAI-1 alleles was analysed by χ^2 or Fishers exact test as appropriate.

4.5 Results

4.5.1 Patient Demographics

Table 4.1 Patient Demographics and Operative Details – Experimental Series II

	Uncomplicated Recovery n = 48	Complicated Recovery n = 34	p value
Age (years)	62.5 (1.5)	64.3 (1.5)	0.43
Sex (M:F)	40:8	24:10	0.17
BSA (m ²)	1.91 (0.03)	1.9 (0.03)	0.81
Weight (Kg)	80.5 (1.9)	79.5 (2.5)	0.7
Hypertension	20 (42)	17 (50)	0.45
Prior MI	20 (42)	7 (21)	0.05
Diabetes Mellitus	6 (12.5)	7 (20.5)	0.45
Prior Aspirin	32 (67)	21 (62)	0.65
Prior Clopidogel	9 (19)	4 (12)	0.39
Tranexamic acid/ Aprotinin	30/1 (65)	13/2 (44)	0.04
CABG	35 (73)	22 (65)	
Valve	11 (23)	9 (26)	
CABG/Valve	2 (4)	3 (9)	0.45
CPB time (min)	95.5 ± 5.1	111.5 ± 4.9	0.034
Aortic Cross Clamp time (min)	55.8 ± 3.4	71.8 ± 4.4	0.0049
Chest tube Blood loss (0-12hrs)	643.3 ± 52	631.1 ± 55	NS
Haemoglobin	8.58 ± 0.22	8.12 ± 0.23	NS
Platelet count	137 ± 7.3	136 ± 8	NS

Values quoted as mean with standard error for continuous variables and number of patients for categorical data, percentages for categorical data in parenthesis. M:F; male: female, BSA; Body Surface Area, CABG; cardiopulmonary bypass graft, CPB; cardiopulmonary bypass.

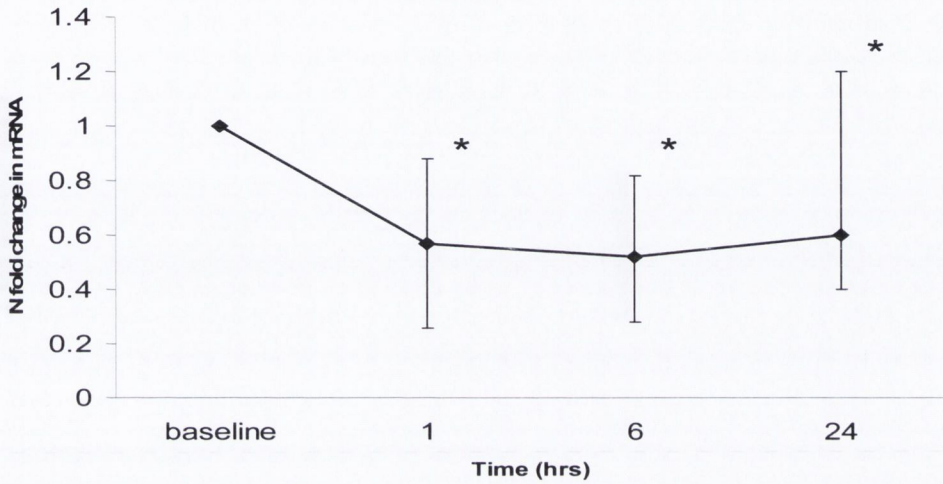
The number of patients receiving clopidogrel, and/or aspirin, was similar for both groups. Three patients received aprotinin and 43 patients received tranexamic acid in the perioperative period (see table 4.1). No patient received both antifibrinolytics. However, more patients in the uncomplicated group received aprotinin/tranexamic acid, $p=0.039$, see table 4.1. Cardiopulmonary bypass time was significantly longer in study patients ($p=0.034$) as was cross clamp time ($p=0.0049$) as shown in table 5.1.

4.5.2 PAI-1 Gene Expression after Cardiac Surgery

Samples were available for PAI-1 mRNA analysis from 82 patients at baseline, in 81 patients 1 hour post-CPB, in 76 patients at 6 hours post-CPB, and in 26 patients 24 hours after CPB. Overall PAI-1 gene expression decreased over time (Figure 5.1). Compared with baseline (where mRNA was assigned a value of 1), PAI-1 mRNA 1 hour after CPB was 0.57 (0.26-0.88) (median, interquartile range, $p=0.002$). At 6 hours post-CPB PAI-1 mRNA was 0.52(0.28-0.82) ($p=0.002$ compared with baseline). At 24hrs post-CPB, PAI-1 mRNA was 0.57 (0.39-1.15), ($p=0.002$, compared with baseline).

