VARIATION IN THE HAEMOSTATIC RESPONSE AND THROMBOTIC RISK: INTERPLAY BETWEEN HAEMOSTATIC FACTORS, PLATELETS AND MONOCYTES

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Abstract

The haemostatic response comprises the interaction of coagulation factors and peripheral blood cells which play a vital role in maintaining vascular integrity. This study begins with the finding that plasma from premature MI subjects has increased endogenous thrombotic potential linked to higher levels of circulating Tissue Factor (TF), suggesting these individuals may have a hypercoagulable phenotype. This study seeks to further understand how cellular interaction, in particular platelet-monocyte interaction, modulates the haemostatic response. Throughout the study, TF and Tissue Factor Pathway Inhibitor (TFPI) were studied as representing the procoagulant and anticoagulant response, respectively.

Study of the effects of activated platelets on monocytes found that whereas direct platelet or platelet-microparticle adhesion to monocytes, and release of platelet soluble mediators induced monocyte gene expression of TF, induction of TFPI was driven solely by platelet soluble mediators. Extension of these studies using gene expression microarray technology found that whereas activation of monocytes via PSGL-1 generates a pro-angiogenic expression profile, platelet soluble mediators significantly enhanced this profile, enabling monocytes to interact with ECM components involved in the wound healing environment of the thrombus, and additionally induced anti-inflammatory, and anti-atherothrombotic genes.

Whether cells within a thrombus act simply as structural and secretory components, or play a more active role involving gene expression is unclear, therefore gene expression array analysis was carried out on thrombi generated *in vitro*. Genes demonstrating significant time-dependent increases included those encoding chemotactic proteins (IL8, CCL2, CXCL1, CXCL2), cell adhesion (ITGAV, ITGA5, ITGB1), regulation of coagulation (THBD, PLAU, SERPINE1), wound-healing (ENDG, SPP1, LAMB3), and regulatory transcription factors (FOS, EGR1, PPARG). Whereas initiation of thrombosis is driven by plasma proteins and facilitated by the platelet surface, this study provides evidence that thrombus resolution may be driven by changes in gene expression within the thrombus that regulate the haemostatic response, thrombus growth, and facilitate wound-healing. These findings could have implications for individuals at risk of plaque rupture, where variation in gene expression may affect not just the formation of an occlusive thrombus but also the rate of resolution.

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PhD thesis

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Statement by the candidate

This thesis is the result of work carried out mainly during the period of registration. It is substantially the original work of the candidate. Where it is not, this is clearly stated in the text.

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Publications arising from this thesis

Presentations

JR Wright, P Ellis, C Langford, NA Watkins, WH Ouwehand, AH Goodall. Demonstration of gene expression within a thrombus: further regulation of the haemostatic response. To be presented at the BCS/BAS meeting, Manchester, UK; June 2010.

JR Wright, U Krishnan, R Farrugia, NA Watkns, P Ellis, C Langford, WH Ouwehand, AH Goodall. Platelet soluble mediators induce a monocyte gene expression profile that is anti-inflammatory, antithrombotic and pro-angiogenic. *J Thrombosis & Haemostasis 2009*; 7 (supp 1) Abstract OC-WE-126

JR Wright, P Ellis, C Langford, NA Watkins, WH Ouwehand, AH Goodall. Increase in mRNA levels of platelet-specific genes within an arterial thrombus. *J Thrombosis & Haemostasis* 2009; 7 (supp 1): Abstract OC-TU-029

JR Wright, JA Appleby, D Chan, AH Goodall. Activated platelets induce Tissue Factor Pathway Inhibitor in peripheral blood monocytes. *J Thrombosis & Haemostasis* 2007;5 (suppl 1): Abstract O-W-028

JR Wright, J Thompson, RK Singh, S Brouilette, J Pasi, NJ Samani, AH Goodall. Increased levels of procoagulant factors associated with increased potential for thrombin generation in patients with premature myocardial infarction. *J Thrombosis & Haemostasis* 2005;3 (suppl 1): Abstract O-R-290

Manuscripts in preparation

Wright JR, Krishnan U, Appleby JA, Chan D, Goodall AH. Collagen activated platelets induce Tissue Factor Pathway Inhibitor in peripheral blood monocytes.

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Wright JR, Ellis P, Langford C, Watkins NA, Ouwehand WH, Goodall AH. Demonstration of gene expression within a thrombus: further regulation of the haemostatic response.

Wright JR, Krishnan U, Farrugia R, Watkins NA, Ellis P, Langford C, Ouwehand WH, Goodall AH. Platelet soluble mediators induce a monocyte gene expression profile that is anti-inflammatory, antithrombotic and pro-angiogenic

List of Abbreviations

ACS	Acute Coronary Syndrome
ADP	Adenosine Diphosphate
AMV	Avian Myeloblastosis Virus
ANOVA	Analysis of variance
APTT	Activated Partial Prothrombin Time
asTF	Alternatively-spliced Tissue Factor
ATIII	Antithrombin III
ATP	Adenosine Triphosphate
B2M	B_2-microglobulin
BMI	Body Mass Index
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
CAD	Coronary Artery Disease
CCL2	C-C chemokine-2
CD	Cellular Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFR	Clot Formation Rate
CFU-GM	Colony-forming unit-Granulocyte Macrophage progenitor
CHD	Coronary Heart Disease
CRP	C-Reactive Protein
CRP-XL	Collagen-Related Peptide (Cross-linked)
CT	Clotting Time
C_T	Cycle Threshold
CTI	Corn Trypsin Inhibitor
CV	Coefficient of variation
CXCL-1	C-X-C chemokine-1
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ETP	Endogenous Thrombotic Potential
F	Clotting factor (suffix ‘a’ denotes activated factor)
FI	Fluorscent Intensity
FITC	Fluorescin isothiocyanate
flTF	Full-length Tissue Factor
FRET	Fluorescence Resonance Energy Transfer
FS	Forward Scatter
GAPDH	Glyceraldehyde 3-phosphatase dehydrogenase

GDP	Guanosine diphosphate
GO	Gene ontology
GPI	Glycosylphosphatidylinositol
GPIb	Glycoprotein Ib
GTP	Guanosine triphosphate
HBS	Hepes Buffered Saline
HCL	Hydrogen Chloride
HDL	High Density Lipoprotein
IL-1B	Interleukin-1β
IL8	Interleukin-8
ITAM	Immunoreceptor tyrosine-based activation motif
IVT	In vitro transcription
LAMP-1	Lysosomal-associated membrane protein-1
LDL	Low-Density Lipoprotein
LiDS	Lithium Dodecyl-Sulfate
LPS	Lipopolysaccharide
MCE	Maximum Clot Elasticity
MCP-1	Monocyte Chemoattractant Protein-1
MI	Myocardial Infarction
MMP	Matrix Metalloproteinase
MPC	Magnetic Particle Collector
MPs	Microparticles
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
OD	Optical Density
OVB	Owren's Veronal Buffer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDI	Protein Disulfide Isomerase
PECAM	Platelet-Endothelial Cell Adhesion Molecule
PMPs	Platelet Microparticles
PPL	Phospholipid
PPP	Platelet-Poor Plasma
PRP	Platelet-Rich Plasma
PS	Phosphatidylserine
PSGL-1	P-Selectin Glycoprotein Ligand-1
PT	Prothrombin Time
RBC	Red Blood cell
ROTEM	Rotational thromboelastrometry
RPE	R-phycoerythrin
rTF	Recombinant Tissue Factor
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SD	Standard Deviation
SMC	Smooth Muscle Cells

SNAP	Synaptosome-associated protein
SNARE	Soluble NSF Attachment protein Receptors
SNP	Single Nucleotide Polymorphism
SS	Side Scatter
STEMI	ST segment Elevation Myocardial Infarction
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TIMP	Tissue inhibitor of metalloproteinases
TLR-4	Toll-Like Receptor-4
TNF-α	Tumour necrosis factor-alpha
TxA2	Thromboxane
UTR	Untranslated region
VAMP	Vesicle-associated membrane protein
VCAM-1	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VWF	Von Willebrand Factor
WHO	World Health Organisation

Gene name abbreviations

ADORA2	Adenosine A2a receptor
AMICA1	Junctional adhesion molecule-like
ANGPTL4	Angiopoietin-like 4
ANTXR2	Anthrax toxin receptor 2
ANXA5	Annexin-V
ATF3	Cyclic AMP-dependent transcription factor ATF3
AVPI1	Arginine vasopressin-induced 1
BMP6	Bone morphogenetic protein 6
C15orf48	Chromosome 15 open reading frame 48
CASP9	Caspase-9
CCL20	Chemokine (C-C motif) ligand 20
CCL3	Chemokine (C-C motif) ligand 3
CCL3	Chemokine (C-C motif) ligand 3
CCL3L1	Chemokine (C-C motif) ligand 3-like 1
CCL3L3	Chemokine (C-C motif) ligand 3-like 3
CCL3L3	Chemokine (C-C motif) ligand 3-like 3
CCL4L1	Chemokine (C-C motif) ligand 4-like 1
CCL7	Chemokine (C-C motif) ligand 7
CCR2	Chemokine (C-C motif) receptor 2
CCR3	Chemokine (C-C motif) receptor 3
CD1D	CD1D antigen

CD244	CD244 natural killer cell receptor 2B4
CD44	CD244 antigen
CD59	Complement regulatory protein
CD84	CD84 antigen
CD9	CD9 antigen
CD9	Tetraspanin CD9
CDCP1	CUB domain containing protein 1
CDK5	Cyclin-dependent kinase 5
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CEBPB	CCAAT/enhancer-binding protein beta
CLEC10A	C-type lectin domain family 10, member A
CLEC4C	C-type lectin domain family 4, member C
CMKLR1	Chemokine-like receptor 1
CMKOR1	Chemokine orphan receptor 1
CMTM3	CKLF-like MARVEL transmembrane domain containing 3
CNTNAP2	Contactin-associated protein-like 2
COL22A1	Collagen type XXII, alpha 1
CTNNB1	Catenin beta, 1
CX3CR1	Chemokine (C-X3-C motif) receptor 1
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCL3	Chemokine (C-X-C motif) ligand 3
CXCL5	Chemokine (C-X-C motif) ligand 5
CXCL6	Chemokine (C-X-C motif) ligand 6
CXCR4	C-X-C chemokine receptor type 4
DDIT3	DNA damage-inducible transcript 3 protein
DNAJB5	DNAj (HSP40) homolog, subfamily B, member 5
DNAJC3	DNAj (HSP40) homolog, subfamily C, member 3
DUSP4	Dual specificity phosphatase 4
EBI3	Interleukin-27 beta chain
EGR1	Early growth response 1
ENG	Endoglin
EPB41L3	Erythrocyte membrane protein band 4.1
EPHX2	Epoxide hydrolase 2
ERAF	Erythroid associated factor
EREG	Epiregulin
ERRFI1	ERBB receptor feedback inhibitor 1
EVA1	Epithelial V-like antigen 1
EVL	Ena-VASP-like protein
F3	Tissue factor
FCER1A	Fc fragment of IgE, high affinity I, receptor 1 alpha

FGL2	Fibrinogen-like 2
FLOT2	Flotillin
FOS	Proto-oncogene c-Fos
FLT4	Fms-related tyrosine kinase 4
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
FOXO3A	Forkhead box protein O3
FUT7	Fucosyltransferase 7
G0S2	G0/G1switch 2
G1P2	Interferon, alpha-inducible protein
GCA	Grancalcin
GNA13	Guanine nucleotide-binding protein alpha-13 subunit
GP9	Platelet glycoprotein IX
GPIBA	Platelet glycoprotein Ib alpha chain
GPIBB	Platelet glycoprotein Ib beta chain
HBD	Hemoglobin, delta
HBEGF	Heparin-binding EGF-like growth factor
HBQ1	Hemoglobin, theta 1
HIF1A	Hypoxia-inducible factor 1-alpha
HMOX1	Heme oxygenase 1
HPSE	Heparanase
HRH1	Histamine receptor H1
ICAM1	Intercellular adhesion molecule 1
IER3	Immediate early response 3
IFNB1	Inteferon beta 1
IL10	Interleukin-10
IL10RA	Interleukin 10 receptor, alpha
IL13RA1	Interleukin 13 receptor, alpha 1
IL17R	Interleukin 17 receptor
IL18R1	Interleukin 18 receptor 1
IL1A	Interleukin 1 alpha
IL1B	Interleukin 1, beta
IL1B	interleukin 1, beta
IL1F9	Interleukin 1 family, member 9
IL1R1	Interleukin 1 receptor, type 1
IL1RL2	Interleukin 1 receptor-like 2
IL1RN	Interleukin 1 receptor
IL23A	Interleukin 23 subunit alpha
IL27RA	Interleukin 27 receptor, alpha
IL4	Interleukin 4
IL6	Interleukin-6
IL8	Interleukin 8
IRAK2	Interleukin-1 receptor-associated kinase-like 2

IRF8	Interferon regulatory factor 8
ITGA2B	Integrin alpha, 2b
ITGAL	Integrin, alpha L (CD11a)
ITGAV	Integrin, alpha 5
ITGB1	Integrin beta 1
ITGB8	Integrin beta 8
KLRG1	Killer cell lectin-like receptor subfamily G
ITM2C	Integral membrane protein 2C
LAIR2	Leukocyte associated Ig-like receptor 2
LAMB3	Laminin, beta 3
LCK	Tyrosine-protein kinase Lck
LILRA2	Leukocyte immunoglobulin-like receptor subfamily A, member 2
LYPD3	Ly6/PLAUR domain containing 3
LYPD3	Ly6/PLAUR domain-containing protein 3
MADCAM1	Mucosal addressin cell adhesion molecule 1
MMP10	Matrix metallopeptidase 10 (stromelysin)
MMP19	Matrix metallopeptidase 19
MS4A2	Membrane-spanning 4-domains, subfamily A, member 3
MX1	Myxovirus resistance 1
MX2	Myxovirus resistance 2
NR4A2	Nuclear receptor subfamily 4, group A, member 2
NRIP3	Nuclear receptor interacting protein 3
OAS2	2’-5’-oligoadenylate synthetase 2
OASL	2’-5’-oligoadenylate synthetase-like
OLR1	Oxidised low density lipoprotein (lectin-like) receptor 1
OSM	Oncostatin M
PALLD	Palladin
PDGF	Platelet-derived growth factor
PHACTR1	Phosphatase and actin regulator 1
PHLDA1	Pleckstrin homology-like domain, family A, member 1,
PLA2G4B	Cytosolic phospholipase A2 beta
PLA2G7	Platelet-activating factor acetylhydrolase
PLAU	Plasminogen activator urokinase
PLAUR	Plasminogen activator, urokinase receptor
PLEKHC1	Pleckstrin homology domain containing family C
PPARG	Peroxisome proliferator-activated receptor gamma
PROK2	Prokineticin-2
PSTPIP1	Pro-Ser-Thr phosphatase interacting protein 1
PTAFR	Platelet-activating factor receptor
PTGS2	Prostaglandin-G/H synthase
PTX3	Pentraxin-related protein
RGS1	Regulator of G-protein signalling 1

RIS1	Ras-induced senescence 1
RSAD2	Radical S-adenosyl methionine domain containing 2
SERPINB2	Serpin peptidase inhibitor, clade B
SERPINE1	Plasminogen Activator Inhibitor-1
SLAMF9	SLAM family member 9
SNCA	Synuclein, alpha
SPP1	Osteopontin
STK17B	STK17B protein
TFPI	Tissue Factor Pathway Inhibitor
TGM	Transglutaminase 2
THBD	Thrombomodulin
THBS1	Thrombospondin 1
TICAM1	Toll-like receptor adaptor molecule 1
TLR6	Toll-like receptor 6
TNF	Tumour necrosis factor
TNFAIP8L1	Tumour necrosis alpha- induced protein 8, like 1
TNFSF8	Tumour necrosis factor superfamily, member 8
TPST1	Tyrosylprotein sulfotransferase 1
TRIB1	Tribbles homolog 1
TRIM5	Tripartite motif-containing 5
TSPAN17	Tetraspanin-17
VCAM1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VWF	Von Willebrand Factor

INTRODUCTION

1.1. Haemostasis

Haemostasis can be defined as the regulatory process which maintains vascular integrity in the event of injury. Under normal haemostatic conditions, this allows a rapid response to endothelial damage by formation of a thrombus. During this process, the procoagulant response is maintained at an appropriate level until the response is quenched by anticoagulant factors, and fibrinolytic factors resolve the thrombus when it is no longer required. However, the haemostatic response can also be initiated in response to damaged endothelium caused as a consequence of disease. One example of this is atherosclerosis, which develops gradually as a result of endothelial dysfunction, lipid oxidation and accumulation, also hypercholesterolaemia, and hyperglycaemia (Yla-Herttuala *et al*, 1989; Cai *et al*, 2000; Menotti *et al*, 2005), exacerbated by external risk factors such as smoking, diabetes, obesity and hypertension (Heeschen *et al*, 2001; Fung *et al,* 2001). Rupture of the plaque exposes the proinflammatory and pro-thrombotic contents, including Tissue Factor, initiating development of a thrombus. Plaque rupture is increasingly recognised as one of the pivotal events prior to myocardial infarction (MI) however the resulting thrombus, and whether it becomes occlusive, may depend on the magnitude of the haemostatic response of an individual to the stimulus. Elevated levels of individual coagulation factors have been reported in association with coronary artery disease and MI, however the data are inconsistent. The Northwick Park Heart Study reported an independent association of Factor VII with cardiovascular heart disease (Meade *et al,* 1986); however this was not reported in the PROCAM or ARIC studies (Folsom *et al,* 1997; Heinrich *et al*, 1984). Other studies

have reported an association between elevated levels of FVIII and coronary risk or recurrent MI (Koenig, 1998; Rumley *et al*, 1999). One of the possible reasons for some of the inconsistencies reported may be due to the fact that haemostasis is regulated by the overall balance of coagulation factors, therefore, an elevated level of one individual factor may be of limited consequence if other factors that drive the procoagulant response are low, or if anticoagulant factors are also elevated to adequately balance the procoagulant response. The global effect of these factors can be assessed by measurement of the endogenous thrombotic potential (ETP) in the plasma, determining the amount of thrombin generated (Hemker *et al*, 1986). This study measures the overall balance of coagulation factors, comparing the endogenous thrombotic potential (ETP) in plasma obtained from subjects who have suffered a premature MI with that of matched, healthy controls.

Whether the haemostatic response is initiated in order to maintain vascular integrity, or in response to plaque rupture, the response comprises the activation and interaction of plasma proteins, and additionally, the activation and interaction of peripheral blood cells. These factors are now considered in more detail.

1.2. Coagulation

Coagulation involves the sequential activation of serine proteases present in the plasma as inactive zymogens, resulting in the formation of a fibrin clot. For several decades, our understanding of coagulation has been based on the classical model of coagulation proposed by Macfarlane (1964), and by Davie and Ratnoff (1964). This model is based on two cascade pathways, the extrinsic and the intrinsic, which converge upon the activation of Factor X (Figure 1.1). The extrinsic pathway is triggered by Tissue

Factor, activating FVII, and leading to activation of FX. In contrast, the intrinsic pathway is triggered by contact activation, acting via FXII, High Molecular Weight kininogen, and prekallikrein, which activate FXI, and in turn, Factors IX and VIII, leading to activation of FX, and the common coagulation pathway. In more recent years, the cellular contribution to coagulation has been incorporated into the model, in

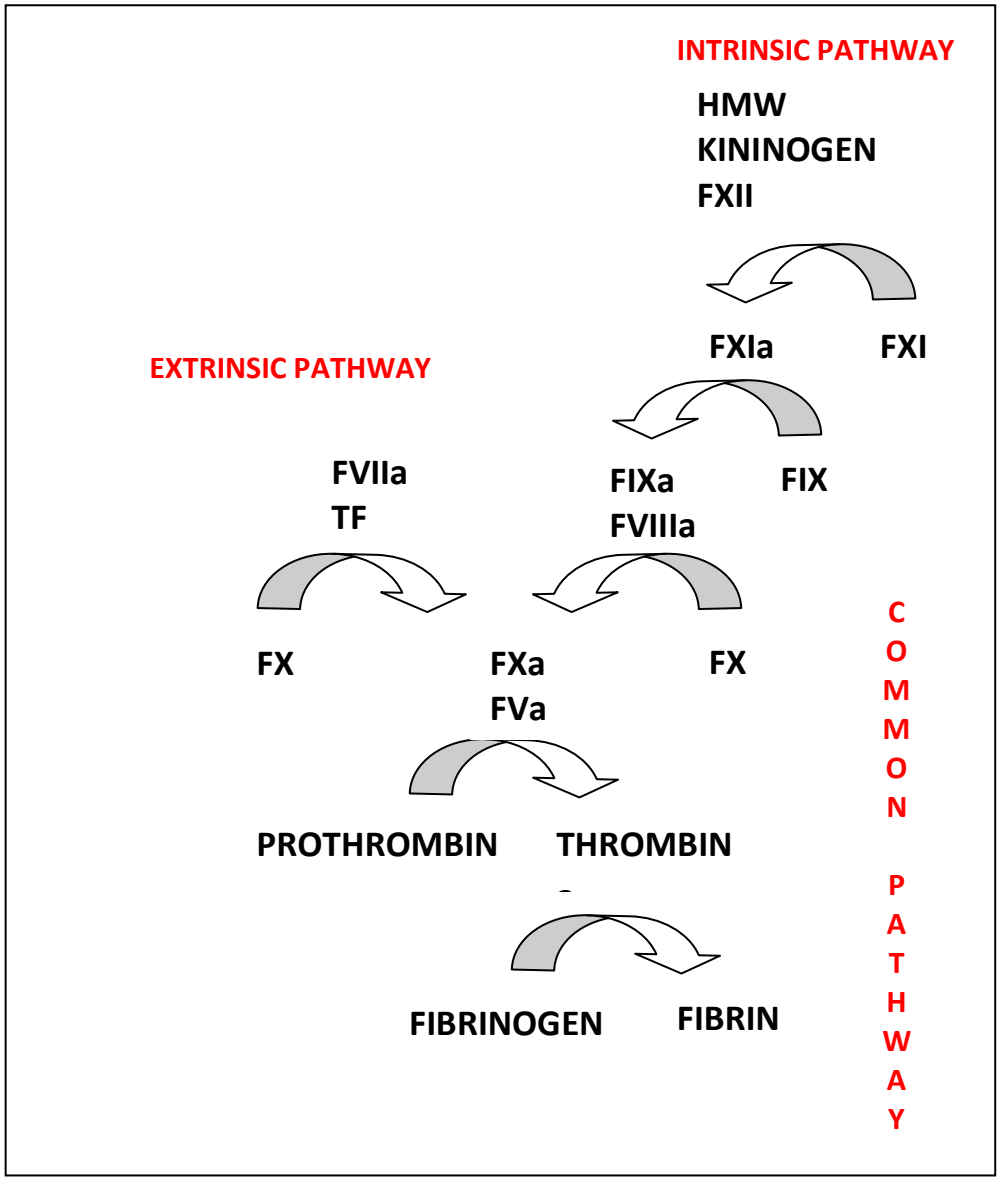


Figure 1.1. Cascade model of coagulation. Illustrating the Extrinsic and Intrinsic coagulation pathways and the common pathway followed upon activation of Factor X. ('a' denotes the active form of the coagulation factor).

particular the provision of a negatively charged phospholipid surface to support coagulation (Walsh *et al*, 1976; Mertins *et al*, 1984; Krishnaswamy *et al*, 1988; Bom *et al*, 1990; and more recently, Zwaal *et al*, 1998; Shaw *et al*, 2007). Therefore, our current understanding of the coagulation process is based on a combination of plasma proteases involved in both the extrinsic and the intrinsic pathways, and the contribution of the cell surface to support thrombin generation. This cell-based model tends to focus on the different stages of coagulation: initiation, amplification, and propagation (Hoffman *et al*, 2001) (Figure 1.2). During the initiation phase, Tissue Factor (TF) is exposed to the circulation. TF is the initiator of coagulation, and is either present within the vasculature due to breach of vessel wall integrity, or released from atherosclerotic plaques upon rupture. Factor VII (FVII) and activated FVII (FVIIa) in the plasma is consequently exposed to the extravascular Tissue Factor (TF), leading to activation of small amounts of FIX and FX, and the subsequent generation of a small amount of thrombin. At the same time, exposure of collagen leads to activation of platelets. These early events allow formation of the initial haemostatic plug, and the small amount of thrombin generated then drives amplification of the procoagulant signal, further activating platelets, and cofactors V and VIII, and FXI on the platelet surface. During the propagation phase, around 95% of thrombin is generated and it is this stage that is responsible for formation of the stable clot. The procoagulant response is regulated by the activity of a number of anticoagulant proteins. In particular, the procoagulant activity of TF is rapidly inhibited by Tissue Factor Pathway Inhibitor (TFPI), which binds to the TF:FVIIa complex and inhibits the activation of FX, thus shutting down the initiation of coagulation.

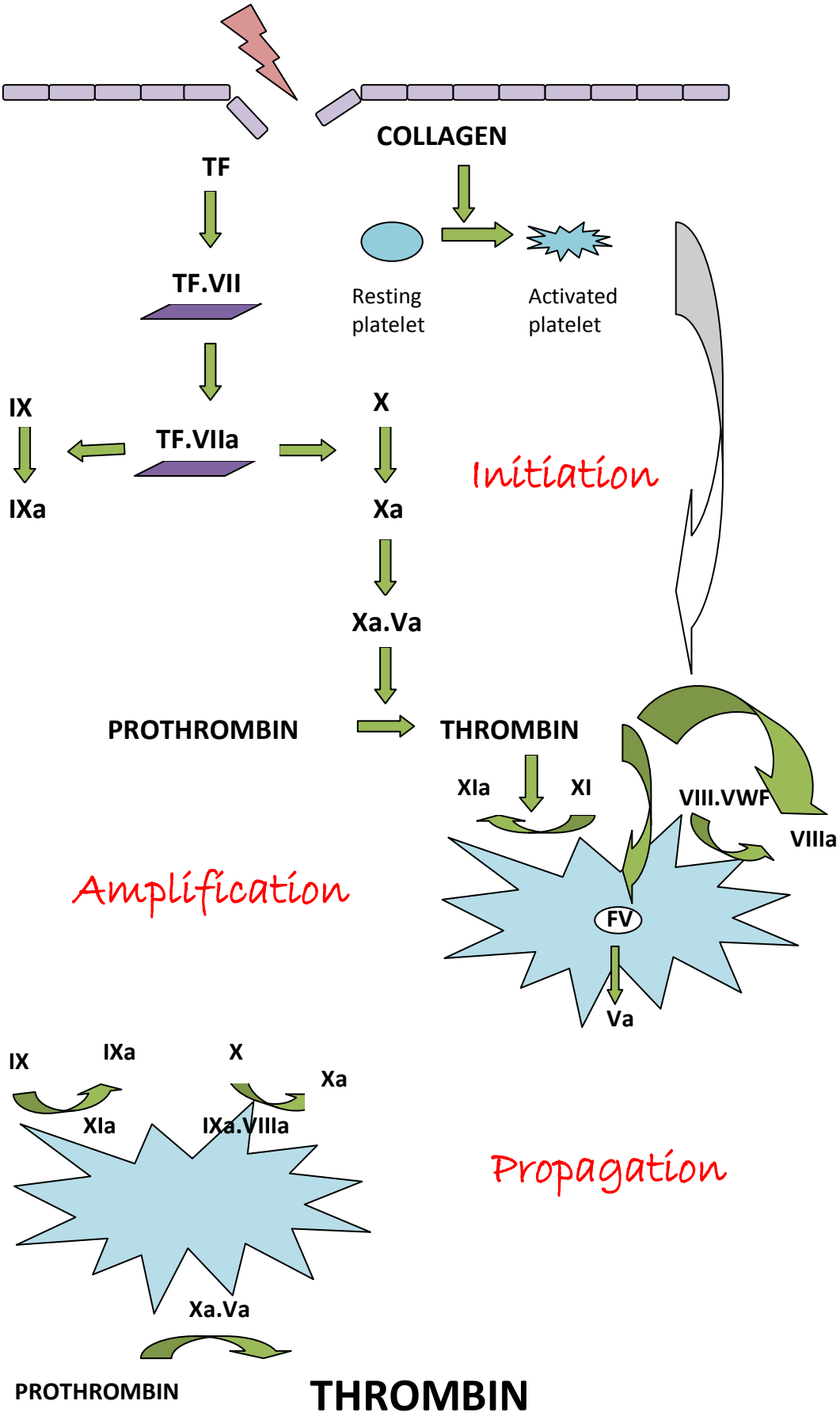


Figure 1.2. Cell based model of coagulation. Illustrating initiation, amplification, and propagation stages of thrombin generation

1.3. Tissue Factor

1.3.1. Structure and function

Tissue Factor, a 47kDa transmembrane glycoprotein, is the initiator of coagulation. It is a member of the cytokine class II receptor family, and comprises three domains. The extracellular domain is a 219 glycosylated polypeptide chain that forms two fibronectin-type III modules (Banner *et al*, 1996), which bind and form a complex with FVIIa. The glycosylation of TF may (Paborsky *et al*, 1989; Waxman *et al*, 1992) or may not (Pitlick *et al*, 1975; Krudysz-Ambio *et al*, 2010) play a role in determining its procoagulant activity. The transmembrane domain is a single chain consisting of 23 residues that anchor the protein to the membrane. The cytoplasmic domain comprises 21 residues, and has been shown to play an important role in intracellular signalling and regulation of TF expression (Mody *et al*, 1997; Dorfleutner *et al*, 2003). The human TF gene is located on chromosome 1 (p21-p22), and contains 6 exons (Mackman *et al*, 1989). Exon 1 encodes the leader sequence, exons 2 - 5 encode the extracellular domain, exon 6 encodes the transmembrane domain, and the cytoplasmic domain (Bogdanov *et al*, 2003) (Figure 1.3).

1.3.2. Cellular distribution

Tissues that have been found to express high levels of TF are heart, lung, uterus and placenta (Pawlinska *et al*, 2002; Erlich *et al*, 1999), with constitutive expression found in epithelial cells which line organs, and astrocytes in the brain (Drake *et al*, 1989; Klein *et al*, 2000). TF is also found in cells in the immediate extravascular space. This is considered to provide a protective haemostatic envelope that limits coagulation to appropriate situations such as at sites of vascular injury, where TF on fibroblasts and

smooth muscle cells (SMC) can be exposed to the circulation (Schecter *et al*, 2000), and coagulation can be initiated.

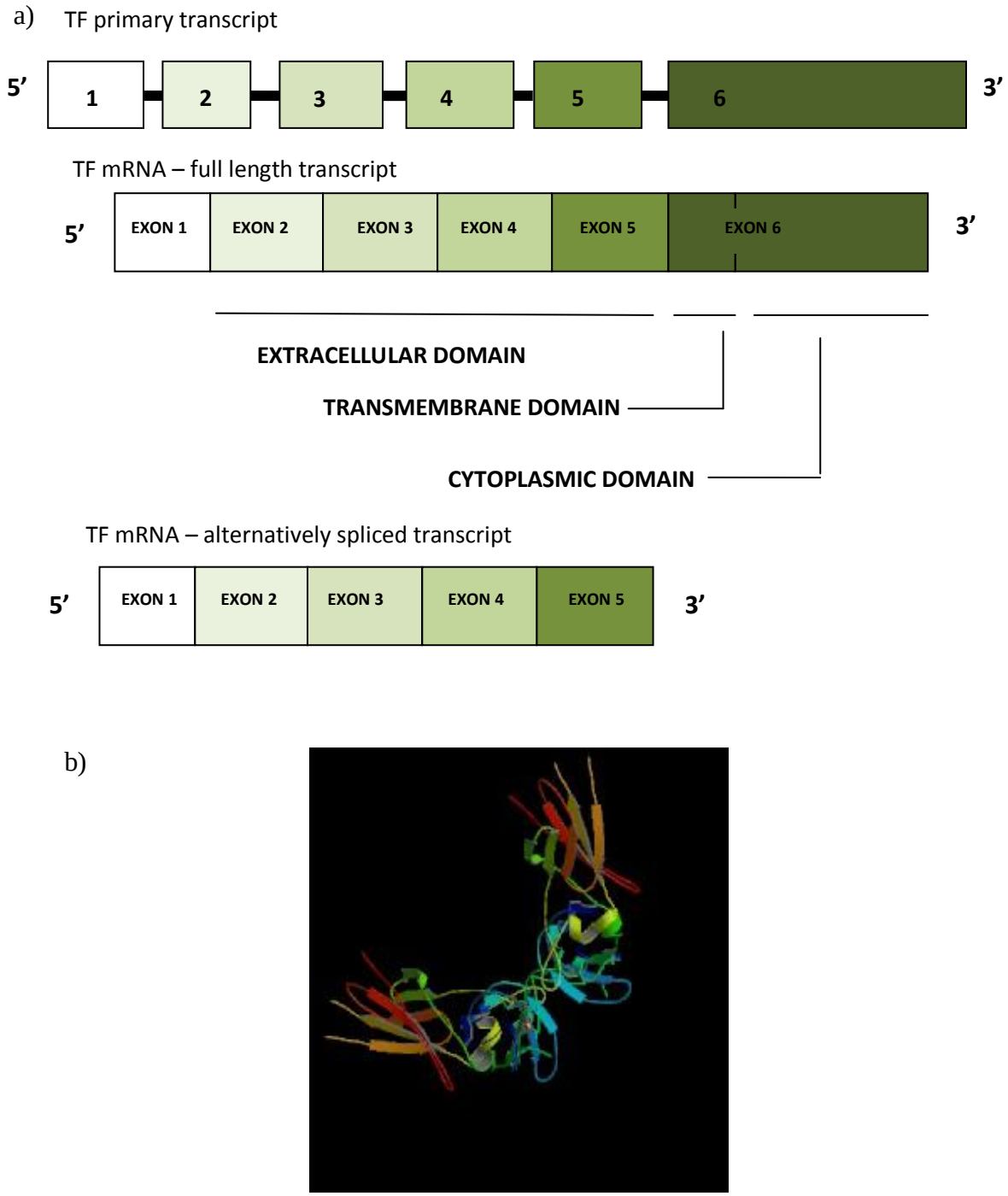


Figure 1.3. Human Tissue Factor mRNA and protein structure. (a) TF mRNA, identifies coding regions for extracellular, transmembrane, and cytoplasmic domains; (b) ribbon structure of TF extracellular domain (source: RCSB Protein Databank (PDB) rendering based on IBOY; Harlos *et al*, 1994).

Studies over the last decade, however, have shown that low levels of TF can also be found within the circulation (Giesen *et al*, 1999). In addition, some cells within the vasculature can be induced to express TF in response to specific stimuli. Vascular endothelial cells do not normally express TF however they can be induced to express TF in response to cytokines such as TNF-alpha, IL-1β or to bacterial lipopolysaccharide (LPS) (Colucci *et al*, 1983; Bevilacqua *et al*, 1986), and following interaction with monocytes (Napoleone *et al*, 1997). Induction of TF in circulating blood cells has been the focus of much debate, with reports claimed and refuted for TF expression in neutrophils (Osterud, 2001; Imamura *et al*, 2002) and platelets (Zillman *et al*, 2001; Siddiqui *et al*, 2002; Schwertz *et al*, 2006; Osterud *et al*, 2006; Panes *et al*, 2007). However, the major source of TF appears to be derived from activated monocytes and macrophages, in response to direct stimulation with, for example, LPS via the CD14/Toll-like Receptor-4 (TLR-4) complex (Brand *et al*, 1991; Guha *et al*, 2001; Osterud *et al*, 2001). TF has also been detected within plasma, and this has been termed 'blood-borne TF' (Giesen *et al*, 2000).

1.3.4. Circulating, 'Blood-borne' TF

The exact cellular origin of blood-borne TF and its function are still under debate. However, the two main sources of TF within the circulation appear to be TF-bearing cellular microparticles (MPs), and a soluble form of TF, generated through alternative splicing mechanisms (Bogdanov *et al*, 2003) (Figure 1.3). The formation and possible role of cellular microparticles is discussed in more detail later, but it has been suggested that during 'normal' circulation, the main source of TF-positive MPs appears to be platelets, however following, for example, inflammatory stimuli, the main source may switch to monocytes (Satta *et al*, 1994; Muller *et al*, 2003; Breimo *et al*, 2005).

Alternative splicing mechanisms generate a soluble TF transcript (asTF) that is lacking the transmembrane domain and has a unique C-terminus. There are conflicting reports as to the function of asTF, with some reporting strong procoagulant activity (Bogdanov *et al*, 2003; Szotoswki *et al*, 2005). In contrast, others have reported it as non-coagulant (Censarek *et al*, 2007) suggesting it may actually inhibit coagulation through binding of FVIIa (Butenas *et al*, 2005), or has an angiogenic function either in promotion of tumour growth or wound healing (Hobbs *et al*, 2007; van den Berg *et al*, 2009).

The presence of TF within the circulation, whether on the surface of cellular MPs or as a soluble protein, presents an apparent anomaly as it would be expected to trigger a thrombotic event, however this does not appear to be the case. It has been suggested, therefore, that much, if not all of the blood-borne TF may exist in an encrypted form (Bach *et al*, 1990; Le *et al*, 1992; Bach *et al*, 1998).

1.3.5. Encryption of TF

It is still unclear what mechanisms are involved in the encryption and decryption of TF and various possibilities including exposure of phosphatidlyserine (PS) have been proposed (Wolberg *et al*, 1999; Henriksson *et al*, 2007). Recent studies by Chen *et al*, (2006) have shown that cryptic TF contains unpaired cysteine thiols that form a disulphide bond upon activation, and Ahamed *et al*, (2006) suggest that regulation of the procoagulant properties of TF occurs via a protein disulphide isomerase (PDI) that targets the cysteine double bond and changes the conformation of TF. PDI appears to enhance procoagulant activity on cellular microvesicles, and therefore may play a role by acting as a chaperone for circulating TF (Versteeg, 2007). It is also possible that encrypted TF is sequestered in lipid rafts, and that influx of extracellular calcium levels leads to reorganisation of the lipid bilayer that makes up the cell membrane resulting in

the decryption of TF (Awasthi *et al*, 2007). Encryption/decryption of membrane TF may depend upon whether it is a monomer or dimer, and it has been suggested that PDI may control the monomerisation, de-encryption process (Bach *et al*, 1997). This process may involve the re-organisation of the cell membrane, with PDI indirectly regulating the transport of lipid domains within the membrane, which specifically affect the externalisation of phosphatidylserine (PS) on the outer membrane (Le *et al*, 1994; Daleke *et al*, 2008). Pendurthi *et al*, (2007) proposed that the majority of cell surface TF exists in an encrypted non-coagulant form, but is able to play a role in cell signalling. Taken together, these data suggest the procoagulant activity of TF appears to depend on its conformation and its localisation to the cellular membrane.

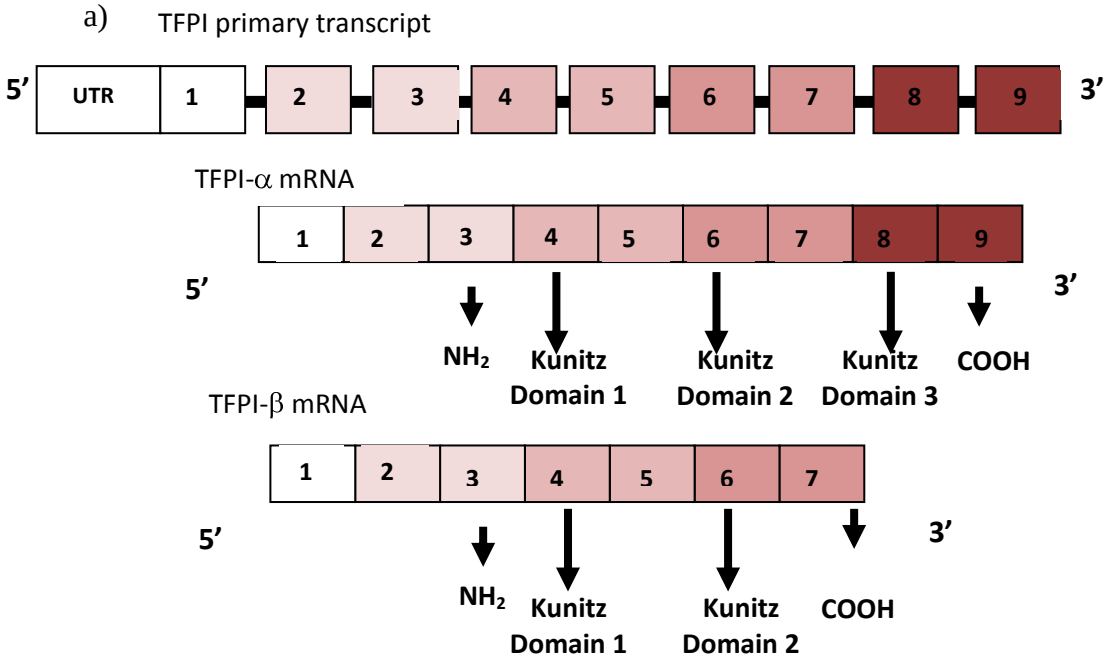
1.4. Tissue factor Pathway Inhibitor

1.4.1. Structure and function

TFPI is the main inhibitor of Tissue Factor (Carson, 1981). The TFPI gene is located on chromosome 2 (2q31-2q32.1), and consists of 9 exons (Girard *et al*, 1991). Exons 1 and 2 encode the 5' untranslated region of TFPI transcript, exons 4, 6, and 8 encode the three Kunitz domains, and exon 9 encodes the 3' untranslated region. There are two known isoforms of TFPI detected in human tissues (Maroney *et al*, 2008). TFPI-α consists of 3 multivalent Kunitz-type domains with an acidic amino-terminal region and a basic carboxy-terminal end (Figure 1.4). Domain one binds to FVIIa, domain 2 binds to and inhibits FXa, and the function of domain3 is as yet undetermined, but may be involved in the association of TFPI with lipoproteins. The C-terminus is required for the binding of TFPI to cell surfaces (Ott *et al*, 2000). The α-isoform of TFPI binds indirectly to the plasma membrane and is thought to be a soluble form of TFPI (Piro *et*

al, 2005). The second isoform of TFPI, known as TFPI-β, consists of the first two Kunitz domains, but then has a unique C-terminus sequence, which allows the

a)



b)

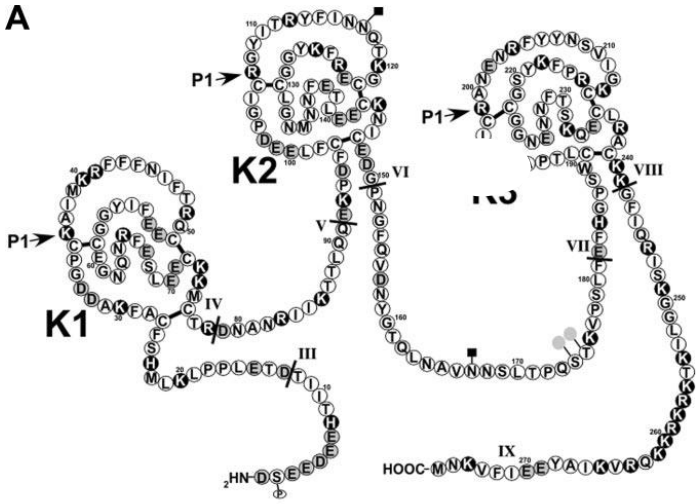


Figure 1.4. Human Tissue Factor Pathway Inhibitor mRNA and protein structure. (a) TFPI mRNA, identifies coding regions for the 3 Kunitz domains of TFPI-α, the predominant form of TFPI, and coding regions for alternatively spliced TFPI-β. (b) Sequence and structure of TFPI-α. (K1, K2, and K3 denote Kunitz domains, and roman numerals depict exon boundaries). The TFPI-β isoform contains Kunitz domains 1 and 2 only, and a unique C-terminal domain. (Crawley and Lane, ATVB; 2008).

β-isoform to bind directly to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor. In studies of endothelial cells, the β-isoform accounts for approximately 20% of the total TFPI, however, it appears responsible for most of the anticoagulant activity in terms of inhibition of activation of FX by the FVIIa:TF complex (Piro *et al*, 2005). In platelets, only the soluble, alpha-isoform of TFPI, has been detected (Maroney *et al*, 2007). Due to the high level of expression of TFPI in endothelial cells, the majority of studies of expression of TFPI isoforms have been carried out in endothelial cell or endothelial cell lines (Piro *et al*, 2004; Maroney *et al*, 2006; Ellery *et al*, 2008).

1.4.2. Cellular Distribution

Approximately 75% of TFPI within the body is associated with endothelial cells, where both isoforms are constitutively expressed on the cell surface (Ellery *et al*, 2008). Additionally, endothelial cells contain an intracellular pool which appears to only comprise the α-isoform, and which can be released into the circulation upon activation by heparin (Lupu *et al*, 1999; Hansen *et al*, 2000). Around 3-5% of TFPI is carried by platelets (Maroney *et al*, 2007), and the remaining 20% is circulating in the plasma. This plasma TFPI is mostly complexed with lipoproteins; free, unbound TFPI accounts for only ~2.5% of the TFPI in normal plasma, but it is this free form that has the highest anticoagulant properties.

1.4.3. Induction of TFPI expression

Under normal physiological conditions, circulating blood cells do not synthesize TFPI, however expression can be induced in several cell types in response to a variety of stimuli. Vascular smooth muscle cells synthesize TFPI mRNA and antigen upon stimulation with serum or growth factors (Caplice *et al*, 1998), and mesangial cells have

been shown to have increased levels of TFPI mRNA in response to thrombin and heparin, whereas there is no change in response to IL-1B or LPS (Yamabe *et al*, 1996). A small number of studies have investigated TFPI expression in monocytes, focusing on response to inflammatory stimuli, with inconclusive results (McGee *et al*, 1994; van der Logt *et al*, 1994; Iochmann *et al*, 1999). However, a recent paper demonstrates upregulation of monocyte TFPI in response to adhesion to fibronectin (Bajaj *et al*, 2007). Changes in monocyte gene expression of TF and TFPI in monocytes in response to different stimuli were explored in detail during this study.

1.5. Platelets

1.5.1. Structure and function

Platelets are small discoid cells, approximately 3µm in size, and play a vital role in the formation of the initial haemostatic plug following endothelial damage. The initial platelet thrombus is generated by platelet adhesion to the damaged sub-endothelium via ligand binding of platelet GPIbα to Von Willebrand Factor (VWF). This is followed by platelet activation and aggregation. Upon activation, platelets release various proteins contained in the intracellular granules, allowing localised delivery of soluble mediators. Platelets are formed in the bone marrow from megakaryocytes. During the transition from megakaryocyte to mature platelet, the megakaryocyte enlarges and undergoes intracellular reorganisation. Development of pseudopods allows formation of pro-platelets, which are eventually released from the cell to become mature platelets (Long *et al*, 1982; Tablin *et al*, 1990; Italiano *et al*, 1999). Also at this point, the megakaryocyte nucleus is extruded from the cell. The platelet life-span is ~ 10 days, and the platelet count within the circulation is usually within the range of 150 – 400 x

10^3/μl. Platelets are eventually removed from the circulation by macrophages in the spleen and liver. It has been assumed that as platelets are anucleate, there is no need for transcriptional components within the cell. However, it has recently been reported that platelets do, in fact, contain splicing components, and also that resting platelets contain unspliced mRNAs in the cytoplasm (Denis *et al*, 2005). These primary mRNA transcripts have been reported to include IL-1β, and the subject of much debate, TF (Schwertz *et al*, 2006), both of which are spliced into their mature form upon platelet activation, enabling translation and protein synthesis to occur.

1.5.2. Platelet agonists and receptors

Platelets exist in the circulation in a resting, non-thrombotic state, with several surface receptors acting to regulate platelet activity. These subdivide into two main categories; receptors that respond to soluble agonists, and adhesion receptors (Figure 1.5).

Many of the receptors for soluble agonist receptors belong to the G-protein coupled, seven transmembrane domain receptor family (Offermans, 2006). These include the receptors for thrombin (PAR1 and PAR4)(Kahn *et al,* 1998; Coughlin, 2005) and ADP ($P2Y_1$, and $P2Y_{12}$)(Hechler *et al,* 1998; Jantzen *et al,* 1999), also to thromboxane (TxA_2, epinephrine, and serotonin (Paul *et al*, 1999; Biegon *et al*, 1990). ADP, secreted from endothelial cells and red cells at sites of vascular injury, is also released from platelet dense granules, and binds to platelet surface purinergic receptors, $P2Y_1$, and $P2Y_{12}$. Activation of the $P2Y_1$ and $P2Y_{12}$ receptors is essential for ADP-induced platelet aggregation (Jin *et al*, 1998). Platelets also possess receptor $P2X_1$, a further purinergic receptor that responds to ATP (Mahaut-Smith *et al*, 2000), which has been reported to regulate platelet response to collagen under conditions of high shear (Oury *et al*, 2002). ADP, TxA_2, serotonin, and epinephrine are often termed weak agonists, as

alone they can only induce weak, reversible aggregation; however, when acting synergistically with other agonists, they can enhance their effect.

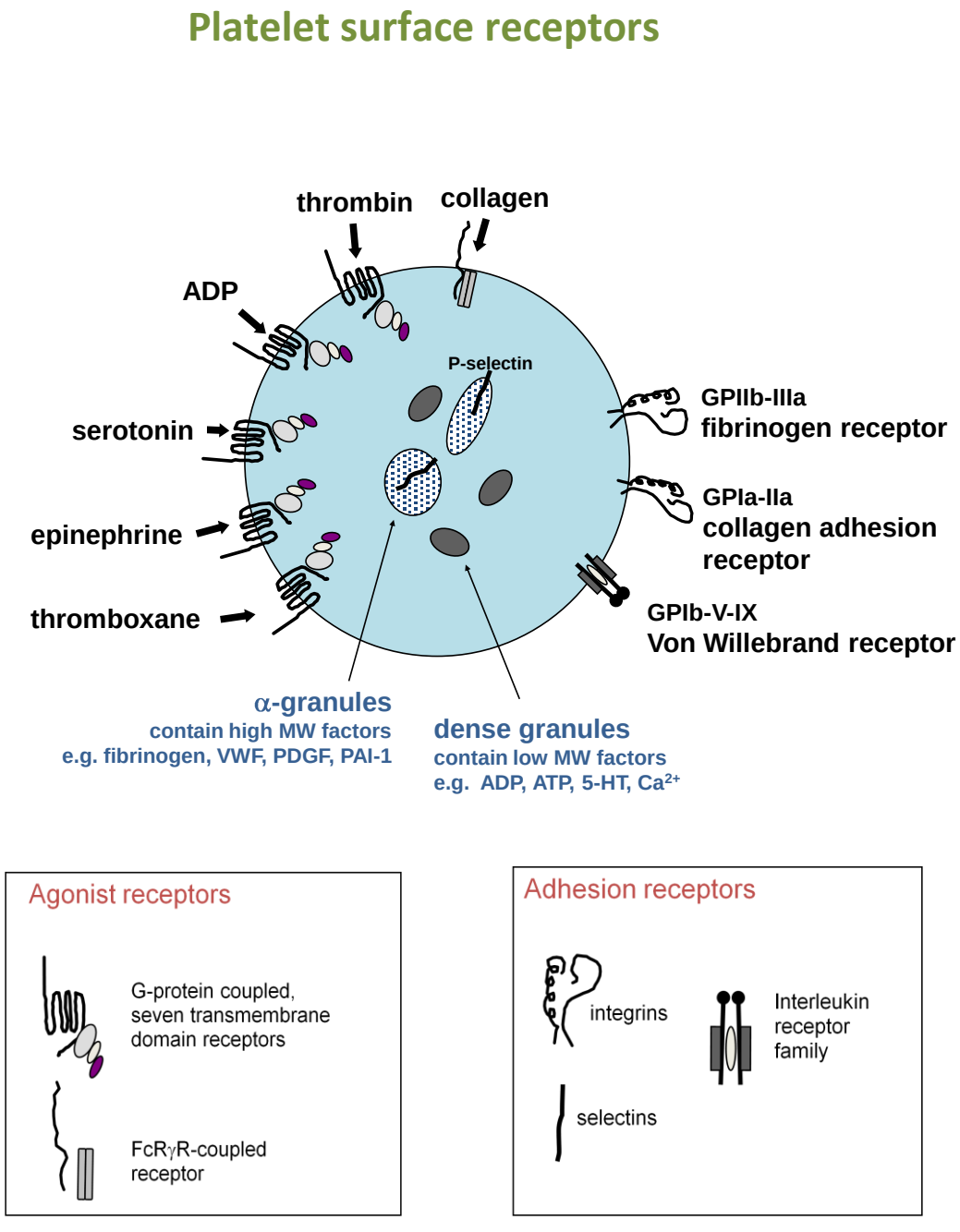


Figure 1.5. Platelet surface receptors. Diagram illustrates agonist receptors and adhesion receptors. (Diagram reproduced with slight modifications, with kind permission from Professor AH Goodall).

Thrombin, a strong inducer of platelet activation, acts via thrombin receptors, PAR1 and PAR4 (Hung *et al*, 1992, Khan *et al*, 1998). PAR receptors are activated when thrombin irreversibly cleaves the N-terminal domain, unmasking a new N-terminal sequence which acts as a tethered ligand (Vu *et a*l, 1991). Ligand-binding of thrombin to a specific thrombin receptor appears to depend upon the concentration of agonist, with PAR1 being the primary receptor at low concentrations of thrombin (Covic *et al*, 2000; Coughlin, 2005). In contrast to the structure of other platelet agonist receptors, the major receptor for collagen is the FcRγ-III-coupled GPVI receptor, with secondary effects induced via integrin receptor α2bβ1 (Knight *et al*, 1999). GPVI is specific to platelets and megakaryocytes. Collagen binding to GPVI results in cross-linking of the GPVI receptor, resulting in activation of ITAM signalling, strong integrin activation, and degranulation. Due to the unique expression of GPVI on platelets, a GPVI-specific collagen peptide was used throughout this study as a platelet-specific agonist.

Platelet-surface integrin receptors, which facilitate adhesion and intracellular signalling, comprise heterodimers of α- and β- subunits. Platelets express three β1 integrins: the collagen receptor (α2bβ1), the fibronectin receptor (α5β1), and the laminin receptor (α6β1); and two β3 integrins: αIIbβ3 and α5β3, which bind fibrinogen, and vitronectin, respectively. Additionally, the platelet surface GPIb-V-IX complex acts as a receptor for VWF. The role of these receptors is considered in more detail below.

1.5.3. Platelet activation

Adhesion

Platelets are recruited to the site of injury and initially form a reversible attachment to sub-endothelial VWF via platelet glycoprotein GPIbα. Following interaction with VWF, platelets undergo a change in morphology which allows them to roll and slide,

facilitating strong contact with the sub-endothelial surface. Formation of membrane tethers reduces forces that may dissociate the ligand binding (Shao *et al*, 1998; Yuan *et al*, 1999; Maxwell *et al*, 2006). Platelets also form cell-cell ligand binding with collagen via the platelet GPVI receptor. The binding of both of these sets of ligands is not sufficient to support firm adhesion, however following these interactions, intracellular signalling leads to activation of additional adhesion receptors. In resting conditions, platelet integrins β1 and β3 are in a low affinity state, but in response to agonist stimulation, shift to a high affinity state, facilitating ligand binding to the exposed extracellular matrix (Elmsley *et al*, 2000), and inducing full platelet activation.

Aggregation

Following platelet activation, intracellular bridges are formed between platelets through the binding of fibrinogen to the now high affinity state integrin receptor αIIbβ3 (Ni *et al*, 2003). Adhesion to the ECM also occurs via the high affinity β1 integrins which bind to collagen (α2β1), fibronectin (α5β1), and laminin (α6β1). Tetraspanins may facilitate the clustering of integrin receptors to optimise ligand binding, and platelet-platelet aggregation may also be stabilised via interactions of JAM-3 and Eph kinases and ephrins (Santos *et al*, 2002; Prevost *et al*, 2002). These interactions commit the complex to irreversible platelet aggregation.

Degranulation

There are three types of platelet granules: α-granules, dense granules and lysosomes. There are approximately 50 – 80 α-granules per platelet and they range between 200 - 500nm in size. The alpha-granules are the major secretory component of the platelet (Harrison *et al*, 1990), and contain a large number of soluble proteins, but also some transmembrane proteins, including coagulation factors (FV, FXI, FXIII), adhesion

molecules, (VWF, P-selectin, fibrinogen and fibronectin), and growth factors (Platelet-derived Growth Factor, Vascular Endothelial Growth Factor, basic Fibroblast Growth Factor, and Epidermal Growth Factor)(Kaplan *et al*, 1979; Ben-Ezra *et al*, 1990; Mohle *et al*, 1997; Galt *et al*, 2002). Some of the proteins contained in the α-granules, such as VWF and P-selectin, are synthesized by the megakaryocyte, whereas other proteins, such as fibrinogen, can be internalised by the platelet via endocytosis from the circulation (Handagama *et al*, 1987; Harrison *et al*, 1989). Dense granules contain high concentrations of small molecular weight molecules such as ATP, ADP, GTP, GDP, calcium and serotonin (Fukami *et al*, 1977), and there are estimated to be between 3 – 9 dense platelet granules per platelet (White 1969).

Lysosomes are small, electron dense granules, around 175 – 250nm in diameter. They contain lysosomal membrane proteins such as LAMP-1, LAMP-2, and CD63, various acid hydrolases, and cathespins D and E (Ciferri *et al*, 2000) (Table 1.1). The packaging of platelet granules has recently become a focus of interest. Studies by Sehgal, *et al*, (2007), investigated the distribution of fibrinogen and VWF within the α-granules, and found that proteins may be packaged into specific zones. These findings support the findings of Italiano *et al*, (2008), who found that proangiogenic factors such as vascular endothelial growth factor (VEGF) and anti-angiogenic factors such as endostatin, were packaged into separate α-granules. Villeneuve *et al*, (2009) have also reported heterogeneity in platelet granule content, reporting specific localisation of various proteins, including matrix metalloproteinases-2 and -9 (MMP-2 MMP-9), tissue inhibitors of metalloproteinases TIMP-1, TIMP-2 and TIMP-4. These studies raise questions as to the mechanisms that orchestrate the packaging of specific proteins into

specific granules, but also raise the possibility that upon platelet activation and degranulation, release of different proteins could occur in response to different signals.

ALPHA GRANULES	ALPHA GRANULES	DENSE GRANULES	LYSOSOMES
αIIb	CCL2	ATP	Acid hydrolase
α6	CCL3	ADP	Cathespin D
β3	CCL5	GTP	Cathespin E
GPVI	COMPLEMENT C3	GDP	Heparinase
PECAM	C4 precursor	Calcium	β-galactosidase
GPIb-IX-V	C1 inhibitor	Serotonin	
CD9	Platelet Factor H	Platelet factor 4	
CD36	VEGF	ADAM-10	
P-selectin	PDGF	ADAM-13	
VWF	FGF	Histamine	
Serotonin	EGF	Platelet activating Factor	
Calcium	HGF	TGF-β	
FV	IGF	Thrombocidins	
FXI	Angiopoietin	IL1β	
FXIII	MMP-1	HMGB1	
FVIII	MMP-2	Thromboxane	
PAI-1	MMP-9	Pyrophosphate	
Antithrombin	Thrombospondin-1	Magnesium	
C1-inhibitor	Angiostatin	Protease nexin-1	
TFPI	Endostatin	Gas6	
Protein S	TIMP-1	TFPI	
Plasminogen	TIMP-4	α1-protease inhibitor	
Fibronectin	LAMP2	HMW kininogen	
Vitronectin	Rap1	α2-macroglobulin	
CXCL1	Albumin	Vascular permeability factor	
CXCL4	β-thromboglobulin	Src	
CXCL5	CD63		
CXCL7	Multimerin		
CXCL8	α2-antiplasmin		
CXCL12	Glut-3		

Table 1.1: Platelet soluble mediators. Compilation of major components of platelet intracellular granules.

Source: King *et al,* 2002; Cell & Developmental Biology: 13, 293-302; Blair *et al*, 2009; Blood Reviews: 23, 177-189; Weyrich *et al,* 2004; TRENDS in Immunology: 25, No.9.; Smyth *et al*, 2009; J Thromb Haemost, 7, 1759-66.

Platelet granule secretion is dependent on membrane fusion which is regulated by SNARE proteins (Soluble NSF Attachment Protein Receptors), and various chaperone proteins that interact with the SNAREs. Platelets have been shown to contain VAMP-3, which is associated with granule membranes, also SNAP-23, which is associated with plasma membranes, and Syntaxin-4 which is evenly distributed throughout platelet membranes (Chen *et al*, 2000; Feng *et al*, 2002). These three SNARE proteins have been demonstrated to form an exocytotic complex in platelets, following activation (Flaumenhaft *et al*, 1999). Chaperone proteins appear to modify SNARE protein function, in particular Rab GDP dissociation inhibitor (Rab GDI), which inhibits release of α-granules but not release of dense granules (Shirakawa *et al*, 2000).

Whatever mechanisms may be involved in this process, release of platelet soluble mediators plays a key role in haemostasis, activating other platelets, and facilitating the localised delivery of soluble factors to the thrombus and the damaged endothelium. In addition, release of chemokines from platelet granules induces infiltration of white blood cells to the injury site, allowing them to become incorporated into the growing thrombus.

1.6. Monocytes

1.6.1. Structure and function

Monocytes originate in the bone marrow where the progenitor cells known as colony-forming unit, granulocyte-macrophage (CFU-GM), give rise to monoblasts which in turn give rise to promonocytes, then monocytes. Newly formed monocytes enter the peripheral blood where they are distributed between circulating and marginating pools (van Furth *et al*, 1986) In a normal healthy adult, the monocyte count is generally

between 0.1 – 0.6 x 10^3/µl, and monocytes have a half-life of up to 70 hours in the circulation (Whitelaw, 1966). Monocytes measure 12 – 15 µm in diameter and contain a nucleus that can be kidney shaped, round or lobulated, and occupies around 50% of the cell area. The cytoplasm contains numerous granules and vacuoles which may comprise several subpopulations. One of the major granule populations are primary lysosomes which contain various hydrolytic enzymes, including acid phosphatase, aryl sulphatase and peroxidase, and therefore have a digestive function (Nichols *et al*, 1973). Following monocytopoiesis, the majority of monocytes migrate to various organs via the extravascular tissues where they differentiate into macrophages (Ebert *et al*, 1939). Peripheral blood monocytes remaining in the circulation may be activated in response to various soluble mediators and cell-cell interactions, and subsequently be involved in mounting inflammatory responses or become incorporated into a thrombus.

1.6.2. Monocyte subsets and plasticity of monocytes

Monocytes are identified by their high expression of surface protein CD14, which forms part of the LPS/TLR-4 complex. Further study of monocyte antigenic markers has revealed that monocytes can be divided into two subsets, with 80 -90% of the monocyte population identified as 'classical' monocytes expressing high levels of CD14 and no detectable CD16 ($CD14^{hi}/CD16^{-}$), and the remaining 10 – 20% classed as 'non-classical' monocytes expressing lower levels of CD14 and positive for CD16 ($CD14^{+}/CD16^{+}$)(Passlick *et al*, 1989). These two subsets, present with a different expression profile of chemokines and chemokine receptors, with ($CD14^{hi}/CD16^{-}$) monocytes expressing C-C chemokines receptors CCR1, CCR2, and low levels of CXCR4 and CX3CR1; whereas ($CD14^{+}/CD16^{+}$) monocytes express CCR5 and high levels of CXCR4 and CX3CR1 (Weber *et al*, 2000). Although both subsets of

monocytes respond to the inflammatory stimulus Lipopolysaccharide (LPS), ($CD14^+/CD16^+$) monocytes exhibit higher expression of pro-inflammatory cytokines, and low or absent expression of anti-inflammatory cytokine IL-10 (Ziegler-Heitbrock *et al*, 1992; Frankenberger *et al*, 1996). Elevated levels of pro-inflammatory ($CD14^+/CD16^+$) monocytes have been reported in patients with chronic kidney disease (Ulrich *et al*, 2010), acute myocardial infarction (Tsujioka *et al*, 2009), and hypercholesterolaemia (Rothe *et al,* 1996). Circulating monocytes also have plasticity, and therefore 'classical' ($CD14^{hi}/CD16^-$) monocytes may differentiate into further subsets depending upon the stimuli they encounter (Sunderkotter *et al,* 2004).

1.6.3. Monocyte activation and induction of gene expression

The monocyte surface membrane contains numerous receptors that are constitutively expressed. These include CD36, a scavenger receptor for oxidized low density lipoprotein (Sparrow *et al,* 1989; Matsumoto *et al,* 1990); CD11b/CD18 which form a receptor complex involved in monocyte adhesion to endothelium (Meerschaert *et al*, 1995); CD14 which, in combination with the Toll-like Receptor-4 complex, enables the monocyte to bind to lipopolysaccharide (Haziot *et al*, 1988; Wright *et al*, 1990; Triantafilou *et al,* 2002), and P-selectin Glycoprotein Ligand-1 which facilitates cellular adhesion (Larsen *et al*, 1990; Sako *et al,* 1993). Engagement of these cell surface receptors with their ligands results in activation of the monocyte through specific intracellular signalling pathways, leading to gene induction, upregulation of surface antigen expression, and release of soluble mediators.

1.6.4. Monocyte role in inflammation

In response to inflammatory stimuli, peripheral blood monocytes are rapidly recruited to the site of infection in response to chemokines such as monocyte chemoattractant

protein-1 (MCP-1, also known as CCL2). Once there, LPS activation of the TLR-4 complex induces gene expression of pro-inflammatory cytokines (*eg*, IL-1β and IL-6), and TF (Gregory *et al*, 1989), and facilitates microbial killing through delivery of antimicrobial factors. Monocytes also play a role in the development of atherosclerosis. Monocytes transmigrate from the circulation into the intimal layer, where they can accumulate lipid, eventually developing into foam cells, and become incorporated into the forming atherosclerotic plaque.

1.6.5. Monocyte role in haemostasis

Monocyte TF has been clearly shown to be induced as a result of direct activation by LPS, however formation of platelet-monocyte aggregates also appear to enhance this expression (Celi *et al*, 1994). Monocytes are recruited into a thrombus (McGuiness *et al*, 2001) in response to chemokines, CCL2/MCP-1, where in addition to delivery of inflammatory mediators and release of chemokines, they may also play an angiogenic role (Anghelina *et al*, 2004).

1.7. Cellular microparticles

Cellular microparticles (MPs) are small, cellular fragments, ranging between 0.1 – 1μm in size, and can be generated following cellular activation or during apoptosis. Reorganisation of cell membrane phospholipids in response to agonist-induced cellular activation or apoptotic stimuli results in increased exposure of negatively-charged phospholipids, including phosphatidylserine (PS), on the outer surface of the cell membrane bilayer. The exact mechanism of microparticle release has not fully been determined, but calcium-dependent reactions have been demonstrated to result in disruption of protein-cytoskeletal binding, leading to budding of membranes and

eventual shedding and release of microparticles (Fox *et al*, 1990; Basse *et al*, 1994; Kunzelmann-Marche *et al*, 2001; Cauwenbergh *et al*, 2006). Cellular MPs have been demonstrated to derive from most cell types including platelets, monocytes, and endothelial cells, and express antigens, activation markers, and intracellular organelles that are characteristic to their cellular origin (George *et al*, 1986; Sims *et al*, 1988; Olas *et al*, 2002). There is growing evidence that certain cellular populations of MPs may be produced in a regulated manner in response to specific stimuli, and perform a specific function, including for example, intercellular transport of receptors (del Conde *et al*, 2005), and transfer of mRNA (Skog *et al*, 2008). In the circulation of healthy individuals it is thought that low levels of platelet-MPs (PMPs) are the predominant MPs present (Berckmans *et al*, 2001), however elevated levels of PMPs, and cellular MPs derived from macrophages, have been associated with coronary artery disease, and MPs derived from macrophages and smooth muscle cells have been reported to be present in the atherosclerotic plaque (Mause *et al*, 2005; Namba *et al*, 2007; Bakouboula *et al*, 2008). Due to the high level of surface PS expression which supports thrombin generation, and increased detection in various pathological conditions, PMPs have been generally associated with having a procoagulant role, however it has also been suggested that PMPs may have an anticoagulant function via inactivation of FVa (Tans *et al*, 1991). Therefore, this study investigates the effect of platelet-MP-monocyte interaction on monocyte gene expression of procoagulant factor, TF and the anticoagulant factor, TFPI.

1.8. Platelet-monocyte interaction

Cell-cell interactions play an important role in the haemostatic process. Following platelet activation and degranulation, P-selectin is translocated from the alpha-granules to the platelet cell surface (Stenberg *et al*, 1985). PSGL-1 is the primary ligand for P-selectin and is expressed on most white blood cells, including monocytes (Moore *et al*, 1992; Sako *et al*, 1993), therefore facilitating formation of platelet-monocyte complexes. Other receptor-receptor interactions then occur between platelets and monocytes that strengthen the complex. The engagement of monocyte PSGL-1 triggers a signalling pathway that activates CD11b/CD18 (MAC-1), and allows this receptor to bind to fibrinogen which is bound to the platelet via $\alpha IIb\beta_3$. This enables platelet CD40 ligand (CD40L or CD154), which is expressed on the platelet surface following platelet activation (Henn *et al*, 1998), to bind to monocyte CD40. CD40L is a member of the TNF family and interaction with monocyte CD40 induces an inflammatory response including release of cytokines, expression of adhesion receptors for ICAM, VCAM, and induction of TF (Bach *et al*, 1997). It is the formation of these secondary ligand interactions which render the platelet-monocyte complex irreversibly aggregated (Evangelista *et al*, 1996) (Figure 1.6).

There are reported to be only small numbers of platelet-monocyte aggregates in the circulation of healthy individuals, however, formation of elevated numbers of platelet monocyte complexes are reported in various disease states, due to increased platelet activation. High blood glucose levels have been reported to lead to increased expression of platelet CD40L and formation of platelet-monocyte aggregates via interaction with monocyte CD40 (Vaidyula *et al*, 2006). Increased levels of platelet-

monocyte aggregates have also been reported in patients with unstable angina and acute MI (Furman *et al*, 2001; Sarma *et al*, 2002).

Platelet microparticles can also form complexes with monocytes via ligand-ligand binding of PMP P-selectin and monocyte PSGL-1, thus activating the monocyte through this signalling pathway, leading to upregulation of monocyte TF. Mice lacking P-selectin or PSGL-1 form thrombi that have only minimal presence of TF (Falati *et al*, 2003); therefore this interaction is important for incorporation of TF and leukocytes into the growing thrombus (Furie *et al*, 2004; Polgar *et al*, 2005).

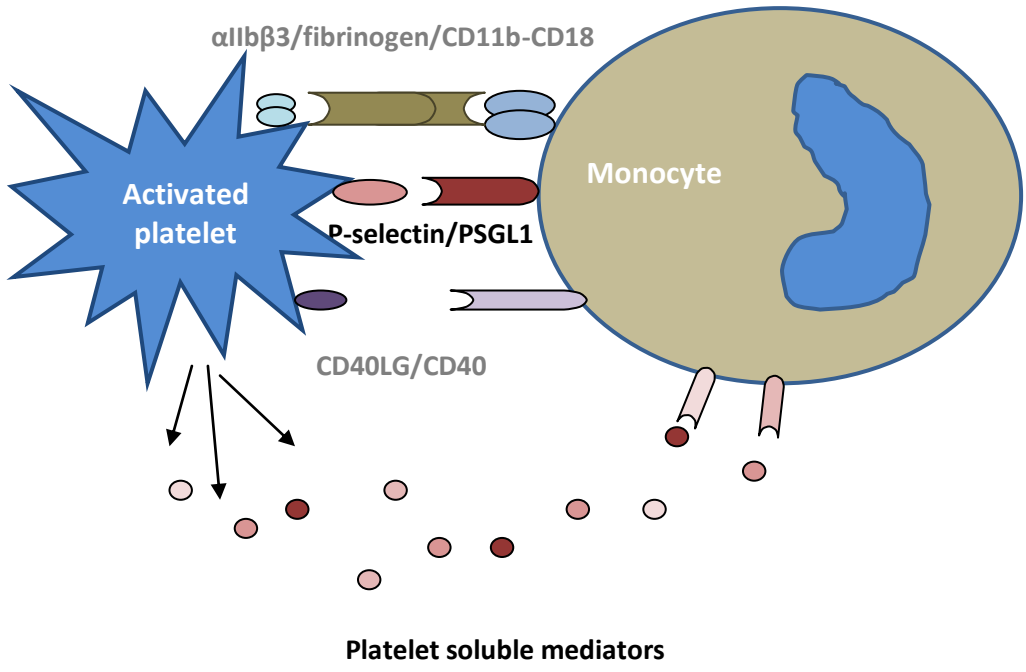


Figure 1.6: Platelet-monocyte interaction. Direct platelet-monocyte interaction through binding primarily through platelet P-selectin and monocyte PSGL-1, with secondary ligand-ligand interactions of platelet αIIbβ3 and monocyte CD11b/CD18 via fibrinogen, and platelet CD40 ligand with monocyte CD40, which render the complex irreversibly aggregated. Indirect platelet-monocyte interaction occurs via release of platelet soluble mediators from platelet intercellular granules which bind to monocyte surface receptors.

1.9. Thrombus formation

Thrombus formation is an accumulative process that involves many of the factors already introduced in this section; primarily the activation of soluble plasma proteins involved in coagulation, and the attraction, activation and interaction of platelets, but also white blood cells, in response to exposure of components of the sub-endothelium. The role of each component, and factors that may regulate or determine thrombus structure, are considered further.

1.9.1. Role of platelets in thrombus formation

Depending upon the shear rate within the vessel, fibrinogen, VWF, or both, will facilitate the initial platelet adhesion to the sub-endothelium. Additional platelets then slide over this initial layer and fibrinogen and VWF, along with other adhesion molecules contribute to the tethering of the platelets. Following formation of the initial platelet monolayer, additional platelets are recruited into the growing haemostatic plug to stabilise it, and prevent premature disaggregation. This process requires integrin αIIbβ3, a ligand for fibrinogen, VWF, and fibronectin, which through activation of intracellular signalling, is now in a high affinity state for binding to ECM, committing the platelets to irreversible aggregation, and induction of full platelet activation (Plow *et al*, 1987; Ni *et al*, 2003).

Studies of platelet accumulation and function during thrombus formation, have suggested that platelets recruited at different stages of thrombus formation may have specific roles. Munnix *et al*, (2007) reported two distinct populations of platelets within the thrombus; pro-aggregatory platelets that form distinct aggregates, with an active conformation of integrin αIIbβ3 that binds fibrinogen, and expressing surface P-selectin, and pro-coagulant platelets with different morphology, appearing in strings at

the edge of the clot, and exposing surface PS, therefore supporting coagulation and thrombin generation. The observation that platelets within the thrombus may be in different states of activation is supported by studies of platelet accumulation within a thrombus, using calcium mobilisation as a monitor, demonstrating that some platelets appeared to be active and adhered at the site of injury, whereas many platelets remained in a resting state (Dubois *et al*, 2004).

A number of platelet adhesion molecules, including SLAM, Ephrins 4, and B1, and Semaphorin 4D, may play a role in determining the structure and stability of the thrombus, as several studies have shown that mice defective in these protein ligands have defective aggregation and delayed thrombus formation (Nanda *et al*, 2005; Prevost *el al*, 2005; Zhu *et al*, 2007). Also Platelet-Endothelial Cell Adhesion Molecule (PECAM) has been shown to regulate thrombus size and rate of formation (Falati *et al*, 2006), and a thiol isomerase enzyme, PDI, secreted from activated platelets and expressed on the platelet surface, may also play a role in thrombus formation through regulation of platelet aggregation (Chen *et al*, 1995; Essex *et al*, 1995; Cho *et al*, 2008; Holbrook *et al*, 2009).

1.9.2. Role of leukocytes in thrombus formation

Activation of platelets and secretion of soluble mediators from platelet granules, leads to delivery of chemokines that are chemotactic for leukocytes. E-selectin ligands, PSGL-1 and CD44, are responsible for the initial capture of the leukocyte to the endothelium, followed by firm adhesion due to the action of integrin binding to ICAM-1 and VCAM-1. Early white cell recruitment to a thrombus is mainly neutrophils, followed by monocytes, and finally a small number of eosinophils, basophils and lymphocytes are incorporated (Shirasawa *et al*, 1971). The infiltration of neutrophils in

the early stages of thrombus growth and presence of IL-8 may play a key role in the structural organisation of the forming thrombus (Wakefield *et al*, 1999; Henke *et al*, 2001). Monocytes have been shown to appear firstly at the edge of the thrombus, eventually becoming evenly distributed throughout (McGuiness *et al*, 2001). This may position them for phagocytosis of damaged ECM material, and removing debris of unwanted cells, eventually creating spaces between fibres. Leukocyte presence within the thrombus also allows delivery of enzymes, including Matrix Metalloproteinases (MMPs), urokinase, and elastase which modulate fibrinolysis (Machovich *et al*, 1990; Moir *et al*, 2002, Goel *et al*, 2003). Monocytes have also been shown to play a role in recanalisation within the thrombus (Leu *et al*, 1987; Moldovan *et al*, 2000).

1.9.3. Role of coagulation proteins in thrombus formation

At the same time as the platelet thrombus begins to develop, formation of a fibrin mesh is generated through activation of plasma clotting factors, leading to generation of thrombin and formation of fibrin (Falati *et al*, 2002). TF, the initiator of thrombin generation may be present at the site of injury, but has also been shown to be present throughout the thrombus, associated with cellular-MPs (Falati *et al*, 2003). Thrombin activity, also detected throughout the thrombus (Baird *et al*, 2003), is inhibited through the action of anti-coagulant proteins, such as TFPI, and antithrombin. The amount of thrombin generated appears to play a role in the organisation and structure of the thrombus, with low amounts of thrombin producing clots with thick strands, and higher concentrations of thrombin resulting in a denser clot, made up of thinner strands (Blomback *et al*, 1994; Wolberg *et al*, 2003). Coagulation factors from the 'contact' pathway may also play a role in thrombus formation, as studies in mice deficient in FXII and FXI were shown to form unstable thrombi (Renne *et al*, 2005; Wang *et al*,

2005). Additionally, FXIII, which is both present in plasma and released from platelet α-granules, is activated by thrombin, stabilising the thrombus through cross-linking of fibrin (Ariens *et al* 2002; Kobbervig *et al*, 2004).

1.9.4. Role of red blood cells in thrombus formation

Red blood cells (RBCs), or erythrocytes, are biconcave discs ~ 8μm in diameter. Their primary function is the transport of oxygen from the lungs to cells, and the return of carbon dioxide from the cells to the lungs, which occurs via haemoglobin. Within the vessel, under conditions of flow, RBCs enhance the opportunity for platelet-vessel wall interaction; however RBCs are also incorporated into the growing thrombus. Within the thrombus they may play a number of roles, including providing additional phospholipid surface for thrombin generation (Peyrou *et al*, 1999). The presence of RBCs may also affect the direction of growth of the thrombus (Mori *et al*, 2008), and influence the fibrin network structure (Gersh *et al*, 2009).

1.9.5. Regulation of thrombus formation

Therefore, thrombus formation is an orchestration of cellular infiltration and accumulation, and sequential activation of plasma coagulation proteins, requiring cellular mechanisms for adhesion, aggregation, activation, and secretion of soluble mediators. The rate of thrombus formation and stability may be dependent upon expression of specific cellular adhesion molecules. In addition, the amount of thrombin generated through activation of coagulation may affect the structure and density of the clot. In turn this could affect the ability of cells, and possibly cellular MPs, to disperse throughout the clot, and may affect the delivery of soluble mediators, and lysis of the thrombus when no longer required. Therefore, regulatory control is required at all stages of thrombus formation.

1.10. Aims of the project

The aim of this study was to examine factors that thrombus formation and stability; firstly by investigating the regulation of coagulation within blood from premature MI subjects and controls; to explore the role of activated platelets in the regulation of monocyte gene expression; and to investigate whether there is regulated gene expression within a thrombus.

Haemostasis is the balance of the actions of procoagulant factors seeking to repair breach of vascular integrity vs anticoagulant factors seeking to limit the response to an appropriate level. Elevated levels of a number of haemostatic factors, including FVII, and fibrinogen, have been linked to increased risk of cardiovascular disease (Meade *et al*, 1986; Folsom *et al*, 1997; Danesh *et al*, 1998). This study begins with an assessment of the balance of pro- and anti-coagulant activity in plasma from subjects who have suffered an MI with a healthy control group, with the hypothesis that subjects who have suffered an MI have a higher procoagulant haemostatic response.

Cellular interaction, in particular platelet-monocyte interaction, plays an important role in the haemostatic process, and monocytes are known to express TF in response to inflammatory stimuli, with expression increasing further when in complex with platelets. TF, the initiator of coagulation, and TFPI, the inhibitor of TF, are two key factors of coagulation, and regulation of haemostasis. Therefore, gene expression of TF and TFPI, as representatives of the procoagulant and anticoagulant response, respectively, were measured during experiments examining how platelet-monocyte interaction might alter the haemostatic balance, and whether gene expression of these two haemostatic factors alters during formation of a thrombus.

The specific aims of this study are set out in more detail below:

- **To compare the thrombotic potential of plasma obtained from subjects that had suffered a premature MI with plasma obtained from a normal healthy control cohort.** Whereas haemostasis assays measuring prothrombin time (PT), and activated partial thrombin time (APTT) can give an indication of initial haemostatic response, it does not give indication of the major thrombin burst generated during formation of a thrombus. The magnitude of the thrombotic reaction to a fissured plaque may be an important factor in determining clinical outcome following an MI. Therefore, measurement was made of the endogenous thrombotic potential (ETP) which measures the global effect of procoagulant and anticoagulant activity in the plasma, and of individual clotting factors.
- **To determine the frequency of two single nucleotide polymorphisms (SNP) within the TF gene, in the premature MI cohort and control group, and examine possible association with plasma TF and ETP.** Gene polymorphisms, *eg* G20210A, the Prothrombin gene variant, have been shown to be linked to increased levels of various haemostatic plasma proteins. There have been several reports suggesting an association between TF gene polymorphism and increased TF activity, however results are inconclusive. The frequency of two SNPs found within the TF gene were determined, -1812CT which is in the promoter region of the gene, and +5466A>G which is in intron 2. Analysis was made of distribution of genotypes in MI and control cohort, and for association of genotype with basal TF expression and ETP in plasma.

- **To measure the expression of monocyte TF and TFPI in response to platelet activation and interaction.** Monocytes are known to produce TF in response to inflammatory stimuli, such as LPS, but have also been reported to express TF following interaction with active platelets. In contrast, there have been varied reports on monocyte expression of TFPI in response to inflammatory stimuli, and it is not known whether they express TFPI in response to platelet activation and interaction. Therefore, a whole blood model was established, using Lipopolysaccharide (LPS) as an inflammatory stimulus, and collagen-related peptide-XL (CRP-XL), which activates platelets, as a haemostatic stimulus, to examine their different effects on monocyte gene expression of TF and TFPI. Further experiments were then carried out to compare the effect of the two mechanisms of platelet-monocyte interaction, that of direct interaction due to platelet-monocyte aggregation and indirect interaction via release of platelet soluble mediators, on monocyte induction of TF and TFPI in an isolated cell system, and to compare the effect of platelet-MP interaction on monocyte gene expression of TF and TFPI.
- **To investigate expression of TFPI isoforms in monocytes.** Two isoforms of TFPI have been detected in endothelial cells, but little is known about expression of the two isoforms in monocytes, particularly in response to activated platelets. To date there have been no reports regarding TFPI isoforms in a clinical cohort. Therefore, expression of alpha and beta isoforms of TFPI were measured in resting monocytes, and in monocytes following exposure to activated platelets, in an MI cohort, in off-spring with two-generational history of MI, and healthy control groups.

- **To investigate whether changes in monocyte gene expression of TF and TFPI are translated into protein.** Experiments were carried out to determine whether changes in monocyte gene expression resulted in changes in plasma levels of TF or TFPI, due to translation of mRNA into soluble protein, and to assess whether these proteins had functional activity. To assess changes to membrane surface expression, a chromogenic assay was used to measure changes in surface procoagulant activity. Investigation was also made to determine the effect of changes in monocyte TF and TFPI gene expression and surface expression, on clot formation.
- **To extend observations on monocyte gene expression of TF and TFPI following exposure to platelet soluble mediators or interaction via P-selectin and PSGL-1, through microarray analysis of monocyte genetic profile following exposure to the differential stimuli.** Microarray studies which investigate monocyte gene expression have mostly focused on macrophage response to inflammatory stimuli such as LPS, or oxidised-LDL, or on monocyte differentiation into macrophages, but to date there have been no published microarray studies on changes in monocyte gene expression profile in response to activated platelets. Therefore, the previous study was expanded to allow identification of key monocyte genes that have altered expression due to direct and indirect platelet-monocyte interaction, *ie*, ligand binding of platelet P-selectin with monocyte PSGL-1, or monocyte interaction with platelet soluble mediators, and evaluate how these changes may alter the monocyte phenotype and therefore its function.

- **To investigate possible changes of gene expression within a growing thrombus.** Despite the many studies of thrombus formation, it is not known whether cells are simply attracted to the site of injury to adhere, provide structure, and release their contents into the wound site, or whether there is an additional, active process involving changes in gene expression of cells incorporated into the thrombus, that further modifies thrombus formation. Therefore, a gene expression microarray study was carried out to investigate changes in patterns of gene expression during early thrombus formation.

CHAPTER 2: MATERIALS AND METHODS

The methods described in this chapter are divided into four main sections: blood collection and description of clinical cohorts; isolation of plasma and plasma assays, including thrombin generation assay and coagulation assays; cell isolation methods and cellular assays, including flow cytometry, rotational thromboelastometry, and thrombus formation using Chandler loop; and finally, molecular methods including RNA isolation, real-time quantitative PCR, and gene expression microarray methodology.

2.1 Blood Collection

2.1.1. Blood collection from normal healthy donors

Peripheral blood was obtained from healthy volunteers with informed consent. Blood was collected by venepuncture via a 21g butterfly needle, without application of a tourniquet, and by clean venepuncture to avoid activation of platelets and coagulation. The first 3mL blood, which was used for cell count, was collected into 3mL Vacutainers^{TM} (Becton Dickinson, Oxford, UK) spray coated with EDTA. All subsequent blood was collected into Vacutainers^{TM} containing 0.105M sodium citrate (1:9 v/v). Blood samples were processed within 10 minutes of collection.

2.1.2. Blood collection from premature MI cohort and controls

PRAMIS cohort

Plasma for assessment of Endogenous Thrombotic Potential (ETP) and coagulation assays was prepared from peripheral blood collected by Dr Ravi Singh, for the PRAMIS study (Platelet Reactivity in Acute Myocardial Infarction). The cases comprised 162 subjects recruited retrospectively from the registries of 3 coronary care

units in Leicester. All had suffered a myocardial infarction according to WHO criteria, before the age of 50 years. Blood was collected at least 3 months post the acute event, and all subjects were in a stable condition. The control cohort comprised 186 subjects recruited from 3 primary care practices located within the same geographical area. All control subjects had no personal or family history of MI. All subjects were Caucasian of Northern European origin. In addition, blood was collected from 20 young healthy males with a strong family history of MI, and 22 matched, healthy controls, with no family history of MI. Blood samples were collected by clean venepuncture via a 21g butterfly needle without tourniquet, into 3.2% (w/v) citrate, centrifuged within 10 minutes of collection. Blood collection from healthy donors and premature MI subjects for all studies was approved by the Leicestershire Research Ethics Committee and all subjects gave written informed consent.

BLOODOMICS cohort

Monocytes for study of monocyte gene expression in premature MI subjects and controls were isolated from peripheral blood, collected by Dr Unni Krishnan, for the BLOODOMICS study. The cases were recruited from the Myocardial Infarction National Audit Project (MINAP), selecting individuals who had suffered an MI before the age of 65 years, and at the time of the acute event, had been admitted to hospital with ST segment Elevation Myocardial Infarction (STEMI). Blood was collected at least 3 months post the acute event, and all subjects were in a stable condition. The control cohort was recruited by advertisement within the hospital premises. All control subjects had no personal or family history of MI. Blood was also collected from young healthy males with a significant family history of MI. A significant family history of premature MI was defined as either parent having had an MI under the age of 55 years

+/- a grandparent having had an MI under the age of 65 years. Case off-spring were matched for age, gender, and smoking status with healthy controls, with no family history of MI. All donors were north European Caucasian. Ethical approval was granted by Derbyshire Research Ethics Committee, February 2007 (Ref 06/Q2401/134). Citrated blood samples were collected into BD vacutainer® glass tubes, and the initial 2mL of blood was discarded. Monocyte isolation commenced within 10 minutes of blood collection, for resting monocytes, and post 4 hour incubation at 37^{o}C in the presence of 500ng/mL CRP-XL.

2.2 Isolation of plasma and plasma assays

2.2.1. Preparation of plasma

Normal healthy donors: Citrated whole blood was centrifuged at 1800 g for 30 minutes at 4^{o}C. Plasma was transferred to 1.5mL microfuge tubes and either used immediately or stored at -80^{o}C, in single use aliquots.

Plasma from PRAMIS cohort: Plasma was prepared in the same way as above, with all plasma stored at -80^{o}C in single use aliquots.

2.2.2. Preparation of plasma pool

4 x citrate tubes were collected from 20 normal healthy donors, and plasma prepared as above. Following centrifugation plasma was removed from all tubes and pooled, then transferred into 1.5mL microfuge tubes for storage at -80^{o}C.

2.2.3. Measurement of Endogenous Thrombotic Potential

A chromogenic method was developed, allowing assessment of the overall haemostatic response in plasma of premature MI subjects and healthy controls. The assay was based on the method of Hemker *et al*, (1986), with slight modifications. The principle

of the method is to measure the overall capacity of the plasma coagulation factors to convert prothrombin to thrombin. Since plasma does not contain any type of blood cell, phospholipid is added to mimic the negatively charged cellular plasma membrane that in whole blood is predominantly provided by activated platelets. Buffer containing a low concentration of recombinant TF (rTF) plus calcium is added to initiate the reaction, and cleavage of a chromogenic substrate reflects the rate of thrombin being generated in the sample. Preliminary experiments were carried out to assess the effect of varying concentrations of substrate, phospholipid and rTF, and determine optimal concentrations to be used in the assay. Following these experiments, the reproducibility of the assay was determined. Experiments were carried out to measure the ETP in 10 samples of pooled plasma, and this experiment was repeated the following day, allowing calculation of intra-assay and inter-assay reproducibility. The final, optimised method was as follows: 96-well plates were set up containing 80µl Plasma, PefaBloc FG [3mg/mL final conc] (Pentapharm GmbH, Basel, Switzerland), Phospholipid [5µg/ml final conc] (91/542, National Institute of Biological Standards and Control), and recombinant TF [1.73ng/mL final concentration] (recombiPlasTin – HemsIL; Instrumentation Laboratory, Warrington, UK). The plate was incubated for 1 minute at 37^{o}C to ensure reagents of all wells were at the same temperature, then thrombin generation was initiated by the addition of 20µl of start solution containing 1:1 volume of 0.1M $CaCl_2$ and 5mM chromogenic substrate H-β-Ala-Gly-Arg-pNA.2AcOH – (Pefachrome TG– Pentapharm GmbH, Basel, Switzerland). The amidolytic activity was determined using an Anthos HTIII microtitre platereader set at 37^{o}C, and the absorbance at 405nm was recorded every 30 seconds for 20 minutes. The OD was then corrected for α-2-macroglobulin using the algorithm described by Rijkers *et al*, (1998).

α-2-macroglobulin forms a complex with free thrombin causing inhibition of thrombin activity. This complex, however, retains amidolytic activity on the chromogenic substrate, therefore a mathematical algorithm is applied to determine the activity due to free thrombin only. To standardise the assay, pooled plasma, prepared from a plasma pool obtained from 20 normal donors, was measured in duplicate on each plate, so that the ETP for each sample could be normalised against the pooled plasma. Stored plasma from two individuals with known low and high ETP were also assayed on each plate as control samples. All samples were assayed in duplicate.

2.2.4. Coagulation Factor Assays

Plasma levels of coagulation factors FII, FVII, FVIII, FIX, FX and FXI were measured in plasma from premature MI subjects and controls, using a Sysmex CA6000 coagulometric analyser (Sysmex, Milton Keynes, UK). The coagulation factors fell into two slightly different groups for analysis and are described in more detail below.

2.2.4.1. FII (prothrombin), FVII and FX

Plasma deficient in FII, FVII or FX (Dade Behring, Milton Keynes, UK), comprising the extrinsic coagulation pathway, resulted in a prolonged thromboplastin (PT) time. A mixture of the factor-deficient plasma and the patient plasma was tested in the PT assay and the result interpreted using a reference curve obtained with dilutions of standard human plasma mixed with deficient plasma. The plasma sample was diluted 1:20 in Owren's Veronal Buffer (OVB- Dade Behring). In a tube warmed to 37^{o}C, diluted plasma was added to factor-deficient plasma, and incubated for 1 minute. Recombinant TF (Innovin-Dade Behring) was added, and the clotting time recorded. The result was read against a reference curve, generated from acommercially available reference

plasma (Dade Behring). The expected range for results was between 70 – 120% of the value of the reference standard for each factor.

2.2.4.2. FVIII, FIX and FXI

Plasma deficient in FVIII, FIX or FXI (Dade Behring), factors comprising the contact pathway, resulted in a prolonged partial thromboplastin time (APPT). Factor-deficient plasma was mixed with diluted patient plasma and 100µl of Actin FS reagent (Dade Behring), a contact pathway activator, was added. Following 6 minutes incubation at 37^{o}C, calcium chloride solution was added and the clotting time recorded. The expected range of results was: FVIII 70 – 150%; FIX 70 – 120%; and FXI 70 - 120% of the value of the reference standard for each factor.

2.2.4.3. Antithrombin III

Antithrombin III (ATIII) was measured on the Sysmex CA6000 analyser, using an automated chromogenic method. ATIII in the sample was converted into an intermediate inhibitor by heparin and inactivated thrombin present in the plasma sample. The residual thrombin content was then determined in a kinetic test by measuring the increase in absorbance at 405nm. Citrated plasma was added to the chromogenic thrombin reagent and incubated for 3 minutes at 37^{o}C. The substrate was added and the change in OD recorded per minute. Results were then determined from a reference curve. The expected range for the results was between 75 – 125% of the value of the reference standard.

2.2.5. TF and TFPI activity assays

The Actichrome TF and TFPI assays are commercially available from American Diagnostica Incorporated (ADI, Greenwich, CT, USA). These kit assays were used to

measure plasma levels of TF and TFPI activity in the PRAMIS premature MI cohort. The same kits were used to measure TF and TFPI activity in plasma obtained from citrated whole blood that had been incubated for up to 6 hours with either CRP-XL or LPS, described in section 2.3.2.

2.2.5.1. Actichrome TF

This assay measures TF procoagulant activity in the plasma. 25µl of each plasma sample was added per well of a 96-well plate and mixed with 25µl FVIIa, 25µl FX, and 50µl assay buffer (pH8.4) and incubated at 37^{o}C for 15 minutes to allow formation of the TF/FVIIa complex, and the subsequent conversion of FX and FXa. 25µl of Spectrozyme FXa substrate was added to each well and incubated at 37^{o}C for 1 hour. The amount of FXa generated was measured by its ability to cleave the substrate Spectrozyme Xa, which releases pNA creating a colour change. The absorbance at 405nm was read against a standard curve generated from known amounts of active TF to determine the TF concentration [pM] of each sample.

2.2.5.2. Actichrome TFPI

This assay measures the ability of TFPI to inhibit the catalytic activity of the TF/FVIIa complex in its course to activate FX and FXa in plasma. Prior to assay, plasma samples and reference plasma were diluted 1:20 in TFPI-depleted plasma. 25µl of diluted plasma sample or reference plasma was then added per well of a 96-well plate and incubated at 37^{o}C for 30 minutes in the presence of 20µl TF/FVIIa. 20µl of FX was added and samples were further incubated at 37^{o}C for 15 minutes. 20µl of EDTA, followed by 20µl Spectrozyme FXa substrate was then added to measure the residual activity of the TF/FVIIa complex. The Spectrozyme FXa substrate is cleaved only by FXa generated in the assay, releasing pNA. The absorbance was read at 405nm and

compared to values obtained from a standard curve constructed using known TFPI activity levels.

2.2.6. Statistical analysis of coagulation assays

Statistical analysis of ETP and coagulation factors in plasma of premature MI subjects and controls was determined by unpaired t-test. Associations of clotting factors with ETP were tested by means of Pearson's correlation coefficient. Statistical analysis was performed using GraphPad PRISM software version 4 (BioData Corp, Horsham, PA, USA). All results are presented as mean ±SD. Statistical significance was defined as $P < 0.05$.

2.3 Cell isolation methods and Cellular assays

2.3.1. Cellular agonists

2.3.1.1. Collagen-Related Peptide-XL

Collagen-Related Peptide-XL (CRP-XL) was used as a platelet agonist in all platelet-monocyte interaction experiments. CRP-XL contains 10 repeats of the Gly-Pro-Hyp sequence found in collagen, and binds specifically to the platelet GPVI collagen receptor (Knight *et al*, 1999). Expression of GPVI is limited to megakaryocytes and platelets, therefore CRP-XL in whole blood will act as a platelet-specific agonist. Optimisation experiments determined that 500ng/mL CRP-XL was sufficient to activate platelets and cause degranulation but not generate platelet-microparticles. In some experiments where platelet-MPs were positively isolated, platelets were activated with 10µg/mL CRP-XL.

2.3.1.2. Lipopolysaccharide

Lipopolysaccharide (LPS) (Sigma, Poole, Dorset, UK) was used to directly activate monocytes in whole blood. LPS, also known as endotoxin, is the major component of the outer membrane of Gram-negative bacteria. Monocytes were activated with moderate concentrations of LPS [200ng/mL] that were sufficient to induce an inflammatory response, but not induce rapid cellular apoptosis.

2.3.1.3. P-selectin-Fc chimera

This chimeric protein comprises an N-terminus of human P-selectin (Trp42 – Ala771), followed by a linking sequence (IEGRDMD), and a C-terminus of Human IgG1 (Pro100 – Lys330)(R&D Systems, Abingdon, UK). A concentration of 10ug/mL P-selectin-Fc chimera was used to bind monocyte P-selectin Glycoprotein Ligand-1, and activate intracellular signalling via this receptor-ligand induced pathway.

2.3.2. Stimulation of whole blood with inflammatory and haemostatic stimuli

Using platelet-monocyte interaction as a focus, a whole blood model was developed to allow investigation of the effect of differential stimuli on monocyte gene expression, on monocyte surface procoagulant activity, and procoagulant activity within the plasma. Citrated whole blood was incubated at 37^{o}C for up to 6 hours in the presence of 500ng/mL CRP-XL (a haemostatic stimulus, which indirectly activated monocytes via the activation of platelets), 200ng/mL LPS (an inflammatory stimulus, which directly activated the monocytes via the CD14/TLR4 receptor complex), or without agonist. Following the incubation period of 1.5, 4, or 6 hours, plasma was prepared as previously described, and monocytes were isolated as set out in the cell isolation methods described in section 2.3.3.

2.3.3. Cell isolation methods

2.3.3.1. Positive isolation of monocytes from whole blood

This method was used for positive selection of monocytes directly from whole blood, prior to extraction of mRNA. Monocyte isolation was performed on citrated whole blood using Dynabeads (Invitrogen, Paisley, UK). These are magnetic beads that are coated with a monoclonal antibody that recognises CD14, a single chain glycoprotein predominantly expressed by monocytes and macrophages. 50µl of CD14-coated Dynabeads [4 x 10^8/mL] were incubated per mL of whole blood, for 10 minutes at 4^oC on a rotary mixer. Each sample was then placed on the Magnetic Particle Collector (MPC) and the supernatant removed. 500µl of cell culture grade Phosphate Buffered Saline (PBS)(Invitrogen), containing 0.3% Bovine serum albumin (BSA)(Sigma)(PBS/BSA 0.3%) was added to each sample. This wash step was repeated, and finally samples were lysed in 1 mL of TRIZOL® reagent (Invitrogen).

2.3.3.2. Isolation of mononuclear cells by density gradient centrifugation

This method was used for experiments investigating the effects of platelet soluble mediators on monocyte gene expression, and also for experiments investigating changes in monocyte surface procoagulant activity. 10ml of citrated whole blood was poured into a 50ml sterile centrifuge tube. An equal volume of PBS/BSA 0.3% was added and the sample mixed gently by inversion. The diluted blood was underlayed with 10ml of Lymphoprep (Nycomed, Oslo, Norway), a density gradient solution (density 1.077±0.001g/ml; osmolality 290±15mOsm), and the sample centrifuged at 800 g. Mononuclear cells were washed in 10ml PBS/BSA 0.3%, and pelleted by centrifugation at 250 g. The mononuclear pellet was then resuspended in 1ml PBS/BSA 0.3% and the number of monocytes counted using a Beckman Coulter AcTdiff^{TM} cell counter

(Beckman Coulter, High Wycombe, UK), which was calibrated daily with quality controls, 4C-ES Cell Controls (Beckman Coulter). Monocyte cell suspensions were adjusted to between $5.0 \times 10^5 - 1.0 \times 10^6$ for all experiments.

2.3.3.3. Platelet isolation

In all experiments using isolated platelets, the platelet population was initially obtained by preparing platelet-rich plasma (PRP). This was achieved by centrifugation of citrated whole blood at room temperature for 20 minutes at 180 g, and transferring the upper platelet-rich layer to a clean tube.

2.3.3.4. Preparation of platelet-soluble mediator-rich plasma

PRP was incubated with CRP-XL [500ng/mL], for 15 minutes at 37^{o}C, to activate the platelets and ensure platelet degranulation. The activated platelet suspension was centrifuged at 1800 g for 15 minutes to pellet the platelets. The soluble mediator-rich plasma layer was transferred to a clean tube and filtered using a 0.2µm acrodisc filter to remove possible contamination of cellular microparticles.

2.3.3.5. Isolation of platelet-MPs

To isolate platelet-MPs, PRP that had been stimulated with a high concentration of CRP-XL [10ug/mL] was centrifuged at 1800 g for 15 minutes to pellet the platelets. The soluble mediator-rich plasma layer was then transferred to a clean tube and centrifuged at 12,000 g for 20 minutes to pellet platelet-MPs which were then washed in Hepes Buffered Saline (HBS)(150mM NaCl, 5mM KCl, 1mM $MgSO_4$, 10mM HEPES; pH 7.4).

2.3.4. Methods for cellular assays

2.3.4.1. Flow cytometry

Flow cytometry was used to assess the efficiency of CRP-XL on platelet degranulation and generation of platelet-MPs. Cytometric assays were carried out on an EPICS profile XL-MCL flow cytometer (Beckman Coulter, High Wycombe, UK). Cells are passed in single file through a laser beam which results in light scattering. The amount of light scattered at narrow angles to the axis of the light beam is known as the forward scatter (FS). This allows the size of the cell to be determined. The amount of light scattered at 90 degrees to the axis of the laser beam is known as the side scatter (SS). This allows determination of cell granularity, therefore it is possible to differentiate between different cell populations that are present in whole blood, eg platelets, monocytes, lymphocytes and granulocytes.

Measurement of cell surface and intracellular antigens can be carried out by using monoclonal antibodies that are conjugated to fluorescent dyes. The dyes emit a coloured light when the laser beam excites them. Various fluorescent intensity filters (FI filters) reflect the light at different wavelengths, allowing several fluorophores to be detected at a time. The intensity and distribution of the fluorescence determines the height and width of the pulse signals. These are then reported as numerical data for further analysis.

P-selectin

Flow cytometric analysis of platelet P-selectin exposure was used to determine platelet degranulation following activation with CRP-XL. To determine the optimal concentration of CRP-XL to be used in this study, 5µl of citrated whole blood was incubated with serial dilutions of CRP-XL [0 – 10µg/mL] in 50µl HBS, containing monoclonal P-selectin FITC antibody (R&D Systems,) or FITC-conjugated mouse

IgG1 isotype control (R&D Systems). Samples were incubated for 15 minutes incubation at room temperature, and subsequently, 5µl of each sample was transferred into 500µl of 0.2% formyl-saline (0.2% v/v formaldehyde in 0.85% w/v isotonic saline solution). Samples were incubated at room temperature for a further 10 minutes, then analysed by flow cytometry. The platelet population was identified by size and granularity by forward and side scatter. This population was then analysed for P-selectin exposure, and expressed as a percentage of the platelet population.

Annexin-V

Flow cytometric analysis of Annexin-V binding was used to determine generation of platelet-MPs following platelet activation with CRP-XL. 5µl of citrated whole blood was incubated with serial dilutions of CRP-XL [0-10µg/mL] in 50µl of HBS containing 2mM $CaCl_2$, Hirudin [100U/mL](Sigma), and monoclonal antibody CD42b-RPE (DAKO, High Wycombe, UK) to identify platelet and platelet-MP populations. An isotype control for RPE was set up using mouse IgG2a-RPE, and 5µl of CRP-stimulated whole blood incubated with HBS without $CaCl_2$ acted as a negative control for Annexin-V binding. Samples were incubated for 10 minutes at room temperature, and subsequently, 5µl of Annexin-V-FITC (Pharmingen, BD Biosciences, Oxford, UK) was added. Following a further incubation for 10 minutes at room temperature, 4µl of sample was transferred into FACS tubes containing 400µl of HBS or HBS/c as appropriate, and the samples were analysed by flow cytometry. The platelet population was identified by size and granularity (forward and side scatter), and the platelet population confirmed by positive binding of CD42b-RPE. This population was analysed for Annexin-binding and expressed as a percentage of the platelet population.

2.3.4.2. Measurement of cell surface procoagulant activity

This method was used to determine changes in procoagulant activity on the monocyte surface following incubation in the presence of CRP-XL-activated platelets, or LPS. Doubling dilutions of rTF (RecombiPlasTin) were made to generate a standard curve. 100µl of a combined reagent comprising Tris Buffered Saline (TBS)[50mM Tris, 150mM NaCl, 0.02% NaN_2, pH 7.4], chromogenic substrate S2765 [3mM] (Chromogenix AB, Milan, Italy), FVIIa [120iu/mL] (Novo Seven, Novo Nordisk Pharmaceuticals, Crawley, UK), $CaCl_2$ (1M), and purified FX [1u/mL] (NIBSC, UK) was added to 100µl of cell suspension and incubated for 1 hour at 37^{o}C. The absorbance was read at 405nm, and the level of procoagulant activity for each sample was determined by plotting absorbance against the standard curve.

2.3.4.3. Rotational Thromoelastometry (ROTEM) measurement of clot formation

The ROTEM analyser (Sysmex) is a modification of the thromboelastography analyser, both of which determine clot formation and lysis in a given sample. Whole blood or plasma is incubated at 37^{o}C in a cup, and a pin within the cup is connected to an optical detector. The pin rotates, and as fibrin forms the impedence of the pin rotation is detected. Various measurements such as clotting time (CT), elasticity of the clot (MCE), and rate of clot formation (CFR) are recorded and these are then displayed in graphic form as well as numerical data. This method was used to examine how changes of monocyte surface TF and TFPI might affect clot formation. Citrated whole blood was incubated with CRP-XL [500ng/mL] or LPS [200ng/mL] (Sigma), and incubated for 4 hours at 37^{o}C. At the end of the incubation period, monocytes were isolated using Lymphoprep and resuspended in PBS buffer. Cells were adjusted to concentration of 1 x 10^5 monocytes/mL. 100µl of cell suspension (intact monocytes) was added to each

ROTEM cup, followed by 100µl of pooled plasma and 20µl of 0.250mM $CaCl_2$. Sample cups were inserted onto the pin immediately after the addition of $CaCl_2$, and sample traces were recorded for 30 minutes.

2.3.4.4. Formation of thrombi in Chandler Loop

The Chandler loop method enables formation of *in vitro* thrombi that have cellular and morphological similarity to arterial thrombi, due to formation at shear rate similar to that found *in vivo*. Shear rate within a normal healthy artery would be expected to fall within a range of 10 – 44 dynes/cm^2. Shear rate within the Chandler loop system is ~20 dynes/cm^2 (Chandler, 1958). Histologically, these thrombi have been shown to consist of a platelet and leukocyte-rich head, and a fibrin and red blood cell-rich tail (Poole, 1959). This method was used to generate thrombi for microarray analysis of changes in gene expression profile during thrombus formation. For each potential thrombus, 900µl of citrated whole blood was recalcified with 50µl $CaCl_2$ [0.25M], and mixed with 150µl 0.9% (w/v) NaCl. 1mL of recalcified blood was placed in Portex vinyl tubing (45cm in length, internal diameter 3mm). The ends of the tube were connected, and all loops assembled on a rotor, and rotated at 36 rpm for up to 6 hours at 37^oC. At each time point, thrombi were removed and washed in 0.9% (w/v) NaCl, and placed in RNA*later®* to stabilise the RNA prior to homogenisation of thrombi for RNA isolation.

2.3.5. Statistical analysis of cellular assays

Analysis of data from cellular assays investigating changes in monocyte surface procoagulant activity and clot formation was determined by paired t-test. Statistical analysis was performed using GraphPad PRISM software version 4 (BioData Corp, Horsham, PA, USA). All results are presented as mean ±SD. Statistical significance was defined as $P < 0.05$.

2.4 Molecular biology methods

2.4.1. RNA isolation

2.4.1.1. Isolation of messenger ribonucleic acid (mRNA) using oligo-dT beads

For the gene expression experiments carried out in whole blood, monocyte mRNA was extracted using Oligo-dT Dynabeads® (Invitrogen). 100µl of Oligo-dT beads® were added per mL of whole blood, and incubated on a rotary mixer for 5 minutes at room temperature. Following incubation, samples were placed on the MPC and the supernatant removed. Samples were washed twice in wash buffer with or without Lithium Dodecyl-Sulfate (LiDS)(Buffer A – 10mM Tris HCl (pH 7.5); 0.15M LiCl; 1mM EDTA; 0.1% LiDS; Buffer B – 10mM Tris HCl (pH 7.5); 0.15M LiCl; 1mM EDTA). Finally samples were resuspended in 20µl RNase-free water (Sigma), and placed on ice ready for RT-PCR.

2.4.1.2. Isolation of total RNA

For gene expression experiments carried out on isolated monocytes, total RNA was extracted using a combination of the TRIZOL® (Invitrogen) phenol-chloroform extraction (Chomczynski *et al*, 1987) and RNeasy® microspin membrane technology (Qiagen, GmbH, Germany). The TRIZOL® reagent is used to lyse the cells and dissolve cellular components whilst still maintaining the integrity of the RNA. Addition of chloroform allows separation of sample solution into aqueous and organic phases, with the RNA being in the aqueous phase. The RNeasy® RNA kit uses a silica-based membrane technology combined with microspin columns to purify the RNA.

For monocyte gene expression studies, monocyte samples were lysed in 1ml of TRIZOL® reagent and incubated for 5 minutes prior to RNA extraction. For thrombus

gene expression studies, thrombi were placed into 2ml of RNA*later*® for 5 minutes to stabilise RNA, then homogenised in 1ml of TRIZOL® reagent and incubated for 5 minutes at room temperature. RNA was then extracted for all samples using the following method. Following 5 minutes of incubation to allow cellular lysis, the cellular-TRIZOL® homogenate was added to a phase-lock gel tube (5Prime, Hamburg, GmbH). 200µl of chloroform was added and the tube shaken for 15 seconds, before standing at room temperature for 2-3 minutes. The sample was then centrifuged at 12,000 g for 15 minutes, separating the sample into aqueous and organic phases. The aqueous phase was then transferred to a sterile eppendorf tube, and an equal volume of 70% ethanol added. The sample was mixed gently by inversion then applied to an RNeasy® column. Following centrifugation at 12,000 g for 30 seconds, the flow through was discarded and 350µl of ethanol wash buffer added to the column. This was then centrifuged at 12,000 g for 30 seconds, and the flow-through discarded. To ensure elimination of contaminating DNA, a DNase digestion was carried out by adding 80µl of DNase 1 (30 kunitz units; Qiagen) to the RNeasy® column and incubating it with the sample for 20 minutes at room temperature. Following a further ethanol wash, 500µl of RPE buffer was incubated on the column for 3 minutes, followed by centrifugation at 12,000 g for 1 minute. This wash step was repeated. Finally, the spin column was transferred to a clean, sterile collection tube and the RNA eluted by adding 20µl RNase-free water and centrifuging the column at 12,000 g for 1 minute. All RNA samples were quantified by Nanodrop quantification (ThermoScientific, Basingstoke, Hampshire, UK).

2.4.2. Reverse Transcription of RNA

For real-time PCR assays, total RNA was reverse transcribed to double-stranded cDNA using cloned Avian Myeloblastosis Virus (AMV) Reverse Transcriptase (Invitrogen). In more detail, for each sample a 20µl reaction-volume was set up containing 1µl oligo-dT_{20} primers (final conc 2.5µM), 2µl 10mM deoxyribonucleotide triphosphate (dNTP) mix (Invitrogen) (10mM each dATP, dTTP, dGTP, and dCTP, 1mM final conc), 250ng total RNA, and RNase-free water to volume of 13µl. To reduce possible inhibition of cDNA synthesis due to secondary structures, samples were heated for 5 minutes at 65^{o}C, then immediately placed on ice. 1µl of cloned AMV RT enzyme (15 units/µl) was subsequently added to each sample, together with 4µl 5X cDNA synthesis buffer (Invitrogen; 250mM Tris-acetate (pH 8.4), 375mM Potassium acetate, 40mM Magnesium acetate), 1µl 0.1 M Dithiothreitol (DTT; Sigma), and 1µl RNase Inhibitor (40U/µl; Invitrogen). The contents of all tubes were mixed gently, then incubated in a PTC-200 tetrad thermal cycler (MJ Research, MA, USA) at 45^{o}C for 60 minutes for reverse transcription, followed by 5 minutes at 85^{o}C for 5 minutes to terminate the reaction. The resulting double-stranded cDNA template was either used immediately for amplification by PCR or stored at -80^{o}C.

2.4.3. Real-time Polymerase Chain Reaction (PCR)

Real-time Polymerase Chain Reaction (PCR), also known as quantitative PCR, allows determination of the amount of target gene present in a sample. A fluorescence threshold is set, and through monitoring the fluorescence emitted during each PCR cycle, it is possible to detect the first significant increase in the amount of PCR product during the exponential phase of the reaction. The parameter C_T (cycle threshold) is

defined as the cycle number at which the fluorescence emission exceeds the threshold. The higher the starting copy number of the target gene within a sample, the sooner a significant increase in fluorescence is detected above the threshold, and the lower the C_T value (Figure 2.1a).

Protocols to measure changes in monocyte gene expression were established using the Relative Quantification method. In this instance the concentration of the target gene is expressed in relation to a reference gene, thus normalising the samples. Initial experiments were carried out using SYBR green dye, later experiments were carried out using TaqMan® probes. The background to the technology and methods for both applications is given in more detail in section 2.4.3.1.

2.4.3.1. SYBR Green I Dye chemistry

SYBR green dye detects PCR products formed during PCR by binding to the double-stranded DNA. As the PCR progresses, and the amount of PCR product increases there is an increase in fluorescence intensity which is proportional to the amount of product. Two main advantages to using SYBR Green dye is that it can be used to monitor amplification of any double-stranded DNA sequence and is cost effective. However, a disadvantage is that SYBR Green is non-specific and will bind to any double-stranded DNA, therefore can generate false positive results. Therefore when using SYBR Green it is desirable to check for non-specific binding by performing a melting curve or dissociation plot. Figure 2.1b illustrates a typical dissociation plot produced when SYBR green has bound to multiple double-stranded DNA products. On the left, the smaller peak has been generated due to the formation of primer dimmers, and on the right, with a higher melting temperature, is the peak generated due to amplification of

the gene of interest. Design of primers with low aptitude for formation of primer-dimers is therefore necessary for SYBR green quantitative real-time PCR assays.

2.4.3.2. TaqMan® chemistry

TaqMan® chemistry involves the use of fluorogenic-labeled probes that are specific to the target gene and therefore reduce the risk of possible detection of non-specific PCR products. The oligonucleotide probe contains a fluorescent reporter dye on the 5' end and a quencher dye on the 3' end. If the target gene sequence is not detected, the probe remains intact and the proximity of the quencher dye limits the fluorescence emitted by the reporter dye through fluorescence resonance energy transfer (FRET). However, if the target gene sequence is detected, the probe anneals next to the target gene sequence and is cleaved by the 5' nuclease activity of the Taq DNA polymerase enzyme within the reaction. The cleavage of the probe, and extension of the target gene sequence template strand increases the distance between the reporter dye and the quencher dye, increasing the signal emitted by the reporter dye.

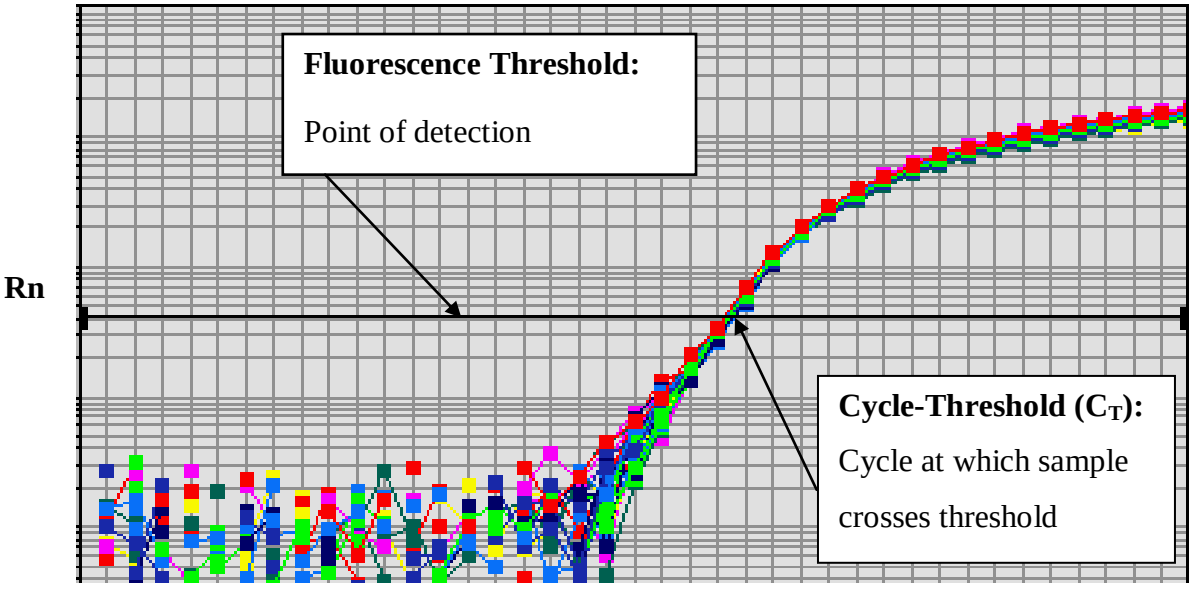


Figure 1a: Amplification and detection of target gene expression by real-time PCR

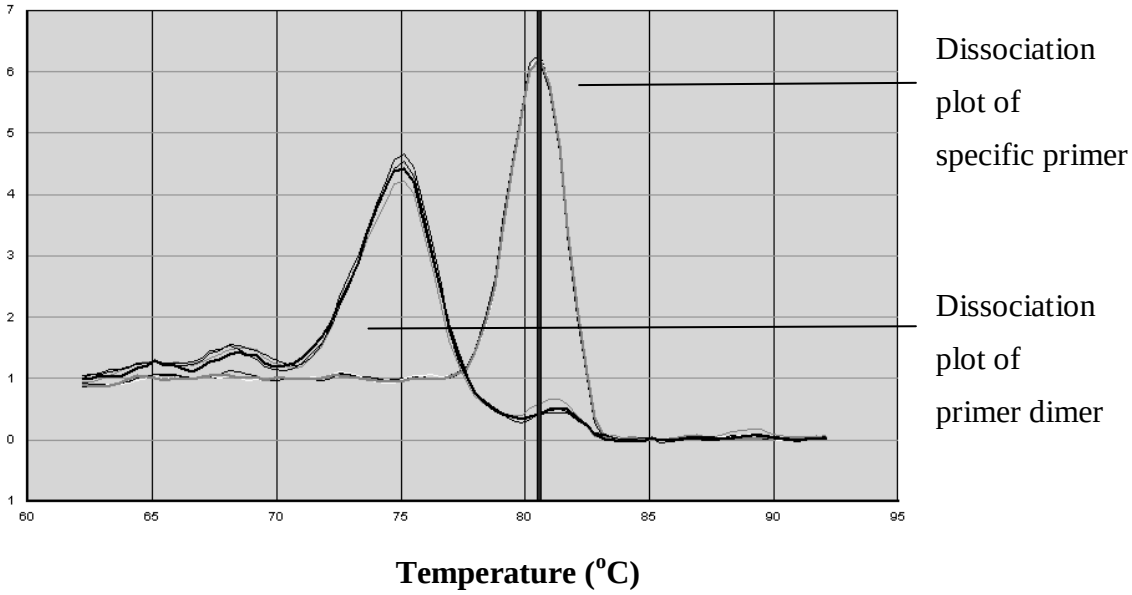


Figure 1b: Dissociation plot of specific and non-specific detection of double-stranded cDNA using SYBR green

As each cycle progresses there is an increase in fluorescence intensity that is proportional to the amount of PCR product.

2.4.3.3. Optimisation of real-time PCR assays

To ensure all real-time PCR experiments were run under optimal conditions, several preliminary experiments were carried out. To determine optimal primer concentration within the PCR reaction, a series of doubling dilution of each primer set was assayed, adding 2µl of each primer solution to a 25µl reaction. To determine optimal sample concentration, a series of 10-fold dilutions of cDNA were set up, and 1µl of sample added to each reaction. All samples were assayed in triplicate. From these experiments it was determined that 1µl of each primer [0.5 µM final conc], and 1µl of cDNA sample was sufficient for a 20µl PCR reaction. Although PCR is an exponential process, the efficiency of a reaction may not be 100%. This can affect the C_T value. The efficiency of the reaction can be determined by making 1:10 dilutions of the cDNA sample and

carrying out PCR for the gene of interest. A standard curve is then generated, and amplification efficiency determined by use of the algorithm: $E = 10^{-1/slope}$.

For real-time PCR assays using SYBR green, 1µl of sample cDNA was amplified in a 25µl reaction using 12.5µl of SYBR green master mix (2x), 1µl each forward and reverse primer [0.5µM], and 10.5µl nuclease-free water. For real-time PCR assays using TaqMan® probes, 1µl of sample cDNA was amplified in a 20µl reaction containing 1µl of TaqMan® gene expression assay 20x solution, 10µl of TaqMan® PCR master mix, and 8µl nuclease-free water. cDNA encoding monocyte TF, TFPI and endogenous control genes were amplified using the cycling conditions listed in Table 2.1. All PCR assays had an efficiency of between 90-110%, which is generally considered acceptable. All real-time PCR assays were carried out using an ABI PRISM 7900HT Sequence detection System (Applied Biosystems, Warrington, UK).

2.4.3.4. Primers and PCR cycling conditions

Table 2.1 gives a detailed list of primers used for each gene of interest and endogenous control genes. Figure 2.2 gives details of PCR cycle conditions.

Table 2.1a: Gene expression assays used in TaqMan real-time PCR assays

GENE	GENE EXPRESSION ASSAY	PRODUCT SIZE
TF	Hs 00175225_m1	118 bp
TFPI	Hs 00196731_m1	126 bp
B2M	Hs 99999907_m1	75 bp

Table 2.1b: Primers used in SYBR green real-time PCR assays

PRIMER	SEQUENCE	ANNEALING POSITION	PRODUCT SIZE
TF			
Forward	5' TTcAAATAAgcAcTAAgTcAggAg 3'	2292 – 2301	264 bp
Reverse	5' cAcTTTTgTTcccAccTg 3'	6391 – 6405	
TFPI			
Forward	5' TgccccTcTTAATgcTgATTcTgA 3'	364 – 387	343 bp
Reverse	5' ATAAccTcgAcATATTccTgg 3'	686 – 706	
TFPI-α			
Forward	5' cATTTgTgAAgATggTccgA 3'	814 – 833	160 bp
Reverse	5' AcAcAATccTcTgTcTgcTgg 3'	953 – 973	
TFPI-β			
Forward	5' cATTTgTgAAgATggTccgA 3'	814 – 833	166 bp
Reverse	5' gAcTTggAAATATgAgcccgc 3'	959 – 979	
18s			
Forward	5' gTggAgcgATTTgTcTggTT 3'	1348 – 1368	200 bp
Reverse	5' cgcTgAgccAgTcAgTgTAg 3'	1528- 1548	
B2M			
Forward	5' AgcgTAcTccAAAgATTcAggTT 3'	125 – 147	91 bp
Reverse	5' ggATgAAAcccAgAcAcATAgc 3'	194 – 215	

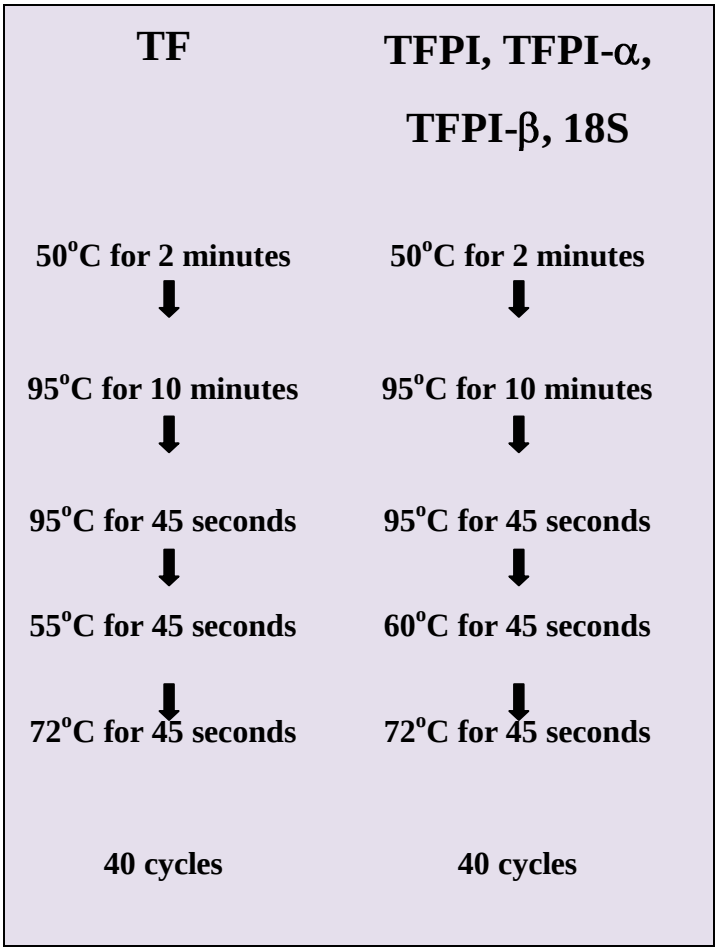


Figure 2.2a: Real-time PCR cycling conditions for SYBR green assays

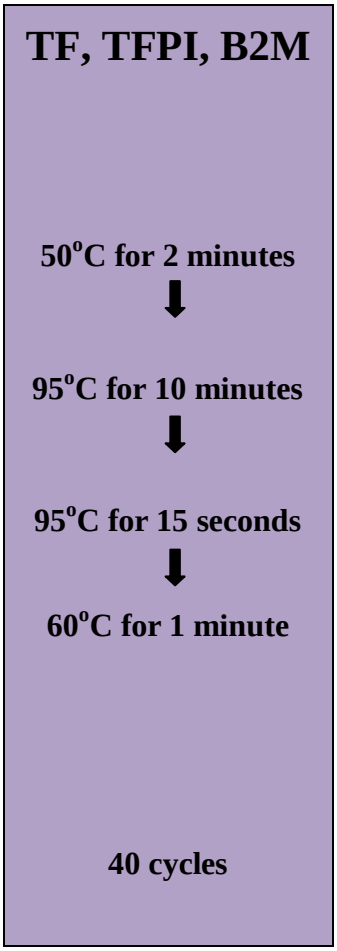


Figure 2.2b: Real-time PCR cycling conditions for TaqMan assays

2.4.3.5. Real-time PCR analysis

The comparative C_T method was used to quantify the amount of PCR product in each sample. This uses the arithmetic formula: $2^{-\Delta\Delta CT}$ (Livak *et al*, 2001). An example of the analysis is given below:

1. Calculate the mean C_T values for 0 hour monocytes (standard) for gene of interest and endogenous control gene (see table below).
2. Calculate the mean C_T value for 4h CRP-stimulated monocytes (treated) for gene of interest and endogenous control gene (see table below).

	C_T for TFPI (gene of interest)	**C_T for B_2M** (endogenous control gene)
0 hour (Standard)	33.85 (mean)	18.17 (mean)
4 hour (Treated)	27.95 (mean)	18.13 (mean)

3. Calculate ΔC_T for Standard and Treated samples.

   = mean C_T for TFPI standard – mean C_T for B_2M standard = 33.85 - 18.17 = <u>15.68</u>

   = mean C_T for TFPI treated – mean C_T for B_2M treated = 27.95 - 18.13 = <u>9.82</u>

4. Calculate $\Delta\Delta C_T$

   = ΔC_T of Treated - ΔC_T of Standard = 9.82 – 15.68 = <u>-5.87</u>

5. Calculate $2^{-\Delta\Delta CT}$ = <u>58.5</u> fold change in monocyte gene expression of TFPI following exposure to activated platelets.

2.4.4. Detection of Single Nucleotide Polymorphism (SNP)

Genotyping of two SNPs within the TF gene was carried out in Deoxyribonucleic acid (DNA) samples from the PRAMIS cohort. DNA was extracted from EDTA anticoagulated blood using PureGene DNA extraction kits (Gentra Systems, Qiagen, Crawley, West Sussex, UK). This had been carried out previously by Peter Braund. Stored DNA samples were genotyped for each SNP in 96-well plates, containing 20ng donor sample DNA, TaqMan Universal Master Mix, sequence-specific primer and TaqMan FAM probe, and DNase-free water. Amplification was carried out by PCR on an MJ Research thermal cycler, using the following cycling conditions: 95^{o}C for 10 minutes, to allow activation of AmpliTaq Gold enzyme; followed by 40 cycles of 92^{o}C for 15 seconds to allow denaturation of DNA, and annealing/extension for 1 minute at 60^{o}C. Following amplification of target DNA, an end point reading was obtained using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) to determine allelic discrimination. Figure 2.3. illustrates a typical fluorescence plot indicating allelic discrimination within a sample group.

2.4.5. Statistical analysis for PCR assays

Analysis of data from experiments measuring changes in monocyte gene expression, examining the difference in gene expression between baseline and following incubation with various stimuli, were determined by paired t-test when comparing two groups, whilst differences between two or more groups were determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-test. Comparison of monocyte gene expression in MI cohort vs control group was determined by unpaired t-test. Statistical analysis was performed using GraphPad PRISM software version 4 (BioData Corp,

Horsham, PA, USA). All results are presented as mean ±SD. Statistical significance was defined as $P < 0.05$.

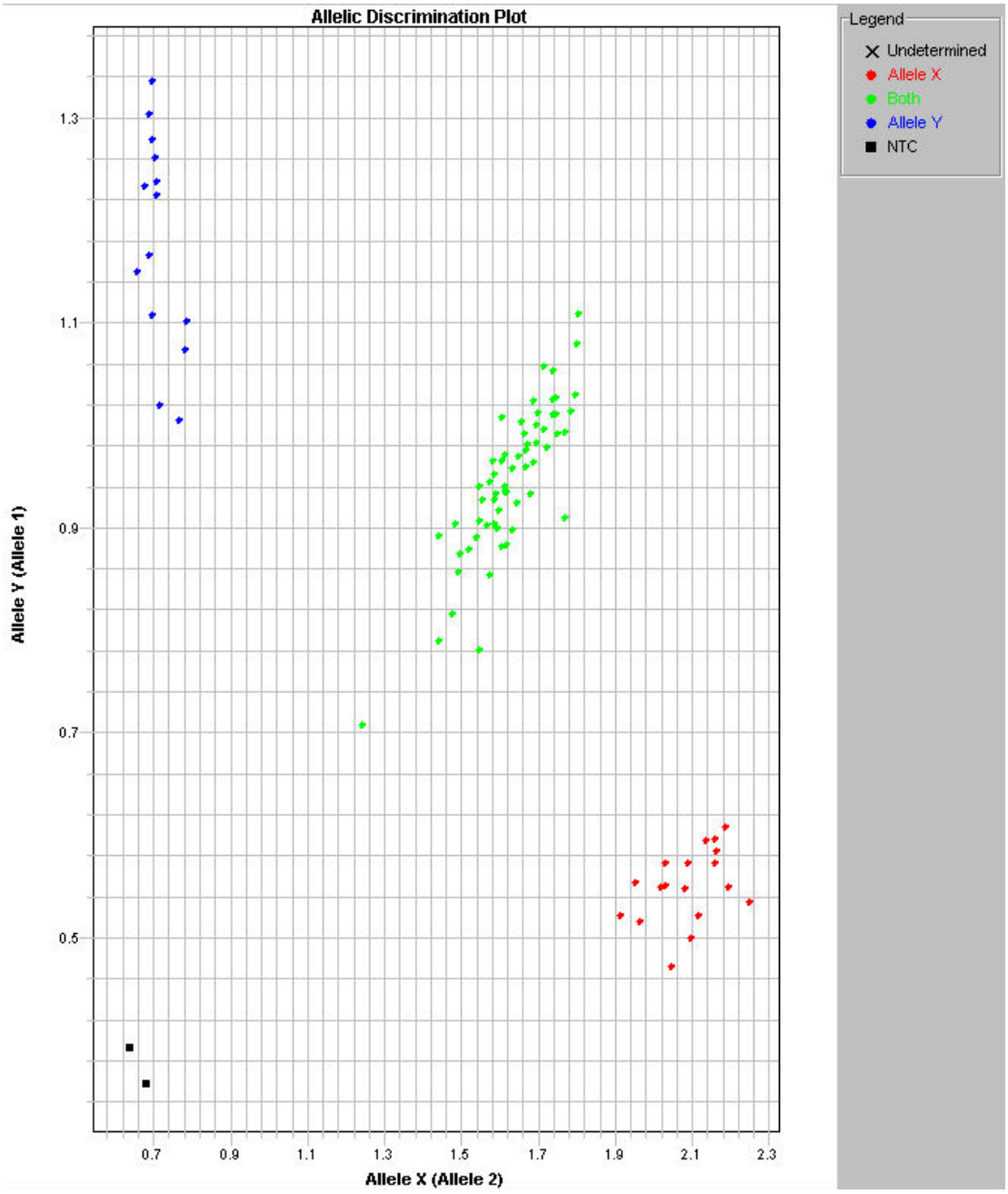


Figure 2.3. Fluorescence plot of allelic discrimination. This figure gives an illustration of a typical fluorescence plot of allelic discrimination. In this instance, samples homozygous for allele Y (TF -1812C, the dominant allele) are represented by the blue dots, samples homozygous for allele X (TF -1812T, the recessive allele) are represented by the red dots, and samples that are heterozygous, containing both alleles, are represented by the green dots. The black dots in the bottom left-hand corner are negative control samples lacking DNA.

2.5. Gene expression microarray methodology

Advances in technology during the last decade have enabled large-scale experiments to be carried out on high density platforms, screening the whole genome for changes in patterns of gene expression. This study used the Illumina® whole genome Human-6 expression BeadChip, version 2 (Illumina, Saffron Walden, Essex, UK), which contains >40,000 probes for genes in the NCBI, RefSeq, and UniGene databases. Illumina's BeadChip technology comprises microspheres hybridised to 50-mer gene-specific oligonucleotide probes, and a short 'address sequence'. The microspheres are assembled into microwells, forming the microchip platform. As the microspheres have assembled randomly into the microwells, the array is then mapped using the 'address sequences' to determine which bead type is in which well. Biotin-labeled cRNA is then hybridised to the BeadChips, followed by image scanning of the BeadChips and data extrapolation for further analysis. The methods involved in all of these procedures are described in more detail below.

2.5.1. *In vitro* transcription (IVT)

Following extraction and quantification of RNA, 100ng of total RNA was amplified using the Illumina® TotalPrep RNA amplification kit (Ambion; Applied Biosystems, Warrington, UK), following the manufacturer's instructions. This method is based on the Eberwine technique (Van Gelder *et al*, 1990). Briefly, the RNA was firstly reverse transcribed to synthesise first strand cDNA. RNA was incubated for 2 hours at 42^{o}C in a master mix containing 1µl oligo-dT primer, 2µl 10X first strand buffer, 4µl dNTP mix, 1µl RNase inhibitor, and 1µl reverse transcriptase enzyme ArrayScript. Second strand synthesis of cDNA was carried out using a second master mix containing 63µl

nuclease-free water, 10µl 10X second strand buffer, 4µl dNTP mix, using 1µl RNAse H to degrade the RNA, and 2µl DNA polymerase, and incubating the samples at 16^{o}C for 2 hours, thus converting the single-stranded cDNA into double-stranded DNA template ready for transcription. The cDNA was purified to remove contaminating RNA, enzymes and salt that might limit *in vitro* transcription. This was carried out by binding the cDNA to a cDNA filter cartridge, using 250µl of cDNA binding buffer, and washing the sample with 500µl ethanol wash buffer. The purified cDNA was then eluted from the filter with nuclease-free water heated to 55^{o}C. The *in vitro* transcription step allows synthesis, biotin labelling, and amplification of cRNA from the double-stranded cDNA. The cDNA sample was incubated overnight at 37^{o}C in the presence of 2.5µl T7 10X reaction buffer, 2.5µl T7 enzyme mix, and 2.5µl Biotin-NTP mix. The reaction was stopped by addition of 75µl nuclease-free water to each sample. The resulting cRNA was further purified by binding the cRNA to a cRNA filter cartridge using 350µl cRNA binding buffer, and washing with 250µl 100% ethanol, followed by 650µl Wash Buffer. The cRNA was eluted from the filter with 100µl nuclease-free water heated to 55^{o}C, ready for hybridisation to the BeadChip.

2.5.2. Chip hybridisation

This part of the sample processing was carried out by Dr Peter Ellis at the Wellcome Trust Sanger Institute, Hinxton, UK. To prepare the cRNA sample for hybridisation, 1.5µg RNA was resuspended in 20µl RNAse-free water and incubated at room temperature for 10 minutes. A hybridisation mix was prepared containing 165µl of pre-warmed Hyb E1 buffer and 99µl formamide, then 40µl of hybridisation mix was added to each cRNA sample. The sample was preheated to 65^{o}C for 5 minutes, and vortexed

briefly to ensure adequate mixing, then 60µl of sample added to the centre of each array. The array cartridges were placed on the BeadChip Hyb wheel in the hybridisation oven and incubated overnight at 55°C. On Day 2 of the hybridisation process, the BeadChip was submerged in a staining dish containing 250mL Wash E1BC solution, then transferred to a waterbath containing High-Temp Wash buffer, and incubated static for 10 minutes. The BeadChip was then re-stained in 250mL Wash E1BC solution and incubated for 5 minutes. The BeadChip was then transferred into 100% ethanol, and incubated on an orbital shaker for 10 minutes. Following a second wash in 250mL Wash E1BC solution, the BeadChip was incubated for 10 minutes with rocking in 4mL Block E1 buffer. Hybridised cRNA was detected using streptavidin-Cy3, where 2mL Block E1 buffer with streptavidin-Cy3 wash was added to a clean staining dish, and the BeadChip incubated for 10 minutes. A final wash step was carried out using 250mL Wash E1BC solution. The BeadChip was then centrifuged at 280 g for 4 minutes, and imaged using Illumina BeadArray Reader.

2.5.3. Gene Expression Microarray Data Analysis

Output files were exported into Microsoft Excel cvs files for further analysis. To remove possible sources of variation between arrays, raw data (mean of probeset expression) was normalised using quantile normalisation, thereby making the distribution of probe intensities for each array in the set of arrays the same. This was achieved using R (Bioconductor). R was also used to perform a paired analysis using Student's t-test, comparing each treated sample with its 4 hour control sample in the platelet-monocyte gene expression studies, or comparing each time point against time 0 in the thrombus array study. Fold change, and P-value with false discovery rate

correction for multiple testing was also calculated (Ploner *et al*, 2005). To identify significant, differentially expressed genes, the output was filtered on fdr2d corrected P-values <0.05, and fold change >2. The transcriptional profile of genes which were significantly differentially expressed was further evaluated by gene enrichment analysis using FATIGO+ (Babelomics v3) (Al-Shahrour *et al*, 2007).

CHAPTER 3: Endogenous Thrombotic Potential – Investigation of the ETP in healthy subjects and patients with premature MI

3.1 Introduction

The haemostatic response to injury involves the sequential activation of many proteins, maintaining a balance between procoagulant factors, which drive the response forward, and anticoagulant factors which quench the response when no longer required. However, the haemostatic response can also be initiated in response to damaged endothelium caused as a consequence of disease, for example, in response to rupture of an atherosclerotic plaque.

Atherosclerosis develops gradually as result of endothelial dysfunction, lipid oxidation and accumulation, also hypercholesterolaemia, and hyperglycaemia (Yla-Herttuala *et al*, 1989; Cai *et al*, 2000; Menotti *et al*, 2005), exacerbated by external risk factors such as smoking, diabetes, obesity and hypertension (Heeschen *et al*, 2001; Fung *et al*, 2001). During progression of atherosclerotic disease, elevated concentration of circulating Low-Density Lipoprotein (LDL) and increased permeability at areas of endothelial damage facilitates LDL to enter the sub-endothelial space. The presence of LDL also leads to increased expression of chemotactic factors, such as MCP-1 and IL-8, which promote infiltration and adhesion of circulating monocytes. Monocytes within the sub-endothelial space eventually differentiate into macrophages, which internalise the LDL, eventually forming foam cells. These foam cells eventually form fatty streaks, and develop into an atheromatous plaque (Fattiotto *et al*, 1984). Due to the accumulation of lipid, cellular debris, inflammatory cells and calcium deposits, the atheromatous plaque develops into a fibrous plaque, with smooth muscle cells (SMCs)

and collagen covering the lipid-rich necrotic core (Huang *et al*, 2001; Ibanez *et al*, 2007; Redgrave *et al*, 2008). SMCs, macrophages, and cellular microparticles within the plaque are a major source of TF. The plaque can become unstable due to the thinning of the fibrous cap, which occurs through the action of proteolytic enzymes and phagocytosis of ECM (Lendon *et al*, 1991; Seino *et al*, 1994; Galis *et al*, 1994; Sukhova *et al*, 1999; Zwaka *et al*, 2001). Finally, rupture of the plaque exposes the proinflammatory and pro-thrombotic contents, initiating development of a thrombus. Plaque rupture is increasingly recognised as one of the pivotal events prior to myocardial infarction (MI) however the resulting thrombus, and whether it becomes occlusive, may depend on the magnitude of the haemostatic response of an individual to the stimulus.

Clotting assays, such as measurement of prothrombin time (PT), and activated partial thrombin time (APTT) can give an indication of initial haemostatic response, but not a measurement of the major thrombin burst generated during formation of a thrombus. The global effect of these factors can be assessed by measurement of the endogenous thrombotic potential (ETP) in the plasma. The aim of this study was to measure individual clotting factors and to compare the thrombotic potential of plasma obtained from subjects that had suffered a premature MI with that of plasma obtained from a normal healthy control cohort. It was expected that in premature MI patients, the atherosclerotic burden is relatively low, and so the thrombotic response may determine the risk of occlusion of the coronary arteries.

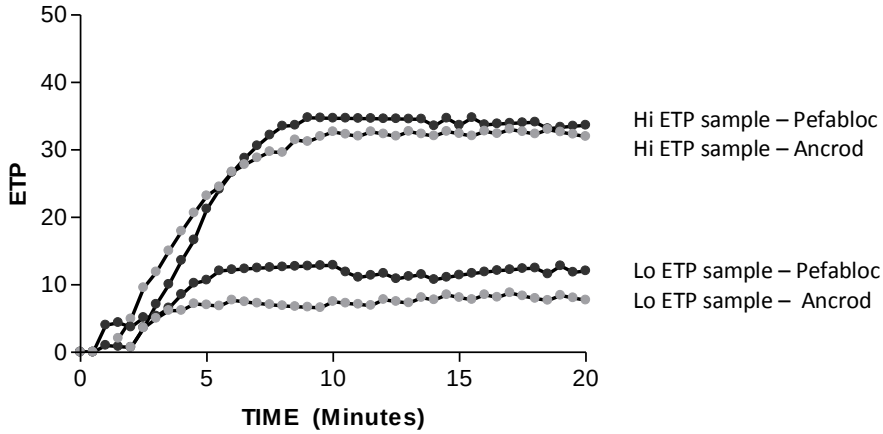
3.2 Development of Thrombin Generation Assay

A chromogenic method was set up, allowing assessment of the overall haemostatic response within platelet-poor plasma (PPP). The chromogenic method assesses the amount of thrombin generated, but a clot cannot be allowed to form as this would increase turbidity and interfere with reading of optical density (OD). To circumvent this problem, the fibrin clot can either be removed prior to assay, or inhibitors added to the sample which prevent fibrin clot formation. Removal of the fibrin clot can be achieved with Ancrod, a purified coagulation enzyme isolated from the Malayan pit viper (*Agkistrodon rhodostoma*), which induces defibrination of plasma through splitting fibrinopeptide A from fibrinogen, without affecting other coagulation factors. Alternatively, PefaBloc® (H-Gly-Pro-Arg-Pro-OH), a synthetic peptide can be used, which inhibits the polymerisation of fibrin. Preliminary experiments were carried out to assess the effects of using the two different methods. PPP from two individuals with known 'low' and 'high' ETP were treated with Ancrod to form a clot which was then removed, or treated with Pefabloc [3mg/mL final conc] to inhibit fibrin clot formation during assay. Results for both approaches were comparable, with ETP for the 'low' sample being 7.60 following ANCROD treatment, and 12.00 in the presence of PefaBloc®. ETP for the 'high' samples were 32.50, and 34.20 respectively (Figure 3.1a).

To assess intra-assay variability, 6 pooled plasma samples were treated with ANCROD and 6 were treated with PefaBloc, resulting in a mean ETP of 27.5 and 32.2, respectively, with %CV of 8.87% in the ANCROD-treated samples, vs 6.32% in the PefaBloc-treated samples (p=0.0280) (Figure 3.1b). Therefore the pattern of ETP measurement within individual plasma samples was comparable whichever method of

defibrination was employed, however use of PefaBloc® resulted in a slightly higher, but significant, ETP measurement. One reason for this could be due to a difference in the kinetics of the reaction taking place in the plasma sample (Hemker *et al*, 2005). *In vivo*, thrombin is generated on a fibrin web as platelets, and cellular microparticles (MPs) which can also promote thrombin generation, bind to the fibres. The PefaBloc®

a)



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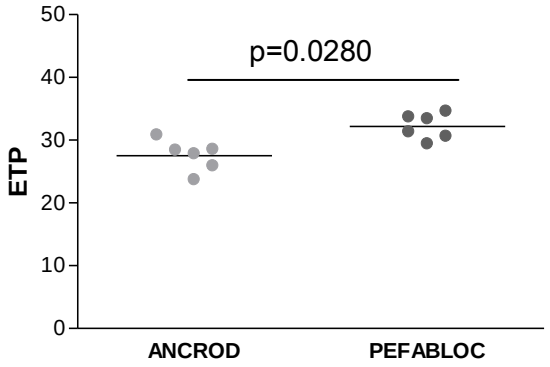


Figure 3.1: Effect of defibrination and inhibitors of fibrin polymerisation on Endogenous Thrombotic Potential. (a) Continuous measurement plot of plasma samples from normal healthy donors with 'low' and 'high' thrombotic potential treated with Ancrod (defibrinator) or PefaBloc® (fibrin polymerisation inhibitor); (b) Mean ETP of pooled plasma samples treated with Ancrod or Pefabloc (n = 6).