The level of PAI-1 mRNA decreased to half the preoperative level (baseline) at 6hrs post CPB, the lowest level recorded in our study. However, even at 24hrs the PAI mRNA level had not returned to preoperative levels.

Figure 4.1. N-fold change in mRNA after cardiopulmonary bypass.



Values expressed as median and interquartile range. N = number of patients, PAI-1 = plasminogen activator inhibitor -1, mRNA = messenger RNA, baseline = preoperative sample. N-fold is the proportional change in PAI-1 mRNA gene expression in the timed samples after cardiopulmonary bypass compared to the baseline. Baseline levels are taken as 1. Comparisons are by Wilcoxon sign rank test. *Wilcoxon sign rank test $p = 0.002$, at 1hr, 6hr and 24hr timepoint compared with baseline.

Table 4.2: N-fold change in PAI-1 mRNA after cardiac surgery compared with baseline values in patients with complicated and uncomplicated recovery.

Group	1hr	6hr
Uncomplicated	0.58 (0.23-0.88) N = 47	0.51 (0.35-0.75) N= 46
Complicated	0.51 (0.32-0.87) N = 34	0.44 (0.21-0.92) N = 33

Values expressed as median and interquartile range. Comparisons are by Wilcoxon rank sum test, N = number of patients, PAI-1= plasminogen activator inhibitor -1, mRNA = messenger RNA, p value = 0.9.

There was no significant difference in PAI-1 gene expression between the uncomplicated and complicated group (p = 0.9), see table 4.2. There was no association between the relative changes in PAI mRNA one and six hours after cardiopulmonary bypass and either the duration of cardiopulmonary bypass and aortic cross clamping.

4.5.3 PAI-1 4G/5G polymorphism and Outcome

The frequencies of the 4G and 5G alleles were 0.54 and 0.46, respectively. Sixteen percent of the patients were homozygotes for the 5G variant, 23% were homozygous for the 4G variant, and 61% were heterozygotes. The allelic distribution was in Hardy Weinberg equilibrium and conformed to previously

reported frequencies in a Caucasian population (Festa et al., 2003). There was no significant difference in allele frequency for PAI 4G/5G polymorphism between the complicated and the uncomplicated recovery groups. Nine patients received transfusion of coagulation blood products after CPB (Table 4.3). Five received both fresh frozen plasma and platelets and four received platelets only. Patients homozygous for 5G were more likely to receive blood coagulation products than those who were not ($p=0.003$). In a multivariate logistic regression analysis of the requirement for transfusion of coagulation products which included PAI-1 genotype, PAI-1 mRNA values, use of antifibrinolytics, and duration of CPB only PAI-1 5G homozygous status was associated with transfusion of coagulation products ($r^2 = 0.16$, $p= 0.003$; odds ratio = 10; 95% confidence interval 2.3-50; and area under a receiver operator characteristic curve 0.722).

Table 4.3: Comparison of 5G/5G genotype to platelets/FFP products transfused

Homozygous for 5G	Yes	No	p value
N	13	69	
Chest tube blood loss in 24hrs after CPB (mls)	1594 ± 174	1285 ± 75	0.18
Received coagulation products	5 (38%)	4(6%)	0.003
Haemoglobin level (g/l)	83 ± 4	85 ± 2	0.6
Platelet level x 10 ⁹ /l	144 ± 12.9	141 ± 5.6	0.8

N = number of patients. Haemoglobin levels and platelet levels = minimum levels obtained within 24hrs post cardiopulmonary bypass. Values quoted as number of

patients with % in brackets for categorical data. Values quoted as mean with standard error for continuous variables.

4.5.4 PAI-1 4G/5G polymorphism and PAI gene expression

Patients were dichotomized into quartiles to identify those with the highest PAI-1 gene expression in the post-CPB period relative to baseline (Table 4.4). Low mRNA levels were taken as the lower three quartiles. An association was found between PAI-1 genotype and PAI-1 mRNA production: no patient homozygous for the 5G genotype but 21% of those homozygous for the 4G allele had PAI-1 mRNA in the upper quartile 1 hour after CPB ($p = 0.01$). However, this association was not observed at the 6 or 24 hr time point.

Table 4.4: 4G/5G genotype frequencies in relation to PAI-1 mRNA 1 hour after cardiopulmonary bypass.

Population based	N	5G/5G	4G/5G	4G/4G	p value
On PAI-1 mRNA Level @ 1hr					
N		13	49	19	
High PAI-1 mRNA	20	0 (0%)	16 (33%)	4 (21%)	
Low PAI-1 mRNA	61	13 (100%)	33 (67%)	15 (79%)	*0.01

Comparisons by Chi square test. N = number of patients, PAI-1= plasminogen activator inhibitor -1, mRNA = messenger RNA, G = Guanine.