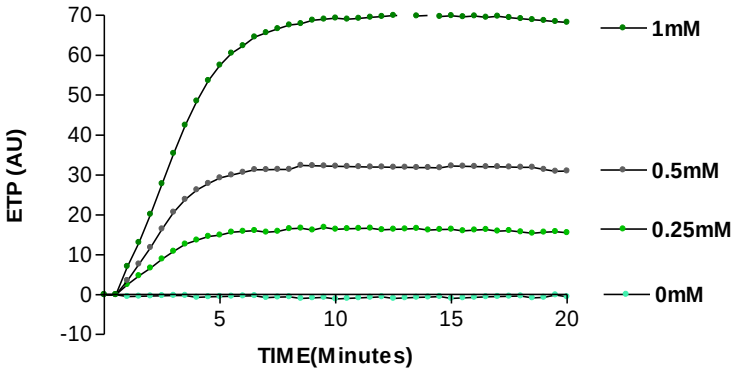
method would inhibit fibrin polymerisation but fibrin fibres would still be present to provide a mechanical support for thrombin generation. However, if the fibrin clot has been removed, the kinetics shifts towards chemical control, measuring residual thrombotic potential of the plasma, therefore perhaps resulting in a lower ETP measurement. Therefore, defibrination of plasma with Pefabloc® was selected due to its closest similarity to the *in vivo* state, and also due to less variability in end-point measurement. It was also technically easier and quicker to add PefaBloc® to each sample than it was to physically remove each clot, thereby increasing the robustness of the method.

Preliminary experiments were also carried out to assess the effect of varying concentrations of the thrombin substrate, recombinant TF (rTF) and phospholipid (PPL), to establish the optimal concentration of each to be used in the assay. The method of continuous measurement of the thrombotic potential requires a thrombin-specific substrate that will bind loosely (has high K_m) and that is not consumed during the reaction (has low K_{cat}). Pefachrome-TG® (H-βAla-Gly-Arg-pNA) is a thrombin-specific substrate that has high K_m and is cleaved slowly. Based on a previous study of thrombin generation, which used a final concentration of 0.5mM of Pefachrome-TG®, (Prasa *et al*; 1997), a substrate concentration range of 0 – 1mM was tested in the current method. Increasing the concentration of substrate in the reaction did not affect the lag time, but did affect the rate of reaction and the ETP (Figure 3.2 - a). From these data, a substrate concentration of 0.5mM was chosen.

Changes in concentration of rTF, generally affected the lag time (Figure 3.2 - b). In the absence of rTF, thrombin generation still took place, presumably due to endogenous,

soluble TF within the plasma, but the lag time was prolonged. Using pooled platelet-poor plasma, addition of rTF at a concentration of 500ng/mL resulted in a lag-time of

a)



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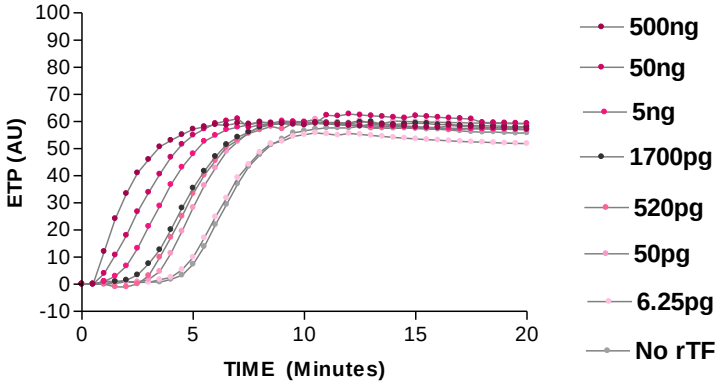

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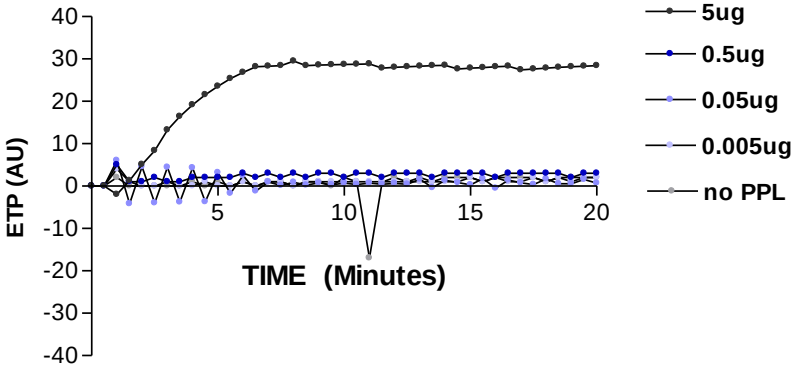


Figure 3.2: Optimisation of method for Thrombin generation assay. Effect of (a) substrate concentration, (b) Tissue Factor concentration and (c) phospholipid concentration on Endogenous Thrombotic Potential of pooled plasma.

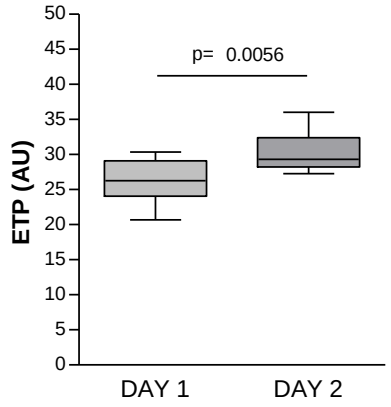
< 1 minute, whereas an rTF concentration of 6.25 pg/mL resulted in a lag-time of ~ 4 minutes, similar to that seen in the sample containing only endogenous TF. For measurement of the overall thrombotic potential, an rTF concentration of 1.7ng/mL was selected. As would be expected, the lag time and ETP in samples from individual donors showed variation which was presumably due to varying concentration of endogenous TF and clotting factors in these samples (data not shown).

In vivo, activated platelets provide a negatively charged phospholipid surface to promote thrombin generation. In these studies, bovine phospholipid (PPL) was added to platelet-poor plasma to mimic the cellular surface. Increasing concentrations of phospholipid from 0 to 10µg/mL were added to pooled platelet-poor plasma in the presence of 1.7ng/mL rTF. Changes in PPL concentration had a marked effect on ETP, with no thrombin generation at PPL concentrations below 5µg/mL (Fig 3.2 - c), thus demonstrating that thrombin generation, and in particular this assay, was dependent on the presence of phospholipid. Therefore a PPL concentration of 5µg/mL was selected for use in the method.

Following optimisation of reagents, the reproducibility of the assay was determined. The ETP was measured in 10 aliquots of pooled PPP, and the intra-assay reproducibility calculated (mean ETP 26.35±2.9; CV 11.16%). This experiment was repeated on a separate day (mean ETP 30.30±2.7; CV 8.83%) (Figure 3.3a). Inter-assay reproducibility was calculated from the ETP measurement for all samples measured over the two days (mean ETP 28.33±3.4; CV 12.03%) (Figure 3.3 b and c). Due to the slight variation in ETP measurement between plates, it was necessary to further standardise the assay. Therefore, during sample analysis, the ETP of pooled PPP was

measured in duplicate on each 96-well plate, so that the ETP for each sample could be normalized, expressing ETP as a percentage of the 'normal' pooled plasma standard.

a)



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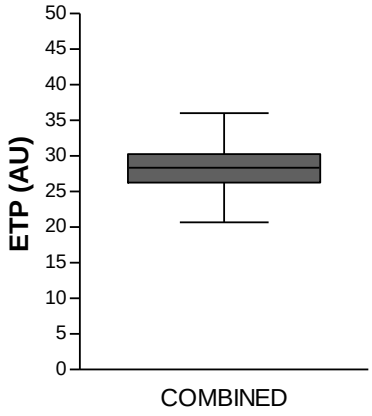


c)

	MEAN ETP	%CV
DAY 1		
INTRA-ASSAY REPRODUCIBILITY	26.35 ±2.9	11.16%
DAY 2		
INTRA-ASSAY REPRODUCIBILITY	30.30 ±2.7	8.83%
COMBINED		
INTER-ASSAY REPRODUCIBILITY	28.33 ±3.4	12.03%

d)

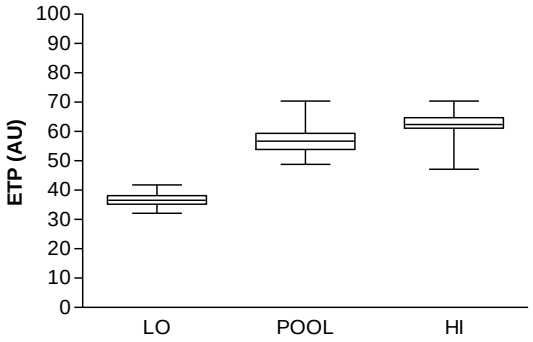


Figure 3.3: Reproducibility of chromogenic Thrombin Generation Assay. (a) Inter-assay reproducibility on two separate days (n = 10); and (b) Intra-assay reproducibility; (c) Table of mean ETP and coefficient of variation for inter-assay and intra-assay reproducibility; (d) Reproducibility of control samples measured in Thrombin Generation assay. Inter-assay reproducibility of plasma control samples measured during study of endogenous thrombotic potential of premature MI subjects and controls. Coefficient of Variation for lo, hi, and pooled plasma control samples was 6.36%, 7.47%, and 8.80%, respectively (n = 27).

Stored plasma from donors with known low and high ETP were also assayed on each plate as control samples (CV 6.36% and 7.47%, respectively; Figure 3.3d).

3.3 Study Design

Premature MI and control group

162 subjects were studied who had sustained an MI before the age of 50 years. Subjects were identified through hospital databases and only those with a verifiable MI, as defined by WHO criteria were recruited to the study. All samples from cases were collected at least 3 months post-MI, therefore samples were not representative of factors that would be elevated due to the acute event. All subjects were matched for age and gender with a control group. The controls were recruited from GP practices from around the county, and were screened for history of heart disease and family history of MI. All subjects were North European Caucasian. Table 3.1 shows the demographics of the clinical cohorts. Many of the factors that have been associated with risk of MI (Koenig 1998), can be seen as being significantly different between the groups, including smoking, BMI, blood pressure, and frequency of exercise. Measurement of factors within the plasma, such as fibrinogen and lipid levels by routine laboratory methods also showed significant difference in levels of HDL, triglycerides and fibrinogen. Although the number of cases with hypertension was significantly higher in the cases than the controls (28.4% v 9.7%; $p<0.0001$), systolic and diastolic blood pressure were significantly lower in the premature MI cohort due to the use of hypertensive agents in the patient cohort. These cohorts were recruited by Dr Ravi Singh, and measurement of fibrinogen and lipids carried out in the routine laboratory (Hetherington *et al*, 2005).

a)

	Cases (n=162)	Controls (n=186)	p-value
Age (years)	47.5 ± 5.7	47.6 ± 5.7	0.8507
M:F (%)	86 : 14	87 : 13	0.9060
Age at time of MI	42.7 ± 5.7	N/A	-
Current/Ex/Non-Smokers (%)	21: 62 :17	18: 33 :49	**<0.0001**
Pack Years	24.5 ± 18.7	9.1 ± 14.1	**<0.0010**
Body Mass Index	29.98 ± 8.05	26.96 ± 4.04	**<0.0001**
BM fasting	5.89 ± 1.62	5.52 ± 0.98	**0.0092**
BP systolic	130 ± 17.4	133 ± 11.6	0.0773
BP diastolic	83 ± 11.2	86 ± 9.9	**0.0076**
Exercise (%) (none/1-2/>3 times /wk)	45:43:12	32:42:26	**0.0022**
Hypertension (%)	28.4	9.7	**<0.0001**
Diabetes mellitus (%)	12.3	1.1	**<0.0001**
Dyslipidaemia (%)	87.7	9.7	**<0.0001**

b)

	Cases (n=162)	Controls (n=186)	p-value
Fibrinogen (mg/dl)	3.13 ± 0.76	2.83 ± 0.61	**<0.0001**
Cholesterol (mmol/l)	5.15 ± 1.24	5.21 ± 1.05	0.6319
Triglycerides	2.04 ± 0.98	1.52 ± 0.86	**<0.0001**
HDL	1.15 ± 0.34	1.36 ± 0.35	**<0.0001**
LDL	3.09 ± 1.00	3.16 ± 0.92	0.5016
CRP (mmol/l)	2.19 ± 3.74	2.04 ± 2.26	0.6354
Homocysteine (mmol/l)	12.61 ± 4.27	11.96 ± 4.14	0.1498
Lp (a)	147 ± 232)	184 ± 225	**0.0120**

Table 3.1: a) Patient characteristics of premature MI subjects and controls; and b) Blood chemistry parameters in premature MI subjects and controls. Premature MI subjects had sustained an MI <50 years, and had verifiable MI as defined by WHO criteria. Samples were collected >3 months post-event. All cases were age- and sex-matched with healthy controls. Values are mean ± SD or n (%).

Young cohort with two-generational history of heart disease

Study of plasma ETP was also carried out in a younger cohort consisting of 20 young healthy males with a strong family history of MI (MI < 50 years of age in a parent and < 65 years in a grandparent) (case offspring) and 22 controls, matched for age and smoking status, with no family history of Coronary Artery Disease (CAD) in the preceding two generations (control offspring). None of the cohort had a history of diabetes, but one subject in each group was receiving treatment for hypertension. Table 3.2 shows the demographics of the Offspring cohorts. Interestingly, the healthy off-spring with the strong family history of cardiovascular disease had significantly higher BMI, and systolic and diastolic blood pressure.

	Case offspring (n=20)	**Control offspring** (n=21)	**p-value**
Age (years)	27.1 ± 6.4	26.1 ± 6.1	0.6000
Current/Non-Smokers (%)	55: 45	36:64	0.7728
Pack Years	8.2 ± 6.9	7.6 ± 7.4	0.8561
Body Mass Index	26.4 ± 4.5	23.8 ± 3.8	**0.0500**
BP systolic	133 ± 12.5	123 ± 8.5	**0.0043**
BP diastolic	84 ± 10.3	72 ± 8.2	**0.0001**
Hypertension (%)	5.0	5.0	1.0000
Diabetes mellitus (%)	0	0	-
Dyslipidaemia (%)	0	0	-
Fibrinogen (mg/dL)	2.95 ± 0.54	2.64 ± 0.54	0.0726

Table 3.2: Patient characteristics of offspring with family history of MI and controls. Case-offspring had a two-generational family history of premature MI, and were age- and sex-matched, also matched for smoking status with control group with no family history of premature MI. Values are mean ±SD or n (%).

3.4 ETP of plasma in controls and premature MI

Measurement of the overall thrombotic potential of plasma obtained from the two groups demonstrated that although the ETP was comparable in the majority of donors from each group, the mean ETP was significantly higher in the cases, (119 ±37.0%) compared to the controls (102 ± 22.8%)($p<0.0001$) (Figure 3.4). To assess the influence of endogenous TF on ETP, plasma samples from the same cohort were measured using the assay above, but without the addition of rTF. Results from the two groups demonstrated that again, the mean ETP was significantly higher in the cases, 97.51 ±23.8% vs 80.88 ± 20.2%; $p<0.0001$, in the control group (Figure 3.5a). There was a strong correlation between the ETP obtained from plasma in the presence or absence of rTF (Cases: $r = 0.7380$, $p < 0.0001$; Controls: $r = 0.5294$, $p < 0.0001$) (Figure 3.5b and c).

Several known risk factors for cardiovascular disease, e.g. smoking, BMI, and fibrinogen level were significantly higher in the premature MI cohort (Figure 3.6). Smoking status showed no significant influence on the level of ETP. BMI did not correlate with ETP in the cases, but showed weak, but significant correlation in the controls ($r=0.2101$, $p=0.0040$). Fibrinogen, however, was significantly correlated with ETP in both groups, (cases: $r=0.4402$, $p <0.0001$; controls: $r=0.2790$, $p=0.0001$) (Figure 3.7).

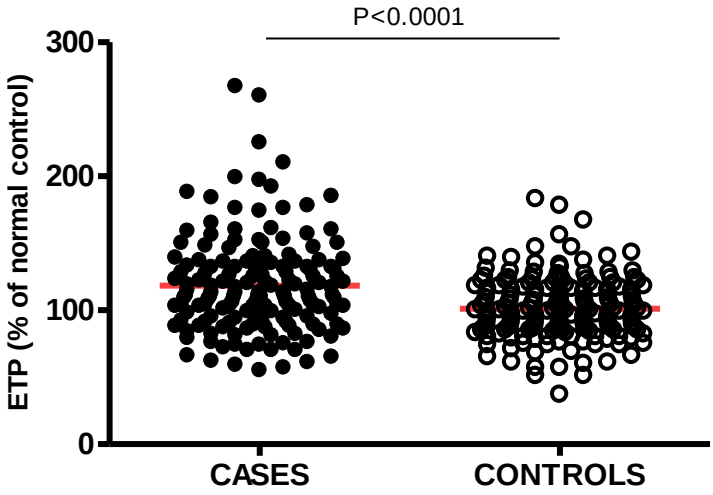
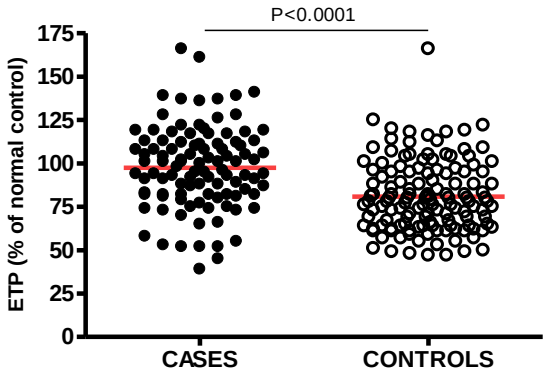


Figure 3.4: Endogenous Thrombotic Potential of plasma in subjects who have suffered a premature Myocardial Infarction and control group. All subjects had acute event aged < 50 years. All samples were collected 3 months post event. Mean ETP was 119 ± 37.0 v 102 ± 22.8% (p<0.0001), for cases and controls, respectively. (Cases n = 162, Controls n = 186).

a)



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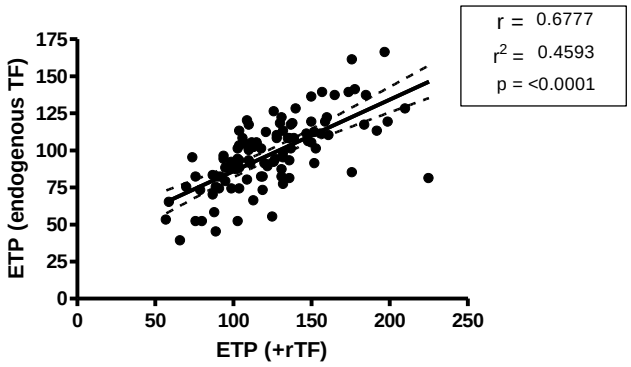

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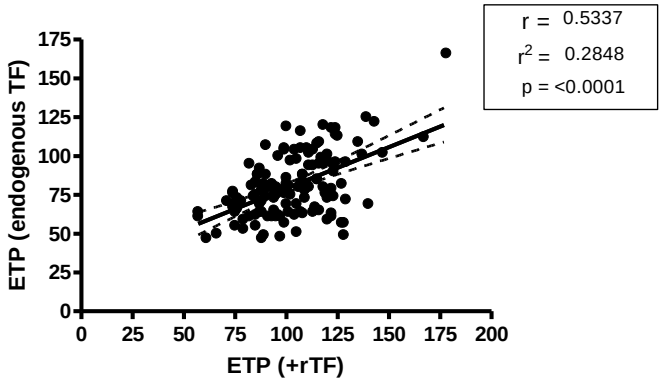
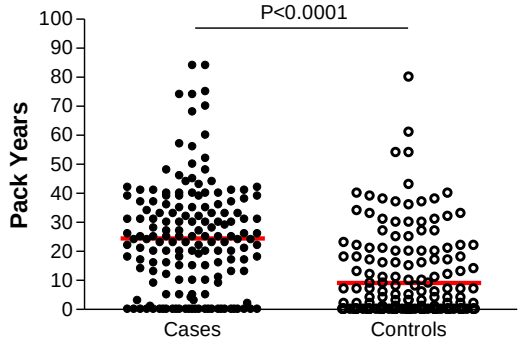


Figure 3.5: ETP initiated by recombinant and endogenous Tissue Factor in plasma from premature MI subjects and controls. a) Mean ETP in plasma from cases and controls, following initiation of coagulation with endogenous TF; b) Correlation of ETP initiated by recombinant TF (rTF) or endogenous TF in plasma from premature MI cases; c) Correlation of ETP initiated by rTF or endogenous TF in plasma from control group.

a)



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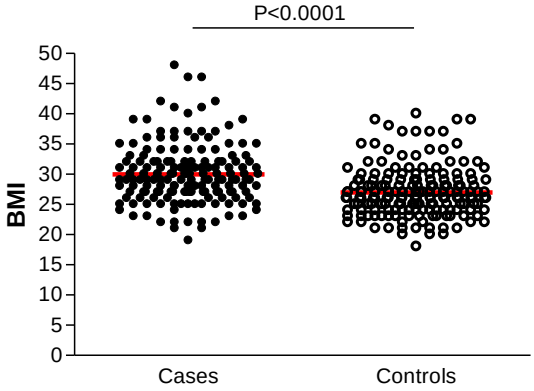

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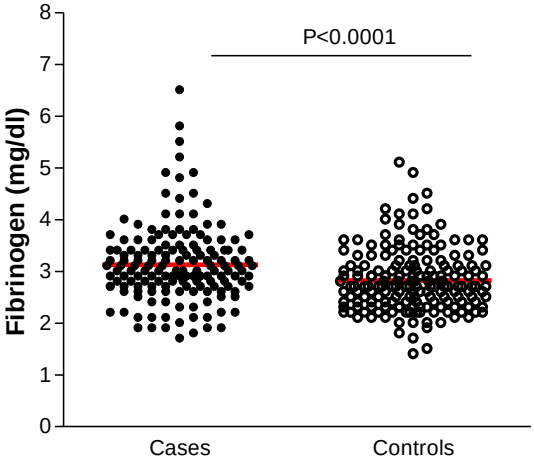


Figure 3.6: Demographical data for Premature MI and control group. (a) Smoking (pack years) mean 24.48 ±1.47 v 9.09 ±1.03; (b) Body Mass Index (BMI) mean 29.98 ±0.40 v 26.98 ± 0.30; and (c) plasma fibrinogen, mean 3.13 ± 0.06 v 2.83 ± 0.04 for cases and controls, respectively.

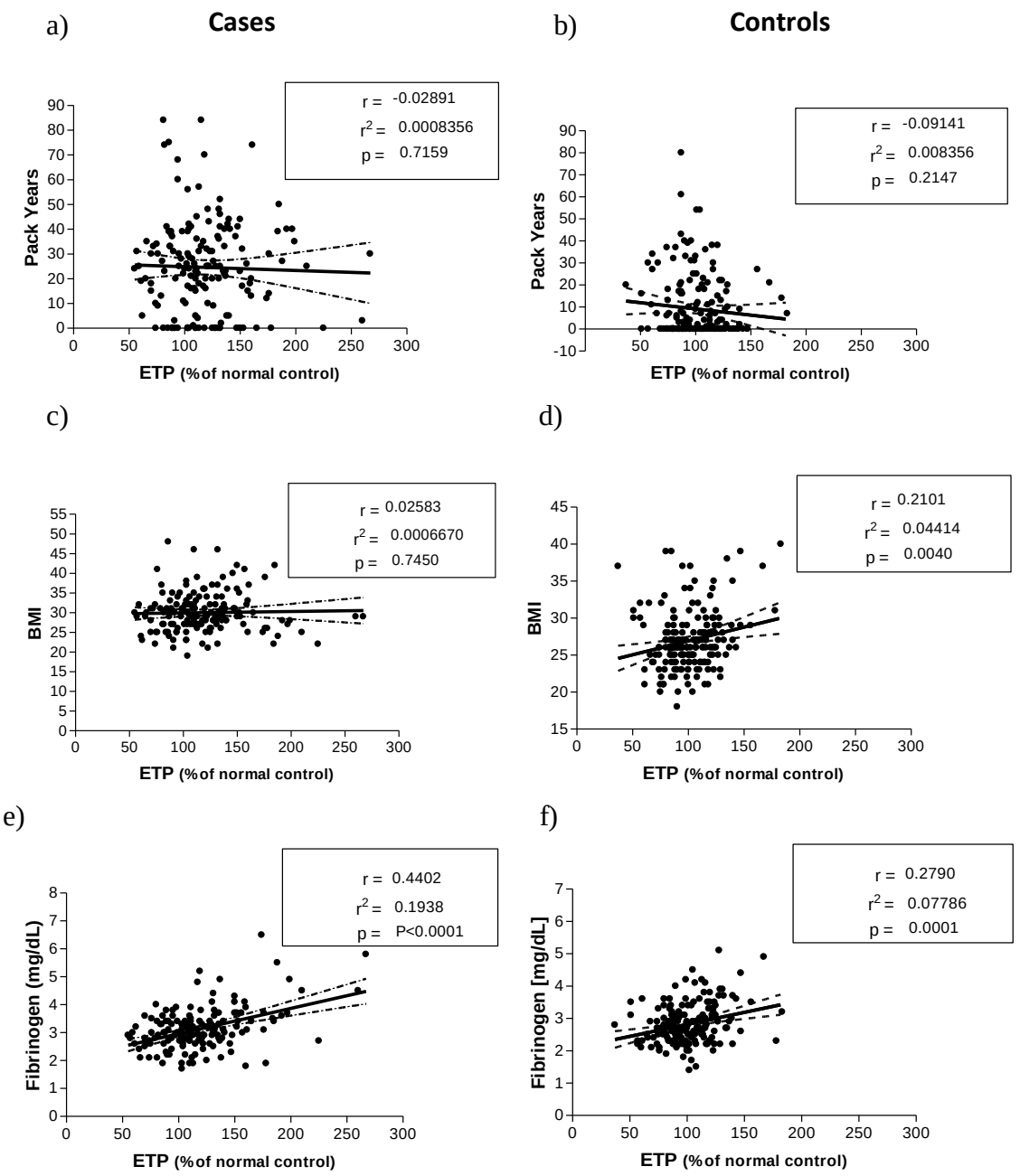


Figure 3.7: Correlation of Endogenous Thrombotic Potential with known risk factors of MI. (a and b) Smoking (Pack years) cases and controls, respectively; (c and d) Body Mass Index (BMI) cases and controls, respectively; and (e and f) plasma fibrinogen, cases and controls, respectively.

3.5 Individual coagulation factors in controls and premature MI

To investigate how the plasma levels of individual clotting factors might contribute to the ETP, clotting factors FX, FIX, FVIII, FVII, FV, and FII were measured on an automated coagulometry analyzer (Sysmex). Plasma levels of TF and TFPI activity were measured using chromogenic assays based on generation of FXa. The results obtained for the two groups were then compared. Although the majority of samples measured were in the normal range for each individual clotting factor, the majority of the procoagulant factors were significantly higher in the cases. In contrast, factors which act as inhibitors to coagulation, such as TFPI and ATIII, were significantly lower in the cases, or showed no difference between the two groups, respectively (Table 3.3). The majority of the procoagulant clotting factors including TF, FII, FIX, FXI, FVIII, and VWF, showed a significant correlation with the ETP in the cases and the control group (Figure 3.8). In contrast, anticoagulant factors TFPI and ATII showed no correlation with the ETP (Figure 3.9).

	Cases (n=162)	**Controls** (n=186)	**p-value**
ETP (% of normal control)	118.4 ± 36.1	101.0 ± 22.7	**<0.0001**
TF [pM]	11.32 ±7.69	9.27 ± 6.37	**0.0070**
FVII [IU]	1.23 ± 0.23	1.23 ± 0.24	0.8518
FX [IU]	1.36 ± 0.22	1.28 ± 0.18	**0.0003**
FIX [IU]	1.33 ± 0.26	1.18 ± 0.21	**<0.0001**
FXI [IU]	1.04 ± 0.29	1.02 ± 0.23	0.4216
FVIII [IU]	1.61 ± 0.56	1.15 ± 0.41	**<0.0001**
FV [IU]	1.28 ± 0.23	1.25 ± 0.23	0.2749
FII [IU]	1.24 ± 0.14	1.21 ± 0.14	**0.0532**
VWF (% activity)	114.9± 40.7	93.64± 35.3	**<0.0001**
TFPI [Units/mL]	1.008 ±0.35	1.090 ±0.36	**0.0330**
ATIII [Units/mL]	1.044±0.11	1.060±0.09	0.1212

Table 3.3: Procoagulant and anticoagulant factors in plasma from premature MI subjects and controls. Figures are mean ± SD. p value determined using unpaired t-test.

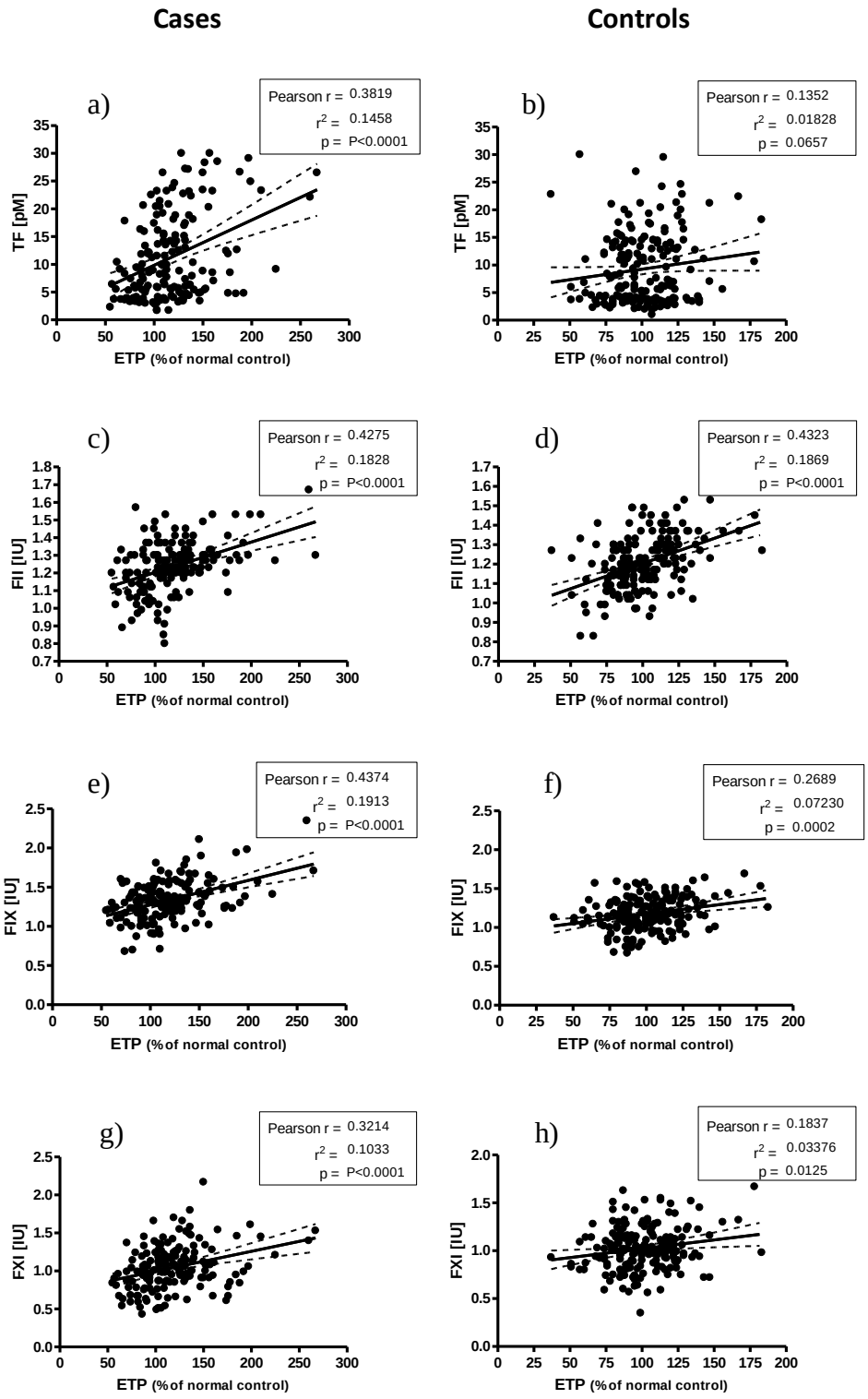


Figure 3.8: Correlations of plasma ETP with clotting factor levels in premature MI subjects and healthy controls. (a) and (b) ETP v TF activity, cases and controls, respectively; (c) and (d) ETP v FII (prothrombin), cases and controls; (e) and (f) ETP v FIX, cases and controls; (g) and (h) ETP v FXI, cases and controls.

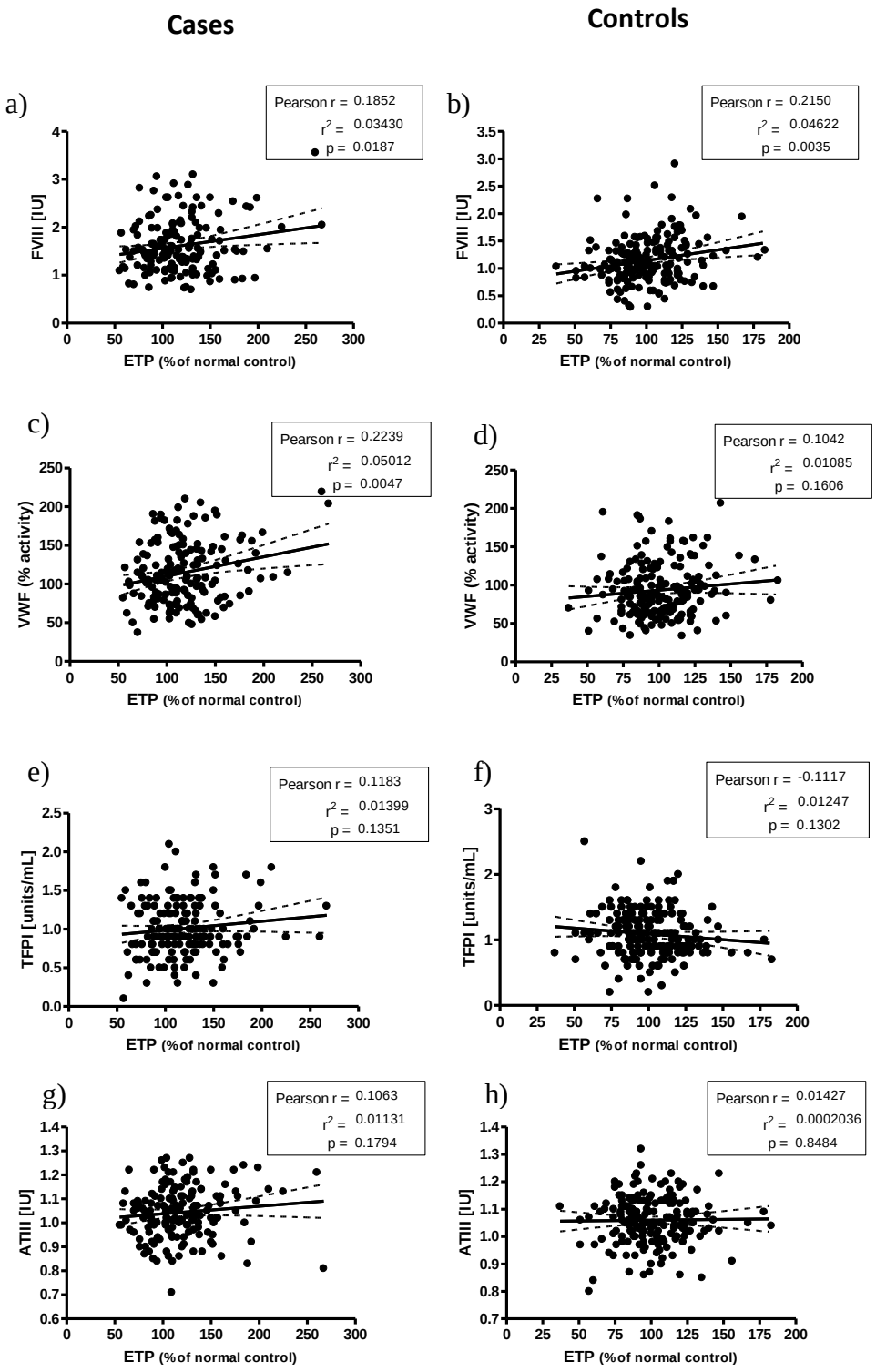


Figure 3.9: Correlation of clotting factors and inhibitors of coagulation with Endogenous Thrombotic Potential in plasma from premature MI subjects and controls. (a) and (b) ETP v FVIII in cases and controls, respectively; (c) and (d) ETP v VWF in cases and controls; (e) and (f) ETP v TFPI in cases and controls; (g) and (h) ETP v ATIII in cases and controls.

3.6 Comparison of ETP in plasma from male and female premature MI patients

Premature MI is rarer in females than males, and the cohorts recruited to this study reflected this trait, with 22 females compared 139 males. Mean ETP for female cases was significantly higher than ETP for the male cases (136±49.7 v 116±33.0, respectively, p=0.0142), whereas mean ETP for female and male controls was very similar (103±20.1 v 101±23.1; p=0.6783). This pattern was also seen for levels of prothrombin (FII) 1.30±0.2 v 1.23±0.1; p= 0.0295), and for fibrinogen, 3.83±1.1 v 3.02±0.6; p=<0.0001) (Figure 3.10). Whereas female controls had significantly lower levels of FX and FV than male controls (FX: 1.20±1.1 v 1.29±1.3; p= 0.0211; FV: 1.14±0.2 v 1.26±0.2; p=0.02), female and male cases had similar levels of both clotting factors (FX: 1.36±1.3 v 1.39±1.3; p=0.4361; FV: 1.30±0.3 v 1.28±0.2; p=0.684). FVIII was higher in both female cases and controls, when compared to FVIII levels in males from each group, although the level of elevation was greatest in the cases (Cases = 1.95±0.8 v 1.56±0.5; p=0.0025; Controls = 1.36±0.5 v 1.11±0.4; p=0.0055) (Figure 3.11). Analysis of other parameters showed that female controls also had lower BMI, lower systolic and diastolic blood pressure, and lower levels of LDL (Figure 3.12 a - d). Both systolic and diastolic blood pressure was lower in females when compared to males, however only reached significance in the control group. There was no significant difference between females and males in event age (p=0.8952) (Figure 3.12 e).

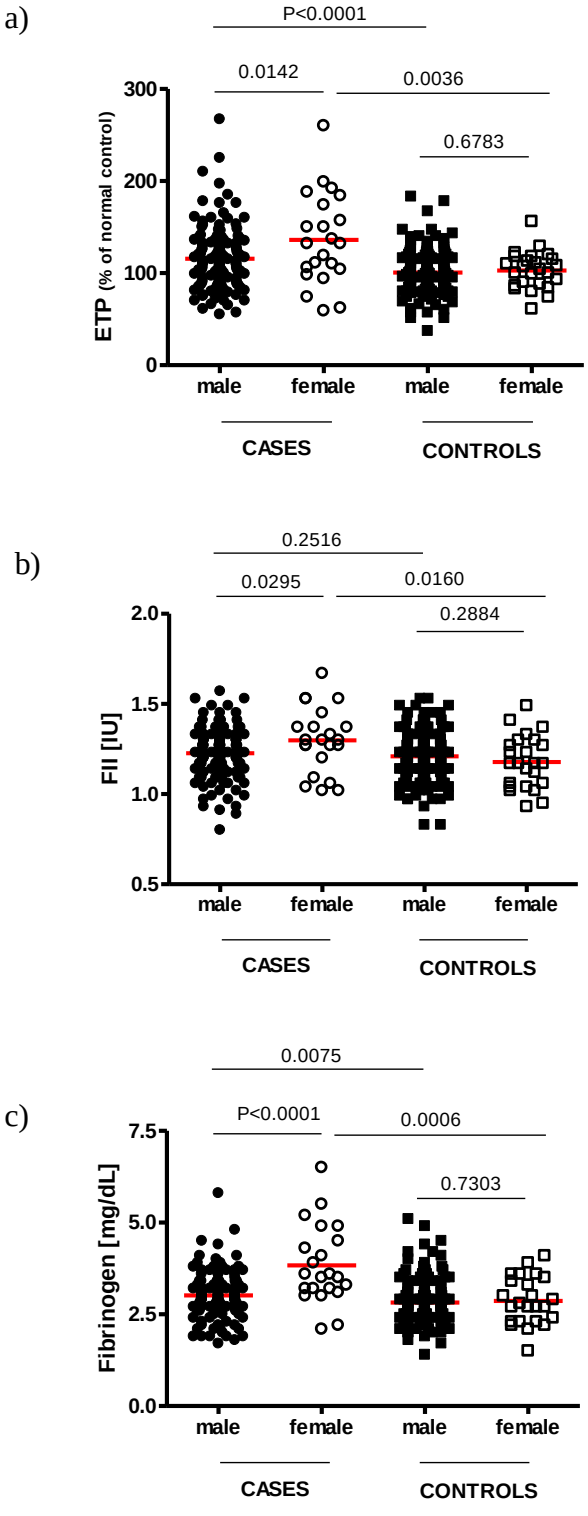


Figure 3.10: Comparison of haemostatic parameters in plasma from male and female premature MI subjects and controls. (a) ETP; (b) FII (prothrombin); and (c) plasma fibrinogen.

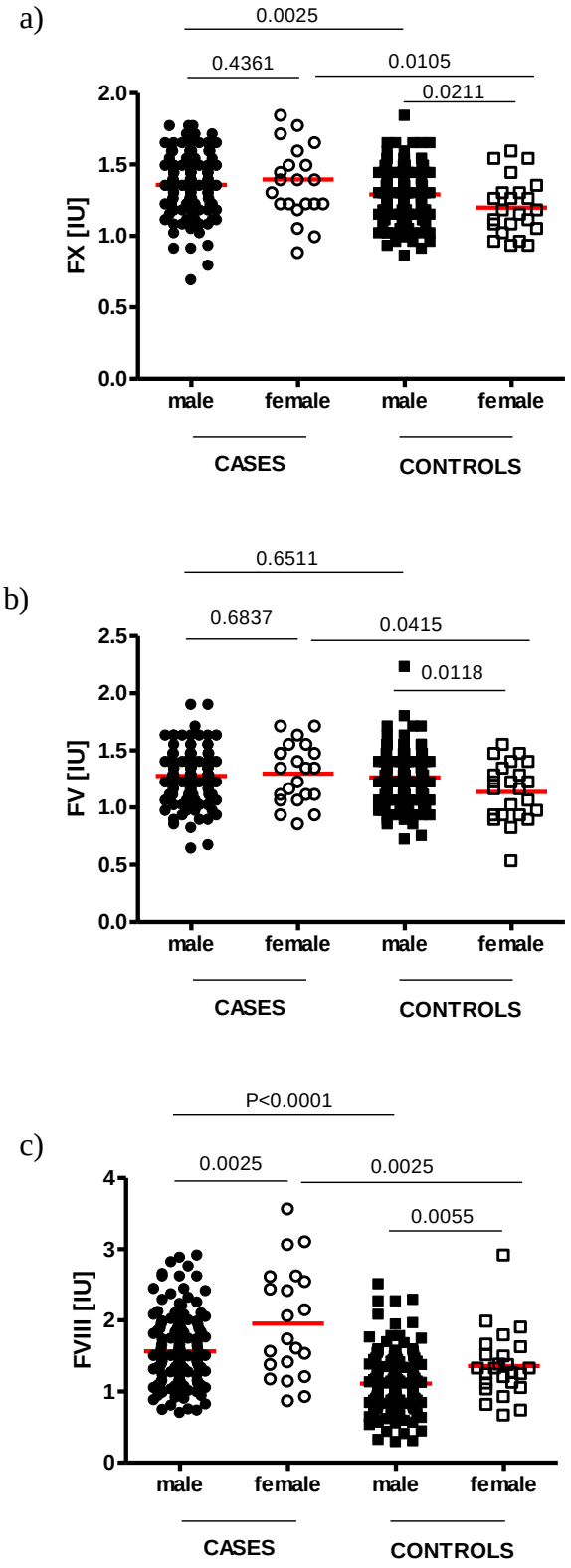


Figure 3.11: Comparison of haemostatic parameters in plasma from male and female premature MI subjects and controls. (a) FX; (b) FV; (c) FVIII.

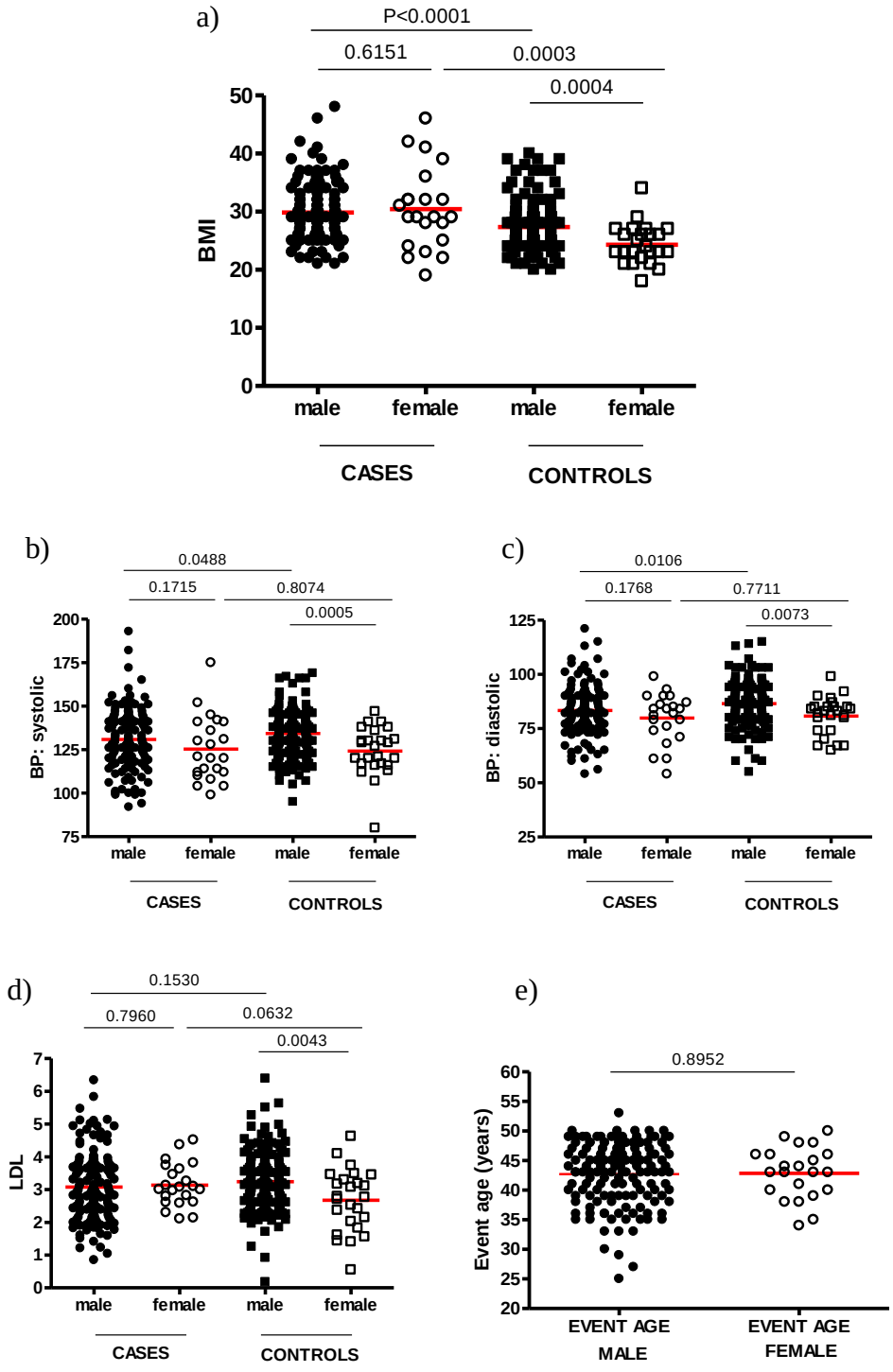


Figure 3.12: Comparison of patient demographical factors in male and female premature MI subjects and controls. (a) Body Mass Index (BMI); (b) Systolic blood pressure; (c) Diastolic blood pressure; (d) Low Density Lipoprotein (LDL); (e) Event age.

3.7 ETP in young cohort with two-generational history of heart disease

Mean ETP in the case offspring was significantly higher compared with control offspring (118.3±21.4 vs 97.6±19.6% p=0.0024) (Figure 3.13), but unlike the parental cohort, there was no significant difference in plasma TF or TFPI activity, and no correlation between ETP and TF, or ETP and TFPI. Within the offspring cohort, 15 case offspring were directly related to parents in the premature MI cohort, and 5 control offspring had parents in the control group of the older cohort; therefore these pairs of donors were studied in more detail. Mean ETP for both parent and offspring cases were elevated (118.5±36.1 vs 117.7±23.17%; p=0.9429). Mean plasma TF and TFPI activity were higher in both offspring cohorts, but only reached significance for TFPI (Figure 3.14 a - c). There was a significant negative correlation of TFPI with age (r= -0.5482; p=0.0025), with TFPI levels falling with increased age (Figure 3.14: d). Analysis of the 15 pairs of cases showed no correlation for ETP or TFPI between parent and offspring. There was a positive correlation for BMI between parent and offspring (r=0.5442; p=0.0545), and this was strongly correlated with ETP in the offspring cases (r=0.7607; p=0.0010)(data not shown). There was no significant difference between the two offspring cohorts in plasma fibrinogen.

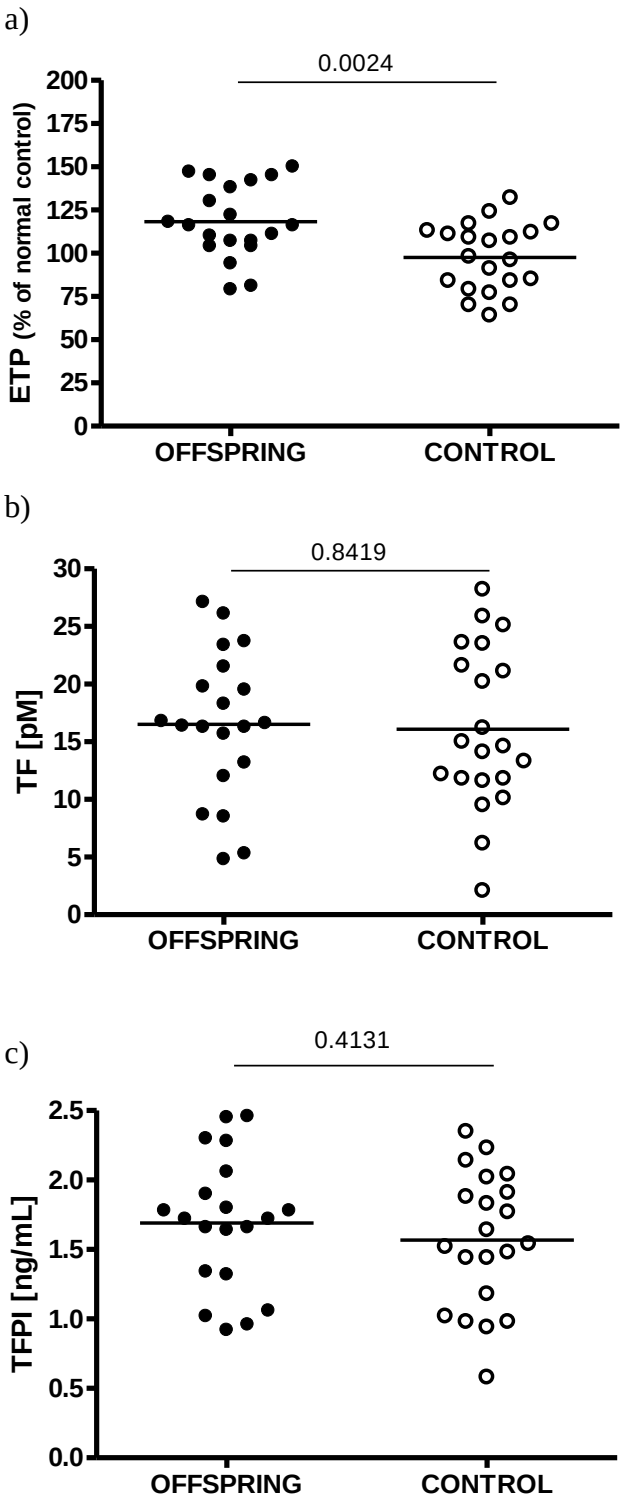


Figure 3.13: Measurement of ETP, TF and TFPI in plasma from offspring with two-generational family history of premature MI and control group. (a) Endogenous Thrombotic Potential (ETP); (b) Tissue Factor (TF); (c) Tissue Factor Pathway Inhibitor (TFPI).

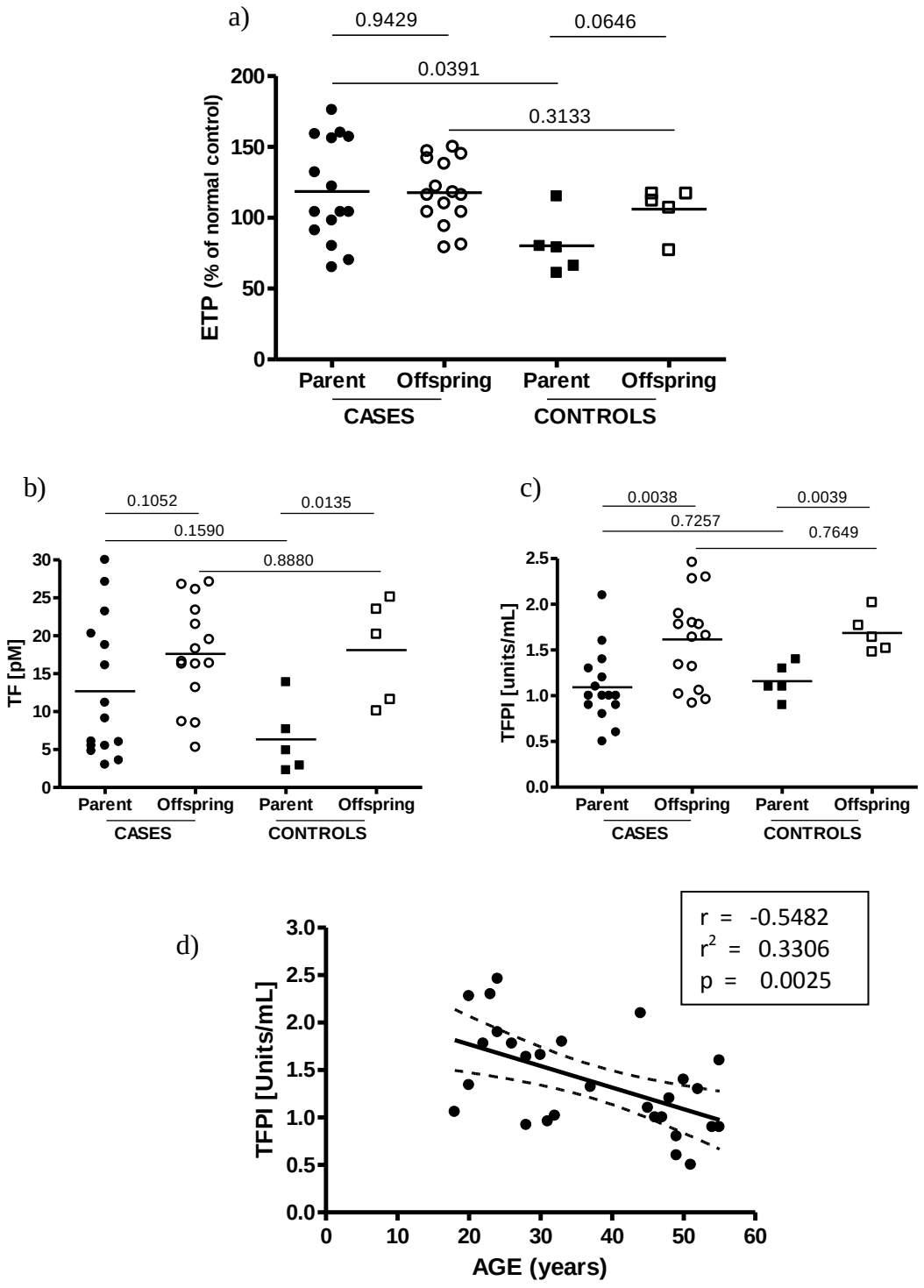


Figure 3.14: Endogenous Thrombotic Potential (ETP), Tissue Factor (TF), and Tissue Factor Pathway Inhibitor (TFPI) in plasma from matched parents and offspring. (a) ETP; (b) TF; (c) TFPI, in parents who have suffered a premature MI and offspring v healthy parents and offspring controls; (d) Correlation of TFPI activity in plasma with age.

3.8 Discussion

Plaque rupture and the contents of the plaque are key factors in the formation of the resulting thrombus, but the overall haemostatic response also plays an important role in determining the clinical outcome. Measurement of the global haemostatic response can be gained through measurement of the amount of thrombin generated, *ie*, the endogenous thrombotic potential of the plasma, using a continuous measurement assay of thrombin generation. This study used a thrombin-specific chromogenic substrate to measure the total haemostatic response of plasma, with the finding that patients who have had a premature MI have a higher capacity to generate thrombin than healthy control subjects with no family history of MI. This suggests that this group of subjects have an increased capacity to react to stimuli such as a fissured atherosclerotic plaque, which *in vivo* could result in the vessel becoming occlusive and lead to MI. As the samples collected from the premature MI subjects were collected >3months post-event, this increased thrombotic response was not due to elevated levels of inflammatory proteins, and therefore was not associated with the acute event. Although there was much overlap in the ETP measurements obtained from both groups, the mean ETP of the cases was significantly higher than the control group. This same trend was seen in the measurements of individual coagulation factors in the plasma, where although the majority of samples fell into the normal range for each coagulation factor, plasma from the premature MI subjects tended to fall at the higher end of the range. A significant number of the clotting factors, including TF, FX, FIX, FVIII, and FII, which drive the coagulation response forward towards thrombin generation were significantly elevated in the premature MI cohort when compared to the control group. In contrast, factors

which have an inhibitory effect, such as TFPI, and Antithrombin III, were higher in the controls, suggesting a more anticoagulant phenotype (Figure 3.15).

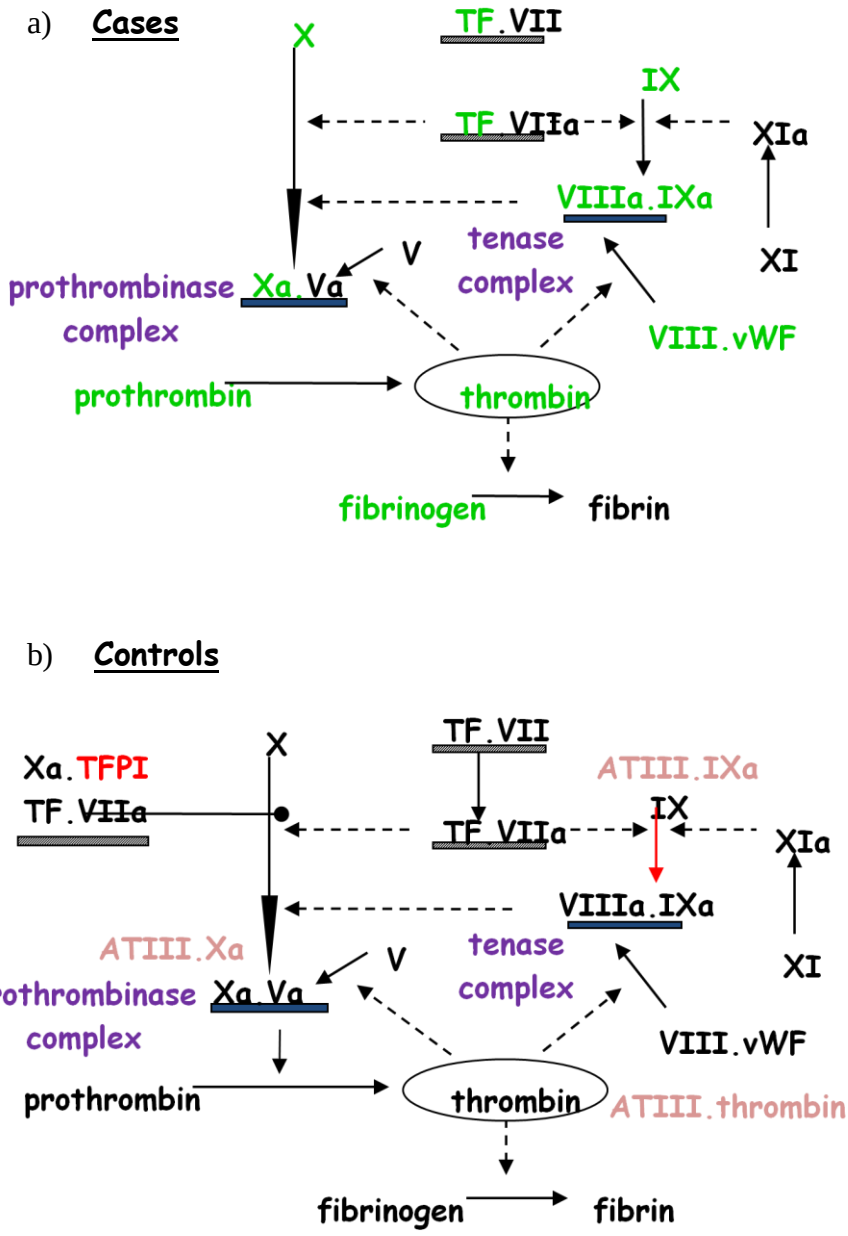


Figure 3.15: Thrombotic response in premature MI subjects and healthy individuals. (a) procoagulant response in premature MI subjects. All coagulation factors in green were significantly elevated in premature MI group, therefore haemostatic response is predisposed to be pro-coagulant. (b) haemostatic response in healthy control group. Factors which inhibit coagulation are in red, and were either higher in control group (TFPI) or at same level as in premature MI group (ATIII), therefore haemostatic response is more regulated.

Overall, the data point to a predisposition to a prothrombotic, or hypercoagulable state in the MI group. This may be due to a heritable phenotype, and this is investigated further in the next chapter. It is important to note that the majority of premature MI patients would be on statins and anti-platelet drugs post acute-event, yet despite this were still able to generate significantly more thrombin than normal healthy controls, and therefore are still at risk of future coronary events.

Low levels of endogenous TF are detected in the circulation; this can be in the form of soluble TF and cellular microparticles (MPs) (Morel *et al*, 2006 review article), the latter of which can be highly prothrombotic (Nieuwland *et al*, 1997; Biro *et al*, 2003; Aras *et al*, 2004; Hron *et al*, 2007). During development of the Thrombin Generation Assay, addition of increasing concentrations of recombinant TF, resulted in a dose-dependent shortening of lag-time, but did not generally affect ETP. However, the concentration of TF may affect the pathway through which thrombin generation is initiated, and along with the collection of blood into citrate tubes, could activate the intrinsic coagulation pathway via activation of Factor XII (FXII) (Luddington *et al*, 2008). There has been much debate recently regarding whether Corn Trypsin Inhibitor (CTI), a FXIIa inhibitor, should be added to samples when analysing thrombin generation (Luddington *et al*, 2004; Tappenden *et al*, 2007; van Veen *et al*, 2008; Spronk *et al*, 2009). Ollivier *et al*, (2009) recently found that addition of CTI to plasma containing low levels of exogenous TF resulted in prolonged lag-time. This effect was not seen at higher doses of TF. Therefore, FXIIa may play a role in initiation and amplification of thrombin generation when TF levels are low, but not when TF levels are elevated. The concentration of rTF added to this assay was sufficient to give variation in lag-time but not affect overall ETP; therefore results obtained should be a

measurement of thrombin generated via the extrinsic, TF-driven pathway of coagulation. Comparison of samples measured without addition of rTF demonstrated a strong correlation with thrombin generated via endogenous TF, suggesting that although TF is required to initiate and drive the haemostatic response, the overall thrombotic potential of plasma was not dependent on the concentration of TF.

Analysis of the correlation of the various coagulation factors with ETP showed that there was a correlation of clotting factor activity with ETP. The strong correlation of Prothrombin levels with ETP was perhaps expected due to it being the precursor of thrombin, and reflecting this, was seen to be significant in both cases and controls. This has also been reported in a recent study of thrombin generation in healthy individuals (Duchemin *et al*, 2008). In this present study, coagulation factors that showed the greatest significant difference between cases and controls were TF, FIX and FVIII. This could lead to increased generation of thrombin through elevated concentration of initiator (TF), and factors that form the Tenase complex (FIX and FVIII), and indeed, all three of these factors showed the strongest correlation with ETP in the cases. This is again supported by the findings of the study by Duchemin *et al*, (2008), observing that reduction of FVIII and FIX resulted in a decrease of ETP by almost two thirds, supporting the suggestion that higher FVIII and FIX levels in the premature MI cohort may support increased thrombin generation. There was no significant difference in FXI levels between the two cohorts, however, it was strongly correlated with ETP in the cases and weakly correlated with ETP in control group. FXI is generally associated with the contact pathway of coagulation, being activated by FXII, but is also activated by thrombin (Naito *et al*, 1991; Gailani *et al*, 1991). Keularts *et al*, (2001) have shown a positive relationship between FXI and thrombin generation. Therefore, in this study,

the correlation of FXI with ETP in the cases may be a measure of increased thrombin generation, amplified via feedback mechanisms which activate FXI, leading to further activation of FIX, cleavage of FVIII from its carrier VWF, all of which are elevated in the plasma from the premature MI group, thus increasing the capacity for Tenase complex formation. Increased thrombotic potential would be augmented further with this mechanism working in parallel with the increased levels of TF available.

MI is less common in younger women in comparison to men of the same age (Colditz *et al*, 1987; Mendelsohn *et al*, 1999; Vitale *et al*, 2009). This could be due to the effects of sex hormones on cells and proteins within the vasculature, with oestrogen levels having a protective effect (Xing *et al*, 2009). Estrogens cause rapid increases in nitric oxide (NO) production and induction of NOS genes (Chambliss *et al*, 2002) and are required for normal vasodilation, as well as affecting blood pressure in males and females (Zhu *et al*, 2002). Sex hormones also appear to affect platelet function. Faraday *et al*, (1997) showed that platelets from male rats displayed greater aggregation; however, platelets from female rats bound more fibrinogen, despite having lower levels of GPIIbIIIa. Platelets from women have also been shown to respond with a greater effect to low dose agonists (Leng *et al*, 2004), and form larger aggregates (Haque *et al*, 2001). Cyclical variation in levels of coagulation factors have been reported, as well as in levels of circulating cellular MPs (Toth *et al*, 2007). Fibrinogen levels have been reported to be elevated in women (Kaptoge *et al*, 2007), although continuous estrogen therapy has been shown to decrease levels of fibrinogen, antithrombin, protein S and Plasminogen Activator Inhibitor-1 (PAI-1) (Mendelsohn *et al*, 1999). In this study, fibrinogen levels were significantly higher in the female cases when compared with the males in that cohort; however there was no difference between

males and female controls. Prothrombin levels also followed the same pattern. Female controls displayed lower levels of FX and FV than males in that cohort, however this difference was not seen when comparing plasma FX and FV levels in the male and female cases. FIX levels were also elevated in female cases. Females from both groups had elevated levels of FVIII compared to males from both groups. Therefore, female cases appear to have increased levels of coagulation components which form the Tenase complex, and the Prothrombinase complex, which cleaves prothrombin (also elevated) into thrombin. The elevated thrombotic potential of the plasma can further fuel the cleavage of the elevated levels of fibrinogen into fibrin, resulting in increased fibrin formation, perhaps contributing to formation of an occlusive thrombus which would result in MI. However, the elevated levels of clotting factors suggest that due to the protective status of sex hormones, the thrombotic potential of plasma in females <50 years needs to be much higher than in males of the same age to provide a risk of MI.

Several of the known risk factors for cardiovascular disease, eg smoking, Body Mass Index (BMI), and fibrinogen level (Kannel *et al*, 1987; Yarnell *et al*, 1987; Lee *et al*, 1990; Scarabin *et al*, 1998; Woodward *et al*, 1998) were significantly higher in the premature MI cohort. Smoking status, in terms of pack years, showed no significant influence on the level of ETP. BMI was significantly correlated with ETP for the control group, but not for the cases. Plasma fibrinogen levels showed positive correlation with ETP in cases and controls. Although smoking status appeared to have no correlation with ETP, smoking has been shown to increase levels of circulating TF (Sambola *et al*, 2003), and therefore may indirectly contribute to an increased thrombotic potential. In the coagulation cascade, fibrinogen is cleaved by thrombin into fibrin, ready for fibrin polymerisation and formation of a clot. Elevated levels of

fibrinogen have been reported to lead to larger thrombi containing a dense structure that is more resistant to lysis (Fatah *et al*, 1992; Scrutton *et al*, 1994; Mills *et al*, 2002). Increased fibrinogen levels are also associated with an inflammatory response, increasing along with other acute phase proteins such as C-Reactive protein (CRP). Fibrinogen levels and CRP are markers of inflammation, but have also been established as biomarkers for increased risk of CHD (Danesh *et al*, 1998; Maresca *et al*, 1999; Koenig *et al*, 2003; Danesh *et al*, 2005). Samples for this study were collected >3months post-MI, therefore, the increased fibrinogen levels are unlikely to be due to the acute event, however they may be significantly higher than in healthy controls due to a higher inflammatory state, generally. But how does this link to ETP? One possibility is that elevated levels of fibrinogen increase plasma viscosity (Lowe *et al*, 1993; McDonagh *et al*, 1997; Folsom, 2001) and that this sterically, supports thrombin generation. A study by Martini *et al* (2009) showed that when fibrinogen synthesis was inhibited, the initiation phase of thrombin generation was also inhibited. The study also showed that when fibrinogen was rapidly degraded, that the propagation phase of thrombin generation was inhibited. Therefore, fibrinogen clearly plays an important role during thrombin generation, although the exact mechanisms of its function are yet to be determined. Obesity has also been shown to be linked to the incidence of cardiovascular disease. This is possibly due to poor circulation, altered lipid profiles, increased insulin resistance and altered metabolic profile (Eckel *et al*, 2005). However, BMI was not correlated with increased ETP in the premature MI subjects in this study, nor was there significant correlation between ETP and lipid parameters, therefore suggesting that the increased thrombotic potential is not reflective of the underlying disease process.

To test this further, ETP was also measured in a small number of healthy subjects with differing familial risk of MI. Individuals whose parents develop Coronary Heart Disease (CHD) at an early age have an increased risk of developing CHD, with reported links to various parameters, including levels of plasma fibrinogen, PAI-1, lipid parameters and leptin levels (Perkins *et al*, 1986; Schildkraut *et al*, 1989; Bara *et al*, 1994; Marenberg *et al*, 1994; Pankow *et al*, 1997; Makris *et al*, 2003). In a pattern reflecting that seen in the older cohort, mean ETP was significantly higher in individuals who had a family history of heart disease. Symptoms of the atherosclerotic process should be relatively low in the healthy offspring from families with a strong history of premature MI; therefore the elevated ETP is not likely to be due to underlying pathological factors due to disease. This suggests that the ETP may have an inherited component which contributes to risk of premature MI and contributed to primary risk of the event. Analysis of related parent-offspring pairs revealed that whilst mean ETP for the two groups was almost identical, there was no correlation of ETP between genetically-linked individuals. 50% of matched pairs of parents and off-spring showed significant correlation of TF activity, therefore it is possible that levels of plasma TF activity may be genetically linked, however this did not result in a similar, inherited pattern of ETP. Therefore multiple factors may play a role in determining the thrombotic potential of plasma.

One of the limitations of this study is that it does not consider the effects of cellular activation and interaction. In normal haemostasis, coagulation is initiated through exposure of TF due to damage of vessel integrity, and activation of platelets occurs via exposure of platelets to collagen. In pathological states such as premature MI, the haemostatic response is triggered by plaque rupture and exposure of its thrombotic

components to the circulation. In both cases, the haemostatic response results in formation of a thrombus, requiring platelet-platelet aggregation, and leukocyte activation and interaction. Therefore the cellular component of haemostasis is an important one, and is considered in more detail in chapter 5.

The method employed in this study used a chromogenic substrate and therefore cannot deal with turbidity in the sample, and therefore cannot give an OD reading in the presence of a fibrin clot, or in the presence of cells. At the time of carrying out the study, this method, or a similar chromogenic sub-sampling method, was generally used to measure the generation of thrombin. Due to the limitations of the chromogenic thrombin generation assay, regarding measurement in the presence of cells and interference of clotting plasma, a commercially available fluorogenic method has recently been developed which is not limited by these parameters. This is an automated method that calculates thrombin activity over time by comparing the cleavage of fluorogenic substrate within a given sample, with the thrombin activity of a calibrator sample (Hemker *et al*, 2002). This method has been used to monitor ETP during anti-platelet and antithrombotic therapy of haemophilia, and MI patients. Administration of therapeutic dosage of recombinant FVIIa increased ETP, whereas high concentrations resulted in a plateau effect (Wegert *et al*, 2005). In a separate study, whereas warfarin reduced the amount of thrombin generated, aspirin had no effect (Brodin *et al*, 2009). In the present study all MI subjects were on aspirin, and those on warfarin were not included in the cohorts for measurement of ETP.

A recent study using this method (Lekhal *et al*, 2009), includes study of thrombin generation in elderly survivors of MI, finding that there was no significant difference in ETP measured in the elderly MI subjects or age- and sex-matched healthy controls.

This may be due to the older age of the study group as thrombin generation has been reported to be increased with age (Dielis *et al*, 2008; Ignjatovic *et al*, 2008; Bernhard *et al*, 2009); also that other factors linked to the disease process itself may play a more prominent role in causing an MI in older individuals. In contrast, a genetic element(s) may be the source of increased thrombotic potential in younger MI subjects with a low atherosclerotic burden. This is supported with the finding of this current study that ETP was significantly increased in the healthy offspring of premature MI subjects. Therefore, measurement of thrombotic potential may be a useful biomarker of increased cardiovascular risk, and further experiments in MI subjects and healthy controls are required to examine the contribution of platelets and MPs to thrombotic potential.

In summary:

- Measurement of the endogenous thrombotic potential of plasma from premature MI patients and controls, demonstrated that the premature MI subjects were able to generate more thrombin.
- This hypercoagualable response was associated with elevated levels of several procoagulant factors, including TF, FX, FIX, FVIII, and FII, that was not compensated by increased levels of the main anticoagulant proteins, TFPI and ATIII.
- Elevated ETP levels were also seen in offspring with a strong family history of premature MI, suggesting a possible genetically determined element.

Limitations:

- Many of the premature MI patients would be taking statins as well as aspirin, therefore therapeutics may mask the 'true' thrombotic potential.

- The study only examines the thrombotic potential of plasma from 'survivors' of premature MI. The study does not address the possible contribution of cellular microparticles to the higher thrombotic potential in the plasma of the premature MI patients.

Future work:

- Examine the possible contribution of cellular microparticles to ETP, examining the source of these microparticles as well as their thrombotic potential.
- Carry out larger study of ETP, and plasma coagulation factors in off-spring with family history of premature MI.

CHAPTER 4: TF gene polymorphism and premature MI

4.1 Introduction

During the last decade, aided by the advancement in genotyping technology, study of genes and gene polymorphism has allowed identification of genetic factors that may contribute to increased cardiovascular risk. Gene polymorphisms have been shown to be linked to increased levels of various haemostatic plasma proteins, for example G20210A, the Prothrombin gene variant (Arruda *et al*, 1997; Franco *et al*, 1999; Burzotta *et al*, 2000), and increased resistance to action of anticoagulant proteins; and Factor V Leiden (Bertina *et al*, 1994; Zöller *et al*, 1994; Folsom *et al*, 2002; Middendorf *et al*, 2004). Several reports have suggested an association between TF gene polymorphism and increased plasma TF activity, or increased cardiovascular risk (Arnaud *et al*, 2000; Donahue *et al*, 2003). Therefore, genotyping was carried out to assess if polymorphism within the TF gene was associated with elevated TF activity in plasma seen in the premature MI cohort, and whether there was also an association with increased endogenous thrombotic potential. There are 6 polymorphisms in the 5'untranslated region (UTR) of the TF gene. Four of these are in complete linkage disequilibrium, determining two haplotypes: -603A/-1208D/-1322C/-1812C, and -603G/-1208I/-1322T/-1812T. For this study, and in order to simplify discussion, the first haplotype is designated as -1812C, and the second haplotype as -1812T. A second SNP selected for analysis was the 5466 A>G polymorphism which is located in intron 2 of the TF gene. Genotyping was performed using allele-specific fluorogenic probes and TaqMan PCR. As part of a separate study undertaken by Dr RK Singh, genotyping of

the premature MI cohort and control group was carried out for 28 SNPs associated with cardiovascular disease including 5 SNPs found in coagulation genes encoding prothrombin, FV, FVII, and fibrinogen (genotyping carried out by Peter Braund). Therefore these coagulation gene SNPs were also analysed for possible association with increased thrombotic potential.

4.2 Results

4.2.1. Analysis of coagulation factor polymorphisms

Analysis of coagulation factor polymorphisms and ETP showed that presence of the G20210A genotype was associated with elevated plasma levels of prothrombin (1.23±0.14 v 1.36±0.18 IU, p=0.1422; 1.20±0.13 v 1.41±0.10 IU, p=0.0002, cases and controls, respectively). Mean ETP was also higher in individuals with the prothrombin variant (118.2±36.0 v 129.3±49.9%, p=0.5993; 100.3±22.7 v 120.1±12.6%, p=0.0226), for cases and controls, respectively (Figure 4.1). There was no association of the fibrinogen gene G-455A SNP with ETP (wt homozygous 122.0±37.3%, heterozygous 112.8±33.9%, minor allele 101.6±26.3% in the cases (p=0.1885); and 102.3±23.0%, 99.1±22.5%, and 96.9±21.0%, (p=0.5962) respectively, in the control group)(Figure 4.2). There was also no association of genotype with ETP for FV, or FVII SNPs. A summary of data for all 5 coagulation factor SNPs can be found in Table 4.1.

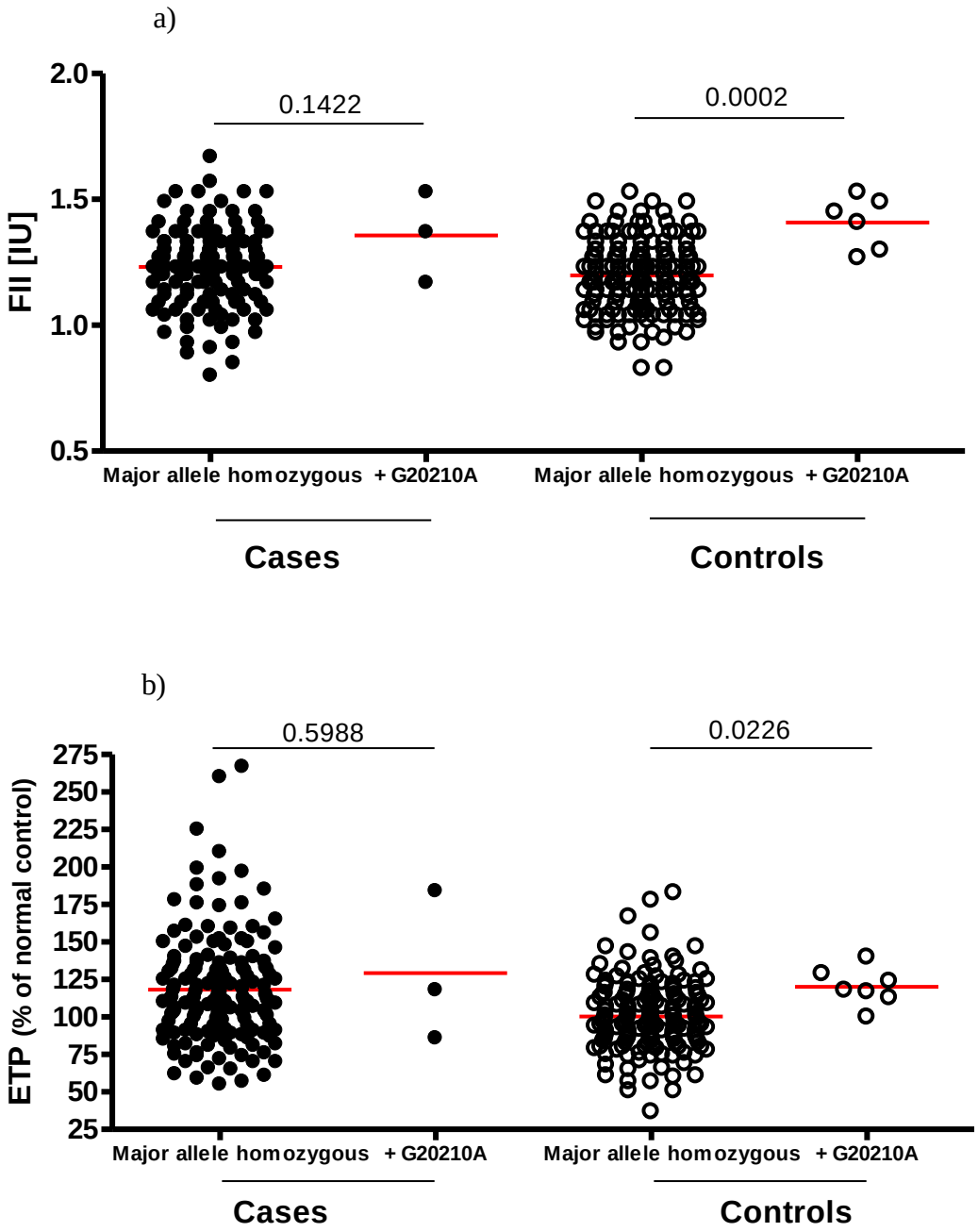


Figure 4.1: Effect of FII G20210A gene polymorphism on FII and Endogenous Thrombotic Potential (ETP). a) FII (prothrombin) levels in plasma from premature MI subjects and controls with and without G20210A polymorphism; b) ETP in plasma from premature MI subjects and controls with and without G20210A polymorphism.

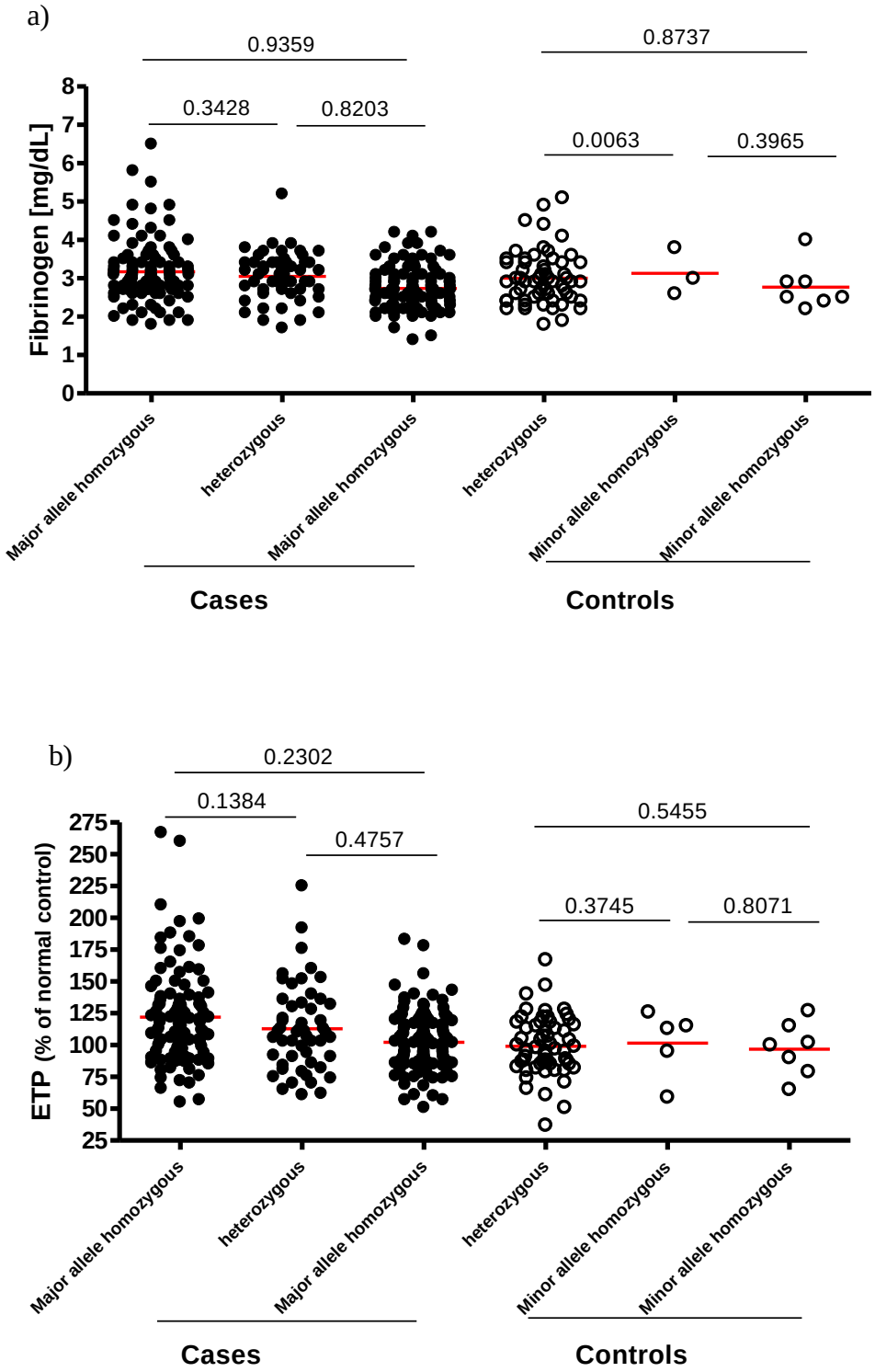


Figure 4.2: Effect of Fibrinogen G-455A gene polymorphism on plasma fibrinogen and Endogenous Thrombotic Potential (ETP). a) fibrinogen levels in plasma from premature MI subjects and controls with and without G-455A polymorphism; b) ETP in plasma from premature MI subjects and controls with and without G-455A polymorphism.

a)

SNP	Population	Major allele frequency	Minor allele frequency
FII	Cases	0.99	0.01
G20210A	Controls	0.98	0.02
FV	Cases	0.98	0.02
506gln	Controls	0.96	0.04
FVII	Cases	0.89	0.11
323ins	Controls	0.89	0.11
FVII	Cases	0.90	0.10
353del	Controls	0.90	0.10
Fibrinogen	Cases	0.81	0.19
G455A	Controls	0.80	0.20

b)

		ETP			
SNP	**Population**	**Major allele**	**homozygous**	**Minor allele**	**p**
FII	Cases	118.2±36.0	129.3±49.9		*0.5993*
G20210A	Controls	100.3±22.7	120.1±12.6		*0.0226*
FV	Cases	119.1±35.6	102.2±15.8		*0.2628*
506gln	Controls	100.6±22.6	106.2±24.7		*0.3907*
FVII	Cases	117.4±36.9	122.3±33.9	110.0±0	*0.4776*
323ins	Controls	100.5±22.0	102.3±25.8	113.5±9.2	*0.6606*
FVII	Cases	117.0±36.8	124.8±33.7	110.0±0	*0.2832*
353del	Controls	100.4±21.7	102.5±27.5	114.3±6.7	*0.6371*
Fibrinogen	Cases	122.0±37.3	112.8±33.9	101.6±26.3	*0.1885*
G455A	Controls	102.3±22.9	99.1±22.5	96.9±21.0	*0.5962*

Table 4.1: Single Nucleotide Polymorphism (SNP) and Endogenous Thrombotic Potential. Table a) Allele frequency for coagulation gene SNPs; Table b) Coagulation gene SNPs and effect on Endogenous Thrombotic Potential (ETP).

4.2.2. Analysis of TF gene polymorphism

4.2.2.1. Allele Frequency

Analysis of the TF C -1812T polymorphism in the premature MI cohort identified 32 CC homozygotes (20%), 92 CT heterozygotes (57%), and 38 TT homozygotes (23%), resulting in allele frequencies of 0.48 and 0.52 for the C and T alleles, respectively. Analysis of the control group identified 54 CC homozygotes (30%), 93 CT heterozygotes (51%), and 36 TT homozygotes (20%), resulting in allele frequencies of 0.55 and 0.45 for the C and T alleles, respectively. Genotype frequency distributions were tested by using the Pearson χ-squared test. Allele frequencies conformed to those predicted by the Hardy-Weinberg equilibrium law, although interestingly, the proportion of -1812T carriers was higher in the premature MI patients than in the control subjects: 80% vs 71%, although not reaching significance ($p = 0.1094$).

Analysis of the TF +5466A>G polymorphism identified 150 AA homozygotes (93%), and 12 AG heterozygotes (7%) in the premature MI patients, resulting in allele frequencies of 0.96 and 0.04 for the A and G alleles, respectively. Analysis of the control group identified 169 AA homozygotes (92%), and 15 AG heterozygotes (8%), resulting in allele frequencies of 0.96 and 0.04 for alleles A and G respectively. Therefore, there was no difference in allele frequency between the two groups. There were no GG homozygotes (Table 4.2a).

4.2.2.2. TF activity and TF gene polymorphism

TF C-1812T

TF activity levels in plasma for all genotypes are shown in Table 4.2b. There was no significant difference in TF activity between the two genotypes, although mean TF activity was lower for the TT genotype when compared to the CC genotype, in both the

cases and the controls (11.05±7.6 vs 9.62±7.2 pM, *p*=0.4242; and 10.05±6.9 vs 9.01±5.6 pM, *p*=0.4512, respectively) (Figure 4.3a and b).

TF +5466A>G

Plasma TF activity was also analysed in relation to the +5466A>G SNP. There was no significant difference between the homozygous AA and heterozygous AG carriers for the premature MI patients (mean TF activity 11.22±7.6 vs 12.62±9.7 pM, *p*= 0.5480, respectively). However, TF activity was significantly lower in the heterozygotes in the control group (mean TF activity 9.51±6.4 vs 6.153.9 pM, *p*= 0.048, respectively) (Figure 4.3c and d).

4.2.2.3. ETP and TF gene polymorphism

TF C-1812T

ETP of platelet-poor plasma was analysed in relation to genotype. There was no significant difference between ETP and genotype in either cases or control cohorts, (Figure 4.4 a and b).

TF +5466A>G

There was no significant difference in ETP for the premature MI cohort (mean ETP 118.3±35.8 v 118.3±43.4% for AA and AG genotype, respectively (*p*=0.9967); nor for the control group (mean ETP 101.4±23.3% vs 96.0±16.6%, for AA and AG genotype, respectively (*p*=0.3776). Therefore there was no association of ETP with TF+5466A>G polymorphism (Figure 4.4 c and d).

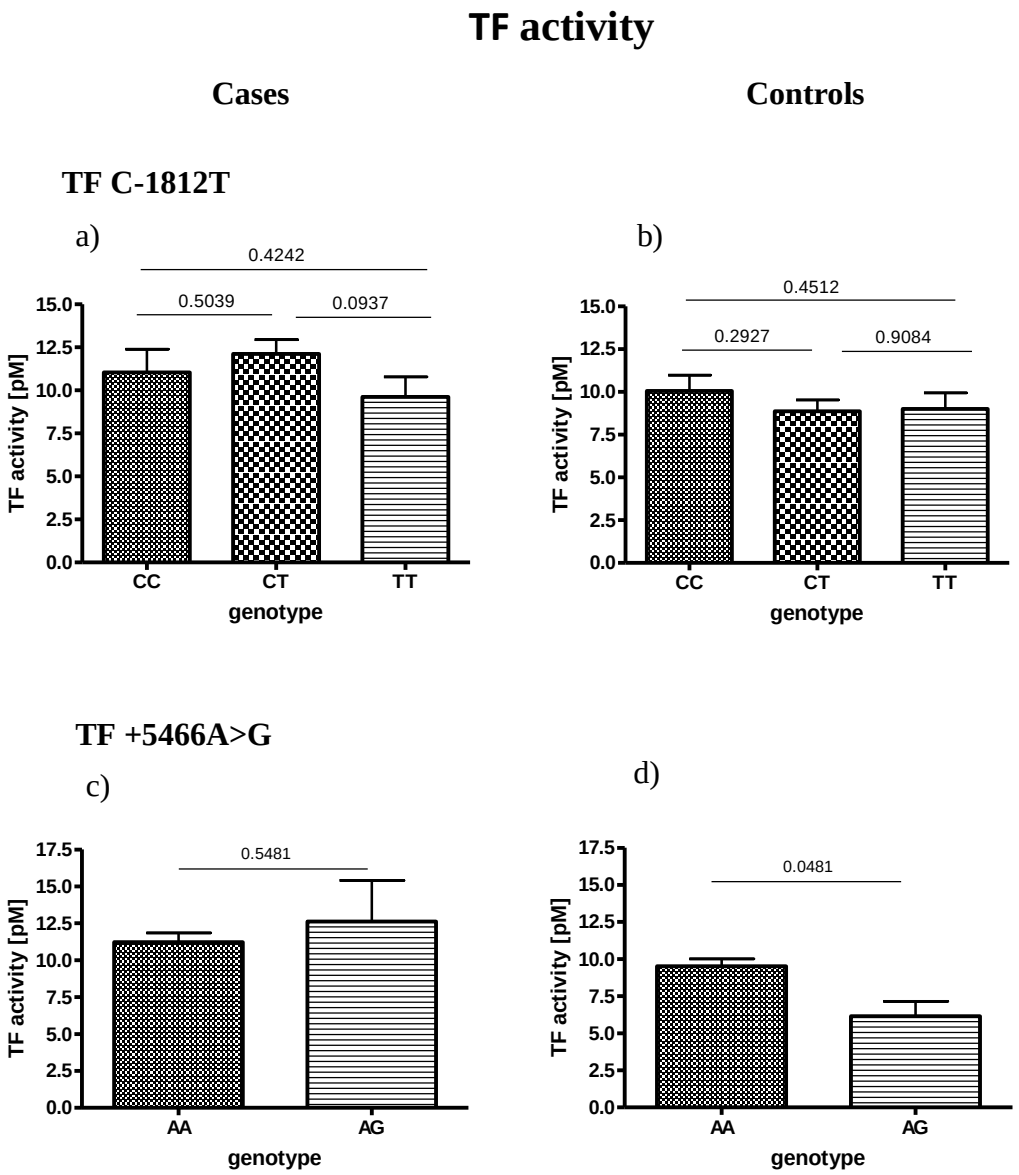


Figure 4.3: Effect of Tissue Factor (TF) gene polymorphism and genotype on TF activity in plasma from premature MI subjects and control group. Figures a and b: Effect of C-1812T polymorphism and plasma TF activity in cases and controls, respectively. Figures c and d: Effect of +5466A>G polymorphism and plasma TF activity in cases and controls, respectively.

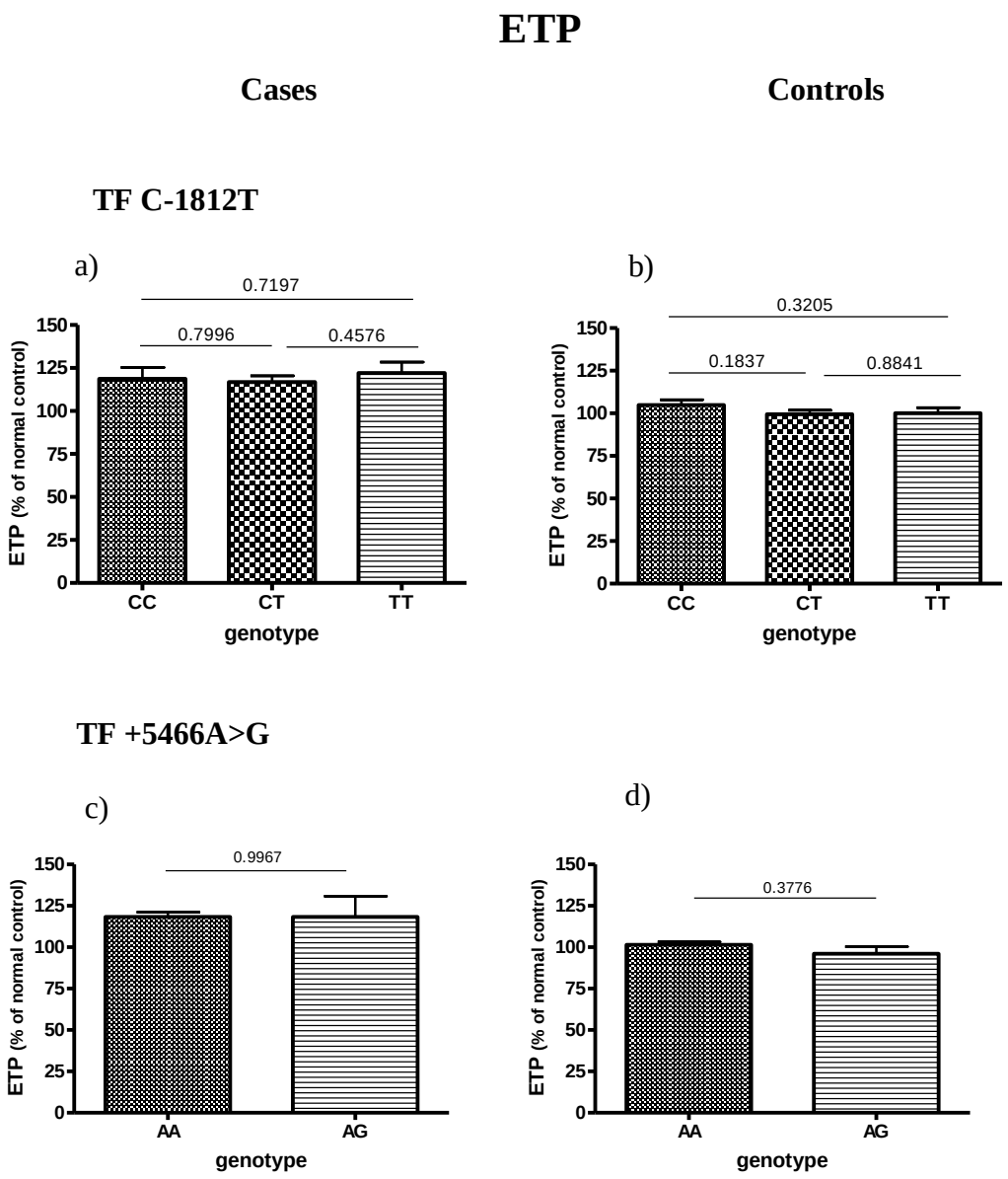


Figure 4.4: Effect of Tissue Factor (TF) gene polymorphism and genotype on Endogenous Thrombotic Potential (ETP) in plasma from premature MI subjects and control group. Figures a and b: Effect of C-1812T polymorphism and ETP in cases and controls, respectively. Figures c and d: Effect of +5466A>G polymorphism and ETP in cases and controls, respectively.

a) Distribution of genotype

SNP	rs	Population	Major allele frequency	Minor allele frequency	Genotype	n (%)	p-value
C-1812T	**958587**	Cases	0.48	0.52	CC	32 (20)	0.1094
					CT	92 (57)	
					TT	38 (23)	
		Controls	0.55	0.45	CC	54 (30)	
					CT	93 (51)	
					TT	36 (20)	

SNP	rs	Population	Major allele frequency	Minor allele frequency	Genotype	n (%)	p-value
+5466A>G	**3917643**	Cases	0.96	0.04	AA	150(93)	0.9435
					AG	12 (7)	
		Controls	0.96	0.04	AA	169(92)	
					AG	15 (8)	

b) Genotype and plasma ETP and TF

	Population	Genotype	TF [pM]	p-value	ETP (% of control)	p-value
C-1812T	Cases	CC	11.05 ±7.6	0.2369	118.7 ±37.4	0.7602
		CT	12.1 ±7.9		116.8 ±34.5	
		TT	9.62 ±7.2		122.0 ±39.6	
	Controls	CC	10.05 ±6.9	0.5400	104.8 ±23.4	0.3715
		CT	8.87 ±6.3		99.41 ±23.6	
		TT	9.01 ±5.6		100.1 ±19.7	

SNP	Population	Genotype	TF [pM]	p-value	ETP (% of control)	p-value
+5466A>G	Cases	AA	11.2 ± 7.6	0.5480	118.3 ±35.8	0.9967
		AG	12.6 ±9.7		118.3 ±43.4	
	Controls	AA	9.5 ±6.4	*0.0480*	101.4 ±23.3	0.3776
		AG	6.1 ±3.9		96.00 ±16.6	

Table 4.2: Analysis of TF SNP polymorphisms. (a) Distribution of genotype; and (b) genotype in relation to plasma TF activity and Endogenous Thrombotic Potential (ETP), in premature MI subjects and controls.

4.3. Discussion

This study found that carriers of the G20210A allele had increased levels of FII, and elevated ETP. These findings are consistent with results reported from previous studies (Rosendaal *et al*, 1997; De Stefano *et al*, 1998; Doggen *et al*, 1998; Simons *et al*, 2000). There were a similar number of G20210A carriers in the premature MI and control group, therefore although the allele confers increased thrombotic potential, it is not the source of the significantly elevated ETP seen in the premature MI subjects. There have been many studies linking polymorphism of the gene encoding the fibrinogen β-chain to arterial thrombosis (Humphries *et al*, 1995; Behague *et al*, 1996; Carter *et al*, 1996; de Maat *et al*, 1998). In this study, the fibrinogen G-455A SNP had no significant association with ETP.

This study also examined two SNPs that occur within the TF gene, -1812CT which is in the promoter region of the gene, and +5466A>G which is in intron 2. In the PRAMIS study population, although frequency of the -1812T allele was more common in the premature MI patients, with an increased number of heterozygotes, this did not reach a level of significance, probably due to the relatively small sample size. In a similar cohort studied by Ott *et al*, (2004), the -1812T allele was significantly higher in MI patients than control subjects, again due to an increase in number of heterozygotes. Therefore study of a much larger cohort is required.

The next question was whether there was an association of the -1812T allele with increased TF activity. The initial picture from studies of the biological effect of TF gene polymorphism is that the -603/-1208I/-1322T/-1812T haplotype combination is linked to elevated levels of TF expression and increased cardiovascular risk, but on closer scrutiny, results are more equivocal. In one of the first studies looking at

association of TF genotype and TF antigen in plasma, lower TF antigen level was associated with the –1812C allele (Arnaud *et al*, 2000). More recently, another study found no significant difference in TF antigen between -1812CC and -1812TT homozygotes (Campo *et al*, 2006). Other studies have looked at levels of mRNA in monocytes, finding that the -1812T allele was associated with increased TF mRNA levels in basal monocytes (Reny *et al*, 2004; Marsik *et al*, 2006). In the study by Reny *et al*, (2004), despite the fact that there was increased expression of TF mRNA, there did not appear to be increased levels of active TF within the circulation as there was no increase in Whole Blood clotting time. This present study also found no significant difference in plasma TF activity, and therefore no association of TF activity in basal plasma with TF-1812 genotype.

So is there any association of the TF-1812 polymorphism with the amount of thrombin generated? There have been no previous studies which investigate this in relation to the polymorphisms associated with the TF-gene promoter region. Analysis of thrombin generation in the PRAMIS cohort showed no significant difference between the CC and TT homozygotes in the premature MI patients or control group. In a recent study investigating whether the TF -1812 polymorphism is associated with the thickness of carotid intima-media, a marker of atherosclerotic progression, it was found that IMT was highest in -1812CC homozygotes (Gertow *et al*, 2009), suggesting that the -1812T genotype may have a protective effect on cardiovascular and atherosclerotic disease progression. This contradicts with the initial finding in this, and other studies, that the -1812T allele is predominant in cardiovascular disease (Ott *et al*, 2004; Donahue *et al*, 2003). There are now an increasing number of studies linking the TF C-1812T promoter polymorphism to risk of MI. Despite finding an increased number of -1812T

carriers in the premature MI population studied, our data does not support a direct effect on plasma TF levels or ETP. Therefore more studies are needed to confirm how this genotype may translate into the biological function(s) that increase this risk.

There have been fewer studies examining the effects of the +5466A>G TF polymorphism, but reports suggest that this polymorphism may be linked to cellular response and cell activation rather than to TF levels under basal conditions, with increased monocyte response to LPS seen in G allele carriers, and increased risk of cardiovascular death in acute coronary syndrome (ACS) patients (Malarstig *et al*, 2005). The data in the present study was obtained only from measurement of activity in basal plasma, and does not incorporate the possible change in procoagulant activity or thrombotic response that may occur following cellular response to stimuli, for example monocyte response to LPS. In the study by Malarstig *et al*, (2005), it was observed that although G allele carriers had the greatest response to LPS, TF activity in resting monocytes was significantly lower in this genotype. In the current study, basal plasma TF activity was also significantly lower in the G allele carriers, but only in the control group, and this did not reflect a similar reduction in thrombotic potential. A recent study has examined the effect of this polymorphism on response to statins, and thrombin formation following vascular injury, finding that G-allele carriers generated thrombin (measured by formation of thrombin-anti-thrombin (TAT) complexes and Prothrombin 1.2 fragments) significantly faster at the site of injury, but that response to statins was far greater, with amount of thrombin generation being significantly reduced (Undas *et al*, 2009). As the plasma samples from all the premature MI subjects were collected > 3 months post event, a large proportion of the individuals would be on statins, therefore this may mask the 'true' differences between thrombotic potential of

the two genotypes. In addition, the study population was fairly small for a genotyping study, and a more comprehensive study should be carried out.

Another possible limitation of this study is that all plasma collected was 'resting' plasma, *ie* collected and centrifuged immediately without incubation with agonist, or activation of cells that could generate TF. During development of the thrombin generation method, variation of phospholipid concentration affected measurement of ETP, therefore the surface membrane of platelets and presence of possible cellular microparticles would affect phospholipid availability, and in turn affect ETP. Platelet activation results in release of soluble mediators from intracellular granules, and affects platelet-platelet and platelet-leukocyte aggregation. In turn this leads to increased availability of negatively-charged membrane surface for coagulation to take place, and an altered vascular environment that also influences changes in cellular gene expression. Therefore the magnitude of the thrombotic response may be altered by the interaction of various cells, which in turn, due to changes in gene expression following activation, may affect the haemostatic balance. The study described in this and the previous chapter examine the thrombotic potential of plasma, but does not consider the effects of cellular activation and interaction. These themes are to be studied further in the next chapter.

In summary:

- There was no association of TF SNP with plasma TF or ETP.

Limitations:

- The population was very small for a genotyping study, and therefore unlikely to result in significant findings.

Future work:

- More comprehensive genotyping study, incorporating all known polymorphisms of coagulation genes, carried out in a much larger cohort, or multiple cohorts to establish the genetic role in producing a hypercoagulable phenotype.

CHAPTER 5: Monocyte expression of Tissue Factor and Tissue Factor Pathway Inhibitor

5.1 Introduction

The results of studies described in the previous two chapters suggest that the level of TF activity in plasma is one of the primary factors regulating the prothrombotic environment in patients with premature MI, and that plasma levels of TFPI are conversely lower. Monocytes are one of the key sources of TF, but the literature is inconclusive as to whether they produce TFPI (van der Logt *et al*, 1994; Iochmann *et al*, 1999). Monocytes produce TF in response to inflammatory stimuli, such as LPS, but have also been reported to express TF following interaction with activated platelets (Celi *et al*, 1994). The concept of how cellular activation and interaction might affect the thrombotic response is an important one. Following platelet activation and degranulation, P-selectin is translocated from the α-granules to the platelet cell surface (Stenberg *et al*, 1985). PSGL-1 is the main ligand for P-selectin and is expressed on most white blood cells, including monocytes (Moore *et al*, 1992; Sako *et al*, 1993), therefore facilitating formation of platelet-monocyte complexes. Although neutrophils are able to form cellular aggregates with platelets, and rapidly infiltrate the thrombus following formation of the platelet plug, it is monocytes that preferentially form heterotypic aggregates with platelets (Rinder *et al*, 1991). There are reported to be only small numbers of platelet-monocyte aggregates in the circulation of healthy individuals, however, formation of elevated numbers of platelet-monocyte complexes have been reported in patients with unstable angina and acute MI (Furman *et al*, 2001; Sarma *et al*, 2002).

Using platelet-monocyte interaction as a focus, a whole blood model was developed to allow investigation of the effect of platelet activation on monocytes, and examine how the balance of TF and TFPI might be affected. Therefore, the first part of this study examined changes in expression of TF and TFPI genes in monocytes, using monocyte response to LPS (an inflammatory stimulus), as a positive control, and comparing this to changes occurring in the monocyte due to platelet activation, a haemostatic stimulus.

5.2. Determination of optimal CRP-XL concentration for platelet activation

The interaction of platelets with collagen is one of the key factors in coagulation and the generation of thrombin, and collagen signalling is mediated through integrin α2β1, and the immunoglobulin platelet glycoprotein VI (GPVI) receptor complex. However it is GPVI that is the main collagen receptor involved in this process (Nieswandt *et al*, 2001). In this study, platelets were activated with a GPVI-specific peptide, Collagen-related peptide-XL (CRP-XL). The cross-linked peptide has the sequence Gly-Cys-Hyp-(Gly-Pro-Hyp)$_{10}$-Gly-Cys-Hyp-Gly-NH_2, and has been demonstrated to activate integrins αIIb$_\beta$3 leading to platelet aggregation, and secretion of platelet intracellular granules (Knight *et al*, 1999).

The optimal concentration of CRP-XL needed to be high enough to cause platelet degranulation, but not so high as to generate large numbers of PMPs. Platelet degranulation can be determined by measurement of surface expression of P-selectin, which is released from the α-granules upon platelet activation and is translocated to the platelet surface. Microparticle generation can be determined by measurement of Annexin-V binding. Activation of the platelet results in flipping of the platelet outer membrane, thus exposing a high concentration of negatively-charged phospholipids, *eg*,

PS. Parts of the membrane bud off and form microvesicles which are highly PS-positive. Annexin-V binds to PS, thus giving indication of the concentration of platelet microparticles in the sample. These changes were measured by flow cytometry, to determine the optimal CRP-XL concentration to use in this study.

The platelet population was identified by forward and side-scatter, and platelet degranulation, measured by expression of P-selectin, was detected at concentrations above 50ng/mL CRP-XL. Expression increased to 74.4% with 500ng/mL CRP-XL, reaching maximum expression at concentrations > 5µg/mL CRP-XL. Platelets staining positive for Annexin-V remained at low levels (<10%) until platelets were activated with concentrations of CRP-XL above 1000ng/mL (Figure 5.1). Therefore, 500ng/mL CRP-XL was selected as optimal concentration for platelet activation in this study. As a positive control, monocytes were additionally activated with a moderate concentration of LPS [200ng/ml](determined in previous studies), that was sufficient to induce an inflammatory response, but would not induce rapid cellular apoptosis.

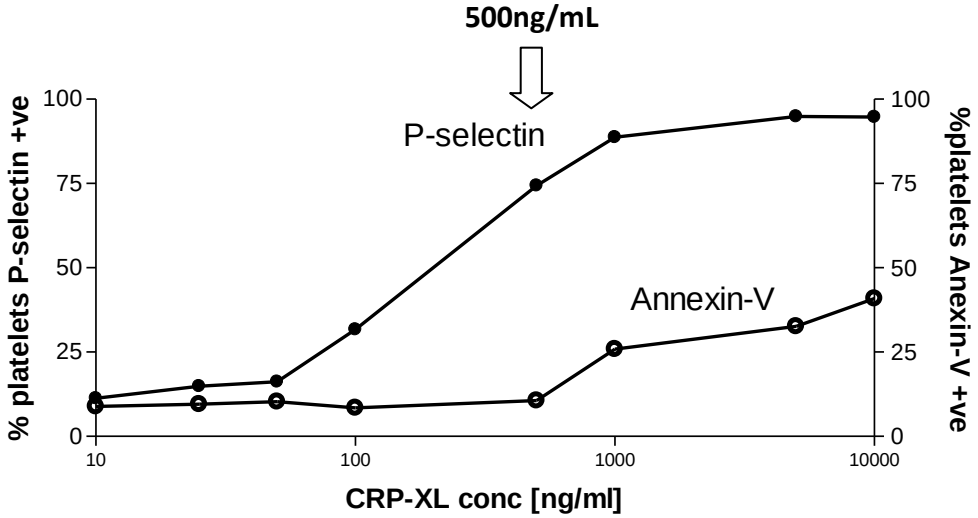


Figure 5.1: Effect of CRP-XL concentration on platelet surface expression of P-selectin and binding of Annexin-V. Platelets were incubated with increasing concentrations of CRP-XL and measured by flow cytometry for P-selectin expression, a marker of platelet degranulation, and for binding of Annexin-V, a marker of phosphatidlyserine (PS) exposure (n = 1).

5.3. Monocyte gene expression of TF and TFPI following activation with haemostatic and inflammatory stimuli

Citrated whole blood was incubated for up to 6 hours at 37^{o}C in the presence of 500ng/mL CRP-XL, 200ng/mL LPS, or without agonist. Monocyte gene expression of TF was rapidly induced by 1.5 hours. In the absence of stimulus, monocyte expression of TF mRNA was measured at significantly higher levels than that of resting monocytes (mean relative expression at 1.5 hours 0.0704; p=0.05). Monocytes that had been incubated in the presence of CRP-XL demonstrated a greater induction of TF mRNA expression (mean at 1.5 hours 0.3025; p=<0.001). LPS had a much greater effect on monocyte gene expression of TF (mean at 1.5 hours 2.487; p=<0.001) (Figure 5.2).

The pattern of changes in monocyte expression of TFPI mRNA was markedly different from that of TF, with activation of the platelet with CRP-XL having the greater effect. Gene expression of TFPI was induced by 4 hours in monocytes from all donors, and at 6 hours either showed increased levels of induction (4 donors) or was beginning to show down-regulated (mean relative expression 0.1810 (p=<0.001); and 1.167 (p= <0.05), at 4 and 6 hours, respectively) (Figure 5.3).

(a) Tissue Factor

TIME	SAMPLE TYPE	MEAN Ct 18S	± SD	MEAN Ct TF	± SD	MEAN ΔCt	± SD	p-value
0 HOUR	**CONTROL**	23.255	1.727	30.3	0.749	7.045	2.152	-
1.5 HOUR	**CONTROL**	24.76	1.524	23.456	0.996	4.369	1.286	0.05
	CRP-XL	24.293	1.187	26.202	0.864	1.909	0.838	0.0005
	LPS	23.074	1.293	22.3	1.22	-0.775	1.324	0.0002
4 HOUR	**CONTROL**	25.831	2.178	30.259	1.007	4.428	2.016	0.0207
	CRP-XL	24.707	2.588	29.23	0.94	4.523	2.724	0.034
	LPS	26.048	4.304	26.496	4.558	0.448	2.59	0.0026
6 HOUR	**CONTROL**	25.111	1.722	29.808	0.85	4.697	1.389	0.0438
	CRP-XL	25.273	2.311	29.625	2.43	4.351	2.535	0.0765
	LPS	25.912	2.916	28.211	3.178	2.299	3.071	0.03

(b) Tissue Factor Pathway Inhibitor

TIME	SAMPLE TYPE	MEAN Ct 18S	± SD	MEAN Ct TF	± SD	MEAN ΔCt	± SD	*p*-value
0 HOUR	**CONTROL**	23.255	1.727	35.138	0.433	11.833	1.712	-
1.5 HOUR	**CONTROL**	24.76	1.524	35.815	1.081	11.06	2.227	0.2232
	CRP-XL	24.293	1.187	31.16	1.642	6.867	2.44	0.0009
	LPS	23.074	1.293	34.559	1.924	11.48	2.234	0.5976
4 HOUR	**CONTROL**	25.831	1.261	33.554	2.262	7.723	3.053	0.1099
	CRP-XL	24.707	2.588	27.835	2.687	3.128	3.247	0.0004
	LPS	26.048	4.304	31.938	2.981	5.89	3.247	0.0026
6 HOUR	**CONTROL**	25.111	1.722	33.566	2.624	8.454	1.749	0.0223
	CRP-XL	25.273	2.311	28.845	5.76	3.572	6.522	0.0226
	LPS	25.912	2.916	32.118	2.604	6.206	3.5	0.0208

Table 5.1. Mean Ct, and mean ΔCt ±SD for monocyte gene expression of (a) TF, and (b) TFPI, following stimulation with CRP-XL-activated platelets [500ng/mL] or LPS [200ng/mL] for up to 6 hours in whole blood. (*p*-value calculated from ΔCt of treated samples compared to ΔCt of 0 hour sample; $n = 6$).

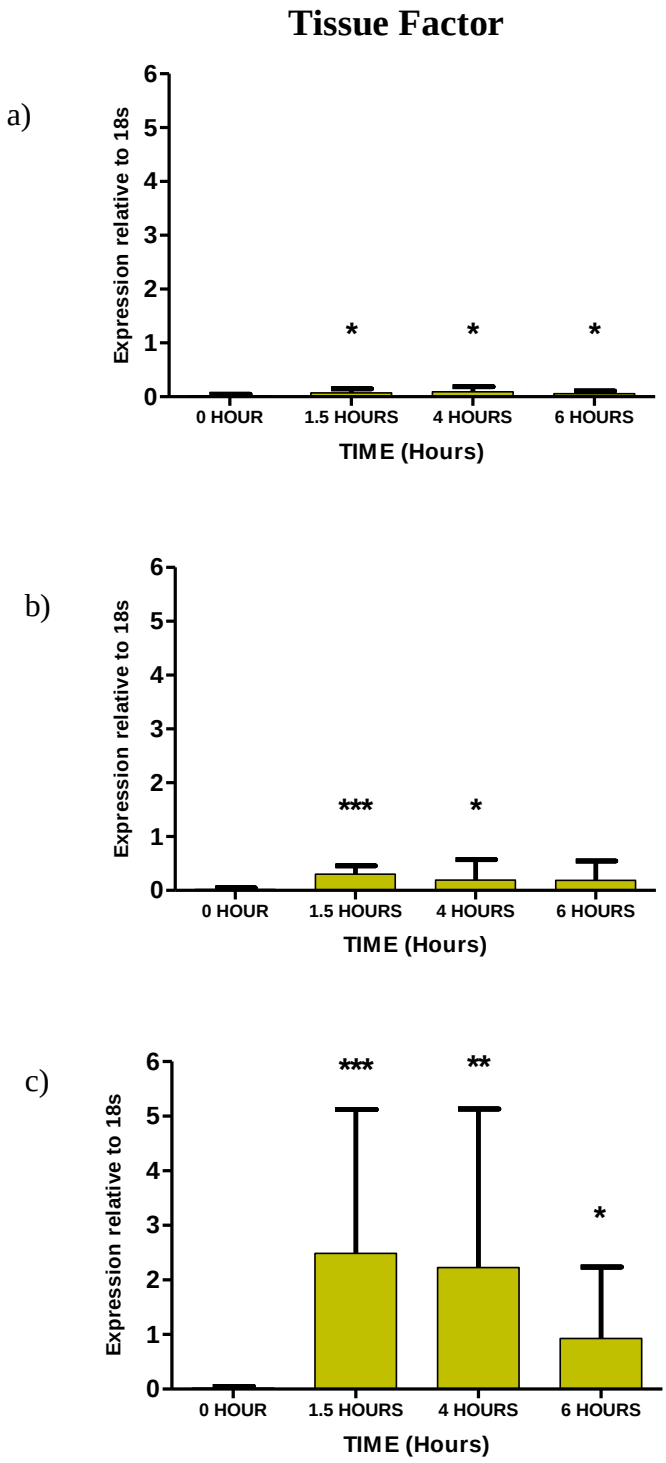


Figure 5.2. Gene expression of monocyte Tissue Factor (TF) following incubation in whole blood with collagen-related peptide-XL (CRP-XL) or lipopolysaccharide (LPS). Citrated whole blood was incubated at 37°C for up to 6 hours (a) without agonist, (b) with CRP-XL [500ng/mL] or (c) LPS [200ng/mL]. mRNA levels were normalised to 18s rRNA and expressed as mean ± SD relative to resting monocytes (n = 6); *P<0.05, **P<0.005, ***P<0.001.

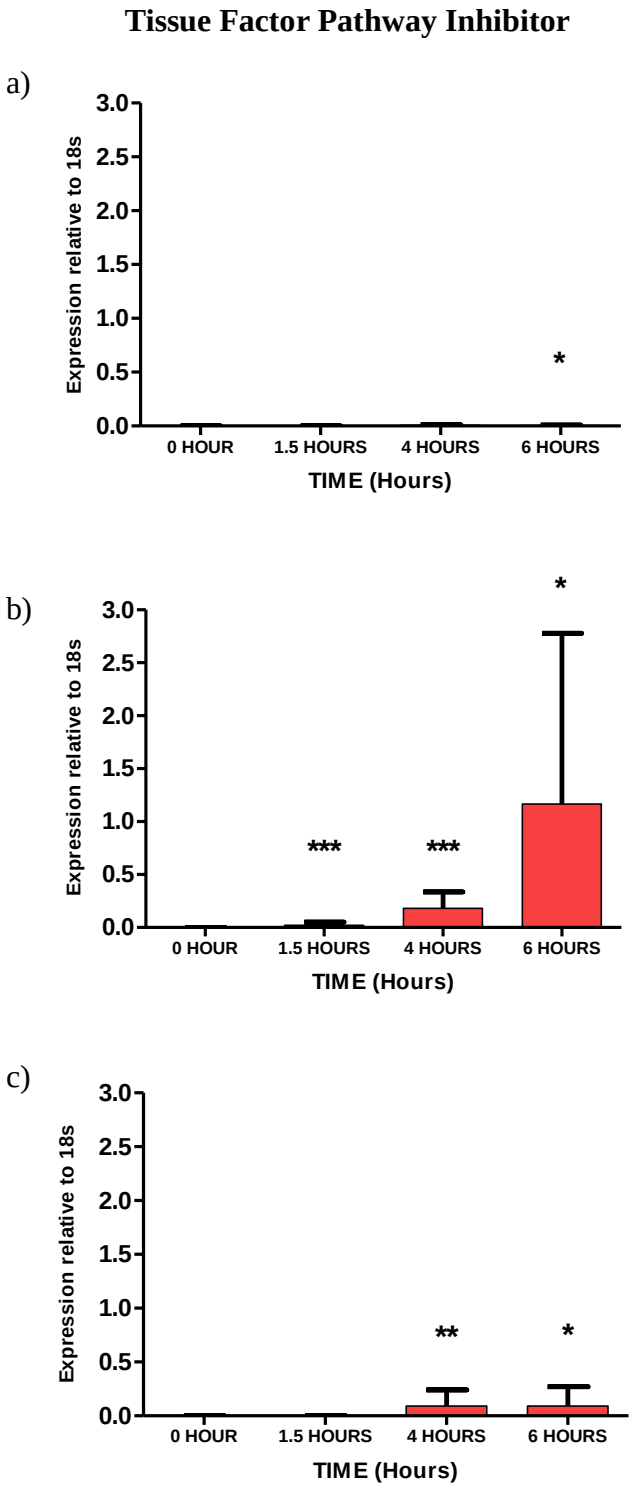


Figure 5.3. Gene expression of monocyte Tissue Factor Pathway Inhibitor (TFPI) following incubation in whole blood with collagen-related peptide-XL (CRP-XL) or lipopolysaccharide (LPS). Citrated whole blood was incubated at 37°C for up to 6 hours (a) without agonist, (b) with CRP-XL [500ng/mL] or (c) LPS [200ng/mL]. mRNA levels were normalised to 18s rRNA and expressed as mean ± SD relative to resting monocytes (n = 6); *P<0.05, **P<0.005, ***P<0.001.

5.4. Translation of monocyte TF and TFPI mRNA into protein

It would be expected that gene induction of monocyte TF and TFPI should lead to translation of the mRNA into protein. Alternative splicing of mRNA allows translation of soluble protein that is released into the circulation, as well as a protein that is integral to the cellular outer membrane. Therefore, experiments were carried out to investigate monocyte gene induction of alternatively spliced TF transcripts in response to platelet activation and LPS. To investigate this further, and to assess whether these proteins might have functional activity, experiments were carried out to measure activity and antigen levels in plasma, and possible changes in procoagulant activity on the monocyte surface.

5.4.1. Monocyte gene expression of alternatively spliced, soluble TF

There have been several splice variants of TF mRNA reported (Bogdanov *et al*, 2003; Chand *et al*, 2006). Monocyte mRNA transcripts for membrane-bound TF contain 6 exons and are generally referred to as full-length TF transcripts. There also exist several shorter transcripts; the most common is a transcript which is missing exon 5. This transcript therefore lacks the sequence for the transmembrane or cytosolic regions of the protein, and therefore appears to code for a soluble form of TF (Bogdanov *et al*, 2003). Using a primer set that traverses exon 5 allows detection of all mRNA transcripts of TF, and when the PCR products are applied to an agarose gel, will allow quantification by densitometry of both full-length TF (flTF), and the shorter transcript (asTF). Therefore, this was explored in monocytes incubated in whole blood as previously at 37^{o}C for 4 hours in the presence of either CRP-XL [500ng/mL] or LPS [200ng/mL].

Using this technique produced a similar pattern to that seen in previous monocyte TF gene expression experiments, with LPS inducing the greatest effect. Densitometry analysis of expression of flTF showed that following 4 hours WB stimulation with CRP-XL, monocytes had a mean expression of 48713± 20052 (p=0.2510), compared to 70920± 38001 (p=0.0902) for LPS-stimulated monocytes. This pattern was repeated in expression of asTF mRNA, with monocytes from WB-stimulated with CRP-XL having a mean expression of 28918± 15882 (p=0.1489) vs 34264± 19265 (p=0.1307) for LPS-stimulated monocytes. The number of soluble transcripts was calculated as a percentage of the total TF mRNA transcripts generated for each sample. The mean percentage of soluble transcript in resting monocytes was 28.8± 5.8% compared with 35.8± 10.3% and 30.6± 6.6% for CRP-XL-stimulated and LPS-stimulated samples, respectively (Figure 5.4). Therefore, although LPS stimulation of monocytes resulted in the greatest induction of TF gene expression, CRP-XL-activated platelets resulted in proportionally higher levels of monocyte asTF.

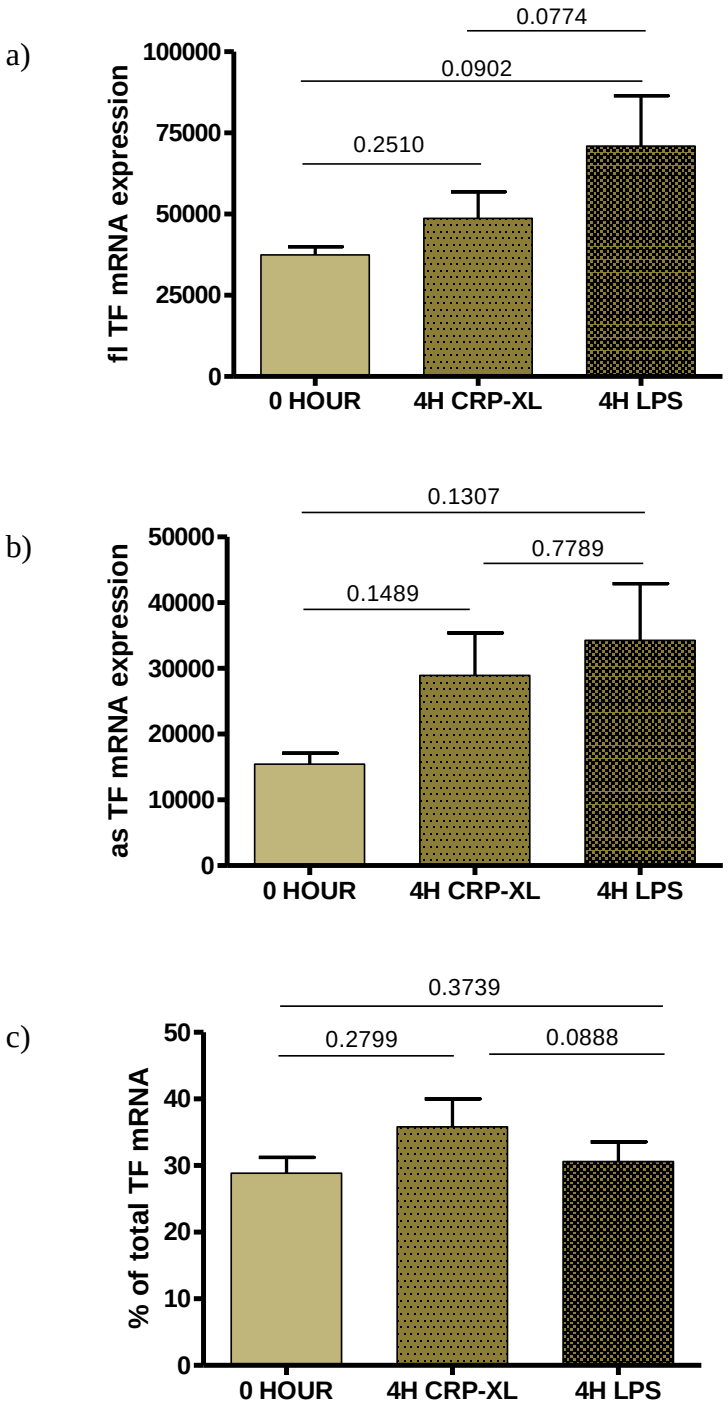


Figure 5.4: Monocyte gene expression of alternatively spliced TF following incubation in whole blood with CRP-XL [500ng/mL] or LPS [200ng/mL]. Graphs show densitometry results of PCR experiments for (a) expression of full-length TF mRNA transcripts; (b) expression of alternatively spliced TF mRNA transcripts; (c) the level of alternatively spliced TF mRNA induced, shown as a percentage of the total amount of TF mRNA transcripts induced in each condition. (Mean ± SEM; n = 6).

5.4.2. TF activity and antigen levels in plasma

Continuing with the model of platelet activation via a collagen peptide, (a haemostatic stimulus) and comparing this with the effect of LPS (an inflammatory stimulus), it was investigated how the different stimuli affected levels of soluble TF in the plasma of 6 normal healthy donors. Activity levels (in terms of FXa conversion), and antigen levels of TF, were measured in plasma obtained from whole blood that had been incubated for up to 6 hours with either CRP-XL [500ng/mL] or LPS [200ng/mL].

Surprisingly, there was no increase of TF activity in plasma from the blood that had been incubated with LPS, however, there was a dramatic increase in TF activity in the plasma isolated from blood incubated with collagen peptide (mean activity at 1.5 hours 23.23± 12.07 pM; p=0.0054), falling slightly but remaining elevated at 6 hours (mean 14.40± 13.24 pM; p=0.0492). This finding was supported by detection of increased levels of TF antigen in CRP-XL-stimulated samples, which increased at 1.5 hours (21.05± 29.34, p=0.3031) slightly further by 4 hours (28.9± 30.63, p=0.1557), and increasing rapidly by 6 hours (57.5± 18.95, p=0.0090) (Figure 5.5).

5.4.3. TFPI activity and antigen levels in plasma

Further assays were carried out to assess whether the TFPI protein may have anticoagulant function. TFPI activity remained at a fairly constant level with only a slight increase over time, and there was no difference in expression in response to the different agonists. However, this was not true for TFPI antigen. Although antigen levels in samples from unstimulated, and LPS samples remained virtually unchanged, TFPI antigen increased significantly in the CRP-stimulated samples by 1.5 hours (mean 73.46± 10.25 ng/mL; p= 0.0174), remaining elevated through to 6 hours (79.01± 15.87 ng/mL; p=0.0513); (Figure 5.6). Therefore, following monocyte gene upregulation of

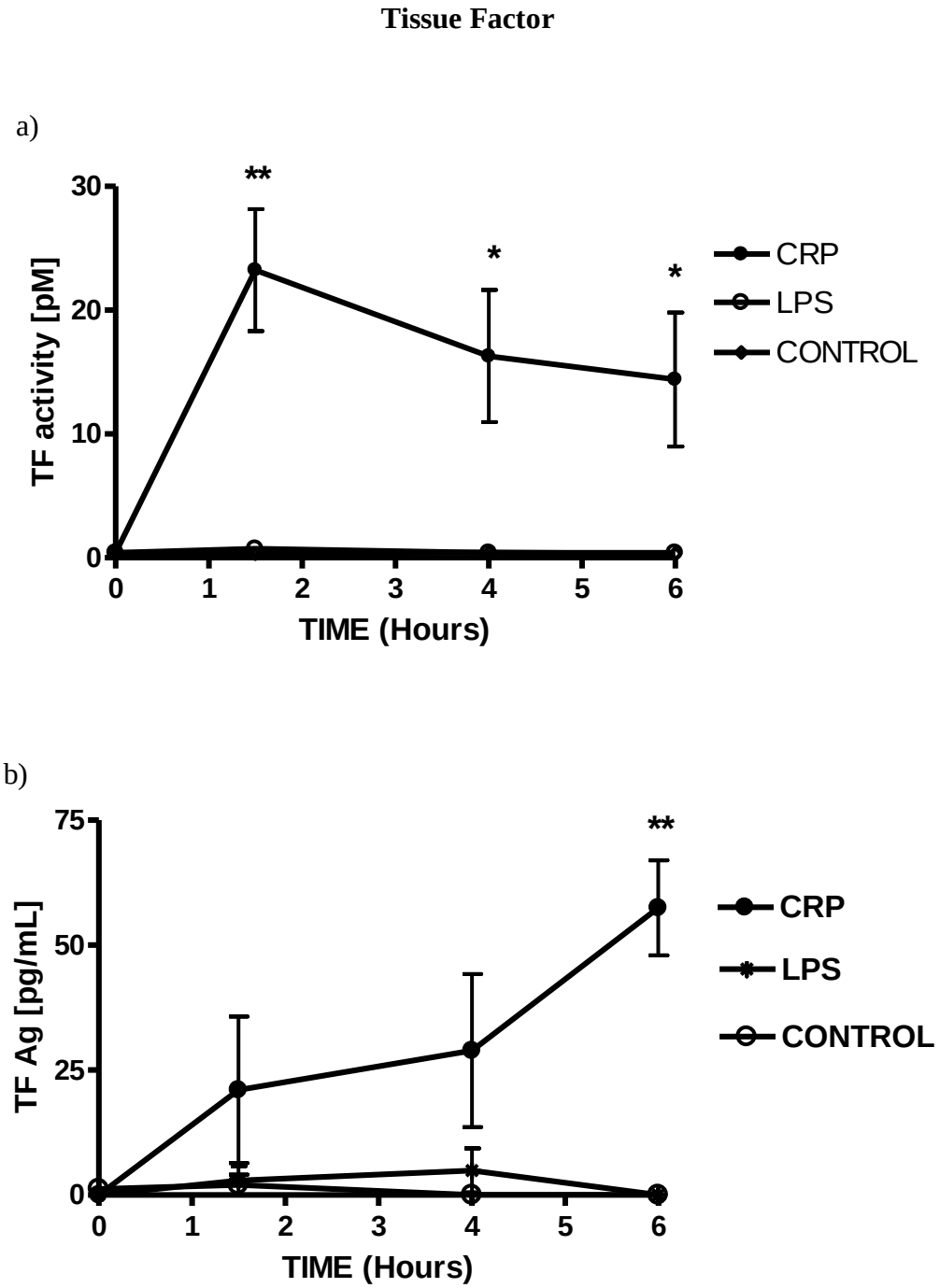


Figure 5.5: TF activity and antigen in plasma following whole blood incubation with collagen-related peptide-XL (CRP-XL), or lipopolysaccharide (LPS). Citrated whole blood was incubated at 37^{o}C for up to 6 hours either without treatment, with 500ng/mL CRP-XL, or with 200ng/mL LPS. (a) plasma TF activity; (b) plasma TF antigen. (Figures are Mean ± SEM; *=<0.05, **=<0.01; *n* = 6).

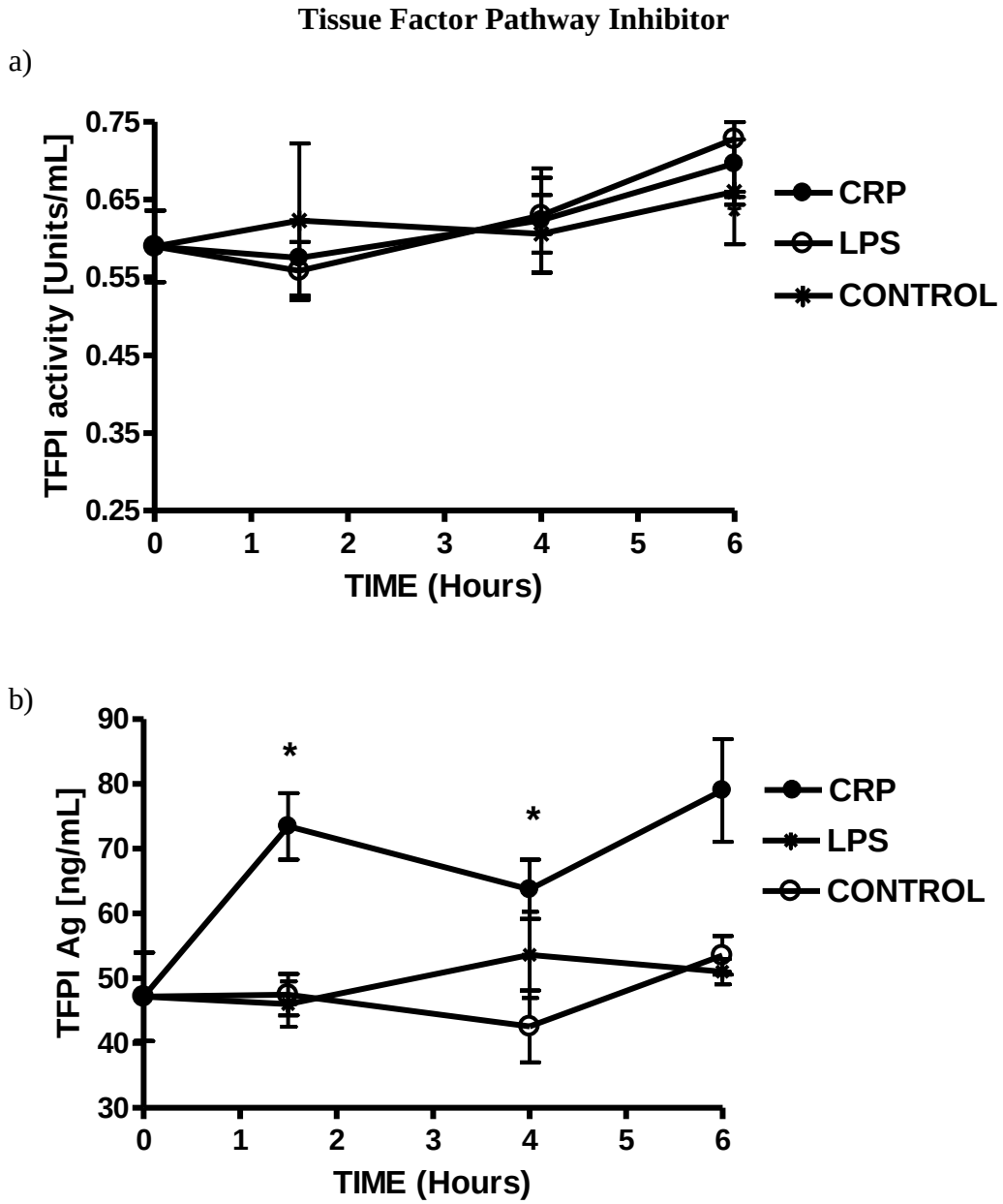


Figure 5.6: TFPI activity and antigen in plasma following whole blood incubation with collagen-related peptide-XL (CRP-XL), or lipopolysaccharide (LPS). Citrated whole blood was incubated at 37^{o}C for up to 6 hours either without treatment, with 500ng/mL CRP-XL, or with 200ng/mL LPS. (a) plasma TFPI activity; (b) plasma TFPI antigen. (Figures are Mean ± SEM; * = < 0.05; *n* = 6).

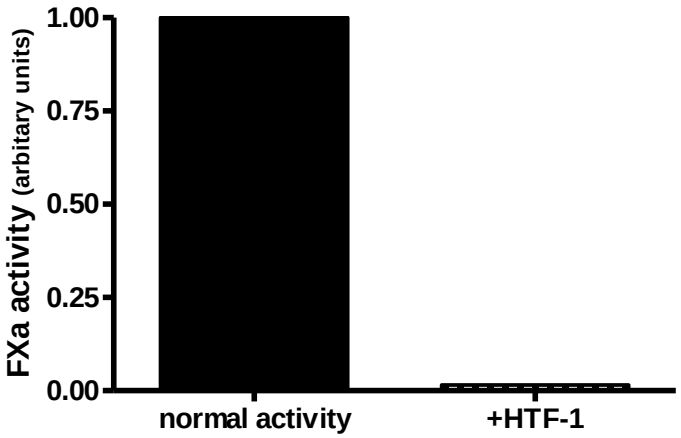
TFPI in response to platelet activation there is an increase in circulating TFPI antigen; however this study found that it does not appear to have anticoagulant activity.

5.4.4. Effect of platelet activation on monocyte procoagulant surface activity

Changes in TF and TFPI expression on the monocyte surface were also investigated. Therefore, measurement of monocyte surface procoagulant activity was made following incubation of isolated monocytes with CRP-XL-activated platelets or LPS, using an assay which measures FXa activity. In the presence of increased levels of active TF on the monocyte surface, procoagulant activity would be expected to be high and FXa activity would be significant. In contrast, if there are increased levels of TFPI on the monocyte surface, procoagulant activity would be expected to be lower, and therefore FXa activation would be inhibited. Procoagulant activity of pooled plasma was designated as 1 arbitrary unit of activity.

To confirm that the assay reflected the level of TF activity in the sample, pooled plasma was incubated in the presence/absence of a TF-blocking antibody (HTF-1). Procoagulant activity in the plasma was almost completely abolished in the presence of HTF-1, reducing 1 unit of activity in pooled plasma to 0.015 units in the presence of anti-TF antibody. As expected, monocytes incubated in PBS buffer demonstrated only residual procoagulant activity and this did not increase with time. Surprisingly, monocytes incubated with LPS showed a similar residual level of surface activity as seen in the control sample. This was contrary to the pattern of expression of TF mRNA following monocyte exposure to LPS. In contrast, following 4 hours incubation, surface procoagulant activity was significantly increased in monocytes that had been incubated in the presence of activated platelets (mean 0.44 units ±0.20; p= 0.0506), remaining elevated at 6 hours (mean 0.39 units ±0.14; p=0.1164); (Figure 5.7).

a)



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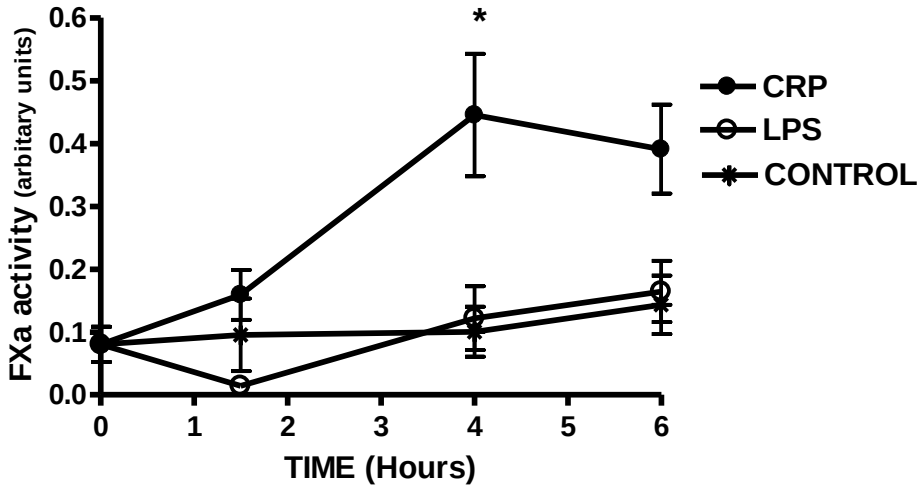


Figure 5.7: Measurement of monocyte surface procoagulant activity.

(a) Sensitivity of the assay to Tissue Factor activity: FXa activity was measured in pooled plasma and designated an arbitrary value of 1 unit. In the presence of anti-TF HTF-1, plasma activity was reduced to 0.015 units (n = 1). (b) Monocyte procoagulant surface: Isolated monocytes were incubated for up to 6 hours at 37^{o}C in the presence of TBS buffer, CRP-XL - activated platelets [500ng/mL], or LPS [200ng/mL]. Data shows amount of FXa conversion supported by the monocyte surface, which is proportional to the amount of TF activity. (Figures are Mean ± SEM; n = 4).

5.4.5. Effect of monocyte procoagulant surface on clotting profile

To further explore how differences in monocyte procoagulant surface activity might affect clot formation. Monocytes were incubated for 4 hours at 37^{o}C, in whole blood in the presence of CRP-XL [500ng/mL] or LPS [200ng/mL]. Monocytes were then washed in PBS, and placed in pooled platelet-poor plasma in a Rotational Thromboelastrometry analyser (ROTEM), and clot formation profiles were monitored. The clotting time for the monocyte samples at baseline or 4 hour unstimulated control was 475 seconds, and 454 seconds, respectively. This was considerably shorter in the monocyte samples incubated with CRP-XL or LPS, with the plasma containing the LPS-stimulated monocytes having the shortest clotting time (277 seconds (s) (LPS) vs 354s (CRP-XL). Clot formation time was longer in the CRP-XL-stimulated monocytes, 146, 211s, 150s, for control, CRP-stimulated, and LPS-stimulated samples respectively, and maximum clot elasticity (MCE) was lower in the clot-stimulated sample, 137, 57, and 121, respectively (Figure 5.8). These results were reflected in the shape of the clot profiles generated from the different samples. Whereas the clots in the unstimulated sample and the LPS-stimulated sample had begun fibrinolysis by 30 minutes, the CRP-stimulated sample had not yet begun this process in the 30 minute time-frame of the experiment (Figure 5.9). To illustrate how the concentration of TF may affect the clotting profile in this method, pooled platelet-poor plasma was incubated with increasing concentrations of rTF. Clotting time decreased in a dose-dependent manner, from 475 seconds (0.125U rTF) to 150 seconds (0.5 U rTF). Maximum clot elasticity also decreased as TF concentration decreased (Figure 5.10).

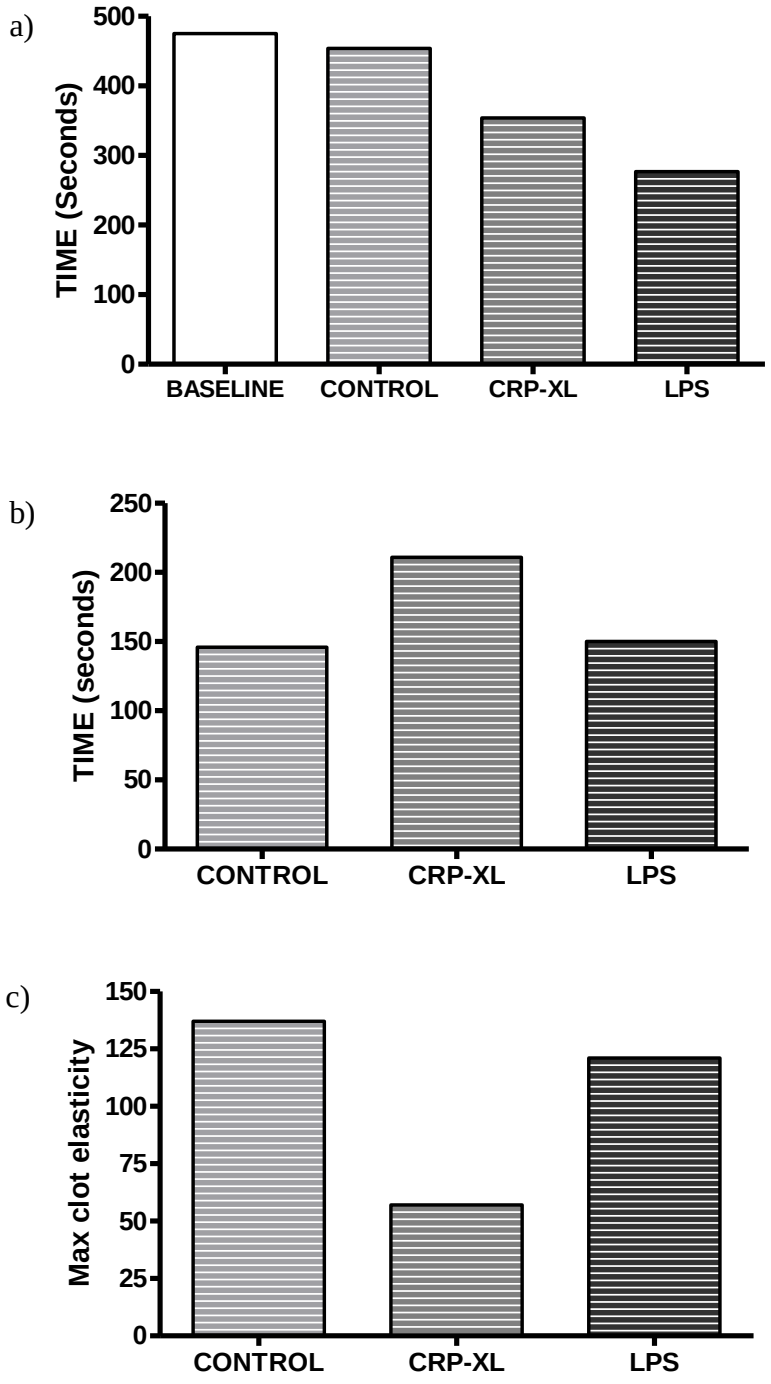


Figure 5.8: Effect of changes in monocyte procoagulant surface on clotting profile. Monocytes were exposed to platelets activated with 500ng/mL CRP-XL or 200ng/mL LPS, and incubated at 37°C for 4 hours. Monocytes were then washed in PBS and added to pooled plasma. Clotting profiles were measured and recorded using Rotational Thromboelastometry (ROTEM) for (a) clotting time; (b) clot formation time; and (c) clot elasticity (n = 1).