High PAI-1 mRNA refers to patients in the group with the highest quartile of PAI-1 mRNA values. Low PAI-1 mRNA refers to the lowest three quartiles.

The figures in parenthesis are a % of each genotype.

4.5.5 PAI-1 gene expression related to cytokine gene expression

There was a significant relation between relative change in PAI-1 mRNA and TNF α mRNA at one hour after cardiopulmonary bypass (Spearman's rho 0.3, p = 0.008). This effect was not apparent at 6 hours or 24hrs after cardiopulmonary bypass. There was no significant relation between changes in IL 10 expression and PAI gene expression after cardiac surgery.

4.6 Discussion

PAI-1 mRNA decreased after cardiac surgery and was not associated with hyperlactataemia or inotrope requirement. PAI mRNA levels remained low 24hrs after cardiopulmonary bypass when compared to preoperative levels. We found a greater reduction in PAI-1 mRNA levels in patients carrying the 5G allele. This finding is consistent with previous studies that have shown lower PAI-1 levels in patients homozygous for the 5G allele when compared to the 4G allele (Burzotta et al., 2003). Transfusion of coagulation products was associated with homozygosity for the 5G variant. There was a significant relation between relative change in PAI-1 mRNA and TNF α mRNA after cardiac bypass. This confirms the relationship between the fibrinolytic and inflammatory cascades. Previous studies have shown that TNF α has an influence on PAI-1 levels.

Excessive postoperative bleeding is associated with increased morbidity and mortality after cardiac surgery. Postoperative bleeding has been linked to platelet dysfunction, impaired coagulation and increased fibrinolysis. Less than 50% of patients who rebleed after cardiac surgery have an identifiable surgical source of bleeding (Moulton et al., 1996). Other factors such as genetic predisposition or an inequilibrium of the haemostatic systems may play an important role. The characterisation of the factors involved in the altered haemostatic balance after cardiac surgery, remains crucial for optimal clinical management and specific treatment of this patient population.

This study identifies homozygosity for the 5G allele as a risk factor for receiving coagulation products post cardiac surgery. A recent study by Jimenez Rivera, associated homozygosity for the 5G allele with excessive bleeding after cardiac bypass (> 1litre within 24hrs post CPB) (Jimenez Rivera et al., 2007). Although we were unable to link the 5G allele with excessive blood loss, an increase in transfusion of coagulation products may represent an equivalent finding. In contrast to our study, none of the patients in Jimenez Rivera's study received anti-fibrinolytics. In addition, this study correlated preoperative levels of PAI-1 antigen with the three genotypes of the PAI-1 4G/5G polymorphism. Because we used a relative quantification method for mRNA analysis, we were unable to compare preoperative gene expression levels.

Future studies on coagulopathy after cardiac surgery are warranted. Our study suggests that the PAI-1 4G/5G genotype should be taken into consideration for any future transfusion algorithms in cardiac surgical patients. In addition, it would be interesting to see if the same pattern of PAI-1 gene expression pertained in general surgical patients without the use of antifibrinolytics or anticoagulants. However, accounting for all the factors influencing fibrinolysis and coagulation in any study would be difficult.

Chapter 5
General Discussion

5.1 General Discussion

This thesis sought to investigate the systemic inflammatory reaction and fibrinolytic response after cardiac surgery from a genetic perspective. The relationship between gene expression, genetic polymorphisms and clinical outcome was analysed. A predominantly anti-inflammatory response was observed after cardiac surgery in patients with uncomplicated initial recovery. In contrast, complicated post-operative recovery was associated with transiently greater TNF α and lesser IL10 gene expression, representing inflammation in response to surgery or cardiopulmonary bypass. We linked an IL10 haplotype to haemodynamic instability within the first 24hrs post cardiac bypass. The fibrinolytic response to cardiac surgery was also investigated with a decrease in PAI-1 mRNA noted after CPB. An association between PAI-1 4G/5G genotype and a propensity to receive coagulation blood products was also observed.