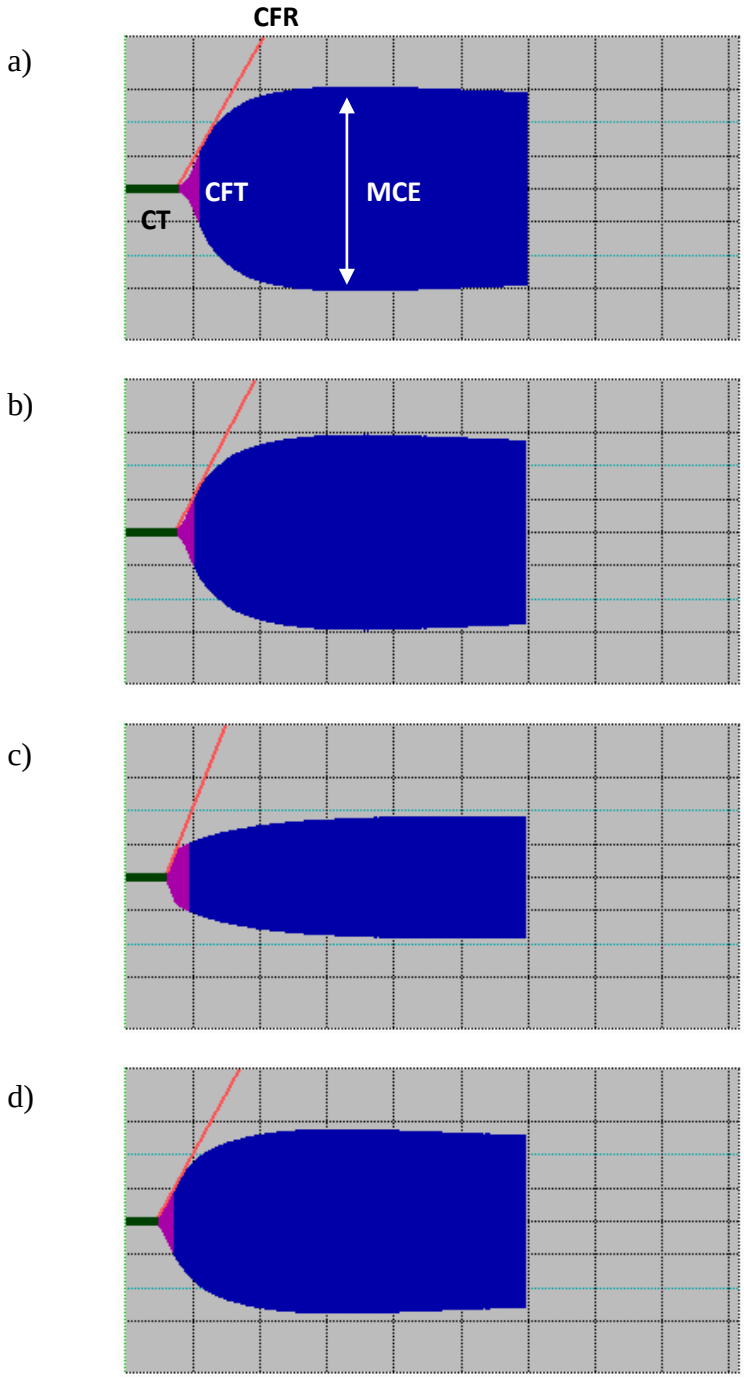


Figure 5.9: Variation in monocyte surface procoagulant activity and clotting profile. Monocytes were exposed to platelets activated with 500ng/mL CRP-XL or 200ng/mL LPS, and incubated at 37°C for 4 hours. Monocytes were then washed in PBS and added to pooled plasma. Clotting profiles were measured and recorded using Rotational Thromboelastometry (ROTEM): (a) resting monocytes (0 hour control); (b) unstimulated monocytes (4 hour control) (c) monocytes exposed to CRP-XL-activated platelets; and (d) monocytes exposed to LPS (n = 1). Abbreviation: CT = Clotting Time; CFT = Clot Formation Time; CFR = Clot Formation Rate; MCE = Maximal Clot Elasticity.

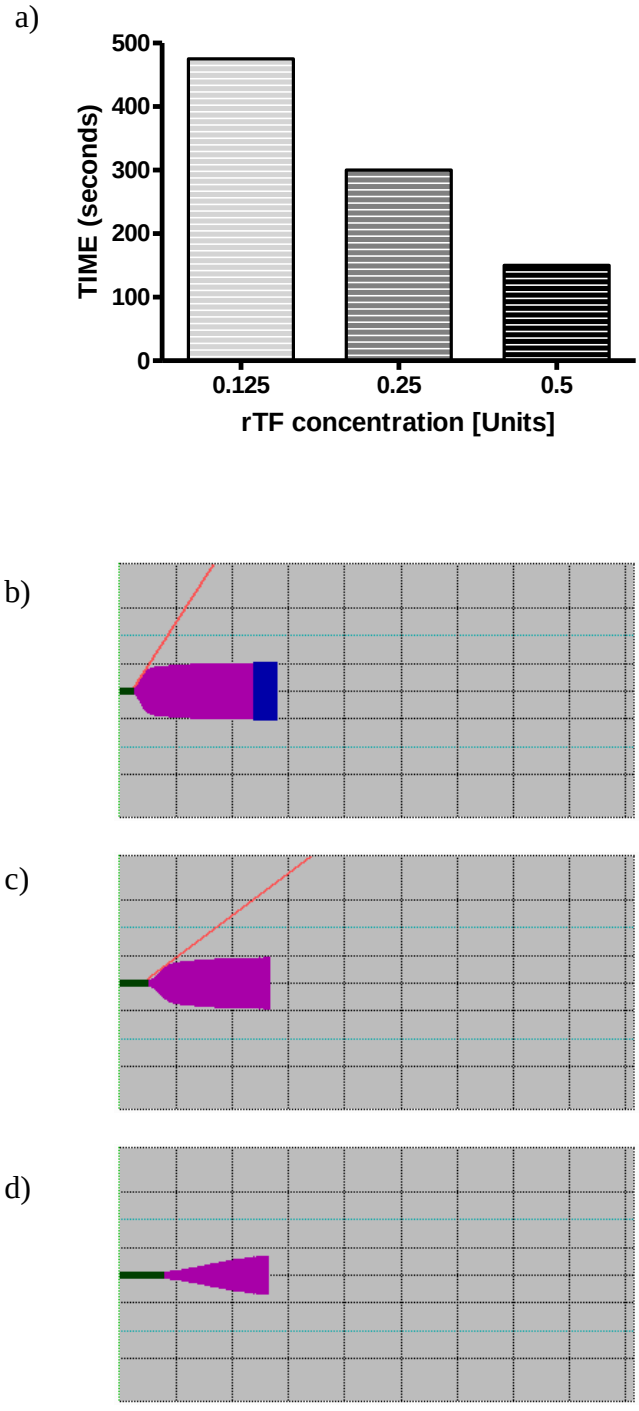


Figure 5.10: Effect of recombinant Tissue Factor (rTF) concentration on clotting profile. Increasing concentrations of rTF were added to pooled plasma and clotting profiles were measured and recorded using Rotational Thromboelastometry (ROTEM). (a) dose-dependent decrease in clotting time with increased rTF concentration; Clotting profile of pooled plasma with decreasing concentration of rTF (b) 0.5 units; (c) 0.25 units; and (d) 0.125 units rTF (n = 1).

5.5. Monocyte expression of TF and TFPI in MI cohort

In the previous chapter, plasma from patients who had suffered a premature MI had elevated levels of endogenous thrombotic potential, which may be due to increased levels of TF activity and to decreased levels of TFPI activity when compared with plasma from normal, healthy controls. This may be due to changes in monocyte gene expression of TF and TFPI, therefore possible differential gene expression of monocyte TF and TFPI was investigated in premature MI subjects and healthy controls.

5.5.1. Study Design

This study of premature MI patients and off-spring with strong family history of MI, was carried out on a second cohort of premature MI subjects, recruited through the BLOODOMICS project. The BLOODOMICS project formed part of the European Union 6th Framework Programme for Research and Development (FP6), and was set up to discover genetic markers for the prediction of thrombus formation in coronary artery disease. Premature MI subjects were recruited from the Myocardial Infarction National Audit Project (MINAP), selecting individuals who had suffered an MI before the age of 65 years. Patients admitted to Glenfield hospital with a premature MI were approached for recruitment of male off-spring. All off-spring were aged between 18 and 40 years. Both cohorts were matched with healthy controls for age, gender and smoking status, recruited by advertisement within the hospital premises. None of the controls were on regular medication, and additionally, control-offspring did not have a family history of premature coronary artery disease. All subjects were north European Caucasian from the same geographic area. All samples were collected into BD Vacutainer® glass tubes. To minimise platelet activation, the initial 2mL of blood was not used for monocyte isolation. Monocytes were isolated immediately, and also following a 4 hour

incubation at 37^{o}C in the presence of 500ng/mL CRP-XL to activate the platelets. Monocytes were isolated with Dynal CD14 magnetic beads, and total RNA extracted as described in section 2.3.3.1. and 2.4.1.2., respectively. All patient recruitment, sample collection, and RNA isolation was carried out by Dr Unni Krishnan, and was funded by the European Union 6th Framework Programme for Research and Development (FP6) (LSHM-CT-2004-503485).

mRNA levels of TF and TFPI isoforms were measured by TaqMan and SYBR green real-time PCR assays, respectively, in resting monocytes and monocytes that had been incubated with CRP-XL-activated platelets. Monocyte mRNA expression of TF was measured in monocytes isolated from 19 premature MI subjects and 19 healthy controls. A sub-group of 11 samples from each population was also measured for TFPI-α and TFPI-β. To further study whether there is a possible inherited element to monocyte gene expression of TF and TFPI mRNA was also analysed in monocytes obtained from 20 healthy offspring with differential family history of MI.

5.5.2. Expression of TF and TFPI mRNA in monocytes from premature MI cohort and controls

Monocytes were isolated from citrated whole blood, either immediately after blood collection, or following 4 hour incubation at 37^{o}C with CRP-XL [500ng/mL] to activate the platelets. TF mRNA levels were significantly higher in resting, unstimulated monocytes isolated from blood samples collected from the MI cohort (mean relative expression = 0.007±0.003 vs 0.011±0.006; p=<0.05). The same pattern was seen in activated monocytes, with TF mRNA levels being significantly higher in the MI cohort (mean = 0.025±0.017 vs 0.050±0.036; p=0.001), (Figure 5.11).

Gene expression of Tissue Factor in resting and activated monocytes isolated from premature MI cohort and healthy control group:

a) **Healthy Controls**

	Resting monocytes			Activated monocytes				
	Ct target gene	CtRef gene	ΔCt	Ct Target gene	Ct Ref gene	ΔCt	ΔΔ Ct	*p*-value
Mean	33.67	26.42	7.25	32.71	27.14	5.57	1.68	<0.0001
S Dev	0.67	0.59	0.61	1.39	1.19	0.90	0.82	

b) **Premature MI**

	Resting monocytes			Activated monocytes				
	Ct Target gene	Ct Ref gene	ΔCT	Ct Target gene	Ct Ref gene	ΔCt	ΔΔ Ct	*p*-value
Mean	31.88	25.21	6.57	30.95	26.39	4.56	2.01	<0.0001
S Dev	0.87	0.60	0.57	1.22	0.88	0.79	0.83	

c)

	Controls	**MI**		
	ΔCt	ΔCt	ΔΔ Ct	*p*-value
resting monocytes	6.57	7.25	-0.68	0.0130
activated monocytes	5.57	4.56	1.01	0.0010

Table 5.2. Mean Ct, ΔCt, and ΔΔCt ±SD for monocyte gene expression in (a) healthy control group, and (b) premature MI cohort. (c) Mean ΔCt and ΔΔCt for TF gene expression in resting and activated monocytes in controls v MI cohort. Monocytes were isolated from citrated whole blood either immediately following blood collection without stimulation (resting), or following 4 hour incubation at 37^{o}C in the presence of CRP-XL [500ng/mL]; *n* = 19 cases, 19 controls. (Ct means Threshold cycle).

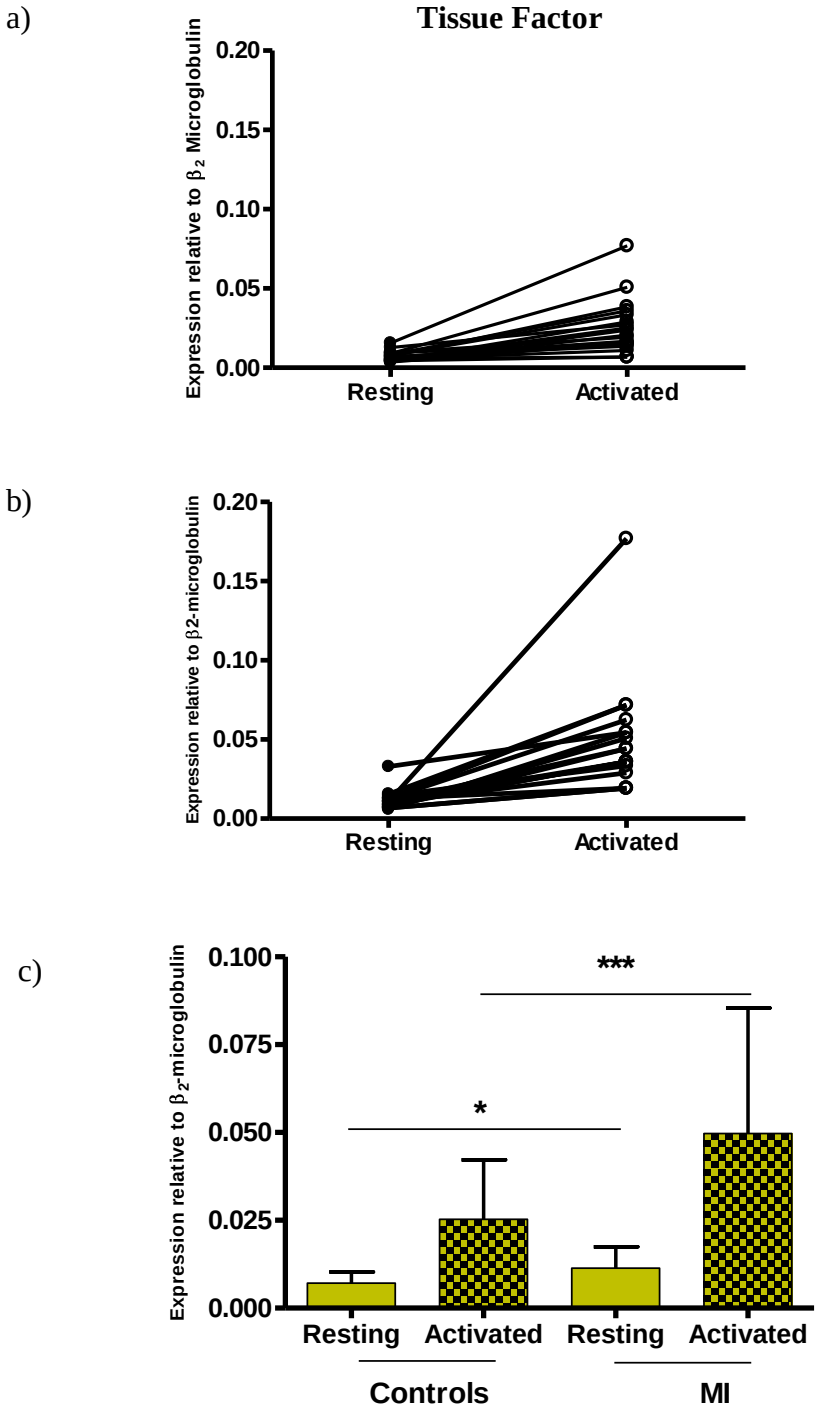


Figure 5.11. Gene expression of Tissue Factor (TF) in monocytes isolated from premature MI cohort and control group. Change in relative gene expression of TF in monocytes pre- and post incubation with CRP-XL-activated platelets, in (a) healthy control group, and (b) premature MI cohort. (c) Expression of monocyte TF gene in resting and activated monocytes from healthy controls and premature MI cohorts. Expression was normalised to β_2-Microglobulin and is expressed as mean ± SD; (n = 19 Cases; 19 Controls), *P<0.05, ***P<0.001.

Samples from the same cohort were analysed for the two major isoforms of TFPI. Levels of the alpha isoform were lower in resting monocytes from the MI cohort (mean relative expression = 0.0007±0.0009 vs 0.0002±0.0007; p= <0.05, for controls and premature MI cohorts respectively)(Figure 5.12). The beta isoform was detectable in only two of the resting monocyte samples from the control group. TFPI-α and -β isoforms were both expressed at lower levels in activated monocytes from the MI cohort. Gene expression of the alpha isoform in monocytes exposed to CRP-XL-activated platelets = 0.151±0.307 vs 0.004±0.005; (p=<0.01), for controls and premature MI cohort, respectively(Figure 5.12). Gene expression of the beta isoform was mean 0.020±0.039 vs 0.00007±0.00009; (p= <0.01), for controls and premature MI cohorts (Figure 5.13).

Gene expression of TFPI-α in monocytes from healthy controls and premature MI cohort:

a) **Healthy Controls**

	Resting monocytes			Activated monocytes				
	Ct target gene	Ct Ref gene	ΔCt	Ct Target gene	Ct Ref gene	ΔCt	ΔΔ Ct	*p*-value
Mean	36.50	24.34	12.26	28.04	22.71	5.33	6.73	0.0038
S Dev	1.41	3.79	3.50	1.97	3.01	2.94	4.47	

b) **Premature MI**

	Resting monocytes			Activated monocytes				
	Ct Target gene	Ct Ref gene	ΔCt	Ct Target gene	Ct Ref gene	ΔCt	ΔΔ Ct	*p*-value
Mean	37.07	21.46	15.61	25.12	18.88	9.24	5.67	0.0035
S Dev	2.82	1.64	3.12	2.12	1.13	2.58	4.44	

c)

	Controls	MI		
	ΔCt	ΔCt	ΔΔ Ct	*p*-value
resting monocytes	12.26	15.61	-3.35	0.0445
activated monocytes	5.34	9.24	-3.91	0.0035

Table 5.3. Mean Ct, ΔCt, and ΔΔCt ±SD for monocyte gene expression in (a) control group, and (b) premature MI cohort. (c) Mean ΔCt and ΔΔCt for TFPI-α gene expression in resting monocytes in controls v MI cohort, and for TFPI-α gene expression in activated monocytes for controls v MI cohort, (n = 11).

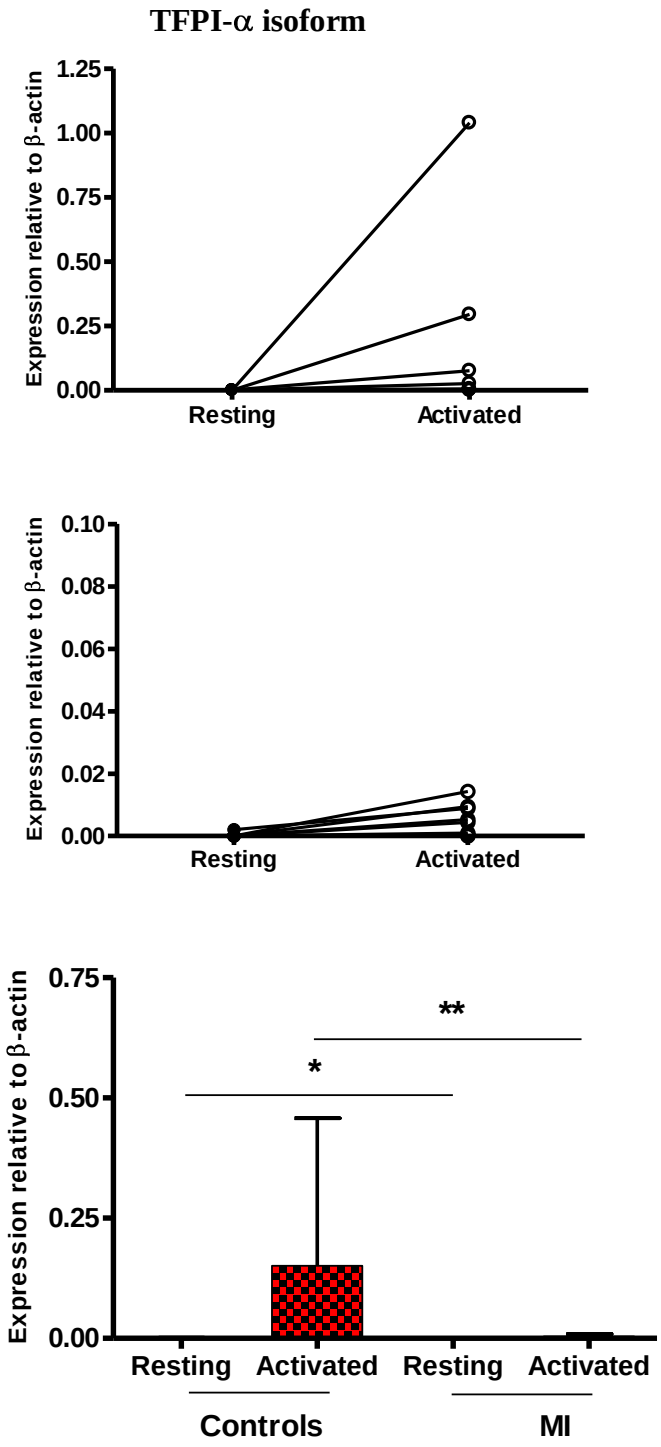


Figure 5.12. Gene expression of TFPI-α in monocytes isolated from premature MI cohort and control group. Change in relative gene expression of TFPI-α in monocytes pre- and post incubation with CRP-XL-activated platelets, in (a) healthy control group, and (b) premature MI cohort. (c) Expression of monocyte TFPI-α gene in resting and activated monocytes from healthy controls and premature MI cohorts. Expression was normalised to β_2-Microglobulin and is expressed as mean ± SD; (n = 11). *P<0.05, **P<0.01.

Gene expression of TFPI-β in monocytes from healthy controls and premature MI cohort:

a) **Healthy Controls**

	Resting monocytes			Activated monocytes				
	CT target gene	CT Ref gene	ΔCT	CT Target gene	CT Ref gene	ΔCT	ΔΔ CT	*p*-value
Mean	37.25	20.35	16.90	32.72	22.71	10.01	3.36	#
S Dev	0.35	1.83	1.48	2.79	3.01	4.17	3.92	

b) **Premature MI**

	Resting monocytes			Activated monocytes				
	CT Target gene	CT Ref gene	ΔCT	CT Target gene	CT Ref gene	ΔCT	ΔΔ CT	*p*-value
Mean	-	24.24	-	34.87	18.87	15.90	-	##
S Dev	-	3.79	-	3.67	1.13	3.46	-	

only 2 resting monocyte samples had detectable levels of TFPI-β (Control group)
no resting monocyte samples had detectable levels of TFPI-β (MI cohort)

c)

	Controls	MI		
	ΔCT	ΔCT	ΔΔ CT	*p*-value
resting monocytes	16.90	-	-	
activated monocytes	10.01	15.90	-5.89	0.0015

Table 5.4. Mean Ct, ΔCt, and ΔΔCt ±SD for monocyte gene expression in (a) healthy control group, and (b) premature MI cohort. (c) Mean ΔCt and ΔΔCt for TFPI-β gene expression in resting and activated monocytes in controls v MI cohort, (n = 11).

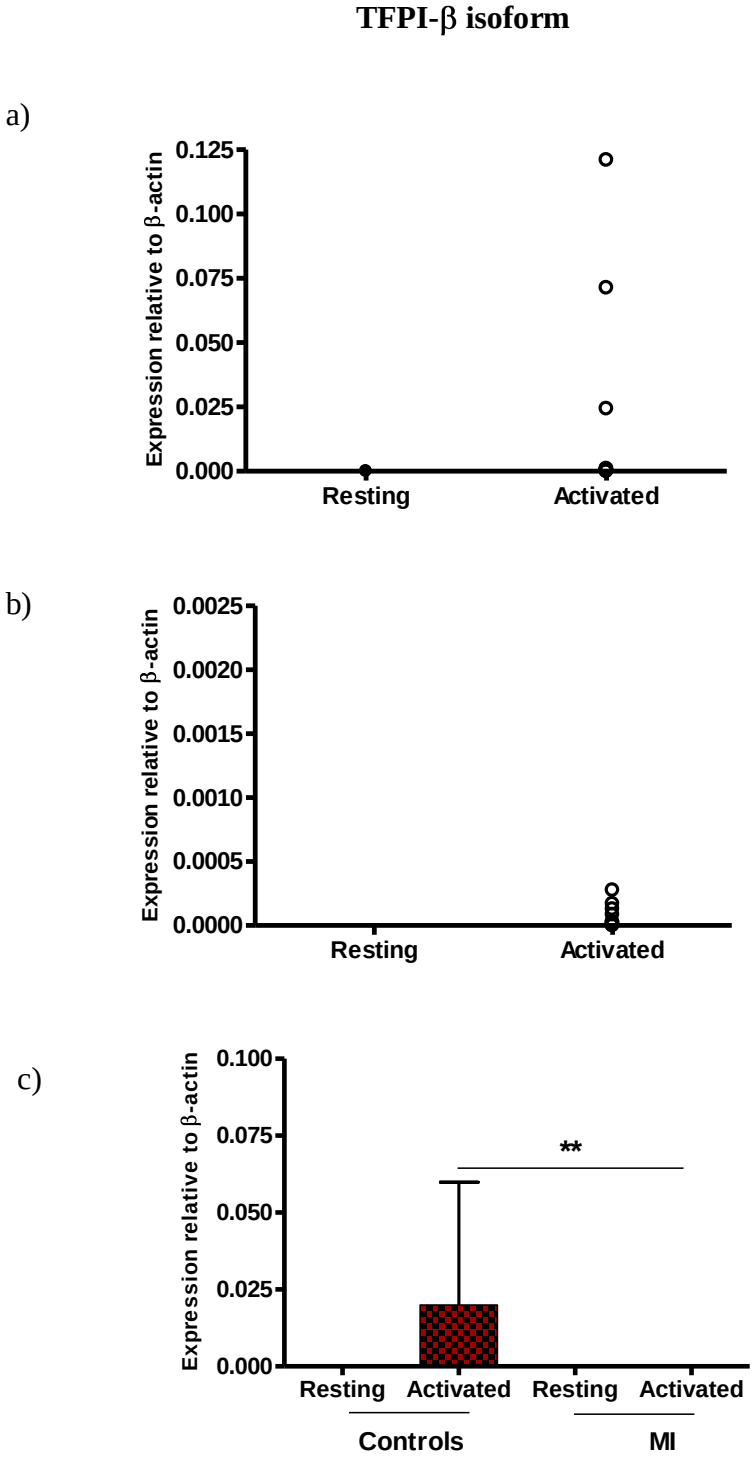


Figure 5.13. Gene expression of TFPI-β in monocytes isolated from premature MI cohort and control group. Change in relative gene expression of TFPI-β in monocytes pre- and post incubation with CRP-XL-activated platelets, in (a) healthy control group, and (b) premature MI cohort. (c) Expression of monocyte TFPI-β gene in resting and activated monocytes from healthy controls and premature MI cohorts. Expression was normalised to β_2-Microglobulin and is expressed as mean ± SD; (n = 11). **P<0.01.

5.5.3. Expression of TF and TFPI mRNA in monocytes from healthy offspring with strong family history of MI and controls

This pattern was repeated when measuring monocyte TF mRNA in monocytes from offspring with family history of MI, age- and sex-matched control group. Resting monocytes of offspring with family history of MI expressed significantly higher levels of TF mRNA (mean relative expression = 0.008±0.005 vs 0.012±0.006; p= <0.01). Activated monocytes of the offspring group also expressed significantly higher levels of TF mRNA (mean = 0.024±0.014vs 0.033±0.019; p= <0.05)(Figure 5.14). In contrast to the older cohorts, there was no significant difference in monocyte expression of TFPI-α or TFPI-β in resting or activated monocytes isolated from off-spring with or without a family history of premature MI. Mean relative expression of the α-isoform was 0.00001±0.00002 vs 0.00005±0.00006 (p=>0.05) for resting monocytes from control vs premature MI off-spring; and 0.086±0.180 vs 0.0240±0.044 (p=>0.05)(Figures 5.15 and 5.16).

Gene expression of TF in monocytes from off-spring with strong family-history of CAD, and off-spring with no family history of MI:

a) **Control off-spring**

	Resting monocytes			Activated monocytes				
	CT target gene	CT Ref gene	ΔCT	CT Target gene	CT Ref gene	ΔCT	ΔΔ CT	*p*-value
Mean	32.75	25.59	7.17	30.50	24.90	5.60	1.57	<0.0001
S Dev	1.148	0.644	0.821	1.158	0.859	1.028		

b) **MI off-spring**

	Resting monocytes			Activated monocytes				
	CT Target gene	CT Ref gene	ΔCT	CT Target gene	CT Ref gene	ΔCT	ΔΔ CT	*p*-value
Mean	31.39	24.86	6.53	30.81	25.74	5.07	1.46	<0.0001
S Dev	0.900	0.603	0.717	0.87	0.71	0.76		

c)

	Control off-spring	**MI off-spring**		
	ΔCT	ΔCT	ΔΔ CT	*p*-value
resting monocytes	7.17	6.53	0.64	0.0017
activated monocytes	5.66	5.07	0.59	0.0117

Table 5.5. Mean Ct, ΔCt, and ΔΔCt ±SD for monocyte TF gene expression in (a) offspring control group, and (b) offspring with family history of MI. (c) Mean ΔCt and ΔΔCt for TF gene expression in resting and activated monocytes in control offspring v MI offspring; *n* = 20 MI offspring, 20 control offspring).

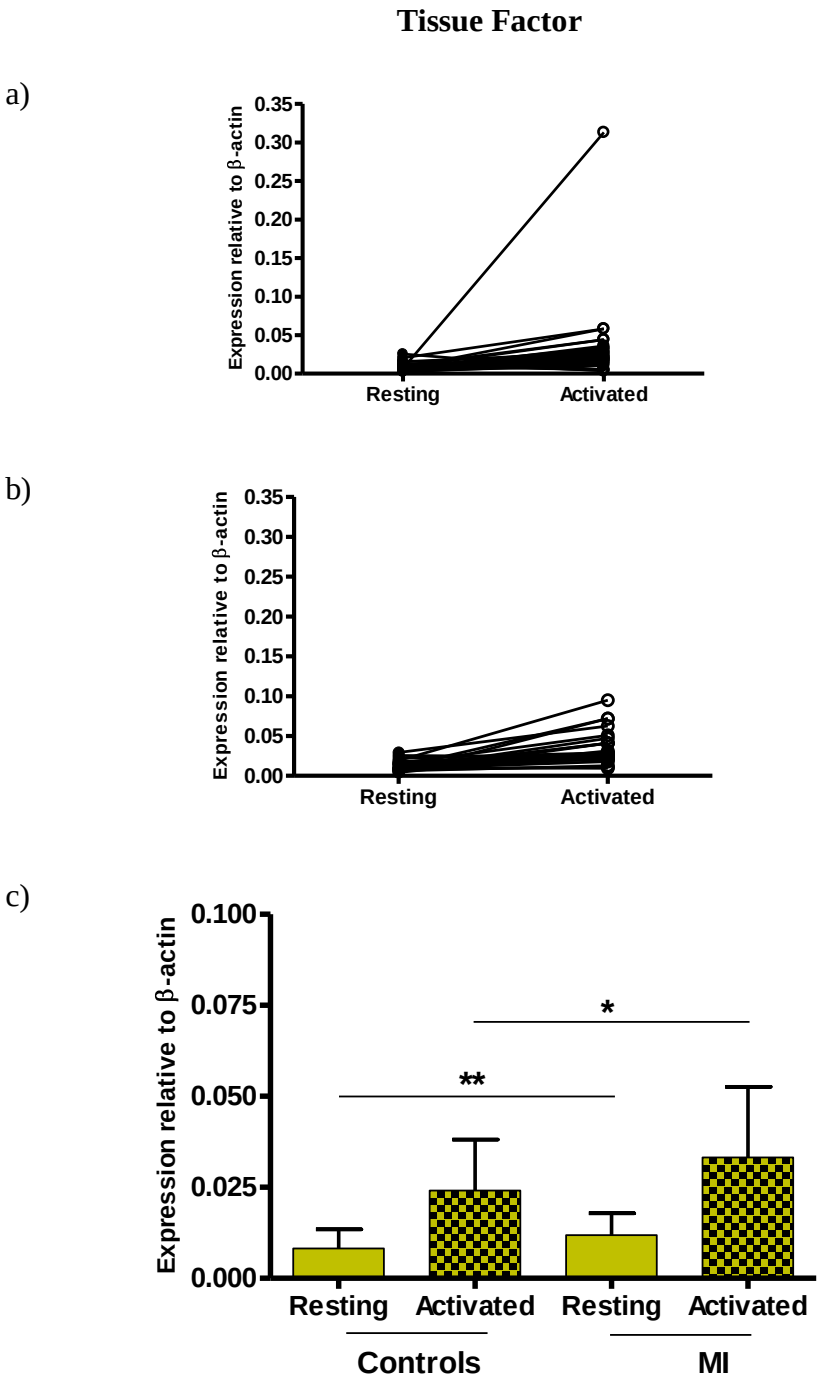


Figure 5.14. Gene expression of Tissue Factor (TF) in monocytes isolated from off-spring with family history of MI and healthy control off-spring. Change in relative gene expression of TF in monocytes pre- and post incubation with CRP-XL-activated platelets, in (a) healthy control off-spring, and (b) off-spring with family history of MI. (c) Expression of monocyte TF gene in resting and activated monocytes from healthy control off-spring and off-spring with family history of MI. Expression was normalised to β_2-Microglobulin and is expressed as mean ± SD; (n = 20 MI off-spring, 20 control off-spring). *P<0.05, **P<0.01.

Gene expression of TFPI-α in monocytes from off-spring with strong family-history of MI, and offspring with no family history of MI:

a) **Healthy Control offspring**

	Resting monocytes			Activated monocytes				
	CT target gene	CT Ref gene	ΔCT	CT Target gene	CT Ref gene	ΔCT	ΔΔ CT	*p*-value
Mean	36.19	20.49	15.70	26.33	19.85	6.48	12.78	<0.0001
S Dev	1.686	2.079	2.482	1.151	1.547	1.594	1.45	

b) **MI offspring**

	Resting monocytes			Activated monocytes				
	CT Target gene	CT Ref gene	ΔCT	CT Target gene	CT Ref gene	ΔCT	ΔΔ CT	*p*-value
Mean	36.9625	20.23	16.93	25.77	21.97	3.80	9.21	<0.0001
S Dev	1.117315	1.254	1.534	0.96	4.00	4.24	3.23	

c)

	Controls	**MI**		
	ΔCT	ΔCT	ΔΔ CT	*p*-value
resting monocytes	15.70	16.93	-1.23	0.3704
activated monocytes	6.48	3.80	2.69	0.0818

Table 5.6. Mean Ct, ΔCt, and ΔΔCt ±SD for monocyte TFPI-α gene expression in (a) offspring control group, and (b) offspring with family history of MI. (c) Mean ΔCt and ΔΔCt for TFPI-α gene expression in resting and activated monocytes in control offspring v MI offspring; *n* = 11 MI offspring, 11 control offspring).

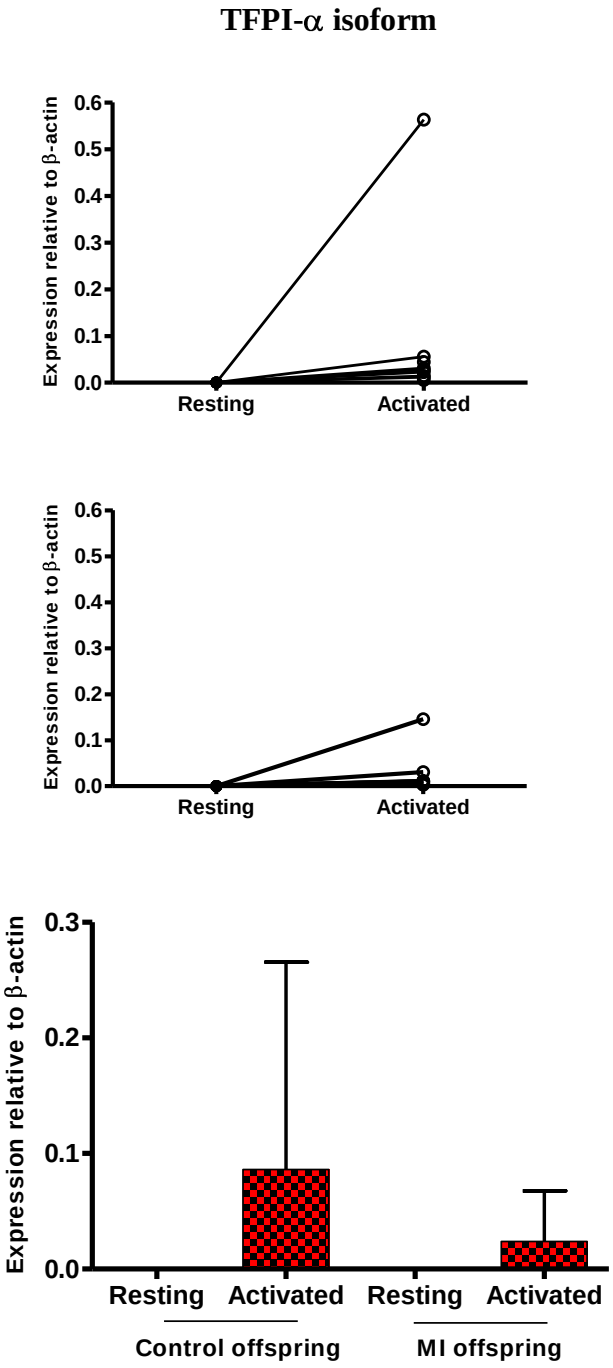


Figure 5.15. Gene expression of TFPI-α in monocytes isolated from off-spring with family history of MI and healthy control off-spring. Change in relative gene expression of TFPI-α in monocytes pre- and post incubation with CRP-XL-activated platelets, in (a) healthy control off-spring, and (b) off-spring with family history of MI. (c) Expression of monocyte TFPI-α gene in resting and activated monocytes from healthy control off-spring and off-spring with family history of MI. Expression was normalised to β_2-Microglobulin and is expressed as mean ± SD; (*n* = 11 MI offspring, 11 control offspring).

Gene expression of TFPI-β in monocytes from off-spring with strong family-history of MI, and offspring with no family history of MI:

a) **Healthy Control offspring**

	Resting monocytes			**Activated monocytes**				
	CT target gene	CT Ref gene	ΔCT	CT Target gene	CT Ref gene	ΔCT	ΔΔ CT	*p*-value
Mean	-	-	-	31.42	21.97	9.45	-	-
S Dev				2.113	4.000	4.345		

b) **MI offspring**

	Resting monocytes			**Activated monocytes**				
	CT Target gene	CT Ref gene	ΔCT	CT Target gene	CT Ref gene	ΔCT	ΔΔ CT	*p*-value
Mean	38.6	20.49	18.34	30.66	19.85	10.81	7.633333	0.0059
S Dev	1.452584	2.079	2.912	2.14	1.55	2.02	1.022758	

c)

	Controls	**MI**		
	ΔCT	ΔCT	ΔΔ CT	*p*-value
resting monocytes	-	18.34	-	-
activated monocytes	9.45	10.81	-0.36	0.9374

Table 5.7. Mean Ct, ΔCt, and ΔΔCt ±SD for monocyte TFPI-β gene expression in (a) offspring control group, and (b) offspring with family history of MI. (c) Mean ΔCt and ΔΔCt for TFPI-β gene expression in resting and activated monocytes in control offspring v MI offspring; *n* = 11 MI offspring, 11 control offspring).

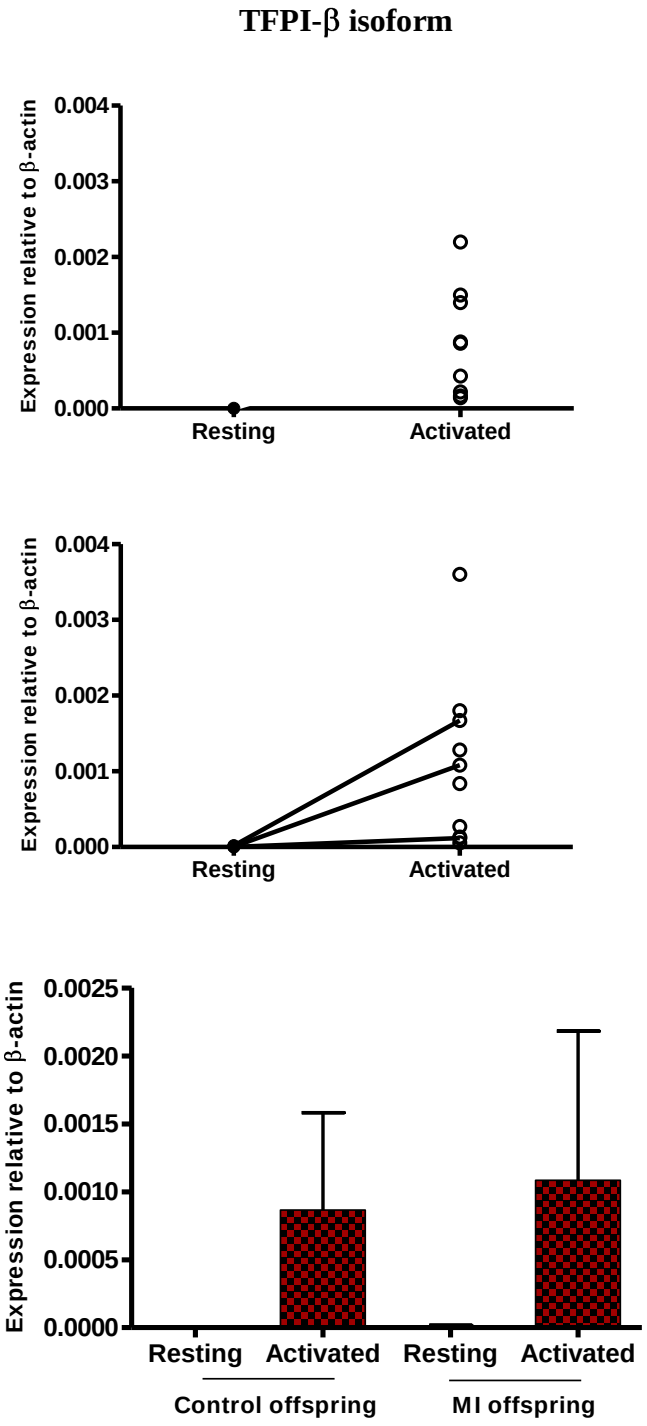


Figure 5.16. Gene expression of TFPI-β in monocytes isolated from off-spring with family history of MI and healthy control off-spring. Change in relative gene expression of TFPI-β in monocytes pre- and post incubation with CRP-XL-activated platelets, in (a) healthy control off-spring, and (b) off-spring with family history of MI. (c) Expression of monocyte TFPI-β gene in resting and activated monocytes from healthy control off-spring and off-spring with family history of MI. Expression was normalised to β_2-Microglobulin and is expressed as mean ± SD; (*n* = 11 MI offspring, 11 control offspring).

5.6. Mechanism of platelet action and monocyte gene expression of TF and TFPI

The experiments carried out in this study so far, have consistently demonstrated that CRP-XL-activated platelets can induce changes in monocyte gene expression of TF and TFPI, resulting in an increased procoagulant surface membrane. The mechanism of platelet action on the monocyte could occur through two routes; firstly through direct ligand-ligand interaction of platelet P-selectin binding to its receptor, monocyte P-selectin Glycoprotein Ligand-1 (PSGL-1), and secondly through indirect action due to the release of soluble mediators following platelet degranulation and the subsequent binding of some of these molecules to monocyte surface receptors. Therefore it was decided to examine the effect of these two mechanisms in relation to changes in monocyte gene expression of TF and TFPI. Mononuclear cells were isolated using Lymphoprep, and washed with PBS. To prepare plasma rich with platelet soluble mediators, platelets in autologous platelet-rich-plasma (PRP) were activated with CRP-XL [500ng/mL] for 15 minutes at 37^{o}C. The platelets were then pelleted, and the soluble-mediator-rich plasma supernatant removed and used to resuspend mononuclear cells. Autologous platelet-poor plasma was prepared and used as a control. P-selectin Fc-chimera, which binds to the monocytes PSGL-1 receptor and thus 'mimics' platelet-monocyte aggregation via P-selectin-PSGL-1, was incubated with the mononuclear cells in PBS buffer, with mononuclear cells in PBS buffer only as a control. All samples were incubated at 37^{o}C for 4 hours, after which the monocytes were positively selected using CD14 magnetic beads and assayed for changes in TF and TFPI mRNA as previously.

5.6.1. Effect of monocyte activation via platelet soluble mediators or PSGL-1

Following incubation of the monocytes for 4 hours at 37^{o}C in autologous plasma in the presence or absence of platelet soluble mediators, there was no significant increase in monocyte TF mRNA (mean relative expression 0.046±0.027 vs 0.038±0.27; p >0.5). In contrast, monocyte activation with platelet soluble mediators resulted in significant induction of gene expression of TFPI (mean 0.008±0.011 vs 0.0305±0.054; p=<0.05); (Figure 5.17a and b). Further study of expression of TFPI isoforms showed that both alpha and beta isoforms were significantly induced following incubation of monocytes with plasma containing platelet soluble-rich mediators. Relative expression of the α-isoform in plasma, and plasma containing platelet soluble mediators was 0.00007±0.00003 vs 0.00057±0.0004, p=<0.001. Relative expression of the β-isoform was 0.00002±0.00002 vs 0.0002±0.0001, p=<0.001; (Figure 5.18a and b).

Monocyte activation via PSGL-1 did not result in significant increased expression of TF mRNA (mean 0.046±0.027 vs 0.069±0.054; p>0.05), for 4 hour control vs 4 hour sample containing chimeric protein (Figure 5.17c and d). There was also no significant induction of TFPI (mean 0.008±0.011 vs 0.033±0.069; p=>0.05) (Figure 5.18c and d). Therefore, the main platelet mechanism of induction for monocyte gene expression of TFPI expression is solely due to the action of platelet soluble mediators.

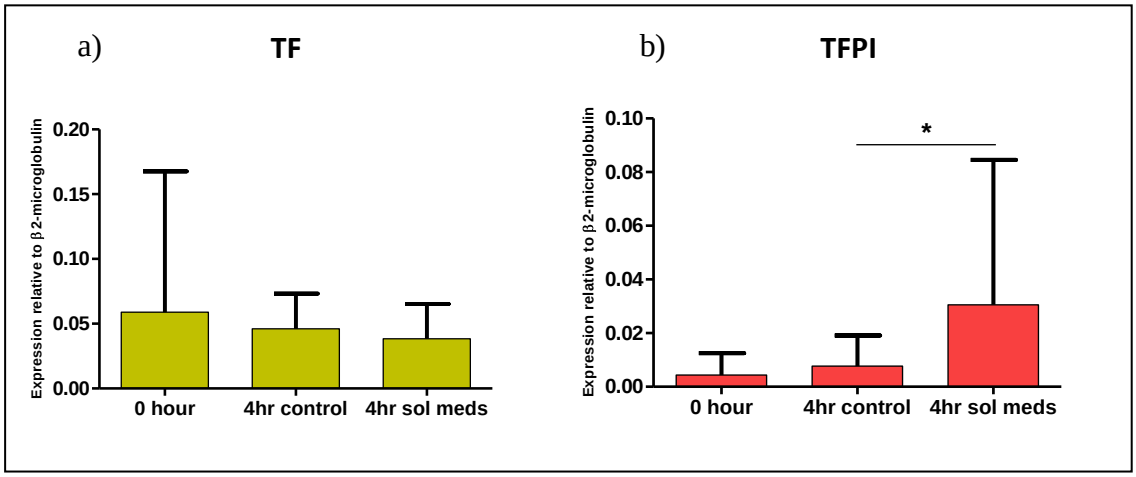
Gene expression of TF and TFPI in monocytes exposed to platelet soluble mediators or P-selectin chimera:

	TISSUE FACTOR			
	0 hour	4h Control	4h Sol Meds	4h Chimera
Mean	5.492	4.405	4.132	4.603
Std Dev	4.107	0.984	2.145	1.461
***p*-value**		0.4579	0.2962	0.6647

	TFPI			
	0 hour	4h Control	4h Sol Meds	4h Chimera
Mean	9.48	7.532	6.162	7.187
Std Dev	4.129	2.021	2.067	2.938
***p*-value**		0.0873	0.0414	0.0347

Table 5.8. Mean ΔCt ±SD for monocyte TF and TFPI gene expression following incubation for 4 hours at 37^{o}C in the presence of plasma containing platelet soluble mediators released following platelet activation and degranulation, or a chimeric protein that binds to monocyte PSGL-1; (n = 5).

Monocyte response to platelet soluble mediators:



Monocyte response to PSGL-1 activation:

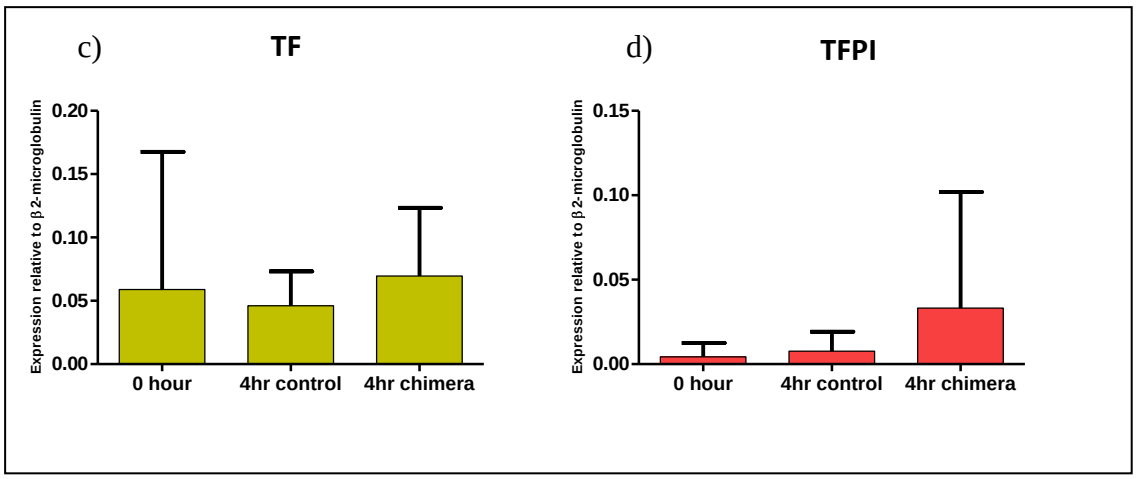


Figure 5.17: Induction of gene expression in monocytes following exposure to platelet soluble mediators or activation via P-selectin glycoprotein ligand-1 (PSGL-1). Isolated monocytes were incubated for 4 hours at 37°C in plasma +/- platelet soluble mediators, or incubated in PBS +/- P-selectin-Fc chimeric protein which binds and activates monocyte PSGL-1. (a and b) monocyte gene expression following exposure to platelet soluble mediators, of TF and TFPI, respectively. (c and d) monocyte gene expression following activation via PSGL-1, of TF and TFPI, respectively. (Mean ± SD; $n = 5$; *$P<0.05$).

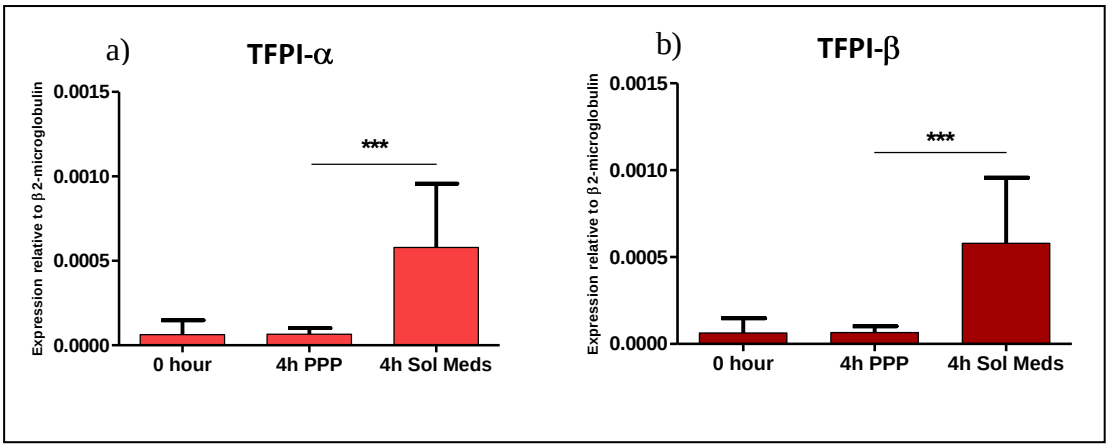
Gene expression of TFPI-isoforms in monocytes exposed to platelet soluble mediators or P-selectin chimera:

	TFPI-α				
	0 hour	4h PPP	4h Sol Meds	4h PBS	4h Chimera
Mean	15.80833	14.06667	11.05	11.98333	14
Std Dev	2.930429	1.093465	1	1.167761	0.801873
***p*-value**		0.1375	0.0156	0.0152	0.1995

	TFPI-β				
	0 hour	4h PPP	4h Sol Meds	4h PBS	4h Chimera
Mean	19.125	15.76667	12.15833	13.625	15.425
Std Dev	1.088194	1.258835	0.74861	2.16766	1.823938
***p*-value**		0.0241	0.0002	0.0138	0.0157

Table 5.9. Mean ΔCt ±SD for monocyte gene expression of TFPI-isoforms following incubation for 4 hours at 37^{o}C in the presence of plasma containing platelet soluble mediators released following platelet activation and degranulation, or a chimeric protein that binds to monocyte PSGL-1; (n = 6).

Monocyte response to platelet soluble mediators:



Monocyte response to PSGL-1 activation:

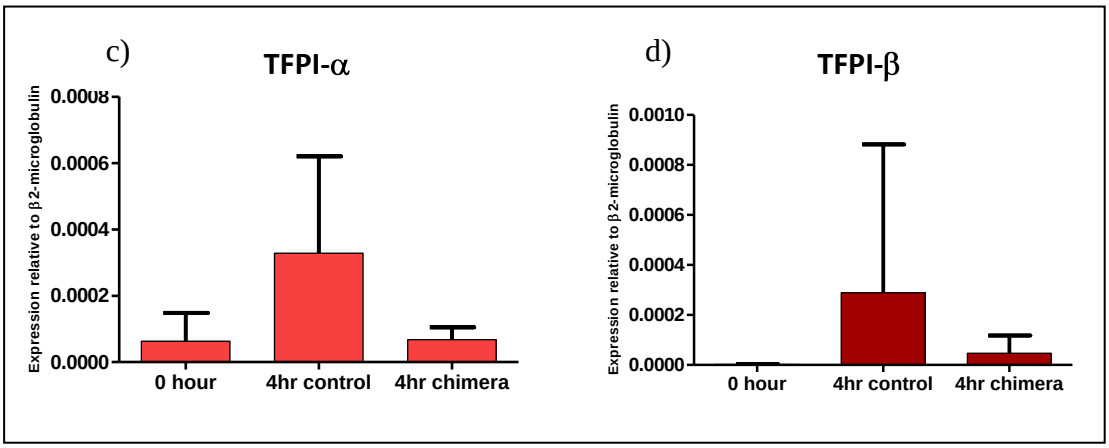


Figure 5.18: Expression of TFPI isoforms in monocytes following exposure to platelet soluble mediators or activation via P-selectin glycoprotein ligand-1 (PSGL-1). (a and b) monocyte gene expression following exposure to platelet soluble mediators, of TFPI-α and TFPI-β, respectively. (c and d) monocyte gene expression following activation via PSGL-1, of TFPI-α and TFPI-β, respectively. (Mean ± SD; *n* = 6; ****P*<0.001).

5.7. Effect of MPs on monocyte gene expression of TF and TFPI

The results of the previous experiment, investigating the effects of the two mechanisms of platelet-monocyte interaction, suggest that the induction of monocyte gene expression of TFPI is due to the action of platelet soluble mediators. However, one further possibility is that induction of TFPI gene expression is due to the interaction of MPs released from the platelets, which, expressing P-selectin, can bind directly to the monocyte via PSGL-1. Although early experiments sought to determine the optimal concentration of CRP-XL which would result in platelet activation and degranulation, without generating large numbers of MPs, it was decided to perform an experiment to test whether platelet-MPs do not play a role in upregulation of monocyte TFPI. Monocytes were isolated, this time using the Miltenyi CD14 microbeads method to ensure pure monocyte-only cell suspension. Platelet-MPs were positively isolated by activating platelets in PRP with a high dose of CRP-XL [5µg/mL] for 15 minutes at 37^{o}C, then pelleting the platelets by centrifugation. The plasma supernatant was then further centrifuged, this time at 13000rpm, to pellet the MPs. These were removed and washed in HBS. Monocytes were then resuspended in filtered autologous plasma containing the isolated platelet-MPs, and incubated for 4 hours at 37^{o}C. Monocytes incubated in filtered autologous plasma or filtered plasma containing platelet soluble mediators were used as negative and positive controls, respectively.

In the presence of platelet-MPs, relative monocyte gene expression of TF measured 0.005±0.003 vs 0.002±0.002 (p= 0.0398), for plasma including platelet-MPs vs plasma only; (Figure 5.19a). To investigate whether inhibiting direct interaction of platelet-MPs with monocytes reduced expression of TF, monocytes were incubated with platelet-MP-rich plasma in the presence of 9-E1, which blocks P-selectin-PSGL-1

interaction. The concentration of 9-E1 used in the experiment had previously been shown to block platelet-monocyte binding (data not shown). However, there was no significant decrease in monocyte TF gene expression (mean 0.005±0.003 vs 0.003±0.002; p>0.05, for absence/presence of 9-E1, respectively (Figure 5.19b). There was no increase of monocyte gene expression of TFPI due to the interaction of platelet-MPs compared to control (mean 0.0006±0.0003 vs 0.0005±0.0002, *p*=>0.5; and this was not affected by blocking P-selectin-PSGL-1 interaction (Figure 5.20). Therefore, gene upregulation of monocyte TFPI is not driven by direct monocyte interaction with platelet-MPs.

5.8. Effect of CRP-XL on monocyte gene expression

The monocyte does not have a GPVI collagen receptor, therefore it should not be stimulated by CRP-XL, however, to verify that the collagen peptide did not stimulate the monocytes directly, isolated monocytes were incubated in PBS with CRP-XL for 4 hours at 37^{o}C. Monocytes incubated in PBS buffer only, or plasma rich with platelet soluble mediators were used as negative and positive control, respectively.

However, there was no significant increase in monocyte gene expression of TFPI as a result of incubation with the collagen peptide CRP-XL (Figure 5.21). This data confirms that the induction of monocyte gene expression of TFPI is not due to direct activation of the monocyte by CRP-XL, but is due to induction of the monocyte by platelet soluble mediators.

Tissue Factor

	0 HOUR	4H PPPF	4H SOL MEDS	4H PPPF + PLT-MPs	4H PPPF + MPs + 9E1
Mean	18.128	9.118	8.133	7.955	8.713
Std dev	0.807	1.404	0.949	0.947	1.164
***p*-value**		0.0031	0.0007	<0.0001	0.0014

Table 5.10. Mean ΔCt ±SD for monocyte gene expression of TF in the presence/absence of platelet-microparticles.

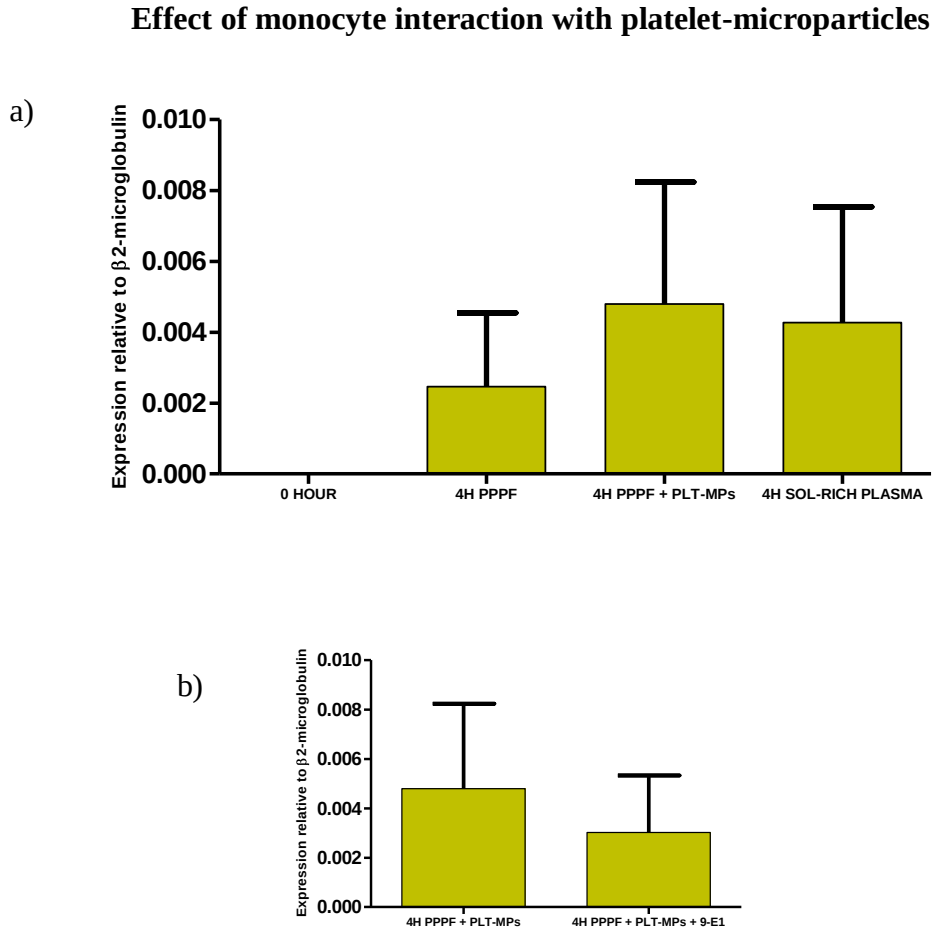


Figure 5.19: Effect of platelet-microparticles (PMPs) on monocyte gene expression of Tissue Factor (TF). (a) Isolated monocytes were incubated for 4 hours at 37°C in filtered, MP-free autologous plasma (negative control); in filtered, MP-free autologous plasma containing isolated platelet-MPs; or in filtered, MP-free autologous plasma containing platelet soluble mediators (positive control). (b) Addition of 9-E1 to inhibit platelet-MP interaction with monocytes resulted in reduced monocyte gene expression of TF. (Mean ± SD; $n = 4$).

TFPI

	0 HOUR	4H PPPF	4H SOL MEDS	4H PPPF + PLT-MPs	4H PPPF + MPs + 9-E1
Mean	19.6	10.9525	9.705	11.1925	10.435
Std dev	0.6188	0.8482	0.6812	0.6689	0.8924
***p*-value**		0.0010	0.0004	0.0007	0.0001

Table 5.11. Mean ΔCt ±SD for monocyte gene expression of TFPI in the presence/absence of platelet-microparticles.

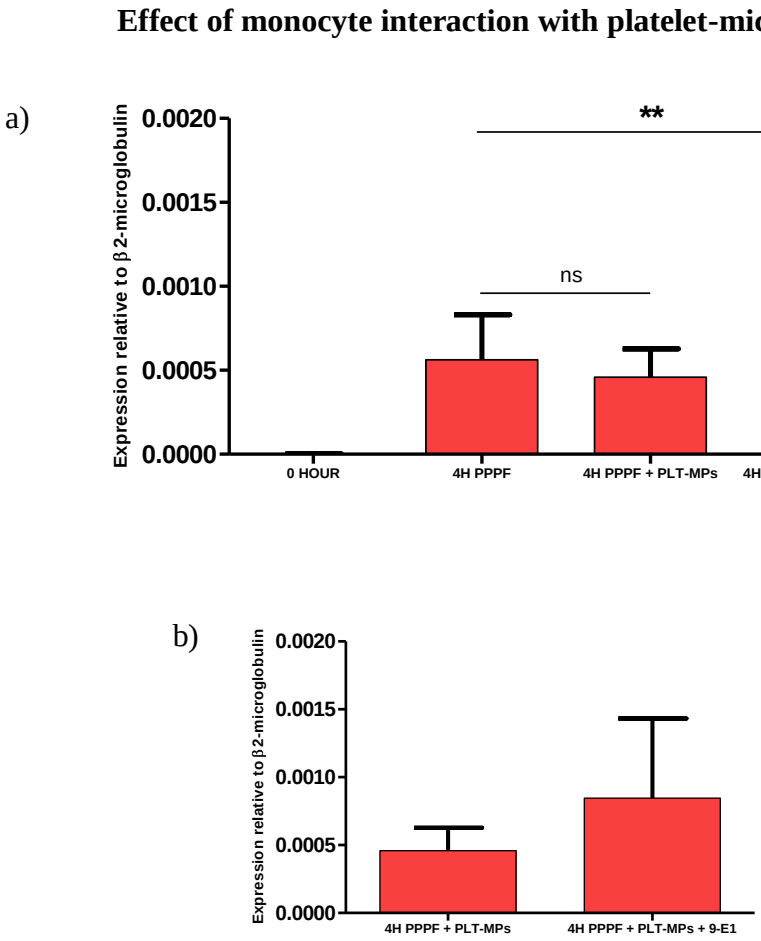


Figure 5.20: Effect of platelet-microparticles (PMPs) on monocyte gene expression of Tissue Factor (TFPI). (a) Isolated monocytes were incubated for 4 hours at 37°C in filtered, MP-free autologous plasma (negative control); in filtered, MP-free autologous plasma containing isolated platelet-MPs; or in filtered, MP-free autologous plasma containing platelet soluble mediators (positive control). (b) Addition of 9-E1 to inhibit platelet-MP interaction with monocytes did not reduce monocyte gene expression of TFPI. (Mean ± SD; n = 4; $^{**}P<0.005$).

Effects of direct stimulation of monocytes with CRP-XL:

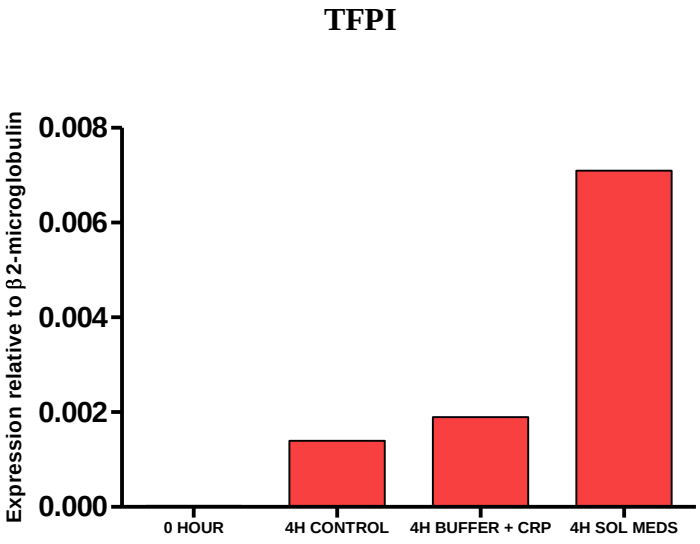


Figure 5.21: Collagen Related Peptide-XL (CRP-XL) is not responsible for upregulation of monocyte TFPI gene expression through direct activation of monocytes. Isolated monocytes were incubated at 37^{o}C for 4 hours in PBS buffer (negative control), in PBS buffer containing CRP-XL [500ng/mL], or autologous plasma containing platelet soluble mediators (positive control). Expression was normalised to β_2-Microglobulin (n = 1).

5.9. Possible candidates for induction of monocyte TFPI

The data from this set of experiments has demonstrated that whereas monocyte gene expression of TF is largely upregulated via interaction of monocyte PSGL-1 with platelet or PMP-P-selectin, it is platelet soluble mediators that are responsible for gene upregulation of monocyte TFPI. There have been reported >300 different factors contained in the various platelet granules, a list of the major components can be found in Table 1.1.

5.9.1. Effect of IL1β and TGFβ on monocyte gene expression of TF and TFPI

Two factors were selected for further study, IL-1β, and TGF-β, both of which are found in the alpha granules of platelets. They were selected because they signal via different pathways, IL-1β activates the NFκβ intracellular signalling pathway associated with inflammation, and TGF-β is known to activate the SMAD pathway, associated with wound healing (Auron, 1998; Rosendahl *et al*, 2001). Isolated monocytes were incubated as previously, for 4 hours at 37^{o}C either in autologous plasma containing platelet releasate only, or in autologous plasma containing platelet releasate with additional presence of TGF-β or IL-1β blocking antibodies.

In the presence of TGF-β blocking antibody, there was a slight increase in monocyte expression of TF mRNA (+16%), whereas in the presence of IL-1β blocking antibody, TF mRNA expression was reduced dramatically but not totally inhibited (-50%). There was no change in monocyte expression of TFPI in the presence of TGF-β blocking antibody, but again, the blocking of IL-1β had an inhibitory effect (-45%) (Figure 5.22).

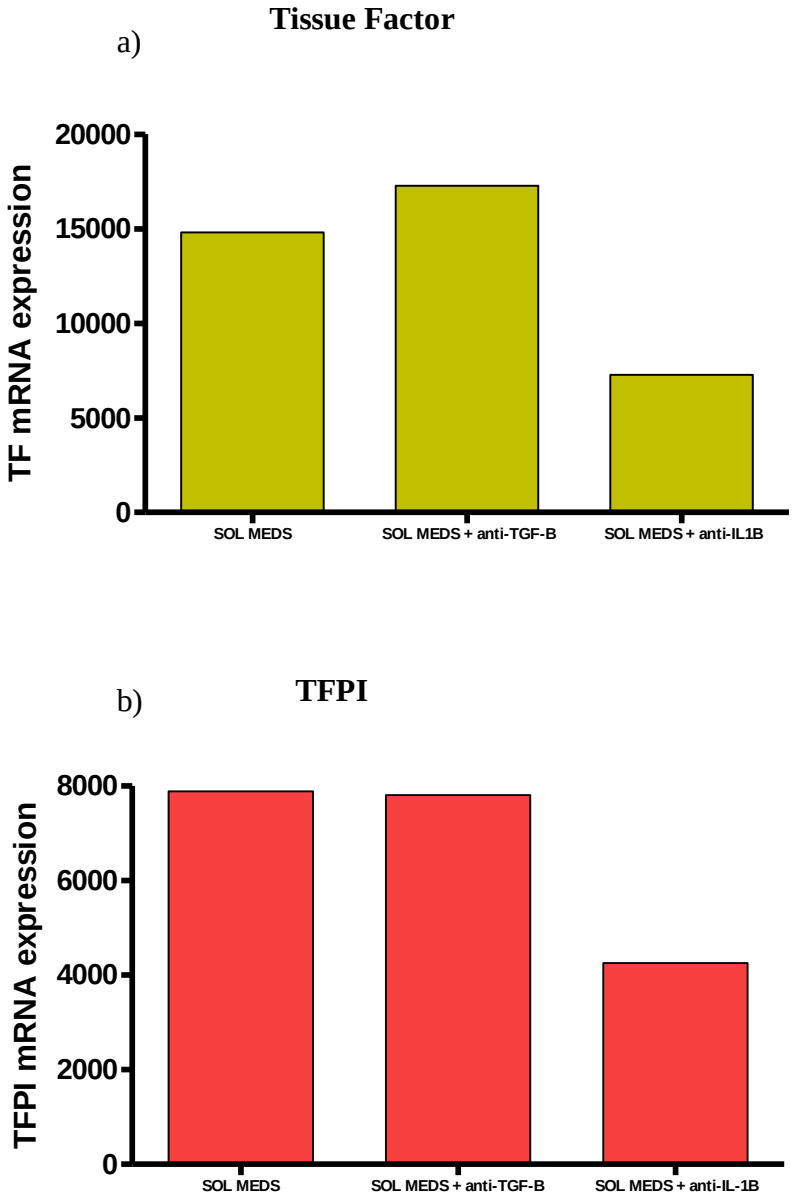


Figure 5.22: Effect of blocking monocyte interaction with TGFβ and IL-1β on monocyte gene expression of Tissue Factor and Tissue Factor Pathway Inhibitor. Monocytes were incubated for 4 hours at 37^{o}C in autologous plasma containing platelet soluble mediators +/- IL-1β, and TGF-β blocking antibodies. Graphs show monocyte gene expression of (a) TF, and (b) TFPI (n = 1).