TNF α , which can be regarded as the prototypic inflammatory cytokine (Beutler and Cerami, 1989), has not been consistently detected in patients undergoing cardiac surgery with cardiopulmonary bypass (CPB). A study by Mitchell investigated the longitudinal time course of TNF α after cardiac surgery (Mitchell et al., 2007). He demonstrated a bimodal increase in TNF α at 2.5hrs after CPB and again at 48hrs (Mitchell et al., 2007). In contrast, McBride et al noted a decrease in TNF α levels after induction of CPB with a subsequent increase in IL10 (McBride et al., 1995). Similar to our mRNA results, a study by Franke found that plasma TNF α levels

decreased after cardiac surgery (Franke et al., 2005). The profound inhibition of TNF α gene expression, with presumed decrease in protein synthesis, which was observed in our study may partly explain why other investigators have failed to detect circulating TNF α protein in similar populations (McBride et al., 1995, Chew et al., 2001). Furthermore, the reported kinetics of TNF α binding of its receptor indicates that a large fraction of TNF α is receptor bound and consequently not detected by ELISA. In addition, plasma levels of cytokines do not necessarily reflect tissue concentrations or tissue activity (Brix-Christensen et al., 2003). Real time PCR offers a unique capacity to quantify quite low concentrations of mRNA however it should be noted that PBMC mRNA may not necessarily reflect protein levels directly due to the possibility that posttranscriptional regulation may occur in these circumstances. Never the less, the change in transcription rate is possibly the most reliable and accurately measured indicator of changing gene expression and may be considered as the best available surrogate for biological activity at the protein level. Although mRNA is eventually translated into protein, one cannot assume that there is a correlation between the level of mRNA and that of protein given posttranslational and other practical considerations. Thus, while the concentration of proteins and their interactions are the true causative forces at a cellular and systemic level, if the secreted proteins bind to receptors with high affinity as in the case of TNF α and IL10, the free protein (e.g. as measured by ELISA) may not provide an accurate index of biologic activity. Indeed in an animal model of cardiopulmonary bypass, plasma cytokine levels poorly reflected mRNA

expression of pro and anti-inflammatory cytokine (Brix-Christensen et al., 2003). Furthermore, as mentioned above, post-transcriptional modification of mRNA may alter the relationship between gene expression and protein production. However, even if mRNA levels do not correlate with proteins, both measurements may yield useful information. More studies are required to examine the correlation between mRNA and plasma cytokine levels.

IL10 is a potent anti-inflammatory cytokine, which acts in part via inhibition of I κ B phosphorylation and reduced nuclear translocation of NF- κ B (Fisher et al., 1996). In an experimental setting, exogenous administration of IL10 is known to attenuate systemic inflammation after endotoxin injection in humans (de Waal Malefyt et al., 1991). However, IL10 mediated immune inhibitory effects may be deleterious in patients with active infection. In contrast, in this study, a post cardiac surgical model, inflammation is precipitated by a sterile non infectious insult, and in this context greater IL10 expression appears to be protective. This response pattern, whereby an anti-inflammatory cytokine response is protective in the context of a sterile insult, has been observed in animal models of systemic inflammation (Schneider et al., 2004, van der Poll et al., 1997). In addition, IL10's release has been associated with improved lung and heart protection (Giomarelli et al., 2003). A previous study has showed that endogenous IL10 inhibits the production of TNF α and nitric oxide and serves to protect the ischaemic and reperfused myocardium in a rat model (Yang et al., 2000). They suggest that endogenous IL10 is essential for survival during prolonged periods of myocardial ischaemia/reperfusion. Similar to

Yang et al, our results demonstrate the benefit of high IL10 in the immediate postoperative period after cardiac surgery.

This study focused on TNF α and IL10, prototypic pro and anti-inflammatory cytokines. These cytokines interact in a complex manner, with TNF α inducing IL10 gene expression, and IL10 acting as a repressor of TNF α gene expression in a negative feed back loop (Oberholzer et al., 2002, van der Poll et al., 1994).

Exogenous IL10 down regulates synthesis of TNF α in human peripheral blood monocytes by inhibiting NF κ B (Shames et al., 1998). The cytokine cascade activated in response to injury consists of a complex biochemical network with diverse effects. IL10 as a modulator of TNF α activity, may have mediated the down regulation of TNF α gene expression in our study thus providing a protective effect. It is noteworthy that significant changes in gene expression of these cytokines were evident almost immediately after cardiopulmonary bypass, even in a relatively low risk cardiac surgical population. However, alternatively, the ratio differences seen in this study may be reflecting prolonged TNF α mRNA production rather than an IL10 effect. Indeed the peak in TNF α gene expression could have occurred during cardiopulmonary bypass and may have been missed. As TNF α is important in initiation of a cytokine cascade, we focused on TNF α gene expression and its principal cytokine antagonist, rather than on downstream cytokines such IL6, and chemo-attractant cytokines such as IL8. Previous studies have shown elevated IL6 and IL8 gene expression after cardiac surgery (Scumpia and Moldawer, 2005).

Further study of a broader group of cytokines is warranted, with a focus on higher risk patients, and over a longer period after cardiopulmonary bypass.

Inter-individual variation in IL10 protein production is well documented, and a considerable proportion of this variability is genetically determined (Shames et al., 2002). We have previously shown that the carriage of specific IL10 alleles has been related to the occurrence of lactic acidosis in post-operative cardiac surgery patients (Ryan et al., 2002). The present study provides data which links complicated recovery after surgery with an index of IL10 gene expression and with an IL10 haplotype i.e. ATA. IL10 -1082A and IL10 -819T are considered to be the low producing alleles. Our findings support this concept, as the frequencies of the low producing IL10 alleles were higher in the complicated group with lower mRNA levels. Unfortunately it was not possible to link IL10 haplotype and mRNA levels as the study was underpowered to achieve this. However, the importance of this IL10 haplotype should be examined in other cohorts to determine its significance.

Genotype is easily ascertained preoperatively and patients with the IL10 ATA promoter haplotype could be targeted for immunomodulative therapy trials in the immediate postoperative period if shown to be of significant influence in future studies. In contrast to other studies, we were unable to correlate the TNF haplotype with clinical outcome. Increased evidence for heritability of the pro-inflammatory state suggests that individual genetic background also modulates the magnitude of the postoperative systemic inflammatory response after cardiac surgery. Patients homozygous for TNF*B2 allele have increased levels of TNF α which predispose to

a detrimental outcome following sepsis (Majetschak et al., 1999, Appoloni et al., 2001, Hou et al., 2004). Polymorphisms in the promoter region of IL6 gene have been associated with significantly higher postoperative plasma IL6 levels and prolonged hospitalization after cardiac surgery (Brull et al., 2001).

The cohort of patients studied had elective and relatively straightforward surgery with no deaths in the complicated population. However, profound hypotension after cardiac surgery may be refractory to inotropic support and intra aortic balloon counter pulsation, with the inevitable sequelae of organ failure, prolonged intensive care stay and excess mortality (Eskdale et al., 1998). The nature of cytokine dysregulation which occurs with prolonged requirement for inotropic support after cardiac surgery has not been fully investigated. In summary, this study shows that the pattern of clinical events during early recovery after cardiac surgery is mediated by a genetically influenced pattern of pro and anti-inflammatory cytokines.

In our second study, we demonstrate a consistent decrease in PAI-1 mRNA production after CPB with the extent of the decrease related to PAI-1 genotype. In addition, we describe an association between PAI-1 4G/5G genotype and a propensity to receive coagulation blood products early after cardiac surgery. Previous studies measuring PAI-1 concentrations after cardiac surgery have produced inconsistent results (Dixon et al., 2005, Chandler and Velan, 2003, Valen et al., 1994). Valen et al found that CPB was associated with an increase in tPA and a decrease in PAI-1 levels (Valen et al., 1994). In contrast, Chandler et al noted a heterogeneous fibrinolytic response after CPB with either an increase or no change

in PAI-1 (Chandler et al., 1995). A portion of this inconsistency might be explained by the assays employed. PAI-1 protein levels in plasma can be assayed as either PAI-1 antigen or PAI-1 activity. However, both methods lack sensitivity (Hoekstra et al., 2004, Declerck et al., 1993), and consequently the methodology of PAI-1 quantification is deficient in terms of a clinically relevant gold standard. This is reflected in the inconsistencies in published data on PAI-1 protein levels post CPB (Dixon et al., 2005, Chandler and Velan, 2003). Quantification of changes in mRNA production, using the technique of RT-PCR, is an exquisitely sensitive measure of gene expression at the mRNA level in comparison to the protein assays (Ramos-Payan et al., 2003). Additionally, RT-PCR may be particularly applicable when assessing the influence of genetic variation on gene expression as the mRNA product is not affected by post-transcriptional modification or regulation, as is the case with the protein product. Furthermore, as already stated, reduced gene expression might be difficult to quantify with a protein-based assay, as inhibition of gene expression may result in undetectable protein levels.

Patient genotype is an important determinant of PAI-1 levels. The 4G/5G polymorphism at position -675 in the promoter region of the PAI-1 gene has been reported to influence basal levels of PAI-1. Patients carrying the 4G allele have a 20% higher basal level of PAI-1 than those carrying the 5G allele (Burzotta et al., 2003). Our methods of measuring change in mRNA without an absolute measurement of mRNA concentrations does not permit a comparison of PAI-1 mRNA values levels with previously published protein levels. However, the

association between carriage of the PAI-1 5G allele and change in PAI-1 mRNA, with greater reduction in PAI-1 mRNA in carriers of the 5G allele may represent an equivalent finding. This is in concordance with previous studies demonstrating lower PAI-1 levels in patients homozygous for 5G (Burzotta et al., 2003, Hermans et al., 1999).