5.9.2. Analysis using predictive software

Using Ingenuity Systems® software, Dr S Amisten performed an analysis of proteins within the platelet secretome that may play a key role in upregulation of monocyte TFPI, focusing particularly of growth factors (GFs), cytokines and chemokines.

The analysis showed 3 possible pathways of upregulation. Pathway one demonstrated a possible key role for Fibroblast Growth Factor-2 (FGF2), a component of the platelet secretome, which may act in parallel with TGF-β, or may be activated by VEGFA, EGF, or PDGFA. A second possible pathway is through direct activation by prothrombin. This pathway may be inhibited by SERPINA1 (α-1 anti-trypsin precursor). Finally, a third possible pathway of activation that may be linked to upregulation of TFPI is through activation of transcription factor KLF2 by PDGFB (Figure 5.23). These possible pathways require testing out *in vitro*.

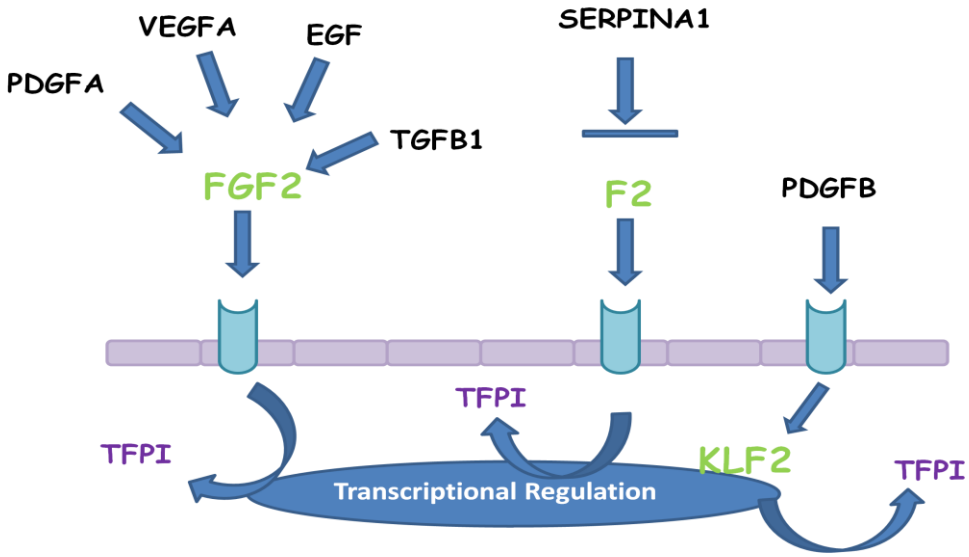


Figure 5.23: Platelet soluble mediators that may be responsible for the gene induction of monocyte Tissue Factor Pathway Inhibitor (TFPI). Pathway 1 involves direct stimulation via Fibroblast Growth Factor (FGF2), which may be augmented by Platelet-derived Growth Factor (PDGFA), Vascular Endothelial Growth Factor-A (VEGFA), Epidermal Growth Factor (EGF), and Transforming Growth Factor-β (TGF-β). Pathway 2 involves induction via a possible negative feedback mechanism involving Prothrombin (Factor II). Pathway 3 involves transcriptional regulation by Kruppel-like Factor-2 (KLF2).

5.10. Discussion

The monocyte response to inflammatory stimuli, such as LPS, has been well documented due to the importance of the role played by the monocyte in the normal immune response, and in progression of inflammatory disease, such as atherosclerosis. Whereas monocyte induction of TF in response to LPS has been reported in many studies (Rivers *et al*, 1975; Edwards *et al*, 1979; Gregory *et al*, 1989), LPS induction of monocyte TFPI expression is more equivocal, ranging from reports of strong LPS effect (McGee *et al*, 1994), through to small effects (Iochmann *et al*, 1999; Rana *et al*, 1988; Nguyen *et al*, 1999), and no significant induced response (van der Logt *et al*, 1994; Ott *et al*, 2001; Bajaj *et al*, 2007). Other studies have focused on the influence of cytokines on monocyte TF and TFPI expression, finding TF induction in response to Interleukin-4 (IL-4) and induction of TFPI in response to IL-10 (Paysant *et al*, 2005). There have been fewer studies examining the monocyte response to haemostatic stimuli, specifically to platelet-mediated responses, and the majority of these have focused on monocyte-platelet aggregation and the expression of TF (Celi *et al*, 1994; Lindmark *et al*, 2000). This has lead to the view that platelets have a pro-inflammatory effect on monocytes. In this study, as expected, the inflammatory stimulus, LPS, was the main inducer of TF gene expression in the monocyte; however it was the effect of the activated platelets, that was responsible for the induction of TFPI gene expression. This was a novel finding, and further experiments were carried out to see if this mRNA was translated into active protein.

5.10.1. Translation of mRNA into protein expression of monocyte TF and TFPI

Despite the expected, significant induction of TF mRNA in response to monocyte stimulation with LPS, for both the full-length membrane-bound TF and for the

alternatively spliced, soluble form, it was surprising that there did not follow a concomitant increase in monocyte surface activity or activity within the plasma. Previous experiments carried out in this laboratory have shown that LPS-stimulated monocytes, stimulated with the same concentration of LPS, express TF surface antigen, when measured by flow cytometry (data not shown). The combination of these experiments suggests that following monocyte activation with LPS, although the TF antigen may be expressed on the surface it does not have procoagulant activity. This suggests that the TF may be either inhibited or encrypted. Encryption of TF is an area of current debate, with various proposals regarding the structure and characteristics of the encrypted non-coagulant, and decrypted procoagulant conformation of TF, and also to the regulatory mechanisms that control the switch from one form to another. There are currently several theories as to the mechanisms of regulation, several of which suggest regulation involves the activity of protein disulfide isomerise (PDI). One possibility involves a disulfide switch, which allows transition between reduced and oxidized states of specific cysteine molecules within the extracellular domain of TF, with PDI regulating the redox switch (Ahamed *et al*, 2006; Chen *et al*, 2006). A further alternative is that encryption/decryption of membrane TF depends upon whether it is a monomer or dimer, with the suggestion that PDI may control the monomerisation, de-encryption process (Bach *et al*, 1997). This process may involve the re-organisation of the cell membrane. It has also been suggested that there is an indirect link of PDI regulating the transport of lipid domains within the membrane, which specifically affect the externalisation of phosphatidylserine (PS) on the outer membrane (Le *et al*, 1994; Daleke *et al*, 2008). This creates a procoagulant environment, and places TF within specific domains where it may be functionally active (Morrissey *et al*, 2008; Popescu *et*

al, 2010). It is also possible that PDI may enhance TF activity when it is complexed with FVIIa (Versteeg *et al*, 2007). So how do these relate to the findings of this current study?

Bach *et al*, (2006), have suggested that although monocyte TF is able to bind FVIIa, it is not functionally active unless it encounters specific flow conditions or intravascular microenvironment. The monocytes in this study are in a closed environment without flow, therefore may not encounter the conditions that might de-encrypt TF, and therefore it may not be possible to assess the levels of soluble TF potential activity released into the plasma, or the total amount of monocyte surface TF expression, without treating the cells or plasma further. What is interesting to note is that this same phenomenon did not occur in monocytes that have been exposed to CRP-XL-activated platelets. Although the level of monocyte TF mRNA expressed in response to platelet activation was much lower than in response to LPS, there was a higher proportion of soluble TF mRNA transcript seen in monocytes following exposure to activated platelets, and this appeared to be translated into protein antigen which had procoagulant activity in plasma. This appears to contradict the previous explanation of the need for flow or specific microvascular conditions. One possible explanation could be the availability of phospholipid on the membrane surface (Le *et al*, 1994; Daleke *et al*, 2008). The concentration of LPS used in these studies was sufficient to induct upregulation of the TF gene within the monocyte, but not high enough to induce rapid cellular apoptosis and generation of microparticles. Therefore, it may be that in the presence of 'low' concentrations of LPS, there is not induction of PS exposure on the monocyte membrane, and therefore the TF remains encrypted. This is supported by the studies of Henriksson *et al*, reporting that high concentrations of LPS [1000ng/mL]

resulted in high procoagulant activity and TF expression due to enhanced PS exposure, in contrast to monocytes treated with low doses of LPS [1ng/mL] (Henriksson *et al*, 2005; Henriksson *et al*, 2006). Monocytes in the current study were activated with 200ng/mL LPS, therefore may be at the lower end of the activation spectrum. The availability of phospholipid may also be relevant to the activity of soluble TF. Therefore, it may also follow, that even if the upregulated alternatively spliced TF mRNA transcripts are translated into soluble protein and released into the plasma, there is not the necessary availability of phospholipid in the plasma, due to lack of microparticles. This idea is supported by a recent study by Ollivier *et al*, (2009), where only very low levels of TF activity were detected in plasma isolated from whole blood stimulated for 5 hours with LPS. In contrast to LPS, where monocytes have been incubated in the presence of platelets that have been activated by collagen or thrombin, there may an increased, but low level, of platelet microparticles which provide the necessary phospholipid that leads to de-encryption of TF.

The major source of TFPI is the endothelium, and therefore a large majority of studies regarding TFPI, including expression of the alpha and beta isoforms, has been carried out on endothelial cells (Bajaj *et al*, 1990; Werling *et al*, 1993). Endothelial cells constitutively express surface TFPI which comprises both isoforms, however an intracellular pool contains only the heparin-releasable alpha isoform (Lupu *et al*, 1999; Hansen *et al*, 2000). Therefore, in response to heparin, all the intracellular pool is released, and a small percentage of surface TFPI-alpha. In response to PLC, the majority of TFPI, alpha and beta, is cleaved from the plasma membrane (Ellery *et al*, 2008).

It is not known whether monocyte TFPI is expressed in a similar manner, *ie*, whether it is expressed on the cell surface, whether it is secreted from an intracellular pool, and how this might relate to anticoagulant activity of the cell and its environment. Therefore, experiments were carried out to investigate whether TFPI mRNA is translated into protein, to determine its cellular location, and whether it has active, anticoagulant activity. Flow cytometric studies of monocyte surface antigen expression of TFPI failed to detect positive staining of membrane-bound monocyte TFPI, even though the TFPI polyclonal antibody was able to positively bind to TFPI on the surface of endothelial cells (data not shown). One possible explanation for this is that the TFPI protein is present on the monocyte surface but is bound to another molecule which sterically hinders the binding of the antibody. Whilst carrying out these experiments, report of TFPI expression in fibronectin-adherent monocytes was published (Baja *et al*, 2007). The study showed TFPI activity and antigen expression in lysates from fibronectin-adherent monocytes, demonstrating the presence and activity of monocyte TFPI, but it is still unclear whether its location is intracellular or expressed on the cell surface. In the current study, measurement of TFPI activity in plasma from whole blood incubated for up to 6 hours with CRP-XL, showed there was no increase in plasma TFPI activity following platelet activation, supporting the notion that the TFPI is membrane-bound. However, in the same sample set, increased levels of TFPI antigen were detected. This suggests that a soluble form of monocyte TFPI may be secreted in response to platelet activation, but does not have anticoagulant activity. Therefore, it may be a truncated form of TFPI, or be bound to lipid which limits its anticoagulant function (Novotny *et al*, 1989; Horie *et al*, 2002; Ohkura *et al*, 2004). Recently, a role for TFPI in lipid metabolism has been suggested due to the finding that the interaction

of lipoprotein with the C-terminus of TFPI may lead to reduced cholesterol levels and also reduced formation of atherosclerotic plaques (Pan *et al*, 2009).

Following on from studies to detect the expression of surface antigen, measurement of FXa conversion on the surface of intact monocytes was carried out to assess the combined effect of changes in TF and TFPI protein on the monocyte surface. Reflecting the results of the TF experiments, monocyte surface procoagulant activity was increased in the presence of activated platelets, but not LPS. Therefore, this again suggests that although LPS has induced TF gene expression, the translated protein is expressed in an encrypted form on the monocyte surface, and that in the presence of activated platelets that can provide the negatively charged phospholipid surface, monocyte surface procoagulant activity is enhanced.

Further study was made to investigate the impact of these differences in monocyte surface procoagulant activity on clot formation. Here, the LPS-stimulated monocytes supported TF activity in terms of shortening the lag-time in comparison with unstimulated monocytes and platelet-activated monocytes, as would be consistent with an increase in surface expression of TF, but did not play a role in altering clot formation rate, elasticity or clot profile. As in previous experiments, it was the monocytes that had been incubated with activated platelets that had the differential effect, resulting in higher clot formation rate, lower clot elasticity, and a more compact clot profile, which may suggest a much tighter, more densely structured clot, but is also more typical of a hypo-coagulable ROTEM® trace (Luddington, 2005). This may reflect the presence of possible TFPI surface membrane protein. It must also be borne in mind that this particular assay is not specific for TF and TFPI, and that other monocytes genes may also be modulated following platelet interaction. Data collected by other researchers

within this department have demonstrated that activated platelets can also induced expression of PAI-1 within the monocytes, through a TGF-β-dependent mechanism. A further factor is that following platelet activation some of the platelets will have formed aggregates with the monocytes, and therefore will be present on the monocyte surface during the assay. Platelets may be able to express TF taken up from the plasma (Lopez-Vilchez *et al*, 2007), or possibly synthesise it *de novo* through mRNA splicing mechanisms within the platelet (Schwertz *et al*, 2007), and therefore could directly contribute to increased surface procoagulant activity.

Time constraints restricted further experiments that might resolve some of the questions remaining unanswered. Therefore, a more detailed study is required to assess the location, isotype, activity, and functional significance of monocyte TFPI protein, and also to confirm the level of expression of encrypted and decrypted TF antigen and activity on the monocyte surface, in response to platelet activation, compared to response to inflammatory stimuli.

5.10.2. Gene expression of monocyte TF and TFPI in premature MI and offspring

Possible variation in TF and TFPI monocyte surface expression may lead to some individuals having monocytes with a higher procoagulant activity, which in turn may increase thrombotic risk. Therefore, study was made of TF and TFPI gene expression in monocytes exposed to CRP-XL-activated platelets from individuals who had suffered a premature MI and matched control group. Study was also made of monocytes exposed to the same stimulus, from a younger cohort with a strong family history of MI, again compared with matched controls. Monocytes isolated from the premature MI cohort, and the offspring with the strong family history of MI had resting monocytes that expressed significantly higher levels of TF mRNA, therefore have circulating

monocytes that may have higher procoagulant activity than healthy controls without family history of MI. This same pattern of expression was seen following monocytes exposure to activated platelets. Study of monocyte gene expression of TFPI showed that the MI cohort had resting monocytes with lower levels of TFPI mRNA. Monocytes from this cohort also had lower expression of both TFPI isoforms following monocyte interaction with activated platelets. Therefore, in this population, the combined effect of elevated TF and lower expression of TFPI may result in resting, and 'platelet-activated' monocytes, that are unable to adequately limit procoagulant activity. Interestingly, this differential expression was not seen in the offspring cohorts. This may mean that monocyte gene expression of TFPI is lower due to therapeutic intervention. This is supported by experiments carried out by Julian Van Capelleveen, in this laboratory as part of a separate set of experiments. Measurement of monocyte gene expression of TFPI was carried out in normal healthy donors, following incubation of monocytes with platelets activated in the presence or absence of aspirin. Results suggested that aspirin reduces the amount of monocyte TFPI mRNA. The pathway by which this occurs is at present unknown, but as the MI cohort would be on aspirin and other medication, it may partly explain the lower level of monocyte TFPI mRNA in the MI cohort.

Whereas there have been many studies looking at the expression of monocyte TF in cardiovascular disease, there have been fewer which look at monocyte TFPI, and none which look at the specific isoforms. In this current study, there was predominantly more α-isoform than β in resting monocytes, with levels of expression comparable to those reported in similar studies carried out in endothelial cells, where the β-isoform has been reported to be present at 5 – 10-fold lower levels than the α-isoform (Maroney *et*

al, 2007). There have been no reports of gene expression of TFPI isoforms in a clinical cohort; therefore the findings of this study are novel. Again, further study is needed to confirm this and understand the mechanisms of the expression of the TFPI-α and -β isoforms in the monocyte, and the changes that may occur due to genetic influence and during development of the disease.

5.10.3. Mechanisms of platelet interaction and monocyte TF and TFPI

Following platelet activation, platelet interaction with monocytes occurs via two main mechanisms, that of direct cell-cell interaction via the receptor-ligand binding of platelet-P-selectin and monocyte PSGL-1, or indirectly through monocyte activation by soluble mediators released from the platelet intracellular granules. Therefore, platelet-induced monocyte gene upregulation of TF, and of particular interest, of TFPI, could be specific to one of these mechanisms. Study of isolated monocytes incubated either with plasma containing platelet soluble mediators, or incubated with P-selectin-Fc chimera, which binds to monocyte PSGL-1, demonstrated that it was platelet soluble mediators that induced monocyte gene expression of TFPI. Again, this was a novel finding, and is supported by a recent study demonstrating induced monocyte TFPI mRNA expression following adhesion to extracellular-matrix component, fibronectin (Bajaj *et al*, 2007). Fibronectin, as well as being a component of the ECM, is also found in platelet granules. Therefore, although the Bajaj study is focused on adherent monocytes, and not on monocyte response to soluble mediators, it does demonstrate that one component of the platelet secretome can induce monocyte gene expression of TFPI. Soluble mediators were found to upregulate both isoforms of TFPI, whereas there was no significant expression of either TFPI-α or TFPI-β due to monocyte activation of PSGL-1. Therefore, it appears that platelet-monocyte aggregation is not required for the

induction of monocyte TFPI gene expression. This leads to two main questions; firstly, can we be sure that the induction of monocyte TFPI is due to platelet soluble mediators and not due to monocyte interaction with platelet-MPs; and secondly, which soluble mediators are responsible for the induction of monocyte TFPI? These questions are discussed in more detail below.

5.10.4. Effect of platelet-microparticles on monocyte expression of TF and TFPI

Following cellular activation, remodelling of the plasma membrane results in exposure of phosphatidylserine on the outer leaflet of the plasma membrane, and redistribution of lipid and protein molecules within the membrane. Further cytoskeletal degradation due to changes in the cytosolic calcium concentration results in release of cellular microparticles into the circulation. Microparticles have been detected from various cells, including monocytes, endothelial cells, and platelets. In the circulation of a healthy individual, platelets are thought to be the main source of MPs and exist only at low levels (Berckmans *et al*, 2001), however increased levels of circulating MPs have been reported in acute coronary syndrome (Mallat *et al*, 2000; Werner *et al*, 2006) atherosclerosis (Mallat *et al*, 1999; Leroyer *et al*, 2007), sepsis (Nieuwland *et al*, 2000), and hypertension (Preston *et al*, 2003; Bakouboula *et al*, 2008). Plasma membrane and intracellular components of the microparticles reflect the cell from which they have been derived. Monocyte–derived MPs have been reported to express large amounts of PSGL-1 and TF, but lack CD45, the common white cell differentiation marker (Del Conde *et al*, 2005). In a profiling study of platelet-MPs it was found that the platelet-MP proteome contained >500 proteins, including cell surface molecules GPIb, integrin receptors, VWF; also various coagulation proteins, including fibrinogen, FV, Thrombospondin, and PAI-1, and vitronectin, TGF-β, and several chemokines (Garcia

et al, 2005). It has generally been thought that MPs are procoagulant, with monocyte-MPs reported to have the highest procoagulant activity due to the expression of TF, however, there have also been reports of MPs expressing thrombomodulin (Satta *et al*, 1994), TFPI (Steppich *et al*, 2005) and endothelial cell protein-C receptor (Perez-Casal *et al*, 2005) and so may also have anticoagulant activity. A recent study by Bernimoulin *et al*, (2009) addresses this, comparing the effect of differential stimuli on monocyte-MP phenotype, demonstrating that different populations of cellular-MPs are produced in response to differential stimuli, and raises the possibility that these MP populations may have specific functions. This current study investigated the interaction of platelet-MPs, generated as a result of platelet activation via collagen-related peptide, on the effect of monocyte gene expression of procoagulant TF and anticoagulant TFPI. Monocyte TF was not significantly increased in the presence of platelet-MPs. In contrast, the presence of platelet-MPs did not increase expression of monocyte TFPI, nor was there any difference in expression when P-selectin-PSGL-1 interaction was inhibited. Therefore these results confirm that in the presence of collagen-activated platelets, TFPI gene expression is induced due to the interaction of soluble mediators released from the platelet secretome upon activation. Therefore, which of these soluble mediators may be the key molecules?

5.10.5. Possible inducers of monocyte gene expression of TFPI

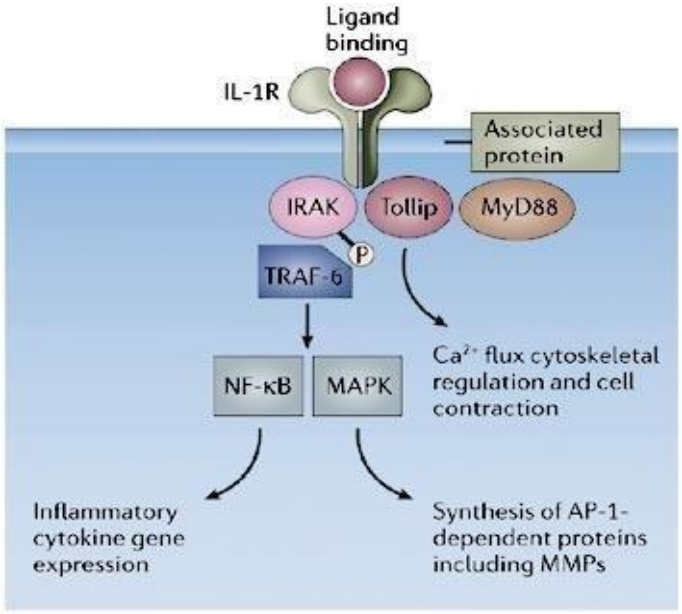
A recent review of platelet proteomic data reported that there are >1100 proteins found in the platelet (Senzel *et al*, 2009), therefore it would be impossible to test all of them. Therefore two soluble mediators found within the platelet secretome which activate different signalling pathways (Figure 5.24), and are present in high levels in the platelet, were selected as possible candidates. These were Interleukin-1β (IL-1β), and

Transforming Growth Factor-β (TGF-β). IL-1β is a pro-inflammatory cytokine that exists as a precursor that is cleaved into its active form by IL-1β-Converting Enzyme (ICE), and then secreted. Platelet IL-1β was first reported by Hawrylowicz *et al*, (1989), and has been reported to induce endothelial secretion of IL-8, and alter chemotactic and adhesive properties of endothelial cells, and induce cytokine production in vascular smooth muscle cells (VSMCs)(Kaplanski *et al*, 1993; Loppnow *et al*, 1998; Gawaz *et al*, 2000). Soluble IL-1β binds to the membrane receptor IL1R1, but requires the receptor accessory protein, IL-1RAcP, to transduce a signal. Receptor binding activates the IL-1 Receptor Activated Kinases, IRAK1 and IRAK2, which in turn lead to activation and recruitment of TRAF6. This activates transcription factors NFκB and c-jun. Gene induction of monocyte TF in response to LPS is known to involve activation of the NFκB transcription factor (Guha *et al*, 2001; Luyendyk *et al*, 2008), and increased expression of TF is associated with the proinflammatory response. Therefore it was expected that inhibition of IL-1β, which also activates NFκB, might lead to a reduction in TF gene expression in the monocyte. Signalling pathways involved in the induction of TFPI are less well documented, and to date there are no reports relevant specifically to the monocyte. Initially it seemed surprising that blocking monocyte interaction with pro-inflammatory IL-1β also resulted in reduced gene expression of TFPI; however it is perhaps reasonable to expect that although a given stimulus may lead to a proinflammatory, or pro-thrombotic response initially, there will also be sequential induction of genes that may counterbalance, or quench that response. Therefore, induction of monocyte TF and TFPI gene expression may be partly through activation of the NFκB pathway, and IL-1β is just one mediator of

activation of that pathway. As expression of neither gene was completely inhibited, the data suggests that gene induction of monocyte TF and TFPI is not solely due to IL-1β.

TGFβ is a cytokine which has numerous roles including regulation of immune response, wound healing, and angiogenesis (Blobe *et al*, 2000). Platelet TGF-β was first identified by Assoian *et al*, (1983), and has been shown to stimulate synthesis of PAI-1 in endothelial cells (Slivka *et al*, 1991). TGFβ binds to Type I and Type II TGFβ receptor complexes. TGF-β signals via two intracellular signalling pathways, firstly via activation of Smad 2 and 3, which form trimers with Smad4 and translocate to the nucleus, where the interaction of co-factors regulate transcriptional responses. The TGFβ receptor complex can also activate a non-Smad pathway through activation of Erk, p38, and JNK MAP kinases, leading to activation of NFκB (Derynck *et al*, 2003; Moustakas *et al*, 2005).

a)



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b)

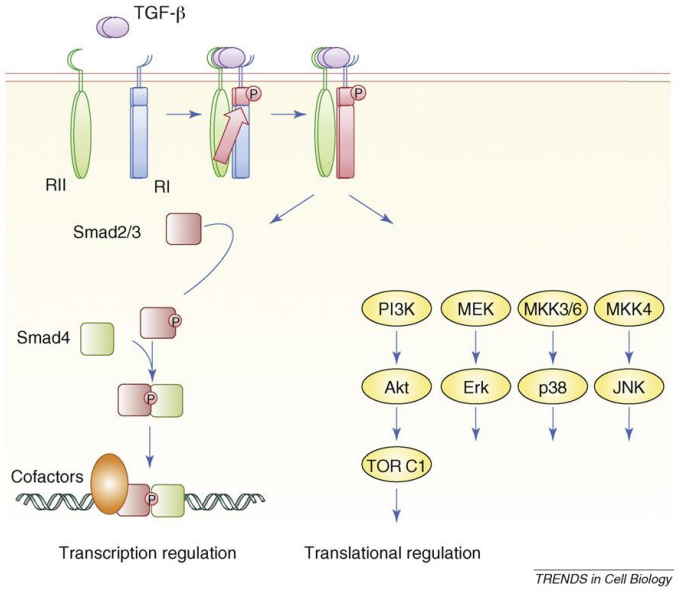


Figure 5.24: Intracellular signalling pathways of Interleukin-1β (IL-1β) and Transforming Growth Factor-β (TGF-β). (a) Soluble IL-1β binds to membrane receptor IL1R1, requiring receptor accessory protein, IL-1RAcP, for signal transduction. Receptor binding activates the IL-1 Receptor Activated Kinases, IRAK1 and IRAK2, followed by activation and recruitment of TRAF6, and finally activation of transcription factors, NFκB and c-jun. (b) TGFβ binds to Type I and Type II TGFβ receptor complexes, which activate two possible pathways. The first via receptor activated Smads (R-SMAD), Smad 2 and 3, and binding of SMAD4, leading to transcriptional upregulation; secondly the TGFβ-receptor complexes can activate Erk, p38 and JNK MAP kinase pathways, resulting in transcriptional upregulation of NFκB.

Therefore, although the SMAD pathway is known not to be involved in gene induction of TF, the alternative pathway of TGF-β signalling leads to activation of NFκB. Therefore it was expected that TGF-β might influence gene expression of TF and TFPI in the monocyte. However, this was not the case, as there was no change of gene expression for either gene when monocyte interaction with TGFβ was blocked. The antibody to TGF-β did, however, inhibit monocytes gene expression of PAI-1, demonstrating that the antibody was capable of binding to its target site. Therefore, this suggests that TGF-β plays no role in gene induction of either TF or TFPI in the monocyte.

Bioinformatic analysis of platelet soluble mediators which may influence gene expression of TFPI revealed three possible pathways that may be involved in induction of monocyte TFPI. The first one comprises the interaction of several growth factors with angiogenic functions, acting mainly via fibroblast growth factor-2 (FGF2), which could be directly responsible for induction of TFPI. FGF2, has a strong affinity for heparin, and regulates the synthesis and deposition of ECM components, therefore playing a pivotal role in reepithelialisation and tissue remodelling (Robson *et al*, 1992; Richard *et al*, 1995; Ornitz *et al*, 2000). Signalling of FGF2 may be further enhanced through the presence of other growth factors, including Vascular Endothelial Growth Factor-alpha (VEGFA), Epidermal Growth Factor (EGF), and Platelet-derived Growth Factor-alpha (PDGFA), which are also found in the granules of platelets, and also play key roles in woundhealing. A second predicted pathway is induction via action of Factor II (Prothrombin). This also is an attractive possibility, as it suggests a negative feedback mechanism regulating the procoagulant response, whereby at a given threshold of prothrombin conversion to thrombin, gene induction occurs of TFPI, the initiator of the coagulation cascade. The third pathway suggests the possible involvement of the transcription factor Kruppel-like Factor-2 (KLF2). KLF2 is an anti-inflammatory, shear-responsive transcription factor, and during the last few years has been shown to regulate quiescence, as well as have atheroprotective and antithrombotic properties in endothelial cells (SenBanerjee *et al*, 2004; Dekker *et al*, 2005; Lin *et al*, 2005; Dekker *et al*, 2006), and may play a role in differentiation of monocytes subsets (Ancuta *et al*, 2009). Therefore, all three activation pathways appear plausible and require further study.

5.10.6. Summary

Monocyte response to inflammatory stimuli, such as LPS, has been well documented (Gregory *et al*, 1989; Guha *et al*; 2001), but less is known about the monocyte response to platelet interaction. The widely-held view is that platelets invoke a pro-inflammatory, procoagulant response in monocytes, acting through binding of platelet P-selectin and monocyte PSGL-1, leading to gene induction of TF and pro-inflammatory cytokines, such as IL-1β and IL-8 (Celi *et al*, 1994; Weyrich *et al*, 1996; Neumann *et al*, 1997; Christersson et al, 2008). Whilst the findings of this study support the role of platelet-monocyte aggregation in monocyte gene induction of TF, the level of induction was low in comparison to the effect of LPS. In addition, activated platelets were found to strongly induce monocytes expression of TFPI, which inhibits TF. Therefore the results of this study demonstrate that platelets actually invoke an anticoagulant response in monocytes.

Further experiments are required to identify the platelet soluble mediators that induce monocyte TFPI, and the transcription factors that regulate this pathway. Experiments are also needed to determine the function of monocyte TFPI, and more specifically, the role of the two TFPI isoforms. However, this study demonstrates the crucial role that platelet soluble mediators may play in modulating the monocyte phenotype. This may prove to have a crucial regulatory role within the thrombus, and therefore these two aspects are explored further in the next two chapters.

In summary:

- CRP-XL-activated platelets induce monocyte gene expression of TFPI.
- Induction of monocyte TFPI gene expression occurs due to monocyte interaction with soluble factors released from the platelet granules, and is not

due to direct monocyte interaction with platelets or platelet microparticles via PSGL-1-P-selectin interaction.

- Human monocytes can be induced to express TFPI-α, and –β mRNA isoforms.
- Monocytes isolated from premature MI subjects express significantly lower levels of TFPI-α mRNA, and significantly higher levels of TF mRNA.

Limitations:

- Insufficient time available to carry out further experiments into monocyte surface protein exposure of TF and TFPI, therefore not possible to draw meaningful conclusions.
- Optimisation experiments should be carried out to confirm activation of monocyte PSGL-1 with the P-selectin-Fc-chimera.
- Experiments using the P-selectin-Fc chimera should also have been carried out using a non-specific-Fc control antibody.

Future work:

- Study of platelet soluble mediators responsible for induction of TFPI – incubation of monocytes with various components of platelet secretome, *eg* recombinant fibronectin, growth factors (especially FGF2), and prothrombin.
- Study of transcriptional regulation of this pathway – focus on *eg*, KLF2, and PPARγ.
- Further study of translation of TFPI mRNA into membrane-bound /soluble protein, by flow cytometry, microscopy, and western blotting. Determination of protein function.

- Further study of TFPI isoforms. Maybe analysis of SNP in TFPI gene and expression of alpha and beta isoforms in the MI and offspring cohort (although numbers small). Experiments investigating possible effect of aspirin on TFPI-β.

CHAPTER 6: The effect of platelet soluble mediators and PSGL-1 activation on monocyte gene expression profile

6.1 Introduction

The effect of platelet activation on monocyte expression of two haemostatic genes emphasises the importance of platelet-monocyte interaction in the regulation of haemostasis. The previous chapter also demonstrates the complexity of the effect of platelet interaction, that of direct cell-cell aggregation and release of intracellular mediators, in the differential activation of intracellular pathways within the monocyte. The platelet 'secretome' is contained within three types of intracellular granules – α-granules, dense granules, and lysosomes. A recent meta-analysis of platelet proteomic studies has suggested that there are >1000 proteins found within the platelet (Senzel *et al*, 2009). The platelet α-granules account for approximately 10% of the platelet volume, and contain chemokines (*eg* CXCL-4, CXCL-1 and CXCL-8) and growth factors (*eg* PDGFβ, FGF2, TGF-β and VEGF), as well as adhesion molecules (*eg* GPIb-IX, von Willebrand Factor, and P-selectin), coagulation factors (*eg* FV, FVIII, FXI, FXIII, fibrinogen, and PAI-1), (Betterle *et al*, 1977; Kaplan *et al*, 1979; Zucker *et al*, 1979; Cramer *et al*, 1985; Harrison *et al*, 1989; Cramer *et al*, 1990; Rendu *et al*, 2001; El Golli *et al*, 2005; Nurden *et al*, 2008; Gleissner *et al*, 2008). The majority of α-granule contents are released into the circulation; however a small number are translocated to the platelet membrane, either because they are integral membrane proteins (P-selectin, GPIb), or bound to the membrane (FV). Dense granules contain metabolites such as ADP, ATP, calcium and magnesium, serotonin, histamine, CD63, and LAMP 2 (White, 1969; Holmsen *et al*, 1979; Nishibori *et al*, 1993; Wojenski *et al*,

1993; Israels *et al*, 1996; Youssefian *et al*, 1997). Platelets also possess lysosomes which contain many enzymes including collagenase, acid phosphatase, heparinase, and Cathespin D and E (Dangelmaier *et al*, 1980; Israels *et al*, 1996; Ciferri *et al*, 2000). Therefore, there is the potential for activation of numerous intracellular signalling pathways within the monocyte, and induction of many genes, through the binding of platelet soluble mediators to monocyte surface receptors.

Microarray technology enables the researcher to obtain a global picture of gene regulation, and therefore this has become a valuable tool for studying changes in gene expression patterns, for example in studies of cell activation and interaction, and studies of disease progression. Microarray studies which investigate monocyte gene expression have mostly focussed on macrophage response to inflammatory stimuli such as LPS, or oxidised-LDL (Shiffman *et al*, 2000; Mikita *et al*, 2001), or on monocyte differentiation into macrophages (Tuomisto *et al*, 2005; Martinez *et al*, 2006), but to date there have been no published microarray studies on changes in the monocyte gene expression profile in response to activated platelets.[1] Therefore, it was decided to expand the previous study, allowing identification of key monocyte genes that have altered expression due to platelet activation, and evaluate how these changes may alter the monocyte phenotype and therefore its function.

6.2 Study design

The purpose of this study was to investigate the changes in monocyte gene expression following exposure to platelet soluble mediators released during platelet degranulation, and to compare these results with alterations to monocyte gene expression induced by the primary interaction that leads to platelet-monocyte aggregation, binding via platelet

[1] A manuscript has recently been submitted from this group (Farrugia *et al*), comparing the differential effects of LPS and platelet interaction on the monocyte transcriptome.

P-selectin and monocyte PSGL-1. Using the same methods as described in the previous chapter, mononuclear cells were isolated from six normal healthy donors, and

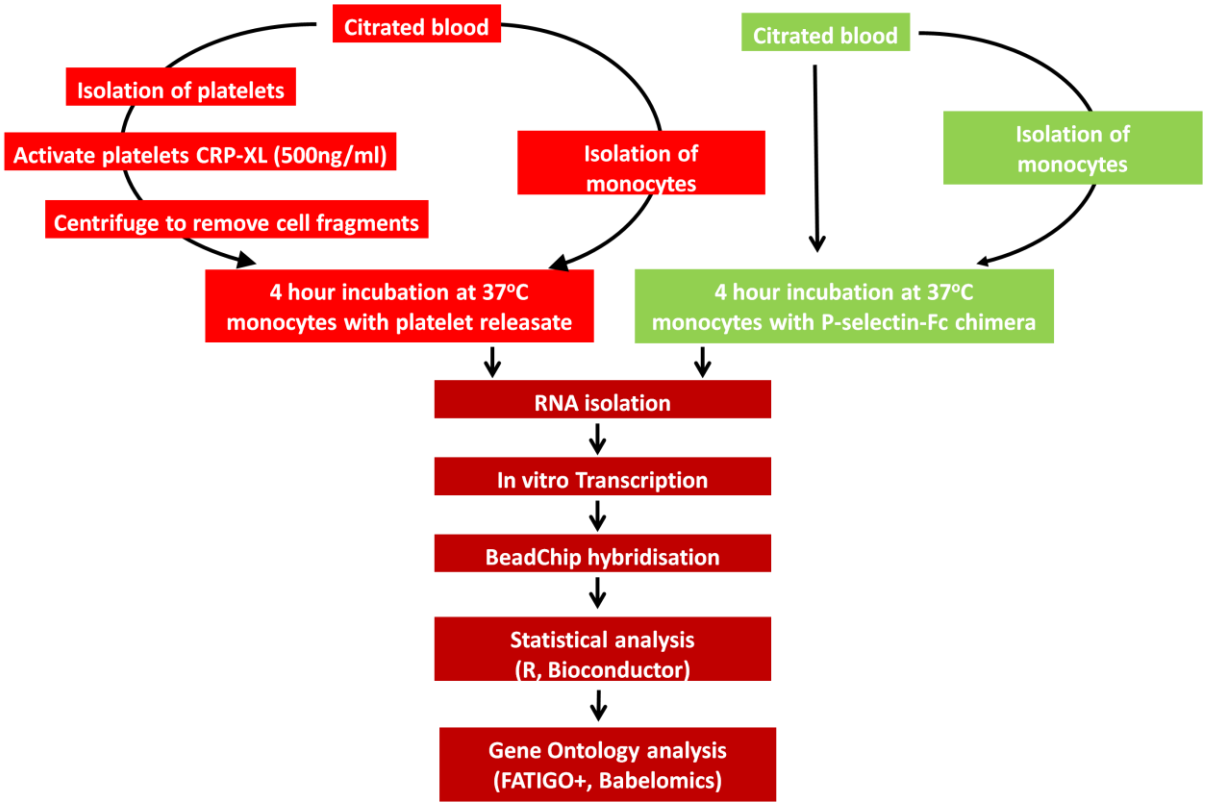


Figure 6.1: Diagram of overview of microarray study investigating the effects of platelet soluble mediators and PSGL-1 activation on monocyte gene expression

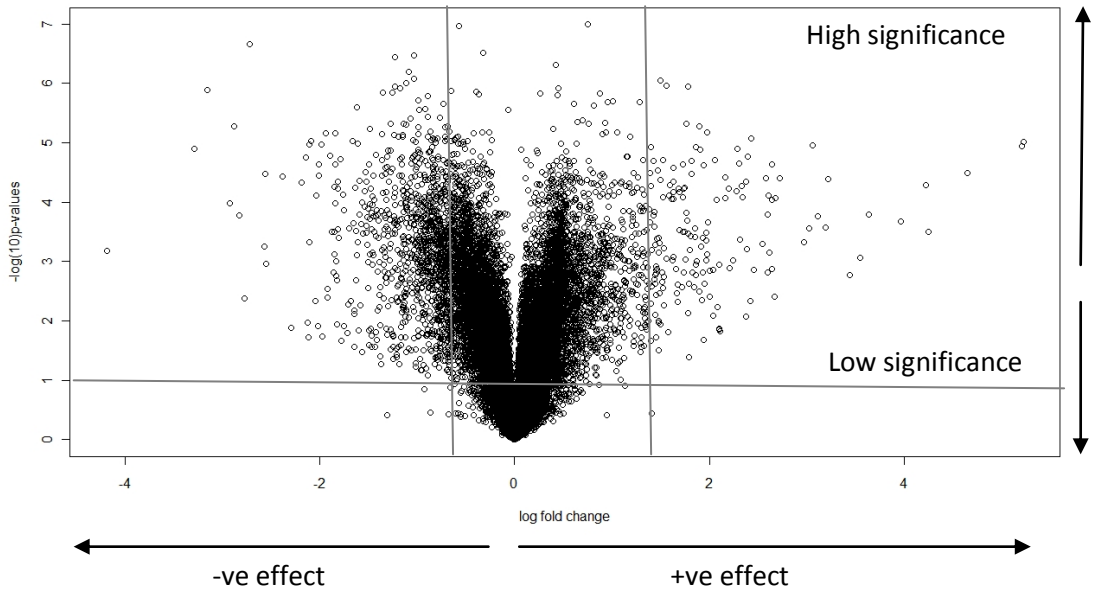
resuspended in autologous plasma containing platelet soluble mediators or in PBS plus P-selectin–Fc-chimera. Mononuclear cells incubated in autologous plasma only, or PBS only, were used as control samples, respectively. CD14+ve monocytes were isolated at baseline and after 4 hour incubation at 37^{o}C, and RNA extracted. Following in vitro transcription (IVT), cRNA was hybridised onto Illumina® Human WG-6 v2 arrays. An overview of the study design is shown in Figure 6.1.

6.3 Results

6.3.1. Initial analysis

The scanned images were imported into BeadStudio (Illumina® Inc) for data extraction, and R (Bioconductor) used to normalise data, perform student's t-test to identify significantly expressed genes, correct for false discovery rate (fdr2d), and generate volcano plots (Figure 6.2). Transcription profiling was carried out, using statistical analyses to identify genes differentially expressed in response to platelet soluble mediators (comparison of 4 hour platelet-poor plasma (PPP) samples vs 4 hour PPP + platelet soluble mediator samples), and in response to monocyte activation via PSGL-1 (comparison of 4 hour PBS samples vs 4 hour PBS + chimera samples). Data was sorted primarily for fdr2d < 0.05; genes with fold change >2 or < 0.5 were considered differentially expressed. Stimulation of monocytes with platelet soluble mediators had the greatest effect on the monocyte transcriptome resulting in changes of gene expression in 397 genes. Stimulation of monocytes via PSGL-1 resulted in changes of gene expression in 143 genes (Figure 6.3). The top 10 up- and down-regulated genes in response to platelet soluble mediators, or PSGL-1 activation can be seen in Tables 6.1 and 6.2, respectively.

a) Platelet soluble mediators



b) PSGL-1

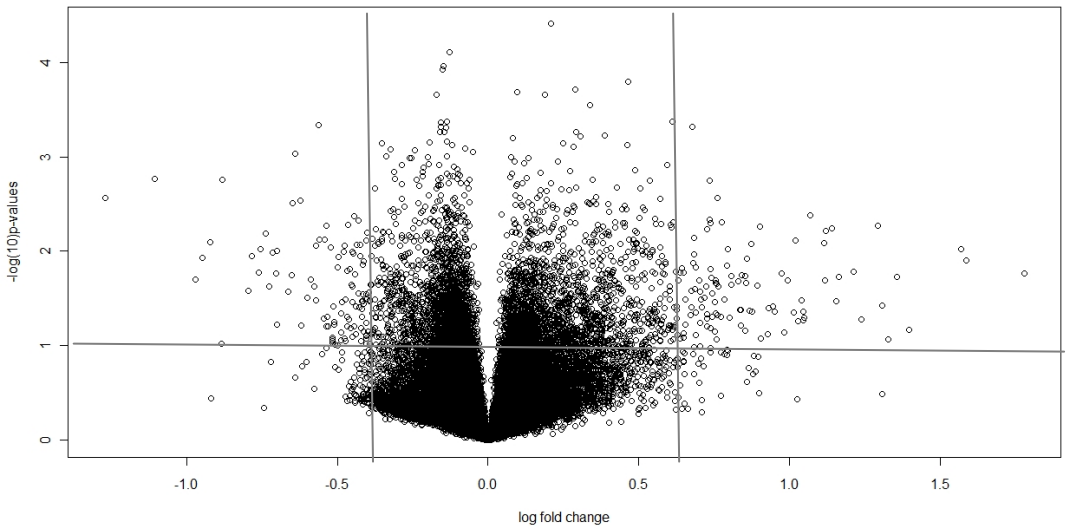
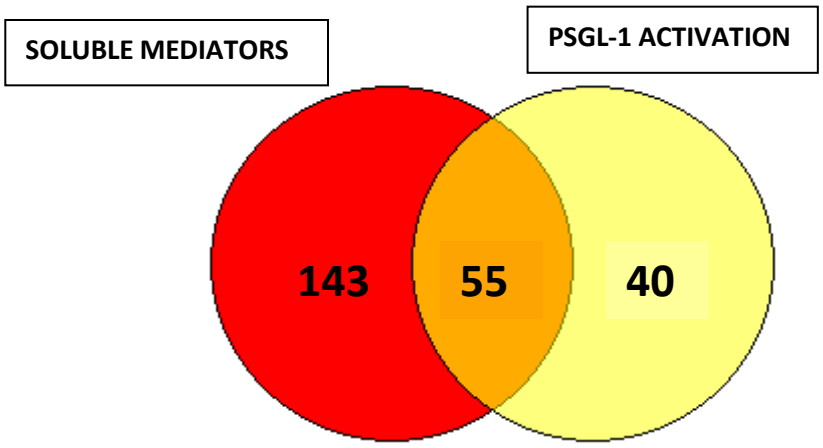


Figure 6.2: Volcano plot of changes in monocyte gene expression profile in response to platelet activation. (a) Plot of changes in monocyte gene expression in response to platelet soluble mediators vs plasma control; (b) Plot of monocyte gene expression in response to PSGL-1 activation vs buffer control. Lines on volcano plots define cut-off points for fold change (x-axis) and p-value (y-axis).

a) Upregulated genes



b) Downregulated genes

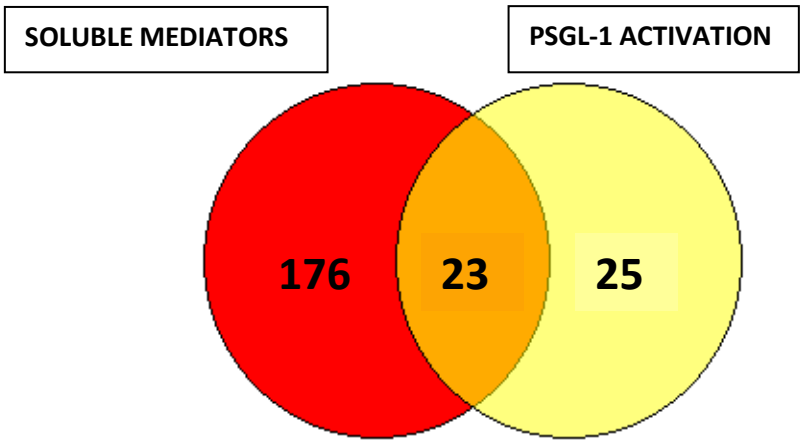


Figure 6.3: Number of monocyte genes induced or downregulated in response to platelet soluble mediators and PSGL-1 activation. Number of genes (a) upregulated, and (b) downregulated > 2-fold change, in response to platelet soluble mediators, PSGL-1 activation or both mechanisms of platelet-monocyte interaction.

(a) Monocyte genes upregulated in response to platelet soluble mediators:

HGNC ID	Description	Fold Change	Fdr2d
CXCL5	C-X-C motif chemokine 5	37.3	0.0016
IL6	Interleukin-6	37.0	0.0014
CXCL1	C-X-C chemokine 1	25.1	0.0020
SERPINB2	Plasminogen activator inhibitor 2	19.0	0.0057
KIAA1199	Putative uncharacterised protein KIAA1199	18.6	0.0021
CXCL6	C-X-C chemokine 6	15.6	0.0045
PTGS2	Prostaglandin G/H synthase 2	12.4	0.0021
CXCL2	C-X-C chemokine 2	11.7	0.0036
CCL7	C-C chemokine motif 7	10.8	0.0081
HEY1	Hairy/enhancer-of-split related with YRPW motif protein 1	9.3	0.0012

(b) Monocyte genes downregulated in response to platelet soluble mediators:

HGNC ID	Description	Fold Change	Fdr2d
CLC	Lysophospholipase	-18.2	0.0013
CCR2	C-C chemokine receptor type 2	-9.7	0.0021
LOC653518	Unmapped	-8.9	0.0021
PDK4	Pyruvate dehydrogenase kinase isozyme 4	-7.6	0.0035
CEBPA	CCAAT/enhancer-binding protein alpha	-7.4	0.0014
GIMAP7	GTPase IMAP family member 7	-7.1	0.0059
HDC	Histidine carboxylase	-6.8	0.0426
LOC653518	Unmapped	-6.6	0.0022
GIMAP8	GTPase IMAP family member 8	-5.9	0.0022
CPA3	Carboxypeptidase A3	-5.9	0.0022

Table 6.1: Monocyte genes showing greatest response to platelet soluble mediators. (a) Upregulated genes; (b) Downregulated genes. (Data analysed by *p*-value and fold change).

Monocyte genes upregulated in response to PSGL-1 activation:

HGNC ID	Description	Fold Change	Fdr2d
EBI3	Interleukin-27 subunit beta	2.5	0.0472
TNF	Tumour Necrosis Factor	2.2	0.0021
LOC643930	unknown	2.1	0.0018
IL23A	Interleukin-23 subunit alpha	2.1	5.30E-08
IL1A	Interleukin-1 alpha	1.8	3.06E-06
PTX3	Pentraxin-related protein PTX3	1.7	0.0036
ICAM1	Intercellular adhesion molecule 1	1.7	0.0348
DCUN1D3	DCUN1 domain-containing protein 3	1.7	0.0397
TLR7	Toll-like receptor 7	1.7	0.0044
TRAF1	TNF receptor-associated factor 1	1.6	0.0009

Monocyte genes downregulated in response to PSGL-1 activation:

HGNC ID	Description	Fold Change	Fdr2d
RGS1	Regulator of G-protein signalling 1	-1.7	8.55E-05
ERRFI1	ERBB receptor feedback inhibitor 1	-1.6	0.04506
ARL4C	ADP-ribosylation factor-like protein 4C	-1.6	3.78E-06
SLC2A14	Solute carrier family 2, facilitated glucose transporter member 14	-1.5	0.0044
HK2	Hexokinase 2	-1.5	0.0039
P2RY5	P2Y Purinergic receptor 5	-1.5	0.0023
AVPI1	Arginine vasopressin-induced protein 1	-1.4	0.0089
ANKRD37	Ankyrin repeat domain-containing protein 37	-1.4	0.0035
GNA13	Guanine nucleotide binding protein alpha 13	-1.4	0.0036
DUSP10	Dual specificity protein phosphatase 10	-1.4	0.0015

Table 6.2: Monocyte genes showing greatest response to activation via PSGL-1. (a) Upregulated genes; (b) Downregulated genes. (Data analysed by *p*-value and fold change).

6.3.2. Gene ontology analysis

The top 200 upregulated and downregulated genes of each sample treatment were then further analysed. Functional Enrichment analysis was carried out using FATIGO+ (Babelomics v3.1) and UniPro, to identify key biological processes that the various genes may be involved in (Figures 6.4 and 6.5). Due to a strong induction of genes associated with chemotaxis (GO:0006935), blood coagulation (GO:0007596), and angiogenesis (GO:0009611), these gene ontology terms were selected for further investigation. In addition, further study was carried out for ontology analysis for cell adhesion (GO:0007155), and cellular lipid metabolic process (GO:0044255), to investigate how the two platelet mechanisms may differentially modulate monocyte adhesion, and lipid homeostasis within the monocyte. Definitions of these ontology classifications are defined in Table 6.3. Genes represented in each category are detailed in Tables 6.4 and 6.5. The results for each ontology term are described in more detail below.

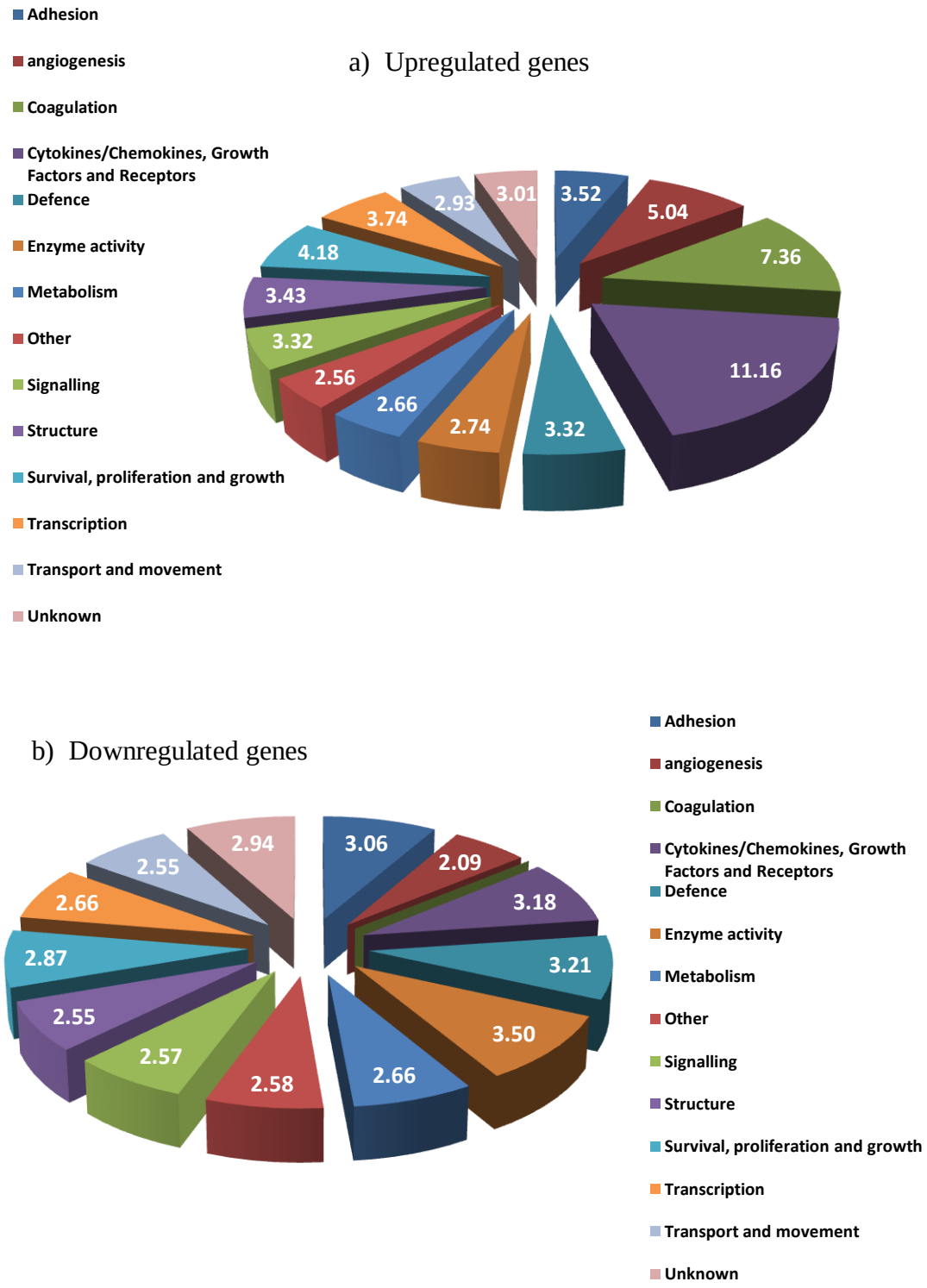
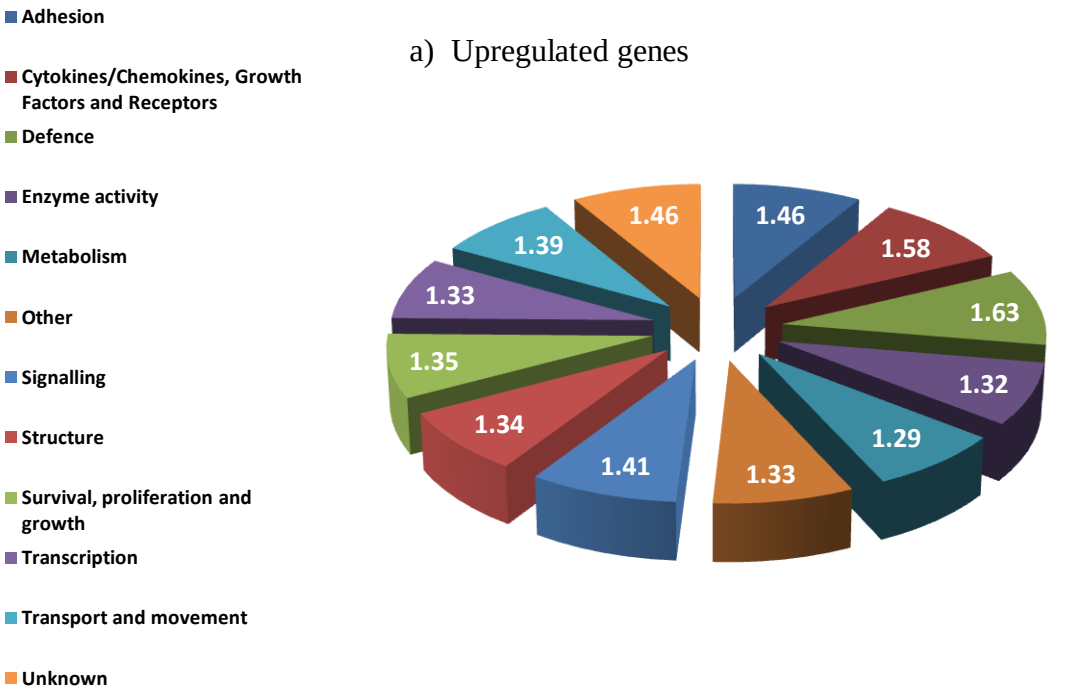


Figure 6.4: Functional distribution of monocyte genes regulated by platelet soluble mediators. Monocyte genes (a) induced and (b) downregulated, by platelet soluble mediators. (Figures represent mean fold change of genes in each category).

a) Upregulated genes



b) Downregulated genes

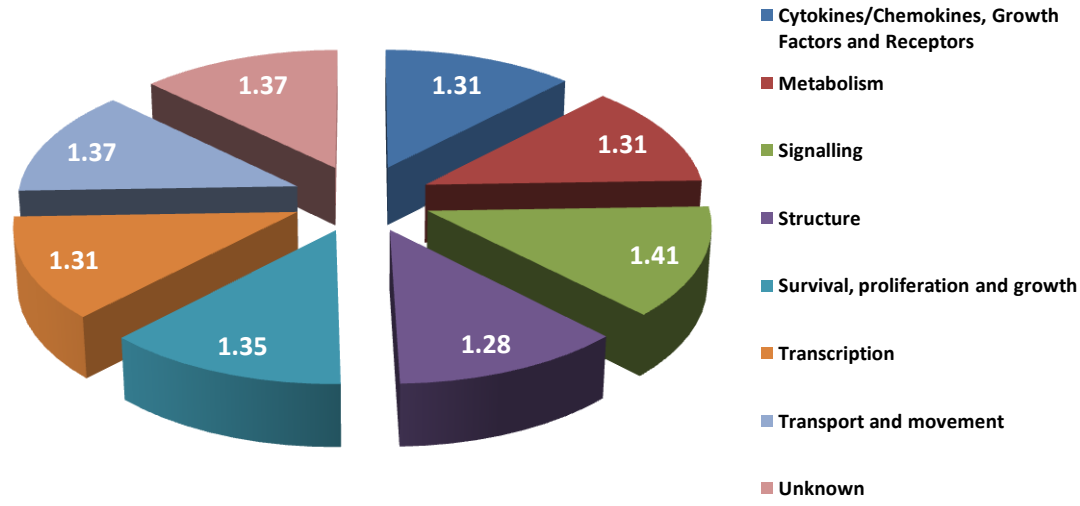


Figure 6.5: Functional distribution of monocyte genes regulated by PSGL-1 activation. Monocyte genes (a) induced and (b) downregulated, by PSGL-1 activation. (Figures represent mean fold change of gene in each category).

GO: CLASSIFICATION	GO: DEFINITION
BLOOD COAGULATION	The sequential process by which the multiple coagulation factors of the blood interact, ultimately resulting in the formation of an insoluble fibrin clot
CHEMOTAXIS	The directed movement of a motile cell or organism, or directed growth of a cell guided by a specific chemical concentration gradient
CELL ADHESION	The attachment of a cell, either to another cell or to an underlying substrate such as the extracellular matrix, via cell adhesion molecules
RESPONSE TO WOUNDING	A change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression) as a result of a stimulus indicating damage to the organism
CELLULAR LIPID METABOLIC PROCESS	The chemical reactions and pathways involving lipids, as carried out by individual cells

Table 6.3: Definition of Gene Ontology (GO) terms. Table shows key gene ontology definitions selected for further analysis, as defined by FATIGO (Babelomics).

GENE SYMBOL		FOLD CHANGE	P-VALUE
	ADHESION		
CDCP1	CUB domain containing protein 1	5.22	0.00062
LAMB3	Laminin, beta 3	4.59	0.00059
CD44	CD244 antigen	4.17	0.00275
LYPD3	Ly6/PLAUR domain containing 3	3.33	0.00059
PLEKHC1	Pleckstrin homology domain containing family C	2.99	0.00079
ANTXR2	Anthrax toxin receptor 2	2.63	0.00053
ITGB8	Integrin beta 8	2.61	0.00236
COL22A1	Collagen type XXII, alpha 1	2.43	0.00011
CD84	CD84 antigen	-4.31	0.00011
CD9	CD9 antigen	-4.02	0.00023
FUT7	Fucosyltransferase 7	-3.52	0.00045
FLOT2	Flotillin	-3.25	0.000279
ITM2C	Integral membrane protein 2C	-3.09	0.002071
ITGAL	Integrin, alpha L (CD11a)	-3.02	0.00044
PSTPIP1	Pro-Ser-Thr phosphatase interacting protein 1	-2.82	0.000023
EVA1	Epithelial V-like antigen 1	-2.78	0.00059
FGL2	Fibrinogen-like 2	-2.35	0.000296
PALLD	Palladin	-2.30	0.000646
CD244	CD244 natural killer cell receptor 2B4	-2.25	0.00019
GCA	Grancalcin	-2.21	0.006467
CDK5	Cyclin-dependent kinase 5	-2.02	0.000009
	ANGIOGENESIS		
PTGS2	Prostaglandin-G/H synthase	12.41	0.00016
MMP19	Matrix metallopeptidase 19	8.34	0.00011
EREG	Epiregulin	5.20	0.00046
TPST1	Tyrosylprotein sulfotransferase 1	4.87	0.00013
BMP6	Bone morphogenetic protein 6	4.11	0.00136
ANGPTL4	Angiopoietin-like 4	4.02	0.00141
SLAMF9	SLAM family member 9	3.18	0.00338
HPSE	Heparanase	3.08	0.00096
MMP10	Matrix metallopeptidase 10 (stromelysin)	2.75	0.00135
HRH1	Histamine receptor H1	2.14	0.00048
HMOX1	Heme oxygenase 1	-2.09	0.00082
	COAGULATION		
SERPINB2	Serpin peptidase inhibitor, clade B	19.0	0.00032
TFPI	Tissue Factor Pathway Inhibitor	6.07	0.00079
F3	Tissue factor	3.97	0.00022
CD59	Complement regulatory protein	2.68	0.00020
CD9	Tetraspanin CD9	-4.02	0.00446
EVL	Ena-VASP-like protein	-2.62	0.00398

Table 6.4a: Selected gene list of mRNA transcripts in monocytes following exposure to platelet soluble mediators: Adhesion, angiogenesis, and coagulation. (- denotes downregulated gene transcript).

	CYTOKINES, CHEMOKINES, GROWTH FACTORS AND RECEPTORS		
GENE SYMBOL		**FOLD CHANGE**	**P-VALUE**
CXCL5	Chemokine (C-X-C motif) ligand 5	37.30	0.00162
IL6	Interleukin-6	36.98	0.00146
CXCL1	Chemokine (C-X-C motif) ligand 1	25.06	0.00199
CXCL6	Chemokine (C-X-C motif) ligand 6	15.64	0.00454
CXCL2	Chemokine (C-X-C motif) ligand 2	11.68	0.00358
CCL7	Chemokine (C-C motif) ligand 7	10.84	0.00807
1L1A	Interleukin 1 alpha	9.16	0.0021
IL1F9	Interleukin 1 family, member 9	6.14	0.00412
IL1R1	Interleukin 1 receptor, type 1	3.63	0.00579
CCL3	Chemokine (C-C motif) ligand 3	3.54	0.02963
CXCL3	Chemokine (C-X-C motif) ligand 3	3.49	0.00354
CCL3L3	Chemokine (C-C motif) ligand 3-like 3	3.45	0.03582
FLT4	Fms-related tyrosine kinase 4	3.43	0.00275
IL10	Interleukin-10	3.34	0.00393
IL1B	Interleukin 1, beta	2.57	0.04116
VEGF	Vascular endothelial growth factor	2.31	0.00452
CCR2	Chemokine (C-C motif) receptor 2	-9.75	0.00013
CCR3	Chemokine (C-C motif) receptor 3	-4.31	0.000476
IL4	Interleukin 4	-3.62	0.001538
IL27RA	Interleukin 27 receptor, alpha	-3.51	0.00059
TNFAIP8L1	Tumour necrosis alpha- induced protein 8, like 1	-2.71	0.00091
CX3CR1	Chemokine (C-X3-C motif) receptor 1	-2.43	0.000601
CMKLR1	Chemokine-like receptor 1	-2.25	0.00074
IL17R	Interleukin 17 receptor	-2.21	0.00063
IL18R1	Interleukin 18 receptor 1	-2.21	0.010658
IL13RA1	Interleukin 13 receptor, alpha 1	-2.15	0.000121
CMTM3	CKLF-like MARVEL transmembrane domain containing 3	-2.12	0.00048
IL10RA	Interleukin 10 receptor, alpha	-2.02	0.000177
PTAFR	Platelet-activating factor receptor	-2.00	0.000176

Table 6.4b: Selected gene list of mRNA transcripts in monocytes following exposure to platelet soluble mediators: Cytokines, chemokines, growth factors and receptors. (- denotes downregulated gene transcript)

GENE SYMBOL	DEFENCE	FOLD CHANGE	P-VALUE
ERRFI1	ERBB receptor feedback inhibitor 1	6.35	0.00398
CMKOR1	Chemokine orphan receptor 1	5.81	0.00051
OASL	2'-5'-oligoadenylate synthetase-like	4.28	0.00114
RSAD2	Radical S-adenosyl methionine domain containing 2	4.24	0.00578
G1P2	Interferon, alpha-inducible protein	3.99	0.00352
TNFSF8	Tumour necrosis factor superfamily, member 8	3.81	0.0004
TNF	Tumour necrosis factor	2.86	0.00224
MX1	Myxovirus resistance 1	2.72	0.0078
OAS2	2'-5'-oligoadenylate synthetase 2	2.71	0.00238
MX2	Myxovirus resistance 2	2.47	0.00063
DNAJC3	DNAj (HSP40) homolog, subfamily C, member 3	2.44	0.000021
DNAJB5	DNAj (HSP40) homolog, subfamily B, member 5	2.41	0.00039
LAIR2	Leukocyte associated Ig-like receptor 2	2.31	0.00068
IFNB1	Inteferon beta 1	2.26	0.00098
TRIM5	Tripartite motif-containing 5	2.13	0.00618
IL1RL2	Interleukin 1 receptor-like 2	2.02	0.00078
FCER1A	Fc fragment of IgE, high affinity I, receptor 1 alpha	-5.85	0.001113
CD1D	CD1D antigen	-5.23	0.00037
IRF8	Interferon regulatory factor 8	-3.55	0.000982
TLR7	Toll-like receptor 7	-3.36	0.000138
MS4A2	Membrane-spanning 4-domains, subfamily A, member 3	-2.84	0.002038
TLR6	Toll-like receptor 6	-2.38	0.00034
TLR8	Toll-like receptor 8	-2.34	0.001299
CLEC4C	C-type lectin domain family 4, member C	-2.19	0.003301
CLEC10A	C-type lectin domain family 10, member A	-2.18	0.001442
TNFAIP8L2	Tumour necrosis factor, alpha-induced protein 8-like 2	-2.16	0.00049

Table 6.4c: Selected gene list of mRNA transcripts in monocytes following exposure to platelet soluble mediators: Defence. (- denotes downregulated gene transcript)

GENE SYMBOL		FOLD CHANGE	P-VALUE
	ADHESION		
ICAM1	Intercellular adhesion molecule 1	1.67	0.10454
LYPD3	Ly6/PLAUR domain-containing protein 3	1.45	0.0018
TSPAN17	Tetraspanin-17	1.25	0.02977
CTNNB1	Catenin beta, 1	-1.28	0.23687
	COAGULATION		
ADORA2	Adenosine A2a receptor	1.49	0.069771
GNA13	Guanine nucleotide-binding protein alpha-13 subunit	-1.39	0.03225
	CYOKINES, CHEMOKINES, GROWTH FACTORS AND RECEPTORS		
EBI3	Interleukin-27 beta chain	2.48	0.03733
IL23A	Interleukin 23 subunit alpha	2.06	0.04318
IL1A	Interleukin 1 alpha	1.78	0.04175
CCL3L1	Chemokine (C-C motif) ligand 3-like 1	1.48	0.29972
IL1RN	Interleukin 1 receptor	1.45	0.04895
CCL3	Chemokine (C-C motif) ligand 3	1.44	0.09188
CCL4L1	Chemokine (C-C motif) ligand 4-like 1	1.38	0.13445
CXCL2	Chemokine (C-X-C motif) ligand 2	1.37	0.09582
CCL20	Chemokine (C-C motif) ligand 20	1.34	0.0601
CCL3L3	Chemokine (C-C motif) ligand 3-like 3	1.33	0.05021
LILRA2	Leukocyte immunoglobulin-like receptor subfamily A, member 2	1.25	0.07662
CXCR4	C-X-C chemokine receptor type 4	-1.31	0.0296
	DEFENCE		
PTX3	Pentraxin-related protein	1.68	0.04931
TLR7	Toll-like receptor 7	1.65	0.06213
TLR8	Toll-like receptor 8	1.40	0.01691
TICAM1	Toll-like receptor adaptor molecule 1	1.31	0.0006

Table 6.5: Selected gene list of mRNA transcripts in monocytes following activation via PSGL-1: Adhesion; Coagulation; Cytokines, chemokines, growth factors and receptors; Defence. (- denotes downregulated gene transcript)

6.3.2.1. Blood Coagulation

Key genes associated with blood coagulation that were upregulated in the monocyte in response to platelet soluble mediators were TFPI, therefore confirming the results of experiments reported in the previous chapter, and F3 (TF). Other genes associated with blood coagulation included upregulation of CD59, the membrane complex attack inhibition factor, and downregulation of EVL, an Ena-VASP-like protein, and tetraspanin CD9. Gene expression altered in response to PSGL-1 activation included ADORA2A, an adenosine receptor, and downregulation of GNA13, a guanine nucleotide binding protein. A summary of these changes in monocyte gene expression of blood coagulation genes can be seen in Figure 6.6. All gene ontology images were generated by Dr Stefan Amisten using Ingenuity Pathway® software (Ingenuity Systems Incorporated (Redwood City, California, USA).

Due to specific interest in monocyte gene expression of TF and TFPI, TaqMan® gene expression assays of TF and TFPI were carried out to validate the array results. Fluorescence Intensity (FI) of a small number of genes that are often used as 'housekeeping genes', *ie*, genes that are expressed constitutively and without fluctuation in response to stimuli, were first analysed for identification an appropriate endogenous control gene for PCR analysis of the samples obtained in this study. FI was very variable for the commonly used housekeeping genes Glyceraldehyde Phosphate Dehydrogenase (GAPDH) and β-actin, and more consistent for TATA Box Binding Protein and β_2-Microglobulin. Therefore, β_2-Microglobulin was selected as endogenous control for normalisation of PCR assay data, and fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Figure 6.7).

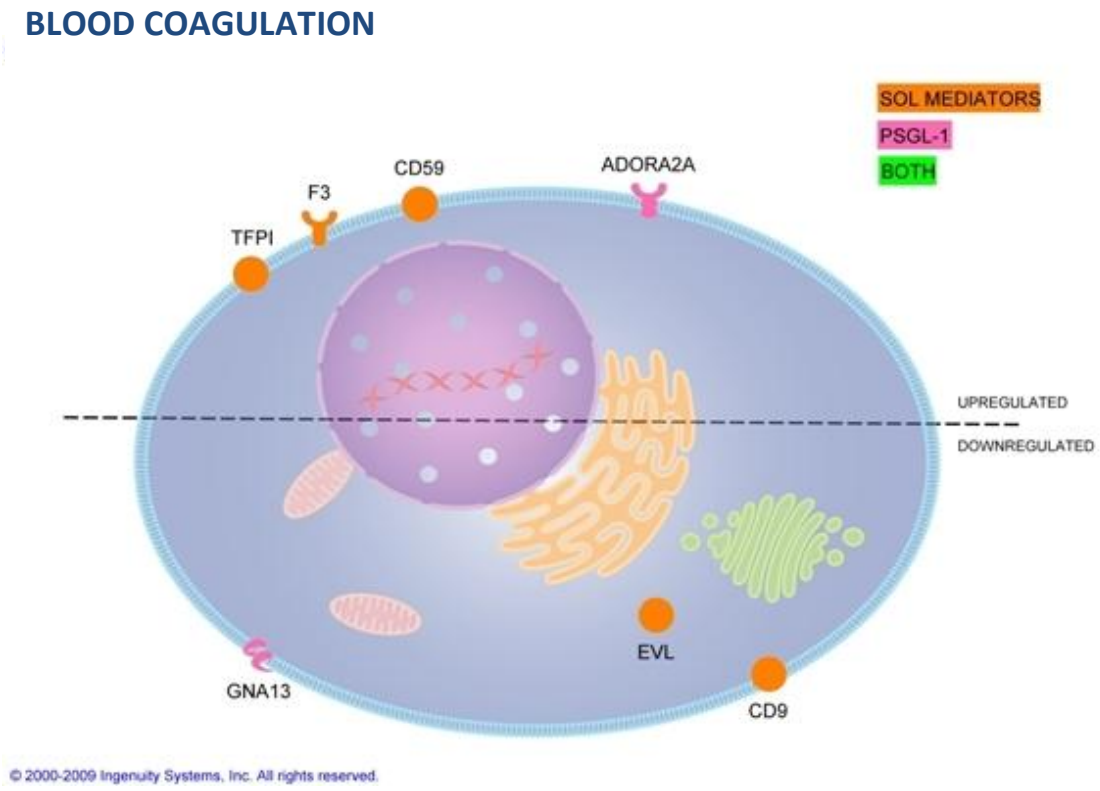


Figure 6.6: Changes in monocyte genes involved in coagulation.