PAI-1 is a pro-thrombotic protein, with lesser PAI-1 levels known to be associated with a bleeding diathesis (Kahl et al., 2003, Minowa et al., 1999). Thus, the lesser PAI-1 gene expression observed in this study would seem more consistent with the propensity towards excessive bleeding commonly seen after cardiac surgery (Cremer et al., 1996). We have also described homozygosity for the 5G variant and an increased tendency to receive pro coagulant blood products. Whilst we did not demonstrate an association between genotype and the volume of peri-operative blood loss, red cell transfusion or haemoglobin levels it is likely that these patients at increased risk of blood loss were treated early and appropriately, therefore not developing post-operative anaemia or requiring excess blood transfusion. One benefit of validating this data set would be to add PAI-1 genotype to the list of factors that increase post-operative haemorrhagic risk thus allowing intervention at an earlier stage, prior to a critical drop in blood volume. Of note, we were unable to link change in PAI-1 mRNA levels with blood coagulation transfusion or bleeding indices. Consistent with current practice, anti fibrinolytic agents were used extensively in this study. These agents, which inhibit plasminogen activator, do not

influence PAI-1 gene expression, but the administration of these medications may have obscured any linkage between PAI-1 gene expression and overt bleeding.

Postoperative bleeding is a common complication after cardiac surgery. It is often difficult to delineate the factors contributing to a coagulopathy after surgery. Our study describes a decrease in PAI-1 mRNA levels after CPB which may contribute to the increased tendency to bleed postoperatively. In addition, this study also suggests an association between PAI-1 4G/5G genotype and a propensity to develop coagulopathy during early recovery after cardiac surgery.

5.2 Assumptions and Limitations

Limitations of our research include the fact that we only measured mRNA production. As discussed in the previous section, mRNA levels do not necessarily reflect cytokine activity. Cytokine activity assays are difficult and laborious and no studies are available using such assays to estimate cytokine activity in patients undergoing cardiac surgery with or without CPB. There are data on plasma levels, but these results need to be interpreted with caution. A focus of this work is to emphasise the utility of mRNA measurement for proteins which are difficult to measure by ELISA. Further more, as previously mentioned, where inhibition of gene expression occurs, protein levels may be undetectable whereas small levels of mRNA can be accurately measured by real time PCR (Bustin, 2000). Our study only measured relative mRNA, comparing postoperative levels with a preoperative baseline. Absolute values would give additional information regarding a) preoperative levels and whether they can be associated with outcome after cardiac surgery, b) whether preoperative “basal” levels are linked to polymorphisms, and c) whether a postoperative cut-off point exists over which all patients will develop SIRS or excessive bleeding post cardiac surgery (similar to an “all or nothing” law). We used relative quantification rather than absolute levels as we were interested in seeing whether PAI mRNA increased or decreased after cardiac surgery rather than absolute numbers. Absolute quantities of PAI mRNA can be obtained by comparison against a PAI-1 standard, for example a known number of copies of a cloned PAI-1 gene, which was unavailable to us at the time of the study and was

considered outside the scope of this work to develop in the time available given other commitments. A decision was therefore taken that relative quantification of mRNA was an appropriate method of measuring mRNA given the objectives of this study and this approach has been widely used in previous studies (Peirce et al., 2001, Girault et al., 2002).

Our study investigated mRNA levels in peripheral blood mononuclear cells, and thus may have failed to obtain a true picture of the inflammatory milieu. We chose peripheral blood mononuclear cells as our focus as monocytes and T cells are the primary source of TNF α synthesis. In addition, IL10 is produced by monocytes and inhibits the production of monocyte derived TNF α . PAI-1 was first identified in human endothelial cells (Ginsburg et al., 1986) and has subsequently been shown to be synthesised in a large variety of cells including vascular smooth muscle, mesangial cells and mononuclear cells (Chomiki et al., 1994, Aljada et al., 2002, Kastl et al., 2006). However, other tissues release cytokines which may contribute to the inflammatory environment after cardiac surgery. Specifically, the myocardium may be an important source of TNF α after myocardial revascularisation (Okubo et al., 2003). Moreover in an isolated heart model, TNF α is produced in response to ischaemia principally by the interstitial cells as opposed to myocytes (Wan et al., 1996). It is unclear how changes in these tissues and in peripheral mononuclear cells are related. This complex inter-relation may only be addressed by serial simultaneous sampling of blood and visceral tissue in an animal model. However it may not be possible to extrapolate results from such an *in vitro*

model into a clinical scenario. Although, we focused our study on mononuclear cells, in the future it would be interesting to study mRNA from specific subsets such as neutrophils and lymphocytes as well as visceral tissue.

We analysed patients for the first 24hrs in terms of haemodynamic stability and hyperlactataemia. We did not look at complications beyond 24hrs. Previous studies have suggested that systemic inflammation may predispose patients to increased risk of nosocomial infection through immunoparalysis. Therefore, high IL10 mRNA may be a marker for haemodynamic stability within the first 24hrs after cardiac bypass but may ultimately lead to an immunoparalysis status that increases the risk of nosocomial infections in the days following cardiac surgery (Allen et al., 2006). A recent article by Sander et al showed that levels of IL10 were significantly increased in patients developing postoperative infections (Sander et al., 2006). Further studies are required to analyse the implications of high IL10 levels and in particular, the incidence of infectious complications in these patients post cardiac surgery. With regard to plasminogen activator inhibitor gene expression, we only focused on bleeding indices within the first 24hrs. We did not investigate thrombotic complications in the postoperative period such as graft occlusion or incidence of stroke. In addition only one polymorphism (4G/5G PAI-1) was investigated in our study. Other polymorphisms affecting the fibrinolytic/coagulation system may predispose patients to a bleeding susceptibility post cardiac surgery. A recent study by Donahue et al reported an association between reduced bleeding after cardiac surgery and the Factor V leiden

polymorphism (Donahue et al., 2003). Welsby et al identified 7 genetic polymorphisms (e.g Tissue Factor Pathway Inhibitor -399 C/T, Prothrombin 20210 G/A) associated with bleeding after cardiac surgery

5.3 Clinical Implications of our findings

After CPB, up to 20% of patients suffer from systemic inflammatory response syndrome (SIRS) that mimics sepsis (Sablitzki et al., 2002). Up to 40% of patients with SIRS develop multiorgan dysfunction syndrome, which is the leading cause of prolonged ICU stay and death and has a mortality of 50-70% (Sablitzki et al., 2002, Brun-Buisson, 2000). Anaesthetists and surgeons have been aware of individual differences to similar surgical insults. Why some patients develop multiorgan failure post cardiac surgery and others do not remains unknown. Our study suggests that genetic factors modulate the propensity of an individual to develop an imbalance of the inflammatory cascade after cardiac surgery. By investigating the fibrinolytic and inflammatory responses to cardiac surgery, this thesis may provide a greater understanding of the molecular basis of a patient's postoperative course and in particular the factors that contribute to the development of a systemic inflammatory response syndrome. Our study identifies an IL10 haplotype that predisposes cardiac surgical patients to a complicated post operative course, thus highlighting the genetic susceptibility of the inflammatory state. Discovering the genetic factors that predispose patients to developing SIRS will help focus our resources on finding a preventative treatment for those most vulnerable to the serious consequences of SIRS. Therefore, predicting those patients genetically vulnerable preoperatively and alleviating SIRS before the onset of complications may prove to be the most efficacious approach to avoiding an irreversible auto destructive inflammatory process.

Many studies identifying risk factors for transfusion of blood products during cardiac surgery fail to assess genetic factors (Arora et al., 2004). Our study suggests that the 4G/5G PAI- polymorphism influences the need for coagulation products post CPB. The decrease in PAI-1 mRNA levels post CPB may also contribute to the increased propensity to bleed after cardiac surgery. A better comprehension of the multifactorial mechanisms of activation of the inflammatory and fibrinolytic pathways may direct a more effective and individual use of the therapeutic options currently available (e.g anti-fibrinolytic agents). Novel approaches incorporating preoperative genetic testing may have the potential to expand our understanding of bleeding after cardiac surgery, and thus alter our future management of transfusing blood products.

5.4 Direction for future research

Clinical manifestations of systemic inflammation after cardiac surgery include bleeding, vasodilatory shock and respiratory failure. An imbalanced cytokine response tilted towards the proinflammatory arm appears to manifest systemically as haemodynamic instability or metabolic derangement as our study demonstrates. This finding is not surprising as proinflammatory cytokines such as tumour necrosis factor α and interleukin 1β are primarily responsible for manifestations of the acute-phase response such as fever and tachycardia. Although a predominantly anti-inflammatory response appears to be advantageous for cardiac surgical patients in the first 24hrs after surgery, these patients may have difficulty defending themselves against a microbial invasion thereafter. Further studies are required to investigate the relationship between cytokine release and the occurrence of late complications after cardiac surgery.