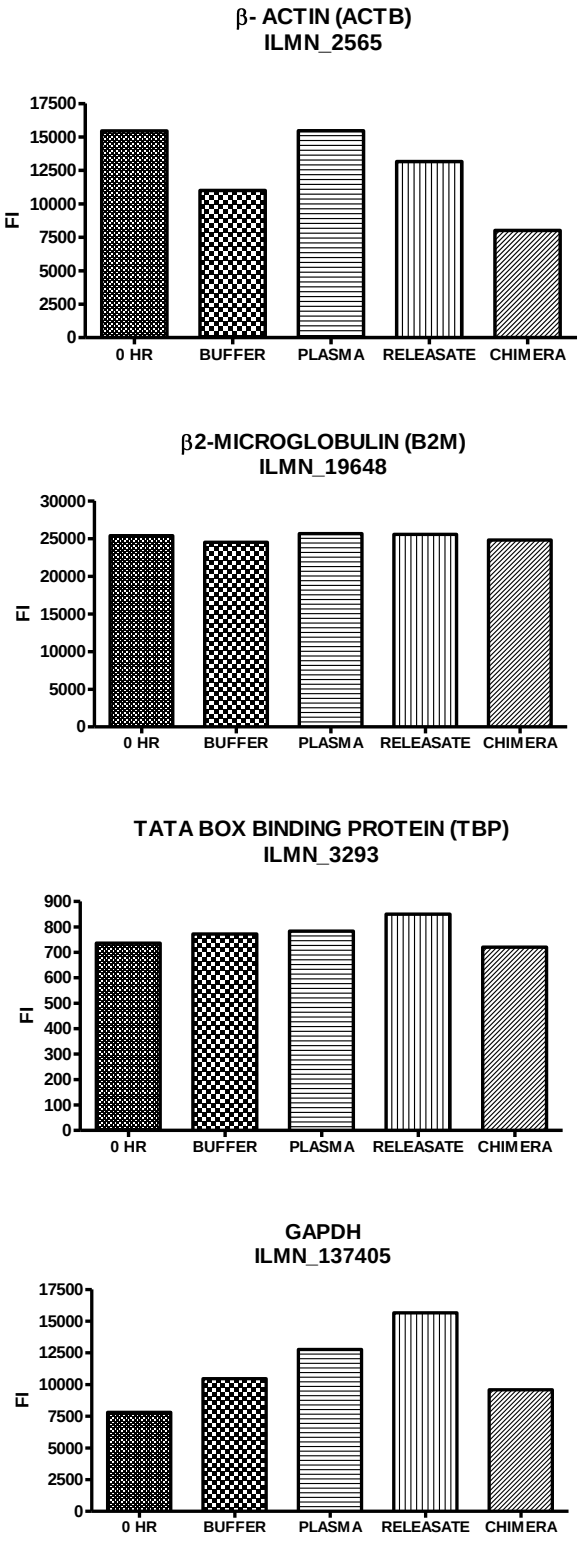
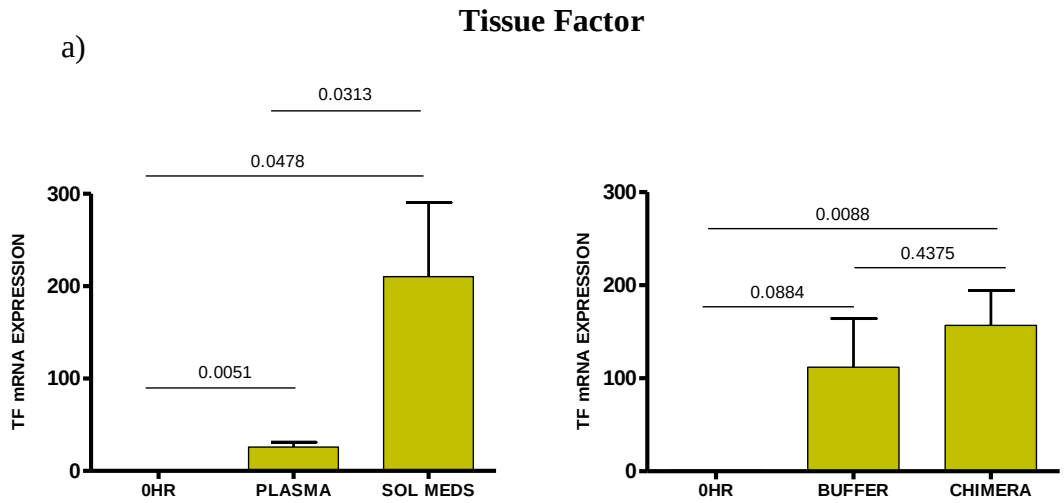


Figure 6.7: Comparison of monocyte gene expression of possible endogenous controls. Fluorescence Intensity was recorded for each 'house-keeping gene' following incubation of monocytes with each stimulus. β2-microglobulin was selected as endogenous control for Taqman PCR validation assays.



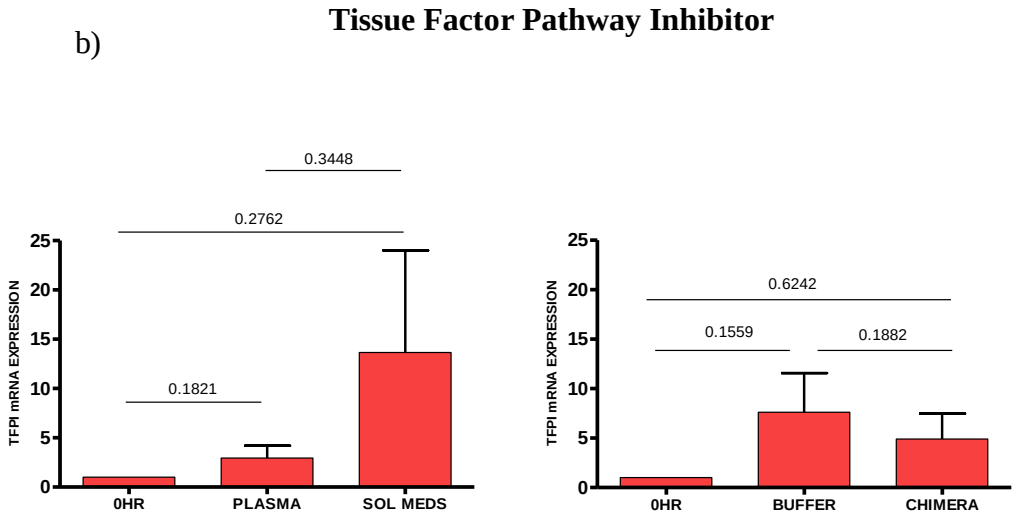


Figure 6.8: Validation of array data for Tissue Factor (TF) and Tissue Factor Pathway Inhibitor (TFPI) using TaqMan PCR. (a) Monocyte gene expression of TF following exposure to platelet soluble mediators or PSGL-1 activation, respectively; (b) Monocyte gene expression of TFPI following exposure to platelet soluble mediators or activation via PSGL-1, respectively. (Figures are Mean ± SEM; n = 6).

Whereas monocyte gene expression of TF was induced by both mechanisms of platelet activation (p=0.0478; and p=0.0088, for soluble mediators v PSGL-1 activation, respectively), monocyte gene expression of TFPI was only induced by platelet soluble mediators, but did not reach significance (p= 0.2762)(Figure 6.8).

6.3.2.2. Chemotaxis

Platelet soluble mediators induced monocyte expression of a large number of chemokines and inflammatory cytokine genes; C-C chemokines upregulated included CCL7, CCL3, and CCL3L3. C-X-C chemokines were significantly upregulated, including CXCL5, CXCL1, CXCL6, CXCL2, and CXCL3. Upregulation of cytokines included IL6, IL10, IL1B, IL1A, and IL1R1. Meanwhile there was downregulation of a number of key chemokine and cytokine receptors including CCR2, CCR3, and CX3CR1, and of IL4. Other genes downregulated in response to platelet soluble mediators in this GO category included PTAFR, the Platelet-Activating Factor receptor, CMTM3 – CKLF-like MARVEL transmembrane domain-containing protein-3, a member of the chemokine family, and CMKLR – chemokine receptor-like-1, an orphan receptor that could be a chemotactic peptide receptor.

A similar pattern of chemokine induction was seen in response to PSGL-1 activation with upregulation of C-C chemokines CCL3L1, CCL3L3, CCL3 and CCL20, and C-X-C chemokine CXCL2. Upregulation of cytokines included Il23A, IL1A, and receptor IL1RN. Meanwhile there was downregulation of CXCR4 receptor.

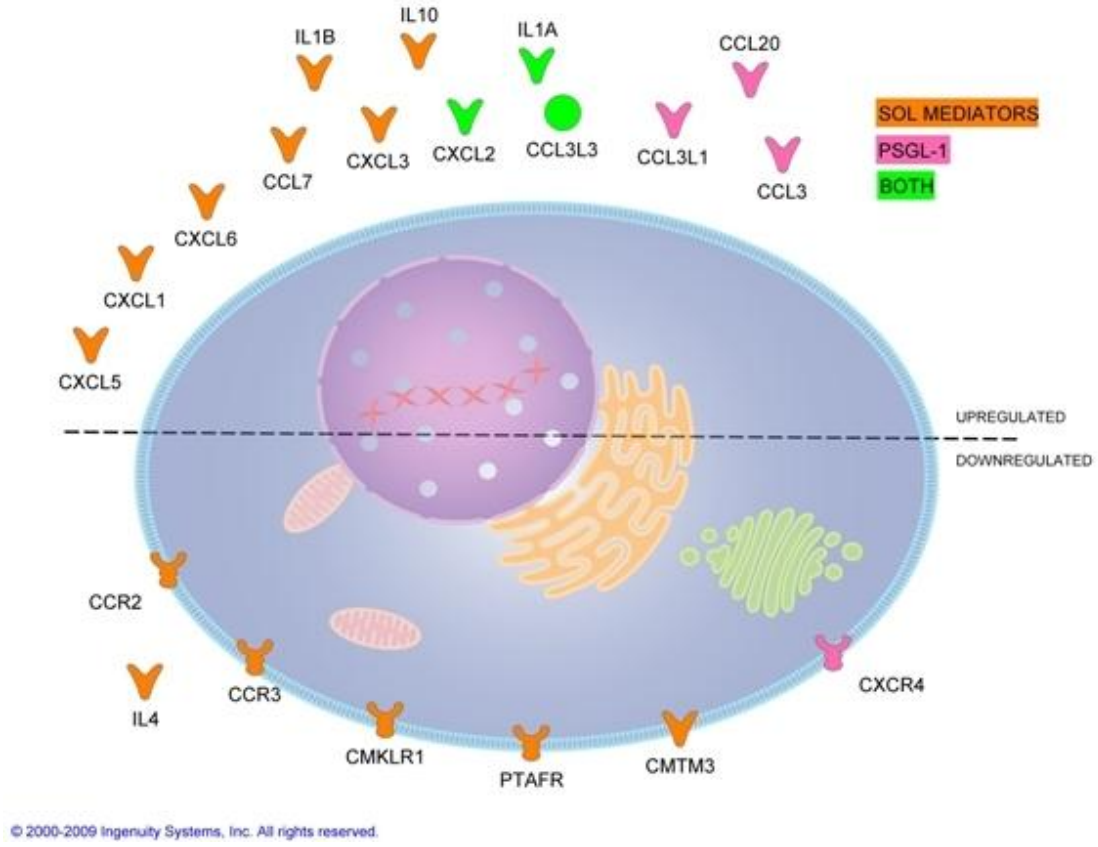


Figure 6.9: Changes in monocyte genes involved in chemotaxis.

Overall there was significant upregulation of genes that code for secreted proteins that induce chemotaxis of various cells, eg neutrophils and monocytes (required for release of superoxide, and metalloproteinases, etc into a wound site and phagocytosis). In contrast there was downregulation of several genes that encoded membrane-bound chemokine receptors. A summary of these changes in monocyte expression of genes involved in chemotaxis can be seen in Figure 6.9.

6.3.2.3. Cell Adhesion

Genes that were specifically upregulated in response to platelet soluble mediators included LAMB3, a subunit of laminin, a major component of the endothelial basement membrane, CD44, an adhesion receptor that has also recently been suggested to be a possible ligand for E-selectin, PLEKHC1, which is involved in the connection of ECM adhesion sites and the actin cytoskeleton, ITGB8, a subunit of the fibronectin receptor, and COL22A1, collagen alpha-1 (XXII) chain which is secreted into the ECM. Genes that were downregulated include CD84, an adhesion receptor involved in homophilic interactions which enhance IFNγ secretion, tetraspanin CD9, ITGAL, also known as CD11a a receptor for ICAM, FLOT2, a scaffolding protein within caveolar membranes, EVA1, which mediates homophilic cell-cell adhesion, PSTPIP1, which is involved in regulation of the actin cytoskeleton, and PALLD, also required for organisation of the actin cytoskeleton. FUT7, a fucosyl-transferase required for expression of sialyl Lewis X antigens on selectin molecules, is also downregulated due to platelet soluble mediators. Interestingly, ICAM1 was specifically upregulated by PSGL-1.

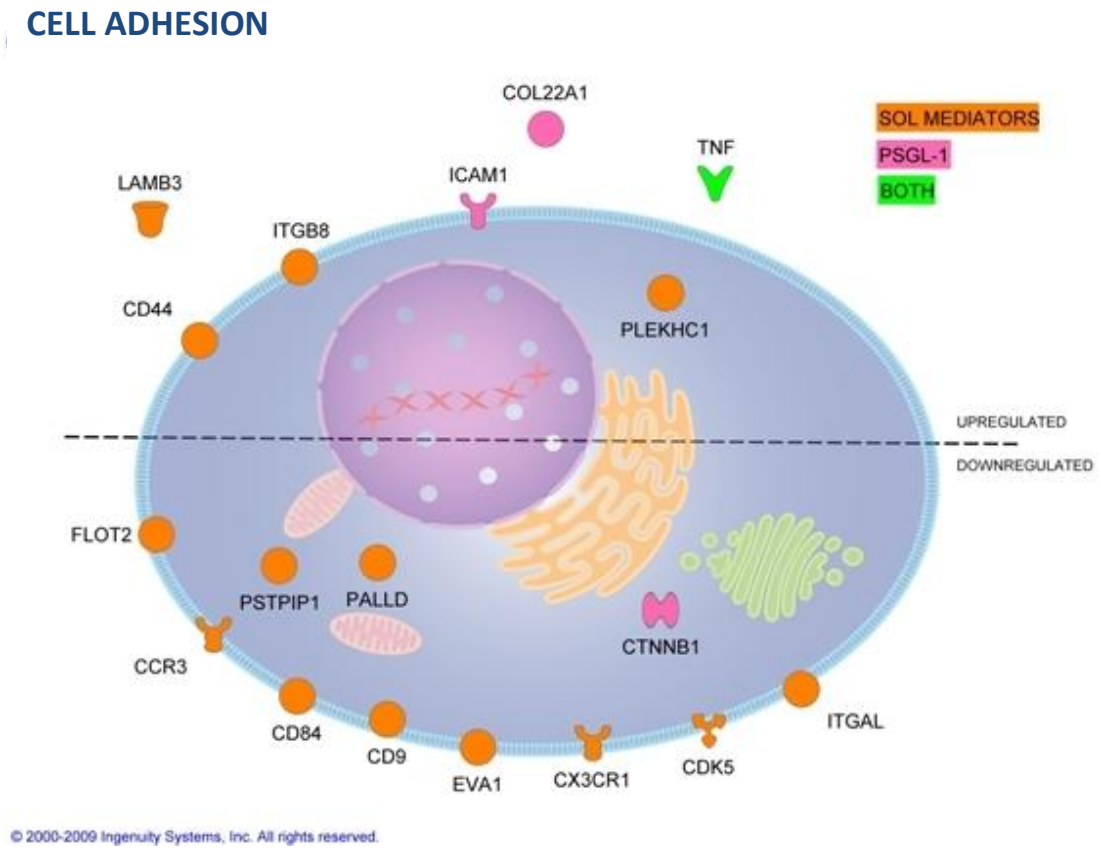


Figure 6.10: Changes in monocyte genes involved in cell adhesion

Therefore, overall there was significant increase in endothelial adhesion receptors and secreted proteins that play a role in maintaining the ECM. Again there was strong downregulation of various cell membrane receptors. A summary of changes in monocyte expression of genes involved in cellular adhesion can be seen in Figure 6.10.

6.3.2.4. Response to Wounding

Response to vascular injury involves various biological processes including luminal narrowing, smooth muscle cell accumulation, inflammatory cell recruitment, adhesion, clot formation and endothelial regeneration. Therefore many of the genes defined as being involved in the wound healing process overlap with the previous categories of blood coagulation, cell adhesion, and chemotaxis. Chemokines and chemokine receptors play a key role in the control of each step of wound healing, and this was reflected in the results of this analysis, with platelet soluble mediators, and PSGL-1 activation inducing monocyte gene expression of a large number of chemokines and inflammatory cytokines. In addition to genes involved in chemotaxis, cell adhesion and coagulation, other genes in this ontology term that were modulated by platelet soluble mediators included upregulation of PTGS2, prostaglandin G/H synthase 2, and HRH1, a histamine receptor, which would allow increased permeability of capillaries; also, EREG, involved in growth factor signalling. There was also upregulation of HPSE (heparanse), a cell surface and ECM degrading enzyme, as well as two matrix metallopeptidase genes which are also involved in ECM degradation (MMP19 and MMP10). BMP6, bone morphogenic protein-6, Angiopoietin-like-4 (ANGPTL4), and TPST1, a tyrosine sulfotransferase were also upregulated, whilst there was downregulation of Heme oxygenase 1 (HMOX1), a stress response gene associated

with the inflammatory response. There was a slight contrast of effects regarding expression of TLR receptors; with platelet soluble mediators leading to downregulation of TLR6, 7 and 8. In contrast, PSGL-1 induced a slight increase in expression of TLR7 and 8.

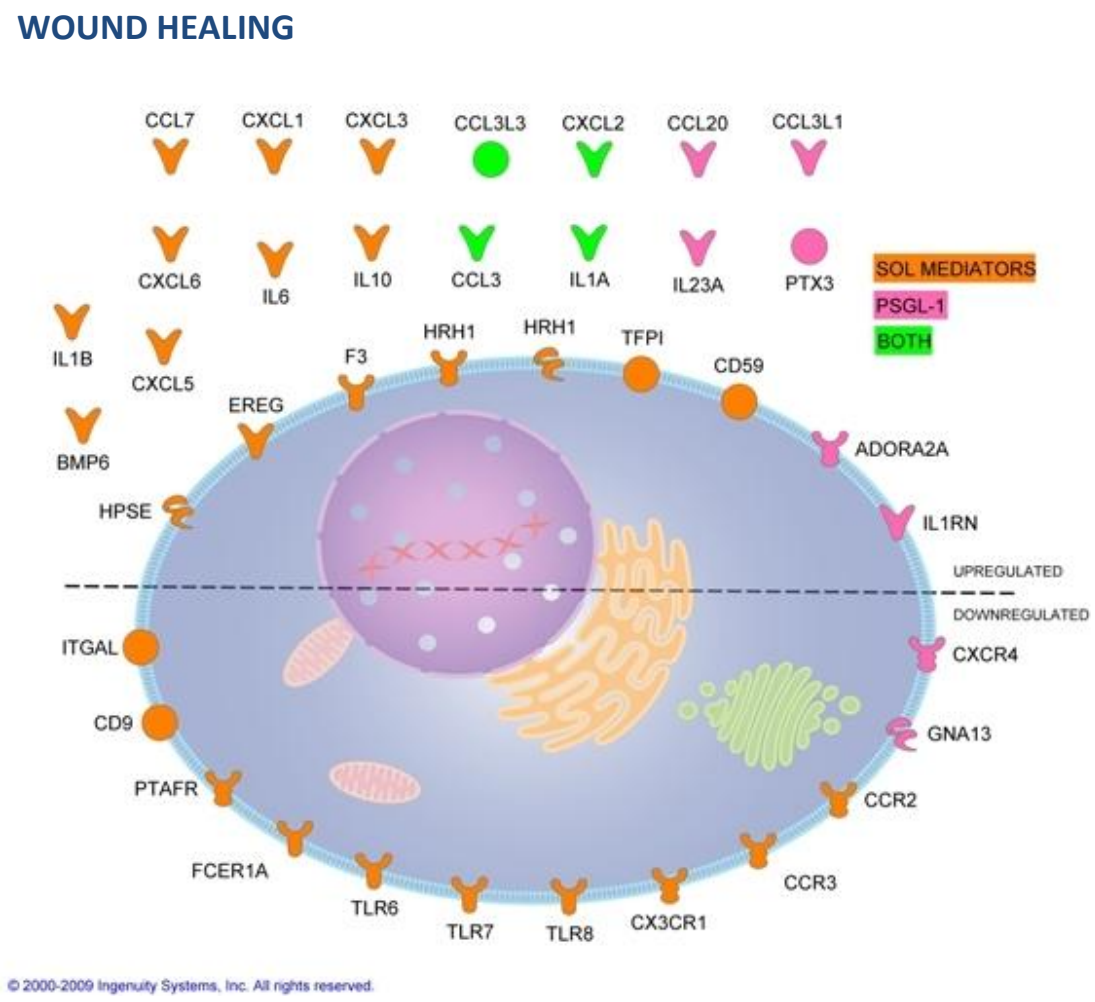


Figure 6.11: Changes in monocyte genes involved in wound healing.

Therefore, changes in monocyte gene expression in response to wounding involved major downregulation of selective chemokine receptors, in particular CX3CR1 and CXCR4, associated with a pro-inflammatory cellular phenotype (Tacke *et al*, 2007), and cellular recruitment and transmigration (Zernecke *et al*, 2005; Mazzinghi *et al*, 2008), respectively. Meanwhile there was a significant upregulation of secreted cytokines and chemokines, coagulation proteins, and proteins involved in ECM interaction and remodelling. A summary of these changes in monocyte gene expression can be seen in Figure 6.11.

6.3.2.5. Cellular Lipid Metabolic Process

Apart from a strong increase in expression of PTGS2, involved in synthesis of arachidonic acid metabolites, and an increase in BMP6, a member of the TGF-β family which has a regulatory function in many intracellular processes, the majority of lipid-related metabolic process genes were downregulated. Those downregulated in response to platelet soluble mediators included Interleukin IL4, a highly significant downregulation of CLC, a lysophospholipase, LYPLA3, also a phospholipase, SLC27A1, involved in transportation of long-chain fatty acids, and UGCG, a ceramide glucosyltransferase involved in glycosphingolipid biosynthesis. Also downregulated was PTGDS, prostaglandin-D2 synthase, PIGB, involved in GPI-anchor biosynthesis, AKR1C3, an aldo-keto reductase family member, GALC, a galactosidase, and OSBPL7, oxysterol-binding protein involved in lipid transport. Monocyte genes downregulated in response to PSGL-1 activation include YWHAH, a signalling adapter

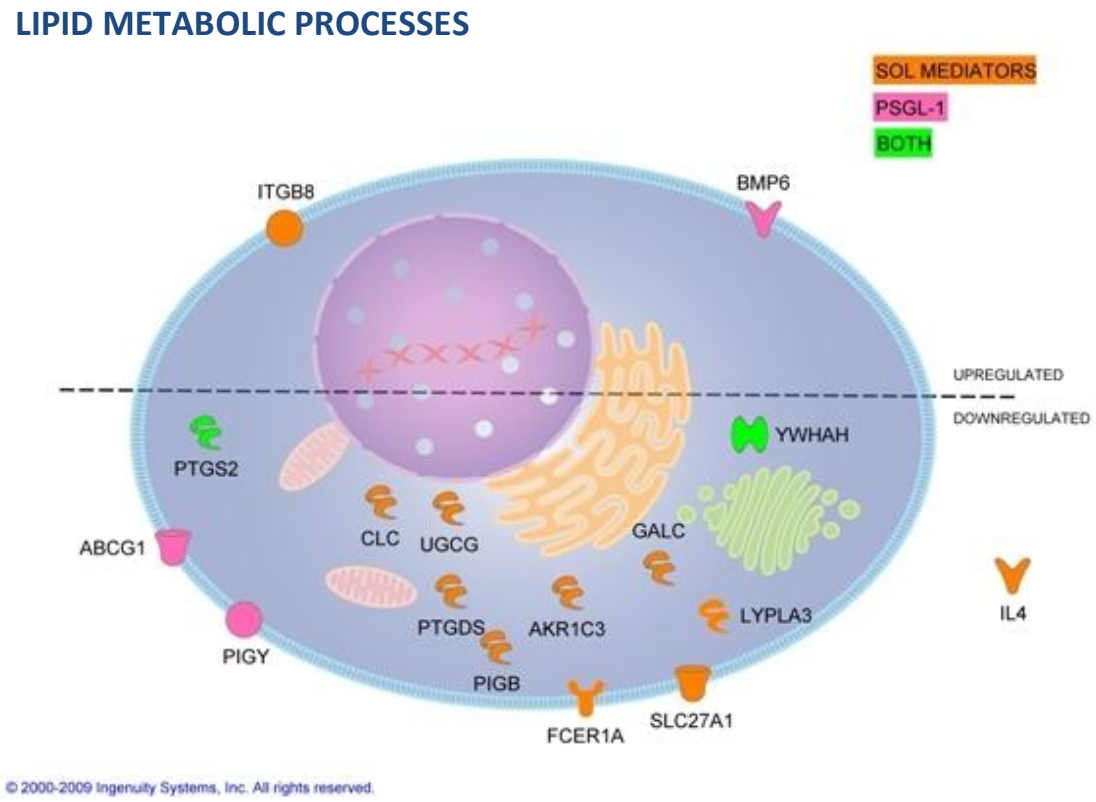


Figure 6.12: Changes in monocyte genes involved in lipid metabolic processes

protein, ABCG1, an ATP-binding cassette family member known to be involved in lipid homeostasis in macrophages, and PIGY, a gene which is again involved in GPI-anchor biosynthesis. Therefore this category clearly demonstrates a different pattern of monocyte gene expression with the majority of downregulated genes being involved in cellular processes within the cytoplasm, particularly in the endoplasmic reticulum (ER). A summary of these changes in monocyte gene expression involved in lipid biosynthetic processes can be seen in Figure 6.12.

6.3.3. Comparison with monocyte gene expression following platelet activation in whole blood isolated from healthy controls and premature MI subjects

The current study was carried out in an isolated cell system specifically to compare the effects of platelet soluble mediators and PSGL-1 signalling on monocyte gene expression; however the results may not reflect changes that occur *in vivo*. In parallel to this study, a gene expression array study was carried out by Dr Unni Krishnan, with the aim of investigating monocyte gene expression in response to activated platelets in whole blood. This study analysed samples from 20 individuals who had suffered an MI under the age of 65, and a group of 20 age and gender-matched healthy controls. Samples from both studies were hybridised to the same Illumina® WG6 v2 platform and were analysed using the same statistical and bioinformatics procedure as used for my own studies, therefore the results of the studies were suitable for comparison. Figure 6.13. gives an overview of the stages of the following preliminary analysis.

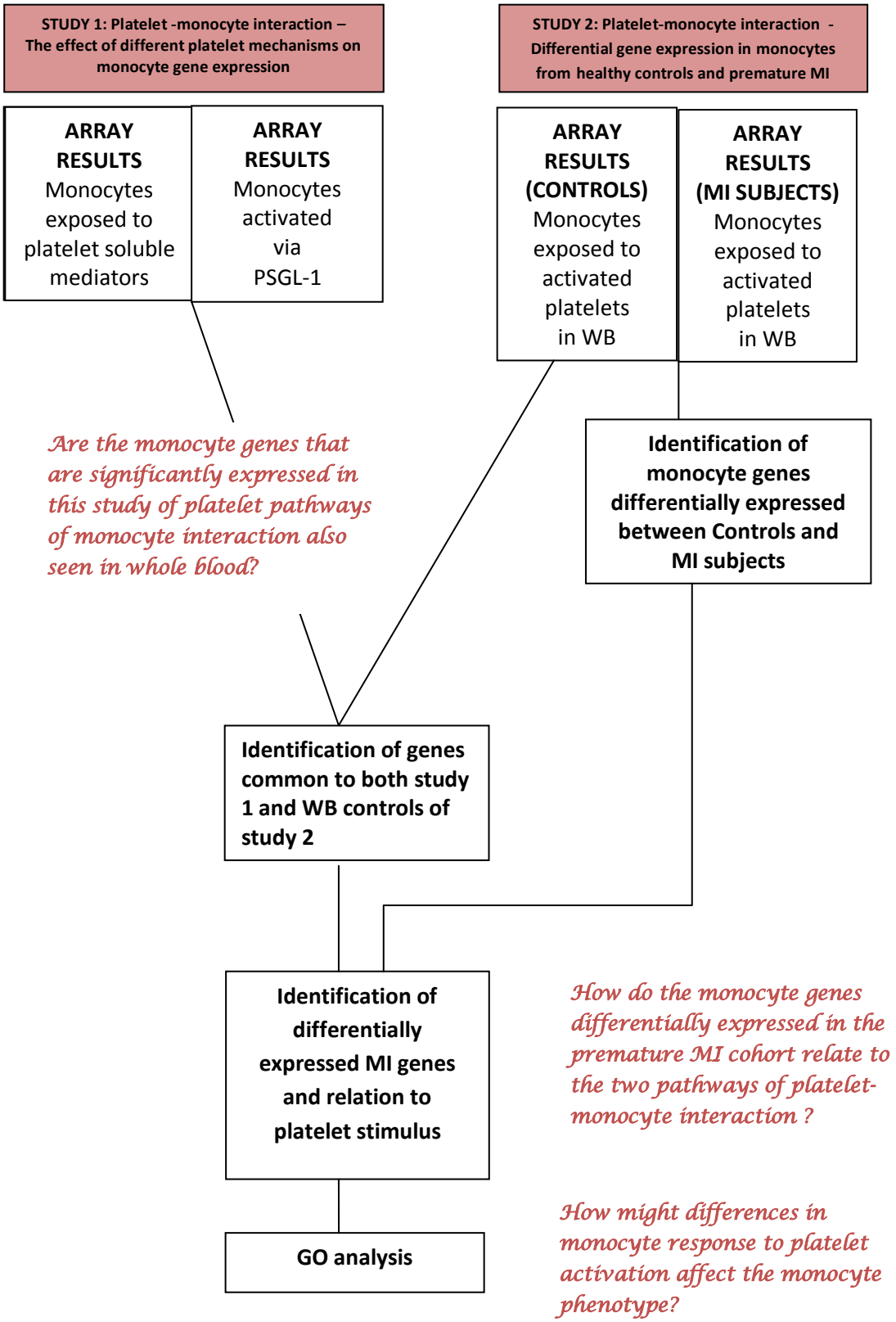
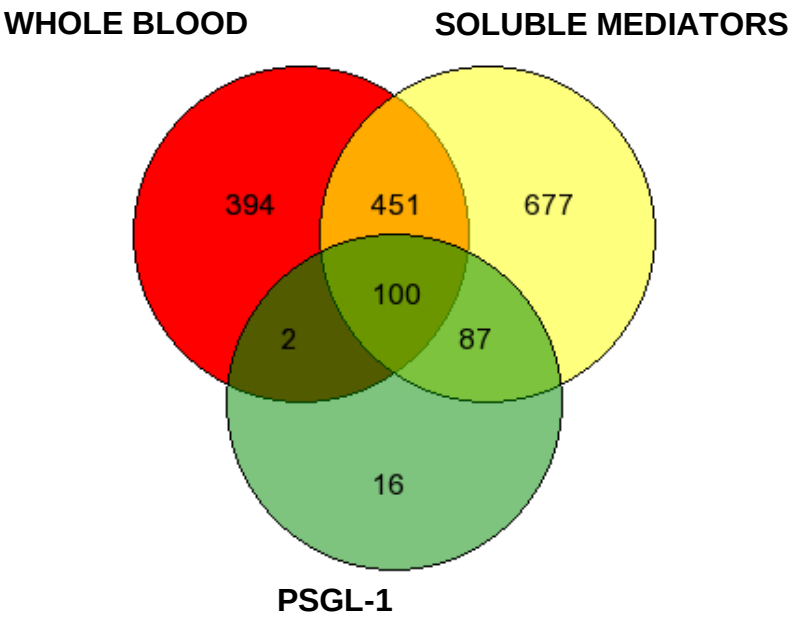


Figure 6.13: Overview of analysis of comparison studies. The first analysis was to compare the results of platelet stimulus study with the results obtained from the analysis of whole blood from healthy controls. The second analysis assigned the stimulus pathway of platelet-monocyte interaction to the differentially expressed genes in the MI cohort. Finally, gene ontology analysis identified how the lower level of response to platelet interaction might affect the monocyte phenotype.

To investigate whether the expression of monocyte genes found to be significantly altered in response to platelet soluble mediators or PSGL-1 activation might also be expressed in whole blood in response to platelet activation, a comparison was made of the significantly expressed genes (fold change >2-fold; $p<0.05$) from the platelet soluble mediators and PSGL-1 study, with the significantly expressed genes of the healthy control cohort from the whole blood study (also fold change >2-fold; $p<0.05$). A total of 1310 (42.3%) monocyte genes that were expressed in response to platelet soluble mediators, and 299 (49.0%) of monocyte genes that were expressed in response to PSGL-1 activation, were also found to be altered in monocytes isolated from whole blood following stimulation by platelets activated with CRP-XL (Figure 6.14). Of the monocyte genes responding to soluble mediators, and also found in whole blood, 551 (42.1%) were upregulated, and 759 (57.9%) were downregulated. A similar pattern of response was seen for monocyte genes in response to PSGL-1 activation and also seen in whole blood, with 102 genes (34.1%) being upregulated, and 197 (65.9%) being downregulated. GO analysis (data not shown) of these genes demonstrated a similar wide range of functional distribution similar to those reported for the functional distribution of monocyte genes regulated by platelet soluble mediators, and PSGL-1 activation (Figures 6.4 and 6.5, respectively), therefore no further analysis was performed on this data.

a) Upregulated genes



b) Downregulated genes

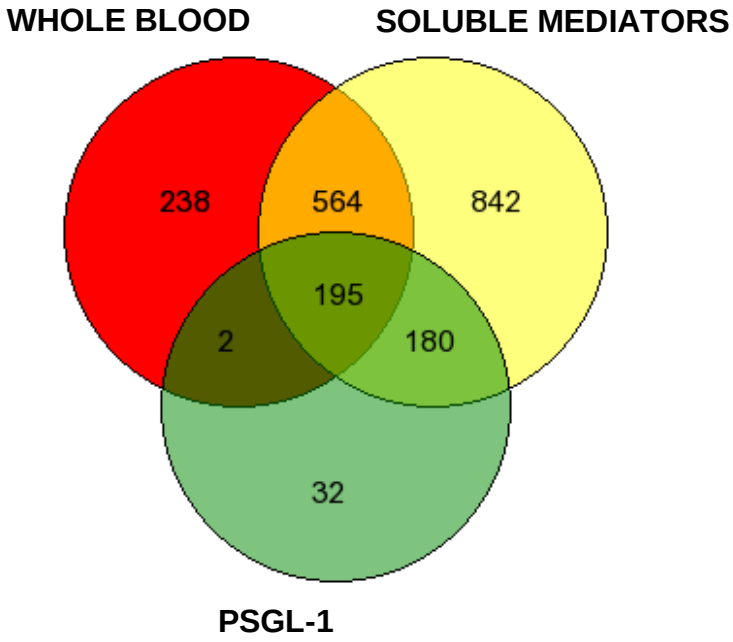


Figure 6.14: Comparison of monocyte gene profile obtained from platelet-activated monocytes incubated in whole blood or an isolated cell system. Genes which were upregulated >2-fold in monocytes following exposure to platelet soluble mediators were compared with a gene list obtained from analysis of monocytes in whole blood following activation of platelet with CRP-XL. Both array studies were run on the same Illumina WG6 v2 platforms

A preliminary analysis was carried out to investigate genes that were differentially expressed between the controls and the premature MI subjects, to determine whether these were restricted to a specific pathway of platelet-monocyte interaction, *ie*, activation by platelet soluble mediators, or via PSGL-1 activation. To achieve this, the list of significantly differentially expressed genes in the premature MI cohort (fold change >2-fold; $p<0.05$) were compared to the 761 genes that were significantly expressed either in response to platelet soluble mediators and/or PSGL-1 activation, and were also significantly expressed in whole blood from the control cohort. A total of 52 monocyte genes that were upregulated in response to platelet soluble mediators were expressed at lower levels in monocytes from MI subjects compared to the matched controls. 21 genes that were down regulated in response to platelet soluble mediators were expressed at higher levels in monocytes from the MI patients. A similar pattern was seen in response to PSGL-1 activation, although the number of genes was much smaller, with only 4 genes upregulated in response to monocyte activation via PSGL-1 being expressed at lower levels in monocytes from the MI patients, and only 1 gene that was downregulated in response to PSGL-1 activation being expressed at higher levels in MI monocytes (Table 6.6).

PLATELET	SOLUBLE	MEDIATORS	PSGL-1	ACTIVATION
upregulated		downregulated	upregulated	downregulated
(n = 52)		(n = 21)	(n = 4)	(n = 1)
BCAR3	KIAA1199	ASGR1	CCL20	TUBA3
BMP6	LOC58489	CAMK1	CXCL2	
CCL7	LOC652175	CBFA2T3	SDC4	
CDCP1	LOC653560	CEBPA	SPHK1	
CMKOR1	LONRF3	CLEC10A		
COL22A1	LONRF3	CREB5		
CRADD	MGC40579	CYP27A1		
CRLF2	MYO10	DPYSL2		
CRLF2	NRIP3	E2F2		
CTSL	OASL	EVA1		
CTSL	PSD3	FGL2		
CXCL2	RASGRP3	FUT7		
CXCL5	RIS1	HSPA1L		
CXCL6	SDC2	IKBKE		
DNAH17	SGNE1	IL17R		
DNAJB5	SLC16A10	IRF8		
DOCK7	SLC4A7	ITGAL		
DUSP4	SMOX	LRRC33		
DUSP4	SPTBN5	LYPLA3		
EDG7	STX1A	RTN1		
FLNB	TFPI	SMAP1L		
FREQ	TFPI			
HPSE	TMEM44			
HRH1	TNFSF8			
IL10	TPST1			
IL1R1	WDR69			

Table 6.6: List of genes differentially expressed in monocytes from premature MI subjects in relation to pathway of platelet-monocyte interaction. Genes are significantly altered >2-fold change, and $p<0.05$. (Further description of these genes is given in Table 6.7).

Table 6.7 : Monocyte genes which are differentially expressed in MI subjects, in relation to platelet pathway of monocyte interaction.

SOL MEDS UPREGULATED GENES (lower in MI monocytes, compared to controls)	
BCAR3	**BREAST CANCER ANTI-ESTROGEN RESISTANCE PROTEIN 3. SIGNAL TRANSDUCTION. (MEMBRANE/INTRACELLULAR).** May act as an adapter protein and couple activated growth factor receptors to a signaling pathway that regulates the proliferation in breast cancer cells. When overexpressed, it confers anti-estrogen resistance in breast cancer cell lines. May also be regulated by cellular adhesion to extracellular matrix proteins.
BMP6	**BONE MORPHOGENETIC PROTEIN 6. SIGNAL TRANSDUCTION. (SECRETED).** Induces cartilage and bone formation.
CCL7	**C-C MOTIF CHEMOKINE 7. CHEMOTAXIS/INFLAMMATORY RESPONSE. (SECRETED).** Chemotactic factor that attracts monocytes and eosinophils, but not neutrophils. Augments monocyte anti-tumour activity. Also induces the release of gelatinase B. This protein can bind heparin. Binds to CCR1, CCR2 and CCR3.
CDCP1	**CUB domain-containing protein 1. CELL ADHESION. (CELL MEMBRANE/SECRETED).** May be involved in cell adhesion and cell matrix association. May play a role in the regulation of anchorage versus migration or proliferation versus differentiation via its phosphorylation. May be a novel marker for leukaemia diagnosis and for immature hematopoietic stem cell subsets. Belongs to the tetraspanin web involved in tumour progression and metastasis.
CMKOR1	**C-X-C CHEMOKINE RECEPTOR TYPE 7. (SECRETED).** Receptor for CXCL12/SDF1. Acts as coreceptor with CXCR4 for a restricted number of HIV isolates.
COL22A1	**COLLAGEN ALPHA-1 (XXII) CHAIN. CELL ADHESION. (CYTOPLASM/ECM/SECRETED).** Acts as a cell adhesion ligand for skin epithelial cells and fibroblasts.
CRADD	**DEATH DOMAIN-CONTAINING PROTEIN CRADD (RAIDD). APOPTOSIS. (INTRACELLULAR)** Apoptotic adaptor molecule specific for caspase-2 and FASL/TNF receptor-interacting protein RIP. In the presence of RIP and TRADD, CRADD recruits caspase-2 to the TNFR-1 signalling complex.
CRLF2	**CYTOKINE RECEPTOR-LIKE FACTOR 2. SIGNAL TRANSDUCTION. (MEMBRANE).** Receptor for thymic stromal lymphopoietin (TSLP). Forms a functional complex with TSLP and IL7R which is capable of stimulating cell proliferation through activation of STAT3 and STAT5. Also activates JAK2. Implicated in the development of the hematopoietic system

CTSL	**CATHESPIN L1. PROTEIN DEGRADATION. (CYTOPLASM/LYSOSOME).** Important for the overall degradation of proteins in lysosomes.
CXCL2	**C-X-C MOTIF CHEMOKINE 2. CHEMOTAXIS/INFLAMMATORY RESPONSE. (SECRETED).** Produced by activated monocytes and neutrophils and expressed at sites of inflammation. Hematoregulatory chemokine, which, in vitro, suppresses hematopoietic progenitor cell proliferation. GRO-beta(5-73) shows a highly enhanced hematopoietic activity.
CXCL5	**C-X-C MOTIF CHEMOKINE 5. CHEMOTAXIS/INFLAMMATORY RESPONSE. (SECRETED).** Involved in neutrophil activation. In vitro, ENA-78(8-78) and ENA-78(9-78) show a threefold higher chemotactic activity for neutrophil granulocytes.
CXCL6	**C-X-C MOTIF CHEMOKIN 6. CHEMOTAXIS/INFLAMMATORY RESPONSE. (SECRETED).** Chemotactic for neutrophil granulocytes.
DNAH17	**DYNEIN HEAVY CHAIN 17, AONEMAL. CELL MOTILITY. (CYTOPLASM).** Force generating protein of respiratory cilia. Produces force towards the minus ends of microtubules. Dynein has ATPase activity; the force-producing power stroke is thought to occur on release of ADP. Involved in sperm motility; implicated in sperm flagellar assembly.
DNAJB5	**DNAJ HOMOLOG SUBFAMILY B MEMBER 5 (HSP40). ? (?INTRACELLULAR).** Involved in protein folding.
DOCK7	**DEDICATOR OF CYTOKINESIS PROTEIN 7. CELL MOTILITY. (GROWTH PROJECTION OF CELL).** Functions as a guanine nucleotide exchange factor (GEF), which activates Rac1 and Rac3 Rho small GTPases by exchanging bound GDP for free GTP. Does not have a GEF activity for CDC42. Required for STMN1 'Ser-15' phosphorylation during axon formation and consequently for neuronal polarization.
DUSP4	**DUAL SPECIFICITY PROTEIN PHOSPHATASE 4 (MAPKP2). CELL SIGNALLING. (NULCEUS).** Regulates mitogenic signal transduction by dephosphorylating both Thr and Tyr residues on MAP kinases ERK1 and ERK2.
EDG7	**LYSOPHOSPHATIDIC ACID RECEPTOR 3. CELL SIGNALLING. (CELL MEMBRANE).** Receptor for lysophosphatidic acid (LPA), a mediator of diverse cellular activities. May play a role in the development of ovarian cancer. Seems to be coupled to the G(i)/G(o) and G(q) families of heteromeric G proteins.
FLNB	**FILAMIN-B. CELL STRUCTURE. (CYTOPLASM)** Connects cell membrane constituents to the actin cytoskeleton. May promote orthogonal branching of actin filaments and links actin filaments to membrane glycoproteins. Anchors various transmembrane proteins to the actin cytoskeleton. Interaction with FLNA may allow neuroblast migration from the ventricular zone into the cortical plate. Various interactions and localizations of isoforms affect myotube morphology and myogenesis.
FREQ	**NEURONAL CALCIUM SENSOR 1. CELL HOMEOSTASIS. (GOLGI)** Neuronal calcium sensor, regulator of G protein-coupled receptor phosphorylation in a calcium dependent manner. Directly regulates GRK1 (RHOK), but not GRK2 to GRK5. Can

	substitute for calmodulin. Stimulates PI4KB kinase activity. Involved in long-term synaptic plasticity through its interaction with PICK1. May also play a role in neuron differentiation through inhibition of the activity of N-type voltage-gated calcium channel
HPSE	**HEPARANASE. CELL MOVEMENT/ANGIOGENESIS. (LYSOSOME/SECRETED)** Endoglycosidase which is a cell surface and extracellular matrix-degrading enzyme. Cleaves heparan sulfate proteoglycans (HSPGs) into heparan sulfate side chains and core proteoglycans. Also implicated in the extravasation of leukocytes and tumor cell lines. Due to its contribution to metastasis and angiogenesis, it is considered to be a potential target for anti-cancer therapies.
HRH1	**HISTAMINE H1 RECEPTOR. CELL SIGNALLING/INFLAMMATORY RESPONSE). CELL MEMBRANE.** In peripheral tissues, the H1 subclass of histamine receptors mediates the contraction of smooth muscles, increase in capillary permeability due to contraction of terminal venules, and catecholamine release from adrenal medulla, as well as mediating neurotransmission in the central nervous system.
IL10	**INTERLEUKIN 10. CHEMOTAXIS/INFLAMMATORY RESPONSE. (SECRETED)** Inhibits the synthesis of a number of cytokines, including IFN-gamma, IL-2, IL-3, TNF and GM-CSF produced by activated macrophages and by helper T-cells.
IL1R1	**INTERLEUKIN-1 RECEPTOR TYPE 1 (CD121A). IMMUNE RESPONSE /INFLAMMATORY RESPONSE. (MEMBRANE).** Receptor for interleukin-1 alpha (IL-1A), beta (IL-1B), and interleukin-1 receptor antagonist protein (IL-1RA). Binding to the agonist leads to the activation of NF-kappa-B. Signaling involves formation of a ternary complex containing IL1RAP, TOLLIP, MYD88, and IRAK1 or IRAK2. Also involved in PDGF receptor binding?
KIAA1199	**PROTEIN KIAA1199. ?. (?CELL MEMBRANE)** Belongs to the TMEM2 family. May be involved in hearing.
LOC58489	Unknown
LOC652175	Unknown
LOC653560	Unknown
LONRF3	**LON PEPTIDASE N-TERMINAL DOMAIN AND RING FINGER PROTEIN 3. (PROTEOLYSIS)**
MGC40579	Unknown
MYO10	**MYOSIN-X. CELL MOVEMENT/STRUCTURE. (INTRACELLULAR?)** Myosins are actin-based motor molecules with ATPase activity. Unconventional myosins serve in intracellular movements. Their highly divergent tails are presumed to bind to membranous compartments, which would be moved relative to actin filaments . Plays a role in regions of dynamic actin.
NRIP3	**NUCLEAR RECEPTOR INTERACTING PROTEIN 3.**

OASL	**59 KDA 2'5'OLIGOADENYLATE SYNTHETASE-LIKE PROTEIN. IMMUNE RESPONSE. (CYTOPLASM).** Does not have 2'-5'-OAS activity, but binds double-stranded RNA and DNA. Induced by interferons.
PSD3	**PH AND SEC7 DOMAIN-CONTAINING PROTEIN 3. CELL SIGNALLING. (CELL MEMBRANE).** Guanine nucleotide exchange factor for ARF6
RASGRP3	**PUTATIVE UNCHARACTERISED PROTEIN RASGRP3. CELL SIGNALLING. (INTRACELLULAR).**
RIS1	**RAS-INDUCED SENESCENCE PROTEIN 1(TMEM158). CELL SIGNALLING. (CELL MEMBRANE).** Receptor for brain injury-derived neurotrophic peptide (BINP), a synthetic 13-mer peptide.
SDC2	**SYNDECAN 2 (HEPARAN SULFATE PROTEOGLYCAN CORE PROTEIN). PROTEIN BINDING. (CELL MEMBRANE).** Cell surface proteoglycan that bears heparan sulphate. Binds cytoskeletal proteins.
SGNE1	**SECRETOGRANIN V. CELL SIGNALLING. (CYTOPLASM/SECRETED).** Involved in neuropeptide signalling.
SLC16A10	**MONOCARBOXYLATE TRANSPORTER 10. CELL TRANSPORT. (CELL MEMBRANE).** Sodium-independent transporter that mediates the update of aromatic acid. Can function as a net efflux pathway for aromatic amino acids in the basosolateral epithelial cells
SLC4A7	**SODIUM BICARBONATE COTRANSPORTER 3. CELL HOMEOSTASIS. (CELL MEMBRANE).** Electroneutral sodium- and bicarbonate-dependent cotransporter with a Na^+:$HCO3^-$ 1:1 stoichiometry. Regulates intracellular pH and may play a role in bicarbonate salvage in secretory epithelia. May also have an associated sodium channel activity
SMOX	**SPERMINE OXIDASE. CELL HOMEOSTASIS. (CYTOPLASM).** Flavoenzyme which catalyzes the oxidation of spermine to spermidine. Can also use N(1)-acetylspermine and spermidine as substrates, with different affinity depending on the isozyme) and on the experimental conditions. Plays an important role in the regulation of polyamine intracellular concentration and has the potential to act as a determinant of cellular sensitivity to the antitumor polyamine analogs. May contribute to beta-alanine production via aldehyde dehydrogenase conversion of 3-amino-propanal.
SPTBN5	**SPECTRIN BETA CHAIN, BRAIN 4. CELL STRUCTURE. (CYTOPLASMIC).** Probably associates with an alpha chain
STX1A	**SYNTAXIN 1A. CELL HOMEOSTASIS. (CYTOPLASM).** Potentially involved in docking of synaptic vesicles at presynaptic active zones. May play a critical role in neurotransmitter exocytosis. Part of the SNARE core complex containing SNAP25, VAMP2 and STX1A. This complex binds to CPLX1. Binds SYTL4 and STXBP6. Found in a ternary complex with STX1A and SNAP25. Interacts with OTOF and LGI3. Found in a complex with VAMP8 and SNAP23. Interacts with VAPA and SYBU.
TFPI	**TISSUE FACTOR PATHWAY INHIBITOR. BLOOD COAGULATION. (SECRETED/CELL MEMBRANE).**

	Inhibits factor X (X(a)) directly and, in a Xa-dependent way, inhibits VIIa/tissue factor activity, presumably by forming a quaternary Xa/LACI/VIIa/TF complex. It possesses an antithrombotic action and also the ability to associate with lipoproteins in plasma.
TMEM44	**TMEM44.**
TNFSF8	**TUMOUR NECROSIS FACTOR LIGAND SUPERFAMILY, MEMBER 8. IINFLAMMATORY RESPONSE. MEMBRANE.**
TPST1	**PROTEIN-TYROSINE SULFOTRANSFERASE 1. INFLAMMATORY RESPONSE. GOLGI.** Catalyzes the O-sulfation of tyrosine residues within acidic motifs of polypeptides
WDR69	**WD REPEAT-CONTAINING PROTEIN 69.**

SOL MEDS DOWNREG GENES **(higher in MI monocytes, compared to controls)**	
ASGR1	**ASIALOGLYCOPROTEIN RECEPTOR 1. ENDOCYTOSIS. (CELL MEMBRANE).** Mediates the endocytosis of plasma glycoproteins to which the terminal sialic acid residue on their complex carbohydrate moieties has been removed. The receptor recognizes terminal galactose and N-acetylgalactosamine units. After ligand binding to the receptor, the resulting complex is internalized and transported to a sorting organelle, where receptor and ligand are disassociated. The receptor then returns to the cell membrane surface.
CAMK1	**CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE TYPE 1. CELL DIFFERENTIATION. (CYTOPLASM)** Calcium/calmodulin-dependent protein kinase belonging to a proposed calcium-triggered signaling cascade involved in a number of cellular processes like transcriptional regulation, hormone production, translational regulation, regulation of actin filament organization and neurite outgrowth. Involved in calcium-dependent activation of the ERK pathway. Recognizes the substrate consensus sequence [MVLIF]-x-R-x(2)-[ST]-x(3)-[MVLIF]. Phosphorylates EIF4G3/eIF4GII. In vitro phosphorylates CREB1, ATF1, CTFR, MYL9, SYN1/synapsin I and SYNII/synapsin II
CBFA2T3	**PROTEIN CBFA2T3 (MYELOID TRANSLOCATION GENE). CELL DIFFERENTIATION/TRANSCRIPTION REGULATION. (NUCLEUS).** Functions as a transcriptional repressor. Regulates the proliferation and the differentiation of erythroid progenitors by repressing the expression of TAL1 target genes. Plays a role in granulocyte differentiation.
CEBPA	**CCAAT/ENHANCER-BINDING PROTEIN ALPHA. TRANSCRIPTION REGULATION. (NUCLEUS).** C/EBP is a DNA-binding protein that recognizes two different motifs: the CCAAT homology

	common to many promoters and the enhanced core homology common to many enhancers.
CLEC10A	**C-TYPE LECTIN DOMAIN FAMILY 10 MEMBER A. ENDOCYTOSIS/IMMUNE RESPONSE. (MEMBRANE).** Probable role in regulating adaptive and innate immune responses. Binds in a calcium-dependent manner to terminal galactose and N-acetylgalactosamine units, linked to serine or threonine. These sugar moieties are known as Tn-Ag and are expressed in a variety of carcinoma cells.
CREB5	**CYCLIC AMP-RESPONSIVE ELEMENT-BINDING PROTEIN 5. TRANSCRIPTION REGULATION. (NUCLEUS).** Binds to the cAMP response element and activates transcription.
CYP27A1	**CYTOCHROME P450 27/STEROL 26-HYDROXYLASE, MITOCHONDRIAL. CELL HOMEOSTASIS. (MITOCHONDRIA).** Catalyzes the first step in the oxidation of the side chain of sterol intermediates; the 27-hydroxylation of 5-beta-cholestane-3-alpha,7-alpha,12-alpha-triol. Has also a vitamin D3-25-hydroxylase activity.
DPYSL2	**DIHYDROPYRIMIDINASE-RELATED PROTEIN 2. CELL STRUCTURE. (CYTOPLASM).** Necessary for signaling by class 3 semaphorins and subsequent remodeling of the cytoskeleton. Plays a role in axon guidance, neuronal growth cone collapse and cell migration
E2F2	**TRANSCRIPTION FACTOR E2F2. TRANSCRIPTION REGULATION. NUCLEUS.** Transcription activator that binds DNA cooperatively with DP proteins through the E2 recognition site, 5'-TTTC[CG]CGC-3' found in the promoter region of a number of genes whose products are involved in cell cycle regulation or in DNA replication. The DRTF1/E2F complex functions in the control of cell-cycle progression from g1 to s phase. E2F-2 binds specifically to RB1 protein, in a cell-cycle dependent manner.
EVA1	**MYELIN PROTEIN ZERO-LIKE PROTEIN 2. CELL ADHESION. MEMBRANE.** Mediates homophilic cell-cell adhesion.
FGL2	**FIBROLEUKIN/FIBRINOGEN-LIKE PROTEIN. CELL SIGNALLING. SECRETED**. May play a role in physiologic lymphocyte functions at mucosal sites.
FUT7	**ALPHA-(1,3)-FUCOSYLTRANSFERASE. CELL SIGNALLING. MEMBRANE.** May catalyze alpha-1,3 glycosidic linkages involved in the expression of sialyl Lewis X antigens.
HSPA1L	**HEAT SHOCK 70KDA PROTEIN 1-LIKE. PROTEIN FOLDING. (?CYTOPLASM).** In cooperation with other chaperones, Hsp70s stabilize preexistent proteins against aggregation and mediate the folding of newly translated polypeptides in the cytosol as well as within organelles. These chaperones participate in all these processes through their ability to recognize nonnative conformations of other proteins. They bind extended peptide segments with a net hydrophobic character exposed by polypeptides during translation and membrane translocation, or following stress-induced damage
IKBKE	**INHIBITOR OF NUCLEAR FACTOR KAPPA-B KINASE SUBUNIT EPSILON. IMMUNE RESPONSE/TRANSCRIPTION REGULATION. (CYTOPLASM).**

	Phosphorylates inhibitors of NF-kappa-B thus leading to the dissociation of the inhibitor/NF-kappa-B complex and ultimately the degradation of the inhibitor. May play a special role in the immune response.
IL17R	**INTERLEUKIN 17 RECEPTOR A (CD217). CELL SIGNALLING. (MEMBRANE).** Receptor for IL17A. Binds its ligand with low affinity, suggesting that additional components are involved in IL17A-induced signaling.
IRF8	**INTERFERON REGULATORY FACTOR 8. TRANSCRIPTION REGULATION. NUCLEUS.** Specifically binds to the upstream regulatory region of type I IFN and IFN-inducible MHC class I genes (the interferon consensus sequence (ICS)). Plays a negative regulatory role in cells of the immune system
ITGAL	**INTEGRIN ALPHA-L (CD11A). CELL ADHESION. MEMBRANE.** Integrin alpha-L/beta-2 is a receptor for ICAM1, ICAM2, ICAM3 and ICAM4. It is involved in a variety of immune phenomena including leukocyte-endothelial cell interaction, cytotoxic T-cell mediated killing, and antibody dependent killing by granulocytes and monocytes.
LRRC33	**LEUCINE-RICH REPEAT-CONTAINING PROTEIN 33. PROTEIN BINDING. (MEMBRANE).**
LYPLA3	**GROUP XV PHOSPHOLIPASE A2. LIPID METABOLISM. (SECRETED).** Has transacylase and calcium-independent phospholipase A2 activity. Catalyzes the formation of 1-O-acyl-N-acetylsphingosine and the concomitant release of a lyso-phospholipid. May have weak lysophospholipase activity.
RTN1	**RETICULON-1. CELL SIGNALLING. (ER).** May be involved in neuroendocrine secretion or in membrane trafficking in neuroendocrine cells
SMAP1L	**STROMAL MEMBRANE-ASSOCIATED PROTEIN 2. ENDOSOME TRANSPORT. CYTOPLASM.** GTPase activating protein that acts on ARF1. Can also activate ARF6 (in vitro). May play a role in clathrin-dependent retrograde transport from early endosomes to the trans-Golgi network

PSGL-1 UPREG GENES (lower in MI monocytes) (lower in MI monocytes, compared to controls)	
CCL20	**C-C MOTIF CHEMOKINE 20 (MIP3-ALPHA). CHEMOTAXIS/ CELL SIGNALLING. SECRETED.** Chemotactic factor that attracts lymphocytes and, slightly, neutrophils, but not monocytes. Inhibits proliferation of myeloid progenitors in colony formation assays. May be involved in formation and function of the mucosal lymphoid tissues by attracting lymphocytes and dendritic cells towards epithelial cells. C-terminal processed forms have been shown to be equally chemotactically active for leukocytes. Possesses antibacterial activity E.coli ATCC 25922 and S.aureus ATCC 29213.
CXCL2	**C-X-C MOTIF CHEMOKINE 2. CHEMOTAXIS/INFLAMMATORY RESPONSE.** Produced by activated monocytes and neutrophils and expressed at sites of inflammation. Hematoregulatory chemokine, which, in vitro, suppresses hematopoietic progenitor cell proliferation. GRO-beta(5-73) shows a highly enhanced hematopoietic activity
SDC4	**SYNDECAN-4. CELL SIGNALLING. (MEMBRANE).** Cell surface proteoglycan that bears heparan sulfate. Thrombospondin receptor activity?
SPHK1	**SPHK1 PROTEIN. CELL SIGNALLING. (?MEMBRANE).** Activation of protein kinase C activity by G-protein coupled receptor protein signalling pathway.

PSGL-1 DOWNREG GENES (higher in MI monocytes) (higher in MI monocytes, compared to controls)	
TUBA3	**TUBULIN ALPHA-1A CHAIN. CELL STRUCTURE. (CYTOSOL).** Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain.

Gene ontology analysis was carried out on these 78 genes to identify how the lower level of monocyte response to platelet interaction may affect the monocytes in the MI

subjects (Table 6.7). This analysis revealed that these genes were mainly divided into 5 categories of function: Endosomal trafficking and endocytosis; Nuclear proteins, transcription and signalling; Structure and motility; Secreted proteins; and, Adhesion and membrane receptor expression (Figure 6.15).

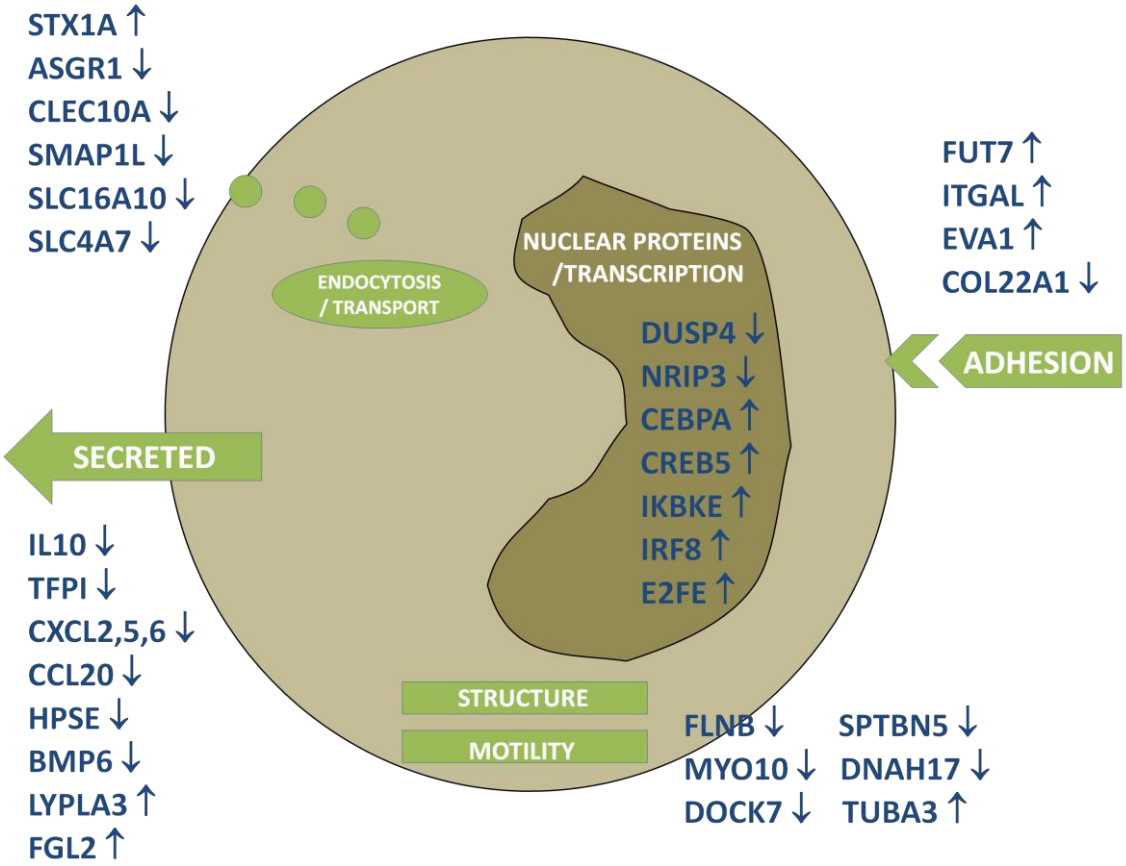


Figure 6.15: Possible phenotype of monocytes in premature MI subjects as a result of reduced response to activated platelets.

This preliminary analysis illustrated how different pathways of platelet activation of monocytes may be differentially regulated in MI patients vs healthy controls. Whether this relates to the underlying disease processes, or to medication, for example anti-platelet therapy with aspirin, or to genetic factors, remains to be determined.

6.4 Discussion

In addition to their role in inflammatory processes, monocytes also contribute to the thrombotic response, becoming incorporated into a thrombus at the site of endothelial injury. Here monocytes come into contact with other leukocytes, and platelets. Platelets preferentially form cell-cell aggregates with monocytes (Rinder *et al*, 1991) and there have been many reports of platelet-monocyte aggregation leading to increased production of monocyte TF, and interleukins IL-1β, and IL-8, (Pinder *et al*, 1985; Celi *et al*, 1994; Weyrich *et al*, 1996; Christersson *et al*, 2008), therefore suggesting induction of a procoagulant, proinflammatory phenotype. Whilst this study also found upregulation of these genes, there was a notable effect of soluble mediators released from the platelet granules upon activation, altering monocyte gene expression to create an anticoagulant, wound healing phenotype.

The main aim of attracting leukocytes into the wound site is the delivery of cytotoxic mediators, and removal of cell debris to facilitate repair of the wound site. This involves the upregulation of a series of ligands which will allow 'capture' of the white cell, adhesion to the endothelium and migration to its destination in the ECM. In this study, the response to platelet soluble mediators caused downregulation of monocyte expression of L-selectin and of FUT-7, a glycosyl transferase which plays a role in the synthesis of functional L-selectin ligands (Smith *et al*, 1996), however there was an

increase in expression of CD44, which has recently been proposed to be a ligand for E-selectin (Katayama *et al*, 2005), therefore allowing monocyte-endothelial attachment via endothelial selectin expression. Monocyte activation via PSGL-1 induced an increase of ICAM-1, and soluble mediators increased expression of ITGAL (CD11a) which would allow further adhesion to endothelial ICAM-1, which binds to leukocyte integrin $\alpha L\beta_2$. The action of platelet soluble mediators induced multiple genes involved in ECM interaction such as increase in receptors for fibronectin, or release ECM components such as laminin and collagen subunits and palladin.

The greatest difference in gene expression between the two mechanisms was in expression of chemokines (Figure 6.17). Chemokines play a vital role in inflammation, but also in various biological functions such as leukocyte trafficking, tumour growth and development, and angiogenesis (Paoletti *et al*, 2005; Rosenkilde *et al*, 2004). Chemokines can be classified into two main groups termed C-X-C chemokines, and C-C chemokines, based on the presence or absence of an amino acid residue between conserved cysteine residues. Chemokines that have been reported to be pro-angiogenic are CXCL1, 2, 3, 5, 6, 7, and 8, and act mainly via the CXCR2 receptor, whereas those reported to be anti-angiogenic include CXCL9, 10, 11, 4 and 13 which act mainly via the CXCR3b receptor (Rosenkilde *et al*, 2004)(Figure 6.16).

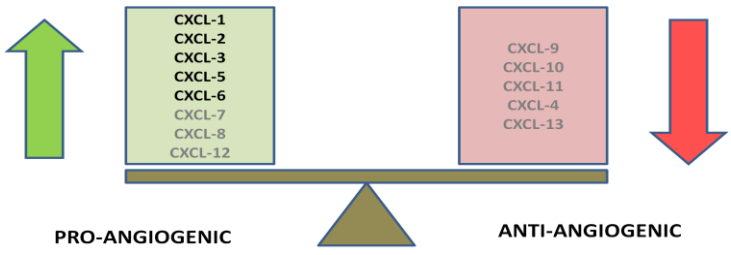


Figure 6.16: Classification of pro- and anti-angiogenic C-X-C chemokines

(Bold script highlights chemokines significantly induced in this study).

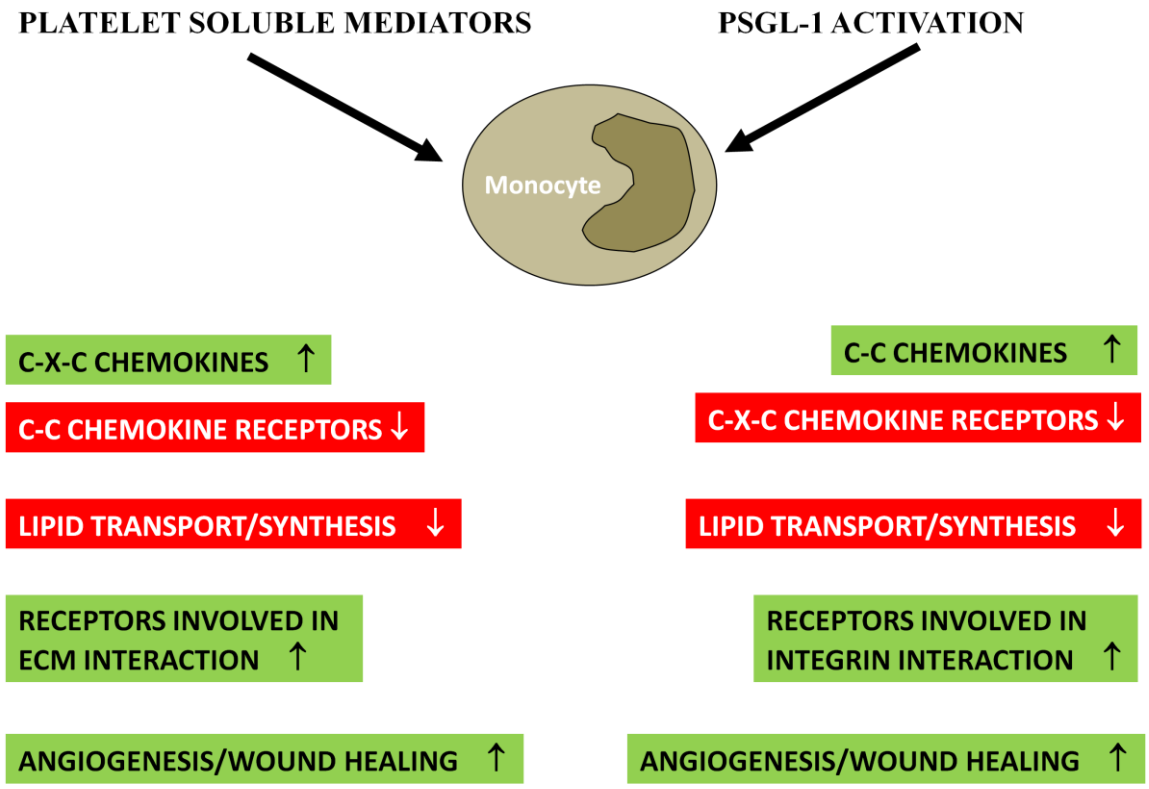


Figure 6.17: Summary of effect of platelet soluble mediators and PSGL-1 activation on monocyte gene expression. (Main difference between platelet soluble mediators and PSGL-1 activation on monocyte gene expression was the induction of C-X-C or C-C chemokines).

In the present study, there was significant upregulation of CXCL1, 2, 3, 5, and 6, all pro-angiogenic chemokines, many of which either attract or activate neutrophils, and were preferentially upregulated in response to platelet soluble mediators. PSGL-1 activation preferentially upregulated expression of C-C chemokines, in particular, CCL3 and CCL20 which would attract circulating monocytes to the site of injury.

In contrast to genes encoding for membrane-bound receptors and secreted mediators seen in the majority of ontology categories considered, expression of genes involved in lipid metabolic processes were mostly intracellular, with many inducing a downregulation of genes involved in the generation of GPI-linked receptors. Platelet soluble mediators induced changes in cholesterol transport. These changes could result in reduced import of long chain fatty acids across the plasma membrane. Therefore future study could be made to measure the effects of these changes, and to determine whether platelet soluble mediators could play a role in limiting foam cell formation.

A further interesting effect of platelet soluble mediators was the change in monocyte gene expression of several members of the Dual Specificity Phosphatase (DUSP) family; inducing upregulation of DUSP 4, DUSP 5, DUSP6, and DUSP16, and downregulation of DUSP10. DUSPs are regulators of signal transduction, acting specifically by dephosphorylation of phosphothreonine and phosphotyrosine residues of Mitogen-Activated Protein Kinase (MAPK) phosphatases, which regulate activation of p38, c-Jun N-terminal kinase (JNK) and Extracellular-signal-Regulated Kinase (ERK), and therefore modulate many signalling pathways, including cell proliferation and differentiation, stress responses and apoptosis (Dickinson *et al*, 2006). DUSPS 4 and 5 are nuclear DUSPs, and have been shown to be induced by growth factors or stress signals (Pearson *et al*, 2001; Murphy *et al*, 2006). Interestingly, DUSP4 was one

of the genes that was expressed at lower levels in the MI subjects. Two array studies carried out in zebrafish, identified DUSP5 as a vascular-specific gene (Xie *et al,* 2005; Sumanas *et al,* 2005), and suggested that DUSP5 has a possible role in vascular development. This has recently been affirmed by Pramanik *et al,*(2009), who have shown that DUSP5 is essential for vascular development in zebrafish, and DUSP5 can be significantly induced in endothelial cells following administration of VEGF (Bellou *et al*, 2009). In contrast, studies in macrophages have found DUSP10 to be induced in response to LPS activation of TLR ligands, and that downregulation may result in change of cytokine production (Zhang *et al,* 2004). Therefore, platelet soluble mediators, eg growth factors such as VEGF, may play an important role in the induction of DUSP signalling pathways, which in turn may play a key role in adapting the monocyte phenotype from pro-inflammatory to pro-angiogenic.