The causative factors for excessive bleeding postoperatively appear to be multifactorial. Studies investigating these factors must consider the interaction of inflammatory, fibrinolytic and coagulation mediators. Our study showed an association between changes in TNF α mRNA and PAI-1 mRNA. The link between these two mediators may be NF- κ B. TNF α appears to have an influence on PAI-1 levels as a recent study has demonstrated that a TNF α inhibitor (infliximab) reduces PAI-1 levels in patients with rheumatoid arthritis (Agirbasli et al., 2006). Furthermore, a TNF responsive enhancer element has been located upstream from the transcription start site of the PAI-1 gene. This region contains a conserved

NF κ B binding site that mediates the response to TNF α and is capable of binding NF κ B subunit p50 and p65 in vitro (Hou et al., 2004). NF- κ B is an inducible transcription factor involved in the regulation of many pro and anti-inflammatory genes. IL10 has been shown to down regulate the production of TNF α via NF- κ B (Shames et al., 1998). Future studies are required to investigate the role of NF- κ B in the systemic inflammatory response after cardiac surgery.

5.5 Final Conclusion

Cardiac surgery elicits the most profound perioperative disturbance and is associated with the highest incidence of adverse outcomes of any surgical procedure. Thus cardiac surgical patients are an ideal population to evaluate the influence of genetic traits on perioperative outcome. The utilization of cardiopulmonary bypass during cardiac surgery induces a systemic inflammatory response syndrome. This syndrome has many different clinical manifestations ranging from mild organ dysfunction to multiorgan failure with lactic acidosis, systemic vascular collapse requiring inotropic support, and respiratory distress syndrome. In addition, postoperative bleeding remains a serious problem following cardiac surgery with an associated increase in morbidity and mortality. A more tailored personalized therapeutic approach based on a patient's inflammatory profile is the way of the future

Appendices

Appendix 1

Interleukin 10 polymorphisms using taqman technology

Polymorphism	Forward primer 3'	Reverse primer 5'→3'	probe allele 1 5'→3'6 FAM	probe allele 2 5'→3'VIC	5'→
IL10-1082	GGAGGCTGGATAGGACCTCC	CACACAAATCCAAGACAACACTATAAG	CTACTTCCCCTTCCCA	CTACTTCCCCTCCCA	
IL10-592	AGCAGCCCTCCATTTTACTTTC	GCCTGGAACACATCCTGTGA	CCTACAGGACAGGCG	TCCTACAGTACAGGCGG	

Appendix 2

Amplifluor primer sequences used to identify TNF α and IL10 polymorphisms

	Allelic specific amplifluor primers	Common reverse primer
TNF α -308	GAAGGTGACCAAGTTCATGCTGGCTGAACCCCGTCCT GAAGGTCGGAGTCAACGGATTGAGGCTGAACCCCGTCCC	GAGGCAATAGGTTTTGAGGGGCAT
TNF α -863	GAAGGTGACCAAGTTCATGCTCGAGTATGGGGACCCCC GAAGGTCGGAGTCAACGGATTGTCGAGTATGGGGACCCCCA	GCCCTCTACATGGCCCTGTCTT
TNF α -857	GAAGGTGACCAAGTTCATGCTCTACATGGCCCTGTCTTCG GTCCAGGGCTATGAAAGTCGAGTAT GAAGGTCGGAGTCAACGGATTCCTCTACATGGCCCTGTCTTCA	
TNF α -238	GAAGGTGACCAAGTTCATGCTCCCCATCCTCCCTGCTCC GAAGGTCGGAGTCAACGGATTCTCCCCATCCTCCCTGCTCT	ACACAAATCAGTCAGTGGCCCAGAA
IL10-819	GAAGGTGACCAAGTTCATGCTGTACCCTTGTACAGGTGATGTAACAGTGAGCAAAGTGGGCACAGAGAT GAAGGTCGGAGTCAACGGATTGTGTACCCTTGTACAGGTGATGTAAT	

Appendix 3

Tumour necrosis factor β polymorphisms

PCR primers	PCR conditions	PCR product size
TNF β1 5'- CCGTGCTTCGTGCTTTGGACTA-3'	95 ⁰ C x 15min, 37 cycles at 95 ⁰ C x 1min, 61 ⁰ C x 1min,	Allele G 586bp and 196bp fragment
TNF β2 5'- AGAGGGGTGGATGCTTGGGTTC-3'	72 ⁰ C x 1min, and then 72 ⁰ C for 60secs.	Allele A 782bp fragment

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E Duggan, M O'Dwyer, E Caraher, D Diviney, E McGovern, D Kelleher, R McManus, T Ryan.

Coagulopathy After Cardiac Surgery May Be Influenced by a Functional Plasminogen Activator Inhibitor Polymorphism.

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