The effect of platelet soluble mediators on monocyte gene expression appeared to have a greater effect than that of platelet-monocyte aggregation, mimicked by activation of monocyte PSGL-1 via the P-selectin-Fc chimeric protein. The platelet degranulate may contain >300 soluble mediators, arising from α-granules, dense granules, and lysosomes stored intracellularly within the platelet. Therefore multiple signalling pathways may be activated via binding of platelet soluble mediators to monocyte membrane receptors. *In vivo*, there may also be differences in soluble mediators released from the platelet granules in response to different stimuli; for example, in response to ADP, there is very little degranulation, whereas in response to collagen and thrombin there is release of alpha and dense granules (Janes *et al*, 1994; Rendu *et al*, 2001). The concentration of agonist may also play a role, as thrombin receptors PAR1 and PAR4 are differentially activated in response to low and high concentrations of thrombin, respectively (Hung *et*

al, 1992; Kahn *et al*, 1999). There have recently been reports of differential packaging of alpha granules, suggesting pro- and anti-angiogenic proteins may be distributed into specific alpha granules (Sehgal *et al*, 2007; Nurden *et al*, 2007; Italiano *et al*, 2008). This suggests that they may also be released differentially, and therefore, the potential influence of platelet soluble mediators on the monocyte may be different depending upon the stimuli. In this study, activation of platelets with 500ng/mL CRP-XL induced maximum platelet degranulation, as shown previously (Figure 5.1), by flow cytometric measurement of platelet P-selectin expression in response to increasing concentrations of CRP-XL. However, it would be interesting to investigate further how platelet degranulation in response to different agonists in different environments, or different concentrations of agonists, affects monocyte gene expression and its phenotype.

In vivo, platelet-monocyte aggregation primarily involves cell-cell interaction via platelet-P-selectin and monocyte PSGL-1 ligands (Ferandes *et al*, 2003). This is closely followed by further platelet-monocyte ligand binding *eg*, monocyte CD11/CD18 and platelet GPIIbIIIa/fibrinogen, which further stabilise the cellular aggregates, and reduce the chance of disaggregation (Evangelista *et al*,1999). In this study, the monocyte is only activated via PSGL-1, which although is the main ligand for platelet-monocyte aggregation, may result in activation of only limited intracellular signalling, as the aggregation of the platelet-monocyte complex may not be committed. Therefore, the changes in gene expression may not be truly reflective of a monocyte that has been committed to form a stable aggregate with platelets, and may explain the low number of significant genes. Further study could be made using similar chimeric proteins to investigate the effect of other platelet-monocyte ligand interactions to compare their effect on monocyte gene expression.

Another approach would be to look at the changes in monocyte gene expression in response to platelet activation in whole blood, which is more reflective of the *in vivo* environment. A microarray study examining just this aspect has been carried out in parallel with this study by Rosienne Farrugia (Farrugia *et al*, manuscript submitted), where whole blood gene expression microarray analysis compared the monocyte response to inflammatory stimuli (LPS) versus the monocyte response to a haemostatic stimuli (CRP-18-activated platelets). The overall finding of the study was that, as expected, LPS induced an inflammatory response in the monocyte, inducing upregulation of pro-inflammatory cytokines typical of an NF-kB-driven response. In contrast, platelet activation induced a monocyte phenotype which was primed for endothelial adhesion, supporting wound healing, and was predominantly PPARγ-driven. These findings support the results of this current study.

To test this further, monocyte genes induced due to individual mechanisms of platelet-monocyte interaction were compared with monocyte genes induced in a whole blood environment following platelet activation with the same stimulus (CRP-XL), and showed a common expression of ~50% of significantly expressed genes. There are several possible reasons why this figure is not greater; firstly *in vivo,* there are other cells present which may release soluble mediators such as ROS, other cytokines, etc, which may counteract the effects seen in an isolated cell system. Other cells, eg neutrophils, or lymphocytes, may also have cell membrane receptors for some of the mediators released from the platelets, therefore reducing the concentration of stimulus that may normally be encountered by the monocyte. A further possibility is that the movement of molecules is sterically slower in the whole blood environment where

many cells, including numerous red cells, are also present, therefore perhaps reducing the opportunity of a soluble mediator encountering and binding to the monocyte.

Further analysis of the 50% of genes that were found to be also significant in the whole blood study revealed that a large number were differentially expressed in monocytes isolated from MI subjects. Four of these genes were related to endocytosis and exocytosis. Firstly, there were lower levels of STX1A, (Syntaxin), which binds with VAMP proteins to facilitate vesicle docking prior to membrane fusion and release of vesicle soluble mediators. Therefore, reduced levels of Syntaxin-1A may result in reduced capacity for vesicle docking, resulting in a lower level of molecule exocytosis from intracellular granules. It would be interesting to investigate whether this lower level of expression also relates to vesicle docking mechanisms in the platelets of these individuals, as this may affect the packaging and degranulation of soluble mediators following platelet activation. Elevated levels were detected for ASGR1 (asialoglycoprotein receptor 1), CLEC10A (C-type lectin domain family 10 member A), and SMAP1L (Stromal membrane-associated protein 2). Increased expression of ASGR1 may result in a higher efficiency of recycling and returning of glycoprotein receptors to the cell membrane. ASGR1 particularly acts on desialyated proteins, therefore increased expression may result in increased expression of cell surface selectin ligands. This may be aided by an increased expression of FUT7 (alpha-(1,3)-fucosyltransferase), which catalyzes the linkages involved in the expression of sialyl Lewis X antigens, and is involved in the synthesis of selectin molecules. Heterophilic, and homophilic cell-cell adhesion may be further augmented by elevated expression of receptors for Intracellular Adhesion Molecule (ICAM) and EVA1 (myelin protein zero-like protein 2).

There was lower expression of several genes involved in intracellular cytoskeletal structure and re-organisation. FLNB (Filamin-B) connects cell membrane constituents to the actin cytoskeleton, and lower levels of FLNB may affect anchorage of transmembrane proteins. Reduced levels of SPTBN5 (Spectrin beta chain, brain 4), may also affect cytoskeletal structure near the cell membrane. Microtubule formation may be altered due to lower expression of DNAH17 (Dynein heavy chain 17), and increased expression of TUBA3 (Tubulin alpha-1A chain). Elevated gene expression of nuclear proteins and transcription factors such as CEBPA, IKBKE, and IRF8, along with lower expression of DUSP4 may be central to the regulation of genes which define the pro- versus anti- inflammatory, angiogenic, coagulant phenotype of the cell. CEPBA plays a major role in myeloid development and commitment to lineage (Cai *et al*, 2006), and the phosphorylatory action of IKBKE leads to degradation of the NFκB inhibitor, therefore possibly maintaining NFκB pro-inflammatory signalling.

Secretion of lower levels of IL-10 and TFPI may contribute to a heightened proinflammatory and procoagulant cellular environment. Reduced levels of chemotactic peptides CXCL2, CXCL5, CXCL6 and CCL20 may result in reduced chemotaxis and activation of neutrophils and lymphocytes at the site of injury. Also expressed at a higher level is FGL2, which has fibrinogen-like activity. Both of these findings may have implications for differences in clot structure, as formation of fibrin fibres, and the incorporation of leukocytes into a thrombus, are important factors in the organisation of the thrombus (Dible, 1958; Davies *et al*, 1975; Feigl *et al*, 1985). The incorporation of fewer white cells results in a fibrinous clot with fewer spaces, and therefore has a more dense structure (Fatah *et al*, 1996; McGuiness *et al*, 2001; Collet *et al*, 2006). Several studies have reported differences in clot structure in patients with

heart disease, noting a more dense clot contains fibres that are thinner, and is more difficult to lyse (Collet *et al*, 2003; Wolberg et al, 2003; Dunn *et al*, 2004; Mills *et al*, 2006).

Therefore, in response to platelet activation, monocytes from premature MI subjects may have a phenotype that is more adhesive, proinflammatory and procoagulant than monocytes in healthy individuals, and may also have impaired mechanisms of intracellular trafficking of molecules. In addition, reduced levels of secreted chemokines may result in fewer leukocytes becoming incorporated into a thrombus at the site of injury, which may in turn affect clot structure and lysis.

The findings of this study also illustrate the importance of the platelet in modulating the plasticity of the monocyte. Monocytes found within the human circulation are generally classified into two main subsets. Approximately 85 – 90% of circulating monocytes are 'classical' monocytes which express high levels of CD14 and CCR2, and low levels of CD16 and CX3CR1. In contrast, 'non-classical' monocytes express high levels of CD16 and CX3CR1 and low levels of CD14 and CCR2 (Geissman *et al*, 2003; Ancuta *et al*, 2003). There has also been reported a small monocyte subset that expresses the angiopoietin receptor Tie2 (De Palma *et al*, 2007). The way these different monocyte subsets respond to stimuli is markedly different, with CD16+ monocytes having a higher inflammatory response to LPS than 'classical' monocytes (Belge *et al*, 2002). An increased proportion of CD16+ monocytes have been reported in coronary artery disease and hypercholesterolemia (Schlitt *et al*, 2004; Rothe *et al*, 1996), although whether this increase is the cause of increased risk of disease or a consequence of the disease progression is still unclear. Circulating monocytes also have plasticity, and therefore 'classic' monocytes may differentiate into further subsets

depending upon the stimuli they encounter (Sunderkotter *et al*, 2004). This is similar to the response seen in macrophages where LPS results in an M1, pro-inflammatory phenotype, whereas other stimuli such as IL4, TGF-β, and IL-10 induce several M2 phenotypes. There have been several studies recently which examine the role of monocyte subsets in the atherosclerotic process with the finding that the different subsets may have different patterns of trafficking, with 'classical' monocytes being preferentially recruited into sites of inflammation (Tacke *et al*, 2006), and non-classical monocytes recently reported to have a role in detoxification of oxidized LDL (Mosig *et al*, 2009). Monocyte subsets with differential expression of CCR2, CCR5 and CX3CR1 have also been shown to have different degrees of accumulation within atherosclerotic plaques (Tacke *et al*, 2007). Therefore during atherogenesis, it is possible that different monocyte subsets could infiltrate at different stages of lesion development and may have a functional role that delays disease progression or may contribute to progression of plaque growth or destabilisation.

Analysis of some of the key markers of monocyte subsets shows that interaction with platelet soluble mediators and cell-cell aggregation via PSGL-1 result in downregulation of CD14, however there is no significant change in CD16 expression. Platelet soluble mediators induce significant upregulation of CCR2, and downregulation of CX3CR1. Therefore analysis of key monocyte markers suggests a classical monocyte but with perhaps lower expression of CD14 similar to that seen in monocyte-derived endothelial progenitor cells (EPCs) (Fujiyama *et al*, 2003), which lose expression of CD14 and CD45, instead expressing endothelial markers and secreting angiogenic growth factors when cultured under angiogenic conditions (Rehman *et al*, 2003; Hur *et al*, 2004). Markers of EPCs such as Tie2, and endoglin were not

upregulated by 4 hours; however there was significant upregulation of many genes associated with wound healing.

Therefore, the findings of this study emphasise how the mechanisms of platelet activation and interaction can modulate the monocyte phenotype, in particular, the strong role that platelet soluble mediators play in utilising the plasticity of the monocyte, attracting it to the site of injury, and inducing a phenotype that is suitable for a role in thrombus formation and wound healing. One question that this raises, is what happens once the cells are incorporated into the growing thrombus? Are they there simply to provide structural support to the initial platelet plug, and deliver cytotoxic mediators, etc, or is there further modulation of gene expression within the thrombus itself? The following chapter investigates these questions.

In summary:

- Monocyte interaction with platelet soluble mediators induces a pattern of gene expression, resulting in a monocyte with a wound healing phenotype.
- This occurs through increased expression of pro-angiogenic (C-X-C) chemokines, and increased expression of receptors facilitating ECM interaction.

Limitations:

- Optimisation of P-selectin-Fc chimera concentration was not carried out prior to sample collection, therefore it is possible that the monocytes were not fully activated by the chimeric protein.
- The study only investigates the platelet secretome following platelet activation with a collagen peptide (CRP-XL). The soluble mediators released from platelet

granules may be different in response to different agonists, and therefore have a different influence on the monocyte phenotype.

Future work:

- Validation of genes of interest by real-time PCR
- Further study of platelet induction of DUSPs; including determination of soluble mediators responsible for gene induction of 4,5,6 and 10, and downregulation of 16; signalling pathways and transcriptional regulation; role of each of these in monocyte function.
- Further study of platelet-monocyte interaction and changes in expression of monocyte adhesion molecules, chemokine expression.

CHAPTER 7: Thrombus formation

7.1 Introduction

The previous chapters have demonstrated that plasma factors play an important role in determining thrombotic potential, and that cellular activation and interaction can alter gene expression, adapting a cell phenotype to one that is suitable for the wound healing environment. But what happens once the cells are incorporated into a thrombus?

In the event of vascular injury, under normal haemostatic conditions, the haemostatic response seeks to stop vascular bleeding, maintain a stable clot that does not become occlusive, and dispose of the clot when it is no longer required. It is well established that this process involves the activation of procoagulant and anticoagulant factors found in the plasma, cellular activation, for example of platelets, to form the initial plug to stop the bleeding, and infiltration of additional platelets and white cells to stabilise the clot (Mustard *et al*, 1962; Poole *et al*, 1963). White cells also deliver soluble mediators such as cytokines, (for example IL-8), chemokines, (CXCL1, CXCL2), matrix metalloproteinases (MMP2 and MMP9), and serine proteases (elastase, and cathespin G), which act to trigger an inflammatory response and degrade extracellular matrix components in the wound site (Kirchhofer *et al*, 1997; Henke *et al*, 2001; Whatling *et al*, 2004). One question this raises is whether the cells are simply attracted to the site of injury to adhere, provide structure, and release their contents into the wound site, or whether there is an additional process involving changes in gene expression in cells incorporated into the thrombus, that further modifies formation of a thrombus? In addition to coagulation, the wound healing environment involves many other processes such as cellular chemotaxis and adhesion, cell signalling, and angiogenesis. All of these processes require regulation over a period of time, and this may involve changes

in expression of specific genes. There have been many studies that have examined the role of, and measured expression of, specific proteins within a thrombus, *eg* TF, PECAM-1, platelet collagen receptor GPVI, thrombospondin-1, ephrins, and Gas6 (Angelillo-Scherrer *et al*, 2001; Falati *et al*, 2002; Kato *et al*, 2003; Prevost *et al*, 2002; Bonnefoy *et al*, 2006; Falati *et al*, 2006). Other studies have examined the structure of a thrombus (Collet *et al*, 2000; Collet *et al*, 2005; Weisel, 2007; Gersh *et al*, 2009), including investigation into hereditary factors and the effect on clot formation and structure (Ariens *et al*, 2002; Dunn *et al*, 2004; Collet *et al*, 2006; Carter *et al*, 2007; Bhasin *et al*, 2008). However, to date, there have been no reports on gene expression patterns within the growing thrombus. This study seeks to investigate how gene expression may alter during early thrombus formation.

7.2 Study Design

The studies described in the previous two chapters have demonstrated how platelets can modulate gene expression through direct cell-cell ligand binding and through release of soluble mediators. The purpose of this study was to investigate whether this also occurs within a thrombus, whether there is regulated gene expression within a thrombus, and if so, how patterns of gene expression change during thrombus formation. Six normal, healthy donors were recruited. Citrated whole blood was recalcified and added to a series of Chandler loops. The blood was rotated within the loop at 37^{o}C for 2, 4 and 6 hours. At each time point, thrombi were removed from the loop and placed briefly into RNA*later®* to preserve the RNA. Thrombi were transferred into TRIZOL® solution and homogenised. Total RNA was isolated, quantified, and amplified by IVT.

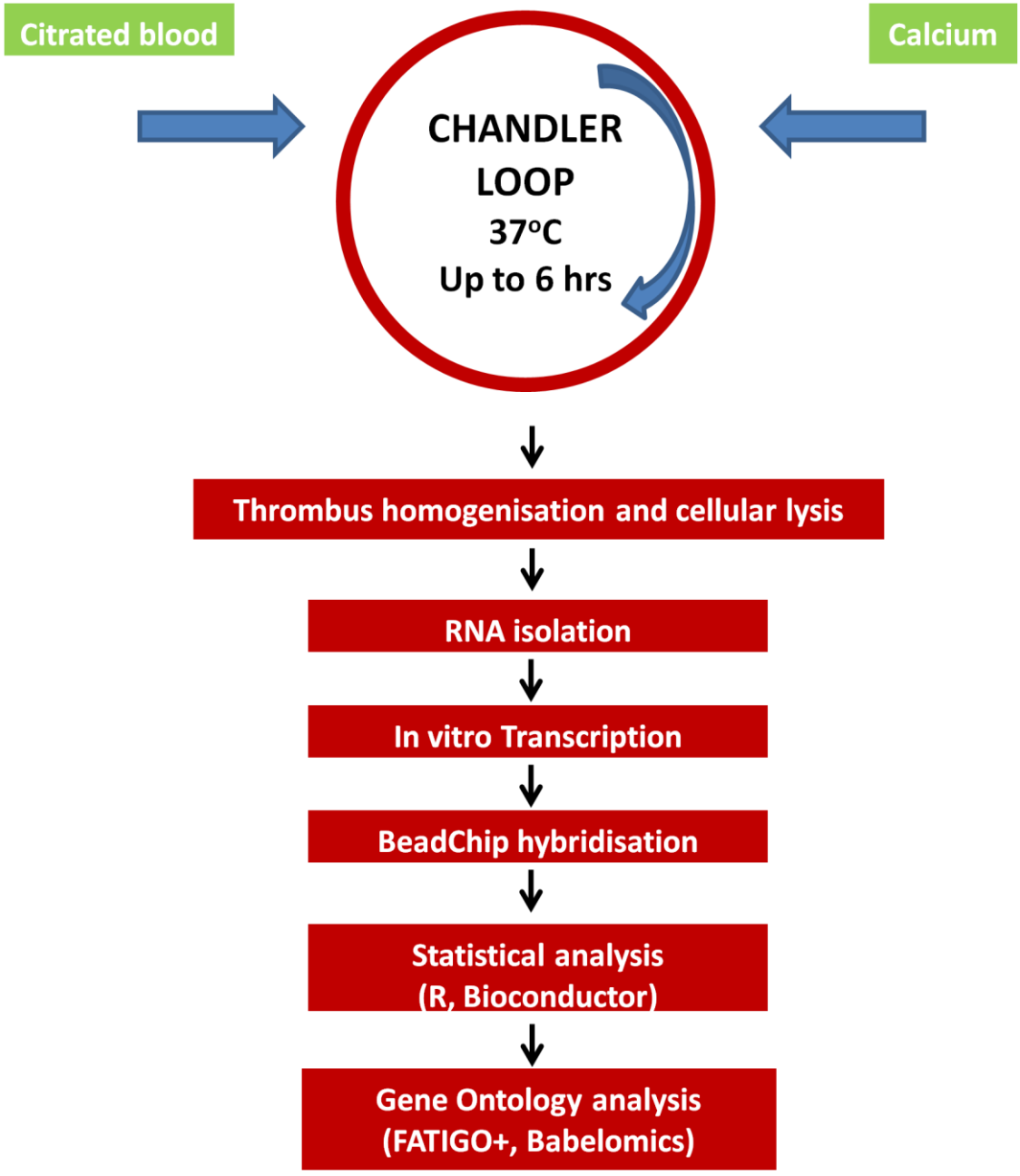


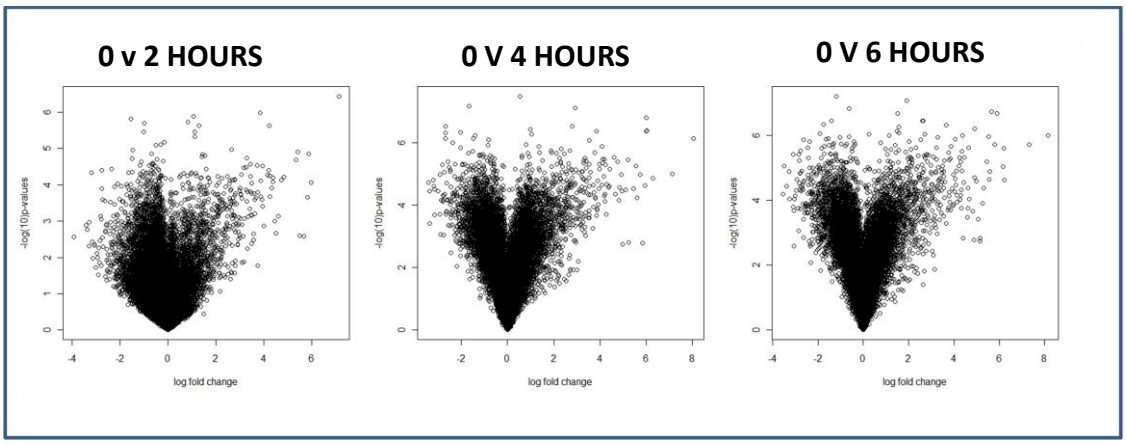
Figure 7.1: Overview of design for thrombus gene expression microarray study

cRNA was then hybridised onto Illumina® Human WG-6 v2 arrays. An overview of the study design is summarised in Figure 7.1.

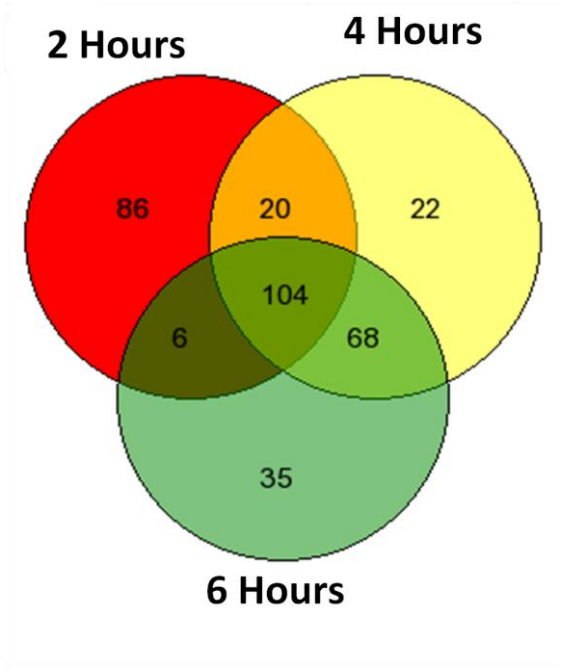
7.3 Results of thrombus gene expression microarray study

Transcription profiling was carried out using statistical analyses to identify differentially expressed genes at each time point. Initial analysis generating volcano plots (Fig 7.2a) demonstrate significant changes in gene expression within a growing thrombus. Transcriptional profiling listed upregulation of 335, 256, and 933 genes at 2, 4, and 6 hours, respectively; and downregulation of 359, 568, and 986 genes at 2, 4, and 6 hours, respectively. The top 225 upregulated and downregulated genes at each time point were selected for further analysis. The web application, Gene Venn, was used to sort the gene lists obtained from each time point, and calculate overlap of expression between time points (Figure 7.2b and c). Functional Enrichment analysis was carried out using FATIGO+ (Babelomics v3.1), and further defined using UniProt. Figures 7.3, and 7.4. show the distribution of the ontological classification of gene expression at each time point, with the figures showing the mean fold change in gene expression for the genes in each term. Tables 7.1, and 7.2 show the top 25 genes induced or downregulated at each time point, listed according to function. Gene ontology terms selected for further analysis included: Coagulation, Cell adhesion, Apoptosis, Angiogenesis, and Transcription factor activity, and are described in more detail below.

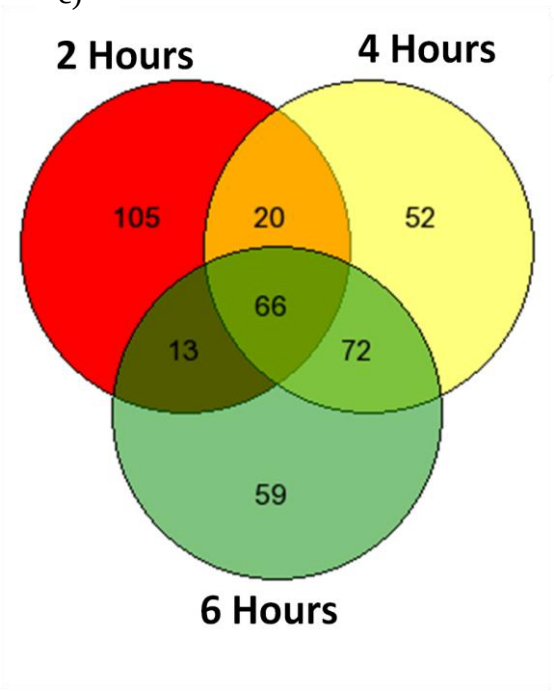
a)



b)



c)



UPREGULATED GENES **DOWNREGULATED GENES**

Figure 7.2: Differential gene expression during thrombus formation.
Venn diagrams showing number of genes (a) Volcano plots showing differential expression of genes during formation of a thrombus; (b) Genes upregulated, and (c) downregulated during thrombus formation.

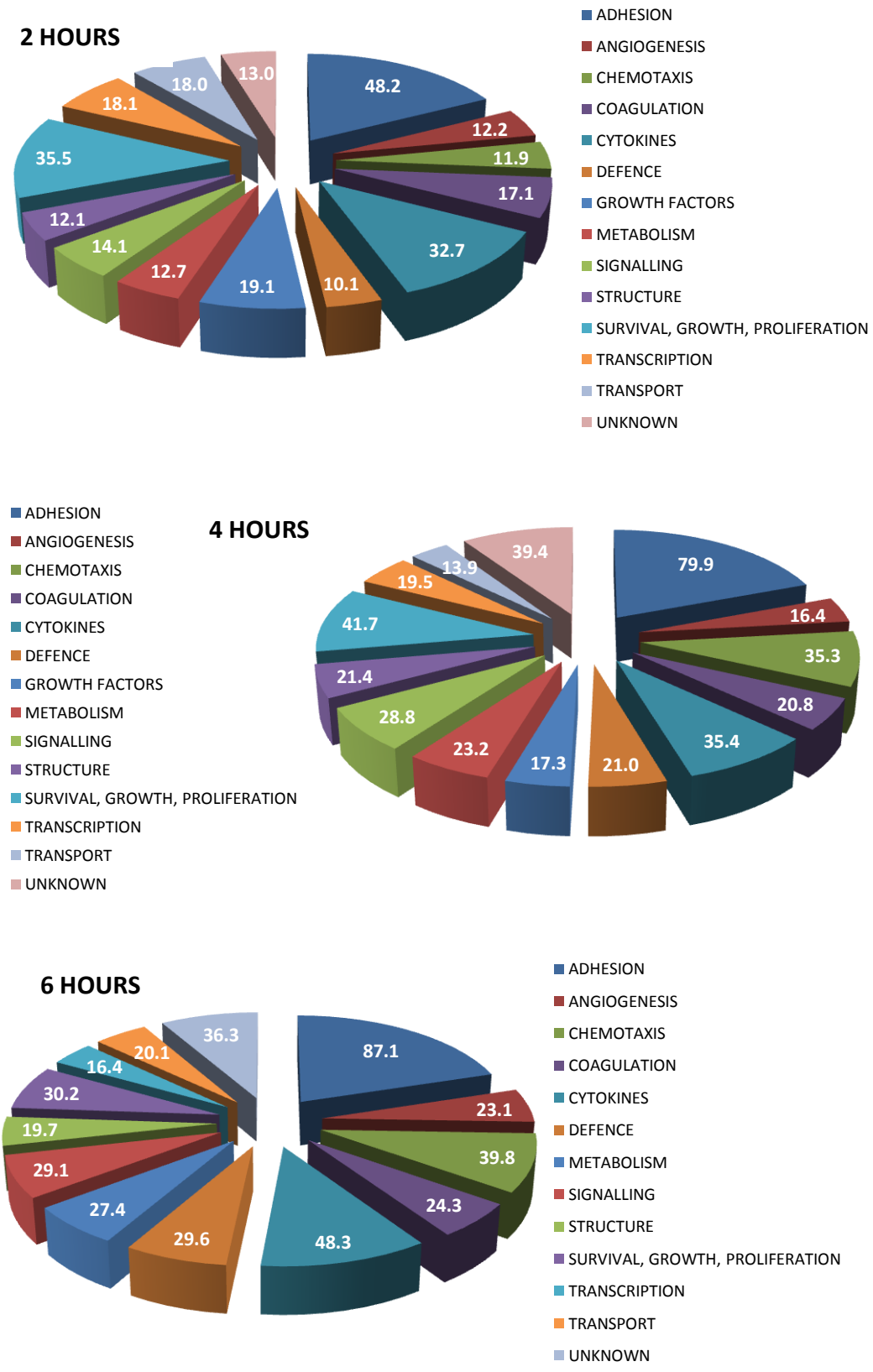


Figure 7.3: Classification of genes upregulated during early thrombus formation. (Figures show mean fold change).

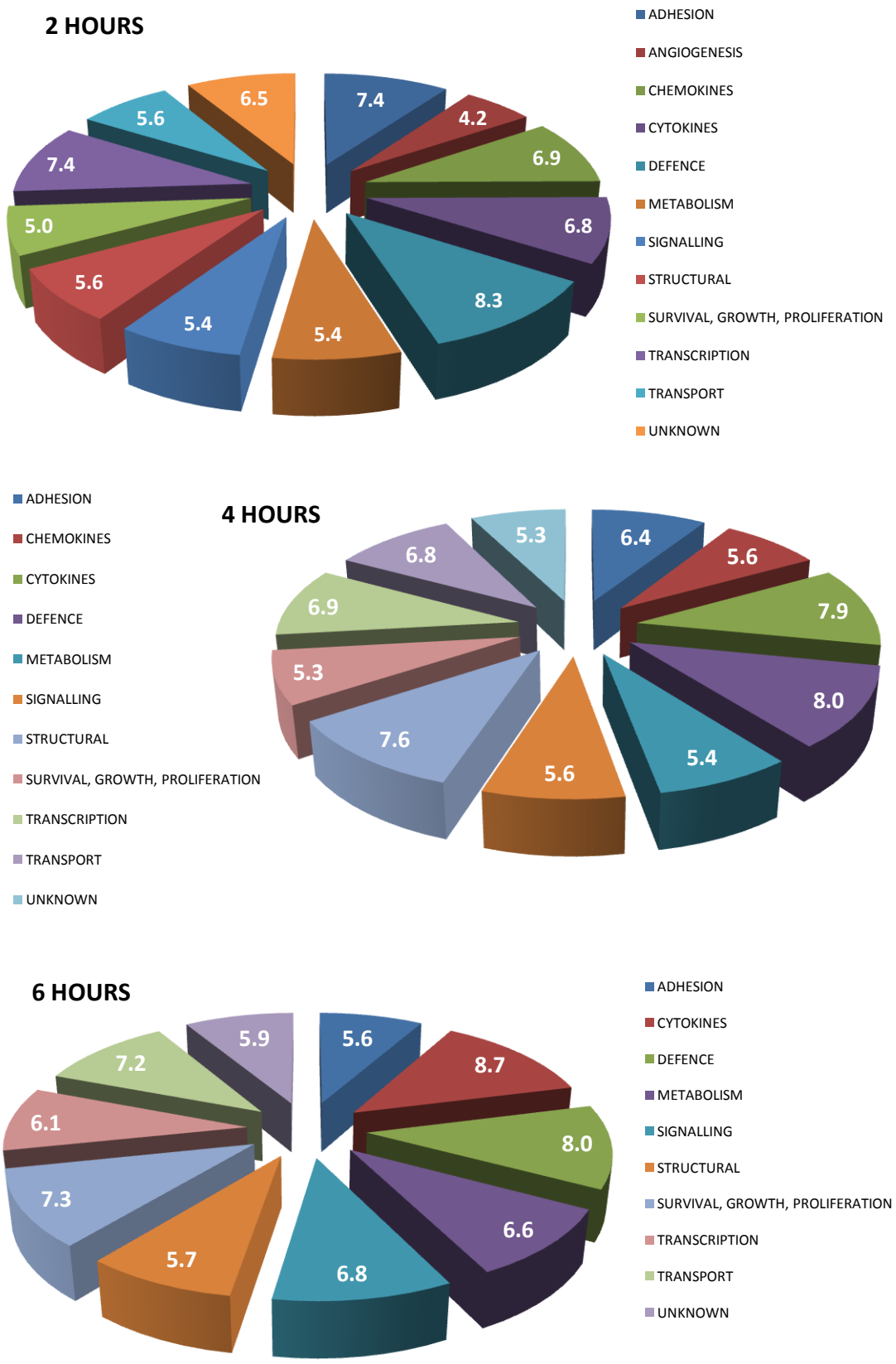


Figure 7.4: Classification of genes downregulated during early thrombus formation. (Figures show mean fold change).

SYMBOL	fold.change	p.value	Definition
			Adhesion
OLR1	138.97	3.57E-07	oxidised low density lipoprotein (lectin-like) receptor 1
THBS1	28.50	6.11E-05	thrombospondin 1
			Coagulation
THBD	19.00	2.13E-04	thrombomodulin
PLAUR	15.19	2.59E-04	plasminogen activator, urokinase receptor
			Cytokines
IL8	62.87	8.51E-05	interleukin 8
IL1B	26.66	7.33E-05	interleukin 1, beta
			Growth factors
HBEGF	19.07	5.67E-05	heparin-binding EGF-like growth factor
			Metabolism
ERAF	21.84	9.92E-04	erythroid associated factor
			Signalling
RGS1	18.76	2.36E-06	regulator of G-protein signalling 1
AVPI1	17.60	5.33E-05	arginine vasopressin-induced 1
IER3	16.01	1.33E-03	immediate early response 3
TRIB1	14.68	7.71E-05	tribbles homolog 1
SNCA	14.50	2.06E-04	synuclein, alpha
PHACTR1	15.39	3.07E-05	phosphatase and actin regulator 1
			Survival, proliferation, and growth
OSM	57.94	1.37E-05	oncostatin M
G0S2	55.87	2.18E-04	G0/G1switch 2
PHLDA1	42.17	1.23E-05	pleckstrin homology-like domain, family A, member 1,
RIS1	22.51	2.16E-03	Ras-induced senescence 1 (RIS1)
CDKN1A	20.89	8.44E-05	cyclin-dependent kinase inhibitor 1A
			Transcription
FOSB	40.51	2.07E-05	FBJ murine osteosarcoma viral oncogene homolog B (FOSB)
NR4A2	20.70	6.42E-05	nuclear receptor subfamily 4, group A, member 2
EGR1	19.92	1.21E-04	early growth response 1
			Transport
HBD	24.31	7.29E-04	hemoglobin, delta
HBQ1	15.30	3.90E-04	hemoglobin, theta 1
			Unknown
C15orf48	18.36	3.93E-05	chromosome 15 open reading frame 48

Table 7.1a: Classification of genes with greatest induction following 2 hours of thrombus formation. (List shows top 25 genes).

SYMBOL	fold.change	p.value	DESCRIPTION
			Adhesion
OLR1	263.64	7.35E-07	oxidised low density lipoprotein (lectin-like) receptor 1
TGM2	141.12	9.87E-06	transglutaminase 2
THBS1	24.46	2.91E-06	thrombospondin 1
			Angiogenesis
ANGPTL4	19.78	2.19E-05	angiopoietin-like 4
			Chemokines
CCL2	62.11	2.39E-05	chemokine (C-C motif) ligand 2
CCL7	48.35	5.77E-05	chemokine (C-C motif) ligand 7
CXCL2	34.23	3.28E-05	chemokine (C-X-C motif) ligand 2
CCL20	30.27	1.03E-05	chemokine (C-C motif) ligand 20
			Coagulation
SERPINB2	28.23	6.89E-05	serpin peptidase inhibitor, clade B (ovalbumin), member 2
PLAU	21.23	2.49E-05	plasminogen activator, urokinase
			Cytokines
IL8	78.67	1.38E-05	interleukin 8 (IL8)
IL1B	31.00	3.57E-05	interleukin 1, beta
			Defence
CLEC5A	27.04	4.11E-06	C-type lectin domain family 5, member A
			Metabolism
CTSL	47.43	5.53E-06	cathepsin L (CTSL), transcript variant 2
			Signalling
DUSP4	43.88	2.98E-05	dual specificity phosphatase 4
RGS1	37.25	3.53E-06	regulator of G-protein signalling 1
NRIP3	28.88	2.72E-05	nuclear receptor interacting protein 3
			Structural
PHACTR1	21.35	7.16E-06	phosphatase and actin regulator 1
			Survival, proliferation, and growth
OSM	64.89	4.03E-07	oncostatin M (OSM)
PHLDA1	63.60	4.26E-07	pleckstrin homology-like domain, family A, member 1
RIS1	57.25	0.001622	Ras-induced senescence 1
G0S2	53.44	1.04E-05	G0/G1switch 2
PMP22	20.75	2.67E-05	peripheral myelin protein 22
			Transcription
FOSB	25.36	4.55E-05	FBJ murine osteosarcoma viral oncogene homolog B
			Unknown
C15orf48	64.38	1.57E-07	chromosome 15 open reading frame 48

Figure 7.1b: Classification of genes with greatest induction following 4 hours of thrombus formation. (List shows top 25 genes).

SYMBOL	fold.change	p.value	Definition
			Adhesion
OLR1	282.30	1.02E-06	oxidised low density lipoprotein (lectin-like) receptor 1
TGM2	160.15	1.91E-06	transglutaminase 2
			Angiogenesis
ANGPTL4	30.38	6.31E-07	angiopoietin-like 4
			Chemokines
CCL2	75.37	2.38E-05	chemokine (C-C motif) ligand 2
CCL20	46.80	2.32E-05	chemokine (C-C motif) ligand 20
CCL7	39.97	4.14E-05	chemokine (C-C motif) ligand 7
CXCL2	38.00	0.000126	chemokine (C-X-C motif) ligand 2
FPRL2	31.72	2.68E-05	formyl peptide receptor-like 2
CXCL1	25.46	9.39E-05	chemokine (C-X-C motif) ligand 1
			Coagulation
SERPINB2	33.40	0.000393	serpin peptidase inhibitor, clade B (ovalbumin), member 2
THBS1	30.19	2.05E-05	thrombospondin 1
PLAU	24.68	4.29E-05	plasminogen activator, urokinase
			Cytokines
IL8	72.06	9.27E-06	interleukin 8
IL1B	24.60	0.00018	interleukin 1, beta
			Defence
CLEC5A	43.60	6.45E-06	C-type lectin domain family 5, member A
			Metabolism
CTSL	55.02	1.93E-06	cathepsin L
RNASE1	24.10	0.000344	ribonuclease, RNase A family, 1
			Signalling
DUSP4	47.71	1.33E-05	dual specificity phosphatase 4
RGS1	45.77	1.09E-06	regulator of G-protein signalling 1
			Survival, proliferation and growth
PHLDA1	60.18	2.09E-07	pleckstrin homology-like domain, family A, member 1
OSM	50.87	1.86E-07	oncostatin M
RIS1	35.50	0.001494	Ras-induced senescence 1
G0S2	28.27	0.00022	G0/G1switch 2
			Transcription
PPARG	24.93	2.39E-06	peroxisome proliferative activated receptor, gamma
			Unknown
C15orf48	73.92	2.54E-06	chromosome 15 open reading frame 48

Figure 7.1c: Classification of genes with greatest induction following 6 hours of thrombus formation. (List shows top 25 genes).

SYMBOL	fold change	p.value	definition
			Adhesion
CD2	-9.76	0.0049	CD2 antigen (p50), sheep red blood cell receptor
			Chemokines
CCR2	-6.88	0.0009	chemokine (C-C motif) receptor 2
			Cytokines
IL7R	-5.84	0.0059	interleukin 7 receptor
IL11RA	-7.78	0.0077	interleukin 11 receptor, alpha
			Defence
GIMAP6	-6.60	0.0003	GTPase, IMAP family member 6
KLRK1	-7.08	0.0034	killer cell lectin-like receptor subfamily K, member 1
GIMAP8	-9.03	0.00004	GTPase, IMAP family member 8
GIMAP7	-10.60	0.0013	GTPase, IMAP family member 7
GIMAP5	-15.17	0.0028	GTPase, IMAP family member 5
			Metabolism
GSDML	-6.08	0.0019	gasdermin-like
CHST12	-5.95	0.0013	carbohydrate (chondroitin 4) sulfotransferase 12
GVIN1	-5.33	0.0022	GTPase, very large interferon inducible 1
ECHDC2	-8.32	0.0043	enoyl Coenzyme A hydratase domain containing 2
			Signalling
PLCG1	-7.04	0.0007	phospholipase C, gamma 1
STMN3	-8.26	0.0047	stathmin-like 3
			Structural
EVL	-6.77	0.00004	Enah/Vasp-like
			Survival, proliferation, and growth
CDC25B	-5.33	0.0015	cell division cycle 25B
DNAJA3	-5.68	0.0085	DnaJ (Hsp40) homolog, subfamily A, member 3
PAQR8	-6.23	0.0003	progestin and adipoQ receptor family member VIII
			Transcription
EOMES	-10.25	0.0017	eomesodermin homolog
ZNF573	-6.71	0.0001	zinc finger protein 573
			Transport
ATP8B2	-6.59	0.0028	ATPase, Class I, type 8B, member 2
			Unknown
LOC653518	-5.86	0.0012	similar to C-C chemokine receptor type 2
DKFZp761P0423	-7.34	0.0055	hypothetical protein DKFZp761P0423
DENND2D	-9.70	0.0011	DENN/MADD domain containing 2D

Figure 7.2a: Classification of genes with greatest downregulation following 2 hours of thrombus formation. (List shows top 25 genes).

SYMBOL	fold change	p.value	Definition
			Adhesion
CD2	-6.43	2.95E-07	CD2 antigen (p50), sheep red blood cell receptor
			Cytokines
LTB	-6.88	7.6E-05	lymphotoxin beta (TNF superfamily, member 3)
IL11RA	-8.97	3.05E-05	interleukin 11 receptor, alpha
			Defence
KLRK1	-5.81	6.12E-06	killer cell lectin-like receptor subfamily K, member 1
GIMAP6	-6.39	4.84E-07	GTPase, IMAP family member 6
GIMAP7	-8.08	3.51E-05	GTPase, IMAP family member 7
KLRG1	-9.21	1.57E-05	killer cell lectin-like receptor subfamily G, member 1
GIMAP8	-10.41	3.56E-05	GTPase, IMAP family member 8
GIMAP5	-10.79	1.89E-05	GTPase, IMAP family member 5
			Metabolism
GSDML	-5.61	9.78E-05	gasdermin-like
ARHGEF3	-6.13	6.91E-05	Rho guanine nucleotide exchange factor
ECHDC2	-7.32	7E-05	enoyl Coenzyme A hydratase domain containing 2
			Signalling
TRIB2	-6.00	4.08E-06	tribbles homolog 2
TUSC4	-6.09	6.54E-05	tumor suppressor candidate 4
PLCG1	-6.41	7.26E-07	phospholipase C, gamma 1
D4S234E	-6.96	7E-05	DNA segment on chromosome 4, 234 expressed sequence
SCAP1	-6.35	1.96E-04	src family associated phosphoprotein 1
			Structure
EVL	-7.62	0.0002	Enah/Vasp-like
			Survival, proliferation and growth
PAQR8	-6.77	5.08E-05	progestin and adipoQ receptor family member VIII
			Transcription
ZNF573	-6.87	4.92E-05	zinc finger protein 573
			Transport
ATP6V0E2L	-8.92	2.08E-04	ATPase, H+ transporting V0 subunit E2-like
ATP8B2	-6.64	8.91E-06	ATPase, Class I, type 8B, member 2
			Unknown
ALS2CR13	-5.61	1.85E-04	amyotrophic lateral sclerosis 2chromosome region, candidate 13
LOC648470	-5.70	6.64E-05	similar to Caspase-4 precursor (CASP-4)
DENND2D	-7.12	5.06E-06	DENN/MADD domain containing 2D

Figure 7.2b: Classification of genes with greatest downregulation following 4 hours of thrombus formation. (List shows top 25 genes).

SYMBOL	fold change	p.value	Definition
			Cytokines
LTB	-6.80	3.77E-05	lymphotoxin beta (TNF superfamily, member 3)
IL32	-7.63	2.29E-05	interleukin 32
IL11RA	-11.71	6.65E-05	interleukin 11 receptor, alpha
			Defence
KLRG1	-7.77	5.11E-05	killer cell lectin-like receptor subfamily G, member 1
NCR3	-8.42	2.07E-04	natural cytotoxicity triggering receptor 3
GIMAP7	-8.57	3.87E-06	GTPase, IMAP family member 7
GIMAP5	-9.76	1.30E-05	GTPase, IMAP family member 5
GIMAP8	-11.30	9.01E-06	GTPase, IMAP family member 8
			Metabolism
ECHDC2	-8.01	7.38E-05	enoyl Coenzyme A hydratase domain containing 2
			Signalling
TRIB2	-6.54	1.25E-05	tribbles homolog 2
TSPAN32	-6.67	5.33E-05	tetraspanin 32
MAP4K1	-6.78	1.42E-05	mitogen-activated protein kinase kinase kinase kinase 1
PLCG1	-6.99	1.48E-05	phospholipase C, gamma 1
SCAP1	-7.11	5.96E-06	src family associated phosphoprotein 1
MS4A6A	-8.29	2.60E-03	membrane-spanning 4-domains, subfamily A, member 6A
D4S234E	-8.55	1.61E-04	DNA segment on chromosome 4, 234 expressed sequence
STMN3	-9.55	9.25E-05	stathmin-like 3
			Survival, proliferation and growth
GZMK	-7.09	3.03E-05	granzyme K
CDC25B	-7.26	3.86E-06	cell division cycle 25B
AHNAK	-7.28	1.73E-04	AHNAK nucleoprotein
PRF1	-7.22	8.75E-04	perforin 1
GZMA	-10.37	3.76E-05	granzyme A
			Transcription
LEF1	-6.82	2.99E-06	lymphoid enhancer-binding factor 1
			Transport
ATP6V0E2L	-8.17	9.39E-05	ATPase, H+ transporting V0 subunit E2-like
			Unknown
LOC202134	-6.91	4.79E-05	hypothetical protein LOC202134

Figure 7.2c: Classification of genes with greatest downregulation following 6 hours of thrombus formation. (List shows top 25 genes).

7.3.1. Coagulation

Key genes involved in coagulation included upregulation of thrombomodulin (THBD), which as the name suggests, limits the amount of thrombin generated by forming a complex with thrombin. There was also upregulation of urokinase plasminogen activator (PLAU) and its receptor (PLAUR) which allows conversion of plasminogen into plasmin, in turn promoting degradation of the ECM by direct digestion and MMPs. This process is tightly regulated by plasminogen activator inhibitor, PAI-1, which was also upregulated (SERPINE1). Previously identified as playing an important inhibitory role, there was upregulation of TFPI, and also Annexin-V (ANXA5) which limits formation of the prothrombinase complex and in turn, limits the amount of thrombin generated (Figure 7.5). Therefore producing a phenotype that was anti-thrombotic and anti-fibrinolytic.

Taqman® expression assay PCRs were carried out to validate the results of the array data with regard to TF and TFPI gene expression. Expression of both genes were seen within the thrombus, with a steady increase in TF over time (mean fold change 27.0±18.2, p= 0.0175; 43.7±23.8, p= 0.0070; and 51.5±78.0, p= 0.1739, at 2, 4 and 6 hours, respectively), compared to a stronger increase in TFPI (mean fold change 13.6±14.1, p= 0.0810; 66.9±95.1, p=0.1506; 168.7±197.7, p=0.0923, at 2, 4 and 6 hours, respectively), perhaps seeking to regulate the procoagulant response (Figure 7.6). Gene expression of the two main isoforms of TFPI was also measured, using SYBR green PCR assays. Mean 2^{-CT} for the α-isoform was 6.63×10^{-10}, p=0.1046; 2.20×10^{-9}, p=0.0802; 8.74×10^{-9}, p=0.0002; at 2, 4 and 6 hours respectively. The β-isoform was expressed at lower levels, with mean 2^{-CT} at 2, 4 and 6 hours measuring 9.79×10^{-11}, p=0.0508; 9.57×10^{-10}, p=0.0452; and 1.15×10^{-9}, p=0.0129, respectively (Figure 7.7).

COAGULATION

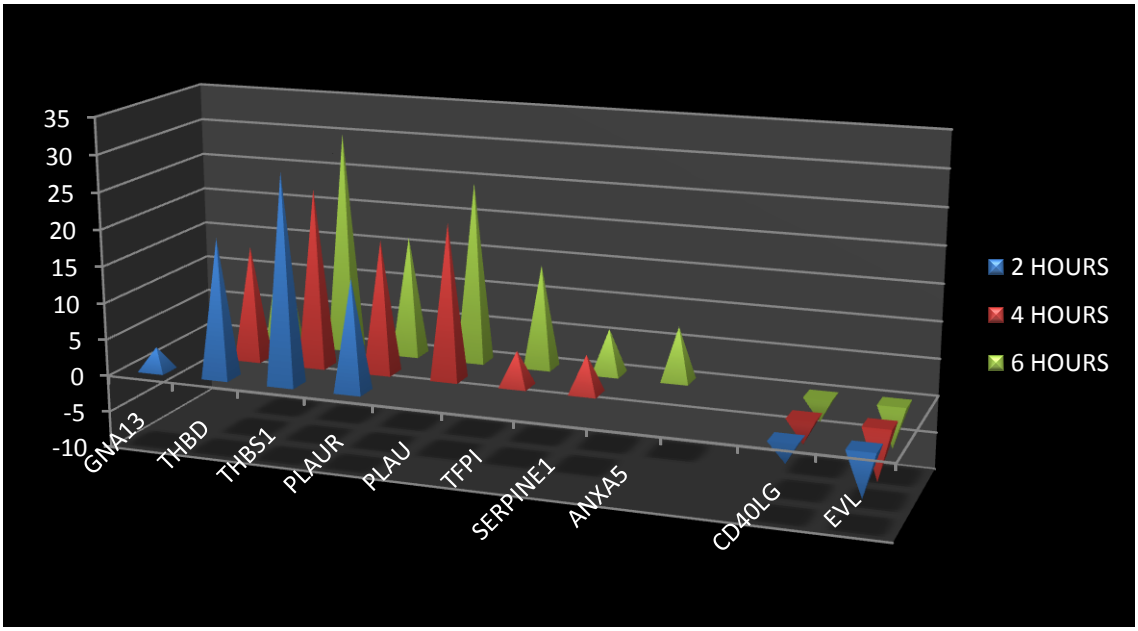


Figure 7.5: Gene expression of coagulation genes during thrombus formation.

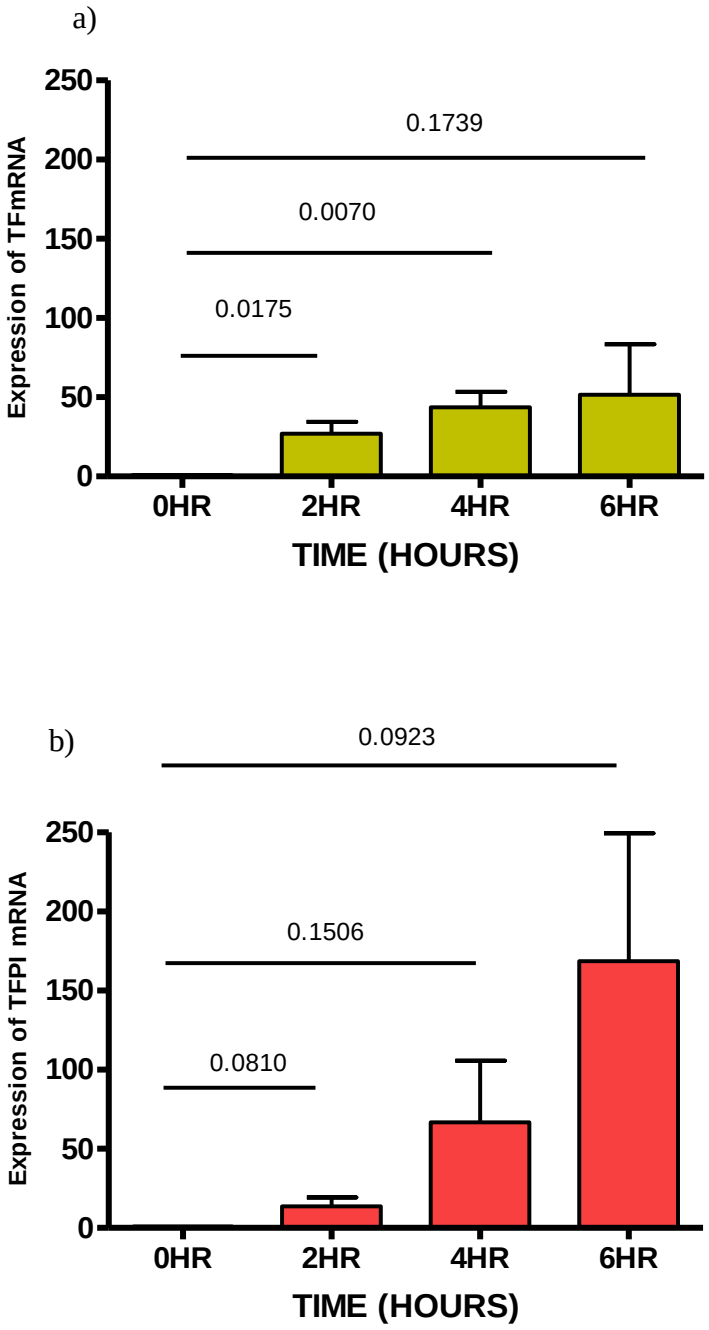


Figure 7.6: Gene expression of Tissue Factor and Tissue Factor Pathway Inhibitor in a growing thrombus. (a) Gradual increase of TF; (b) Strong induction of TFPI. (Figures are Mean ±SEM; *n* = 6).

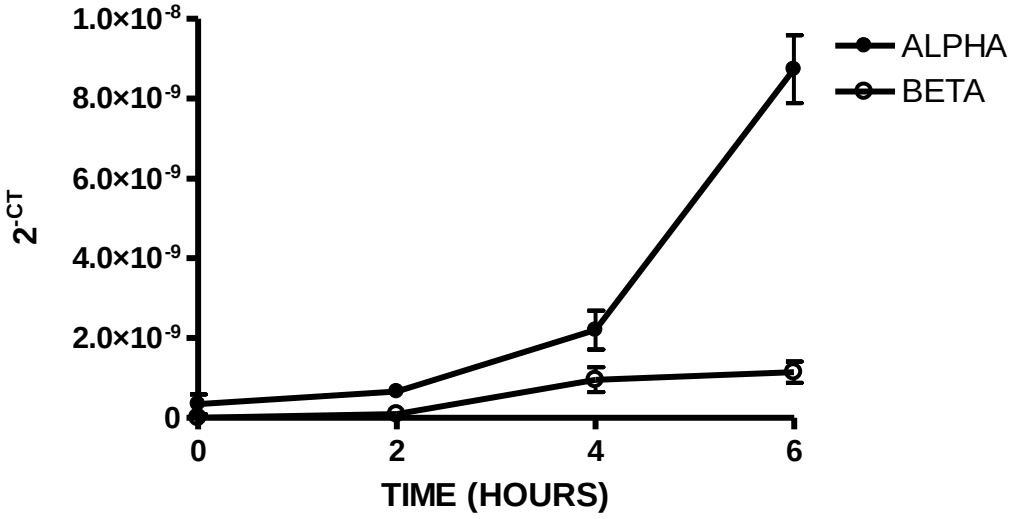


Figure 7.7: Gene expression of TFPI-isoforms in a growing thrombus. Graphs show changes in gene expression of (a) TFPI-α, and (b) TFPI-β, in thrombi isolated from 6 normal, healthy donors. (c) Mean gene expression for TFPI-α and β isoforms ± SEM (*n* = 6).

7.3.2. Cell Adhesion

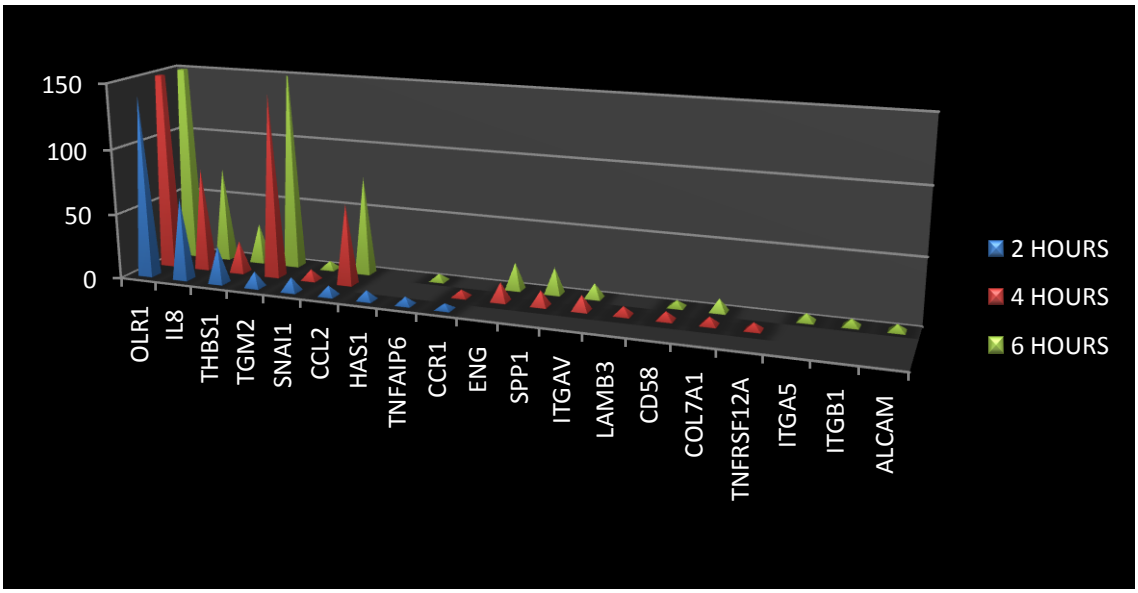
Genes that underwent major upregulation included IL8, which is involved in chemotaxis of neutrophils, and may be required for organisation of the thrombus (Wakefield *et al*, 1999); and thrombospondin (THBS) which mediates cell adhesion via binding to ECM components such as fibrinogen, fibronectin, laminin and collagen. There was also increased expression of transglutaminase-2 (TGM2) which catalyses the cross-linking of proteins, and therefore possibly confers stability to the forming thrombi; and CCL2 (also known as MCP1) which is a strong chemoattractant for monocytes, and is generally associated with the early phase of arterial injury (Goede *et al*, 1999). By 4 hours, there was induction of endoglin (ENG), a receptor for TGF-beta family members. This has been reported to be upregulated during monocyte to macrophage transition, and may play a role in the binding of endothelial cells to integrins (Walshe *et al*, 2009). There was upregulation of several genes involved in cell-extracellular matrix (ECM) interaction including osteopontin (SPP1), which binds hydroxyapatite, and also upregulation of its receptor, ITGAV. LAMB3, one part of the trimeric protein laminin-5, was also upregulated. This is a major component of the basement membrane of the ECM, and mediates attachment and organisation of cells. Also upregulated was COL7A1, another basement membrane protein that forms anchoring fibrils that may contribute to membrane organisation and adherence by interacting with ECM proteins such as type IV collagen. Upregulation of CD58, a ligand of T-lymphocyte CD2 glycoprotein, may regulate induction of various biological functions such as cytokine production, particularly IL-8. Finally, by 6 hours, a third group of genes was upregulated, including two that are subunits for the fibronectin

receptor, an ECM component (ITGA5 and ITGB1); and ALCAM which is an adhesion molecule, required for optimal activation of T-cells.

Genes that were downregulated during the early period of thrombus formation included T-cell differentiation markers CD6 and CD2, also CD40LG, which is expressed on activated T-cells and platelets, and CX3CR1, the fractalkine receptor. A second group of genes, including flotillin (FLOT) which acts as a scaffolding protein within caveolar membranes; ITGAL, (also known as CD11a) a receptor for ICAM 1, 2, 3, and 4, and involved in numerous leukocyte-endothelial cell interactions; integrin ITGB7, that interacts with adhesion molecules MADCAM1, VCAM1 and ECM component fibronectin; and CD47, an adhesion receptor for THBS1 on platelets and modulator of integrins; were all downregulated by 4 hours. This was followed by downregulation of AMICA1, a junctional adhesion molecule involved in recruitment of leukocytes to inflammatory sites; CNTNAP2, involved in potassium channel localisation; and MFGE8, a lactadherin precursor involved in binding of integrin β3 to apoptotic cells. Also downregulated was IL32, a proinflammatory cytokine that induces monocyte to macrophage differentiation; and EVA1, a transcription factor, that is possibly involved in T-cell differentiation (Figure 7.8).

CELL ADHESION

a) Upregulated genes



b) Downregulated genes

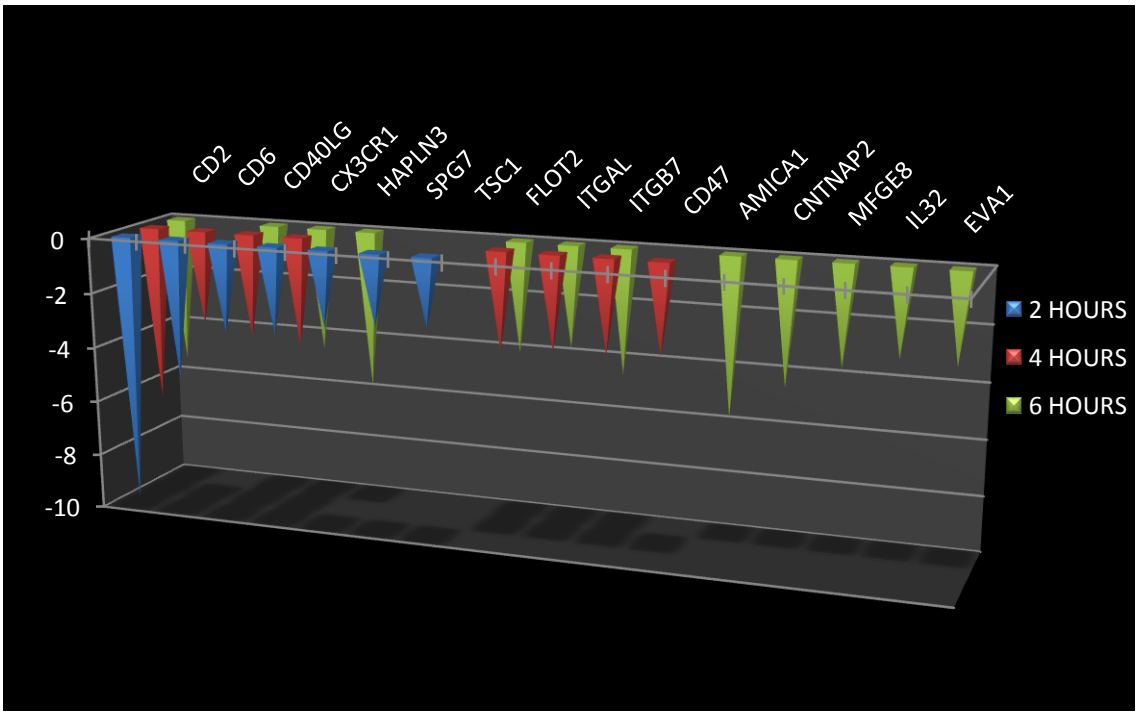


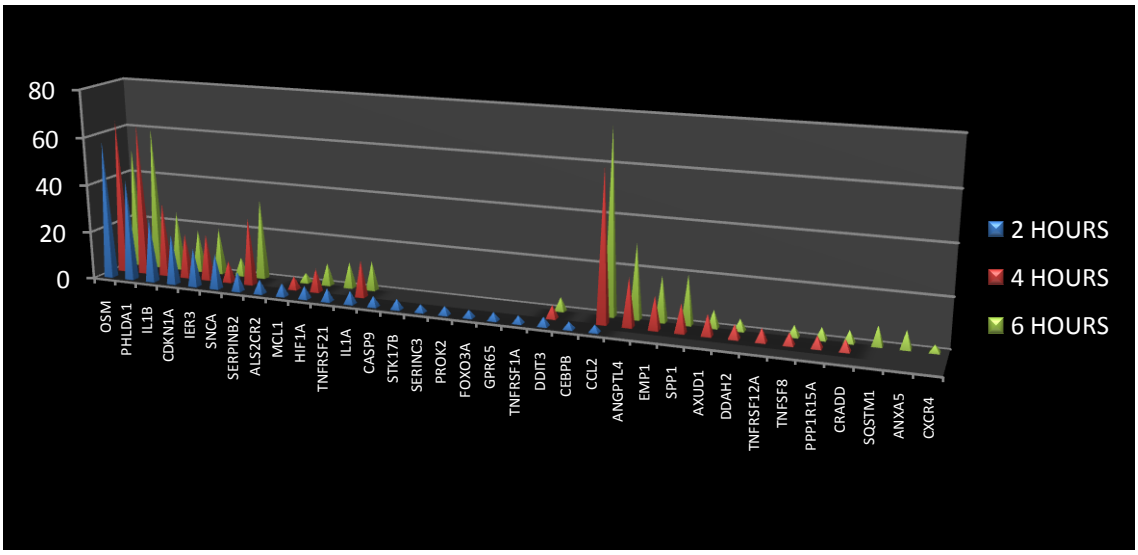
Figure 7.8: Changes in expression of genes involved in cell adhesion during thrombus formation. (a) upregulated genes; (b) downregulated genes.

7.3.3. Apoptosis

Gene ontology analysis showed upregulation of several transcription factors that play a role in regulation of apoptosis, including CEBPB, IER3, HIF1A, and FOXO3A, all of which can both induce or suppress apoptosis (Wu *et al*, 1998; Schilling *et al*, 2001; Buck *et al*, 2001; Ranjan *et al*, 2006; Okada *et al*, 2007; Fulda *et al*, 2007; Mei *et al*, 2009; Blake *et al*, 2010). Reflecting this, there was upregulation of CASP9, and DDIT3 (CHOP!), which are pro-apoptotic genes, and CDKNIA which inhibits cell cycle progression. On the other side of the balance there was upregulation of PAI-2, PROK2, and CCL2 which are associated with cell survival (Dickinson *et al*, 1995; Roca *et al*, 2009; Duan *et al*, 2009), and MCL1, a member of the anti-apoptotic Bcl-2 family (Figure 7.9). In total there was upregulation of 36 apoptosis-related genes, this was a balanced upregulation of pro and anti-apoptotic genes (47.2% vs 52.8%). 22 apoptosis-related genes were downregulated, 81.8% were pro-apoptotic genes vs 18.2% anti-apoptotic genes.

APOPTOSIS

a) Upregulated genes



b) Downregulated genes

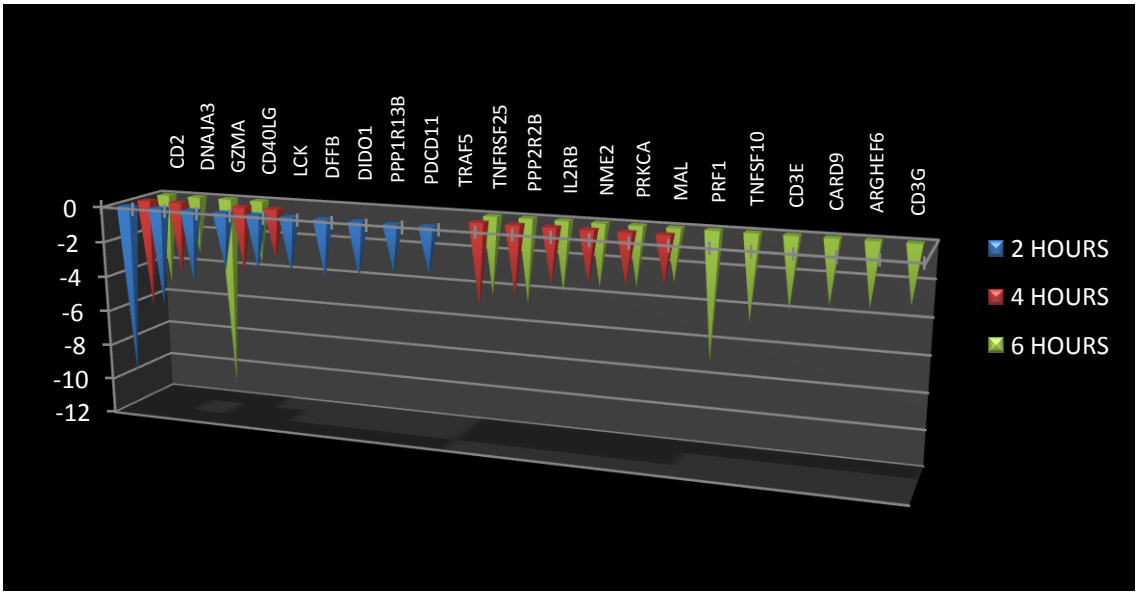


Figure 7.9: Changes in expression of genes involved in apoptosis during thrombus formation. (a) upregulated genes; (b) downregulated genes.

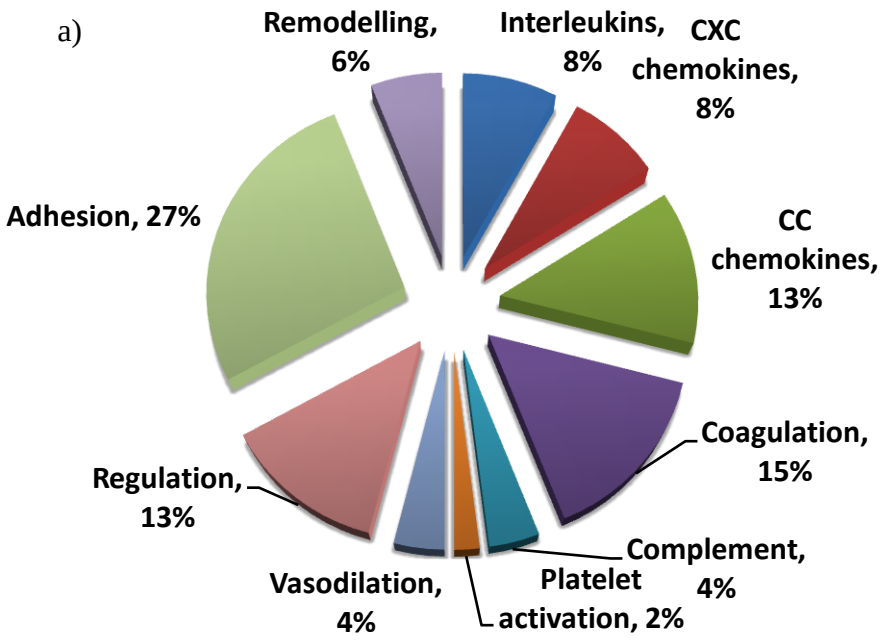
7.3.4. Wound healing

As observed in the study described in chapter 6, there was upregulation of many cytokines, including IL-8, and many IL-1 family members (IL1B, IL1RN, IL1A and IL1R1); C-X-C chemokines (CXCL1, CXCL2, CXCL5), and CXCR4 receptor; and C-C chemokines (CCL7, CCL2, CCL3L3, CCL3 and CCL20). Other genes in this category included an increase in genes encoding vasodilators prostaglandin synthase and adrenomedullin (PTGS2 and ADM); increase in angiogenic, vascular remodelling genes (TNFAIP6, PROK2), and regulatory genes (FOS, BMP6, IRAK2, KLRG1). Meanwhile, there was downregulation of CCR2, CX3CR1 and IL23A which would result in modulation of cytokine and adhesion molecule expression (Boring *et al*, 1997; Aggarwal *et al*, 2003; Ghilardi *et al*, 2004). There was also potential for reduced platelet activation via PAF (due to upregulation of PLA2G7); and downregulation of genes involved in breakdown of arachidonic acid (PLA2G4B, and EPHX2)(Figure 7.10).

7.3.5. Transcription Factor Activity

Further analysis of gene ontology results showed that there was upregulation of 20 genes, and downregulation of 13 genes, with transcription factor activity. Key transcription factor genes that were significantly upregulated were EGR1, NR4A2, FOS, ATF3, PPARγ, and HIF1A. Whereas EGR1, NR4A2 and FOS peaked at 2 hours and then declined, ATF3, PPARγ, and HIF1A increased further (Figure 7.11). EGR2 was induced at 4 hours and remained at a similar level of expression at 6 hours.

a)



b) **WOUND HEALING**

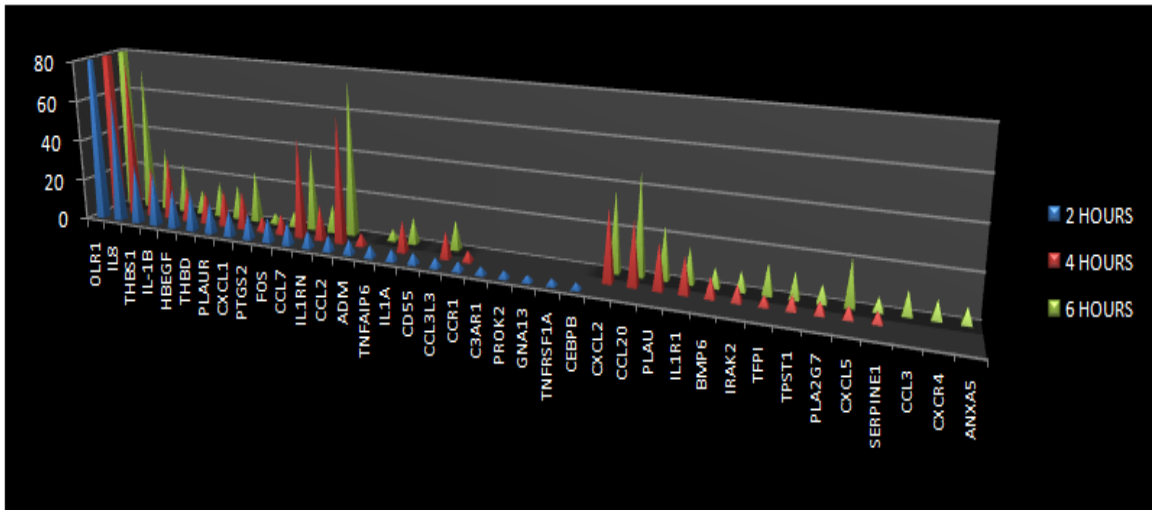


Figure 7.10: Expression of genes involved in wound healing in a growing thrombus. (a) Classification and distribution of wounding healing genes induced during thrombus formation; (b) Wound healing genes upregulated during thrombus formation.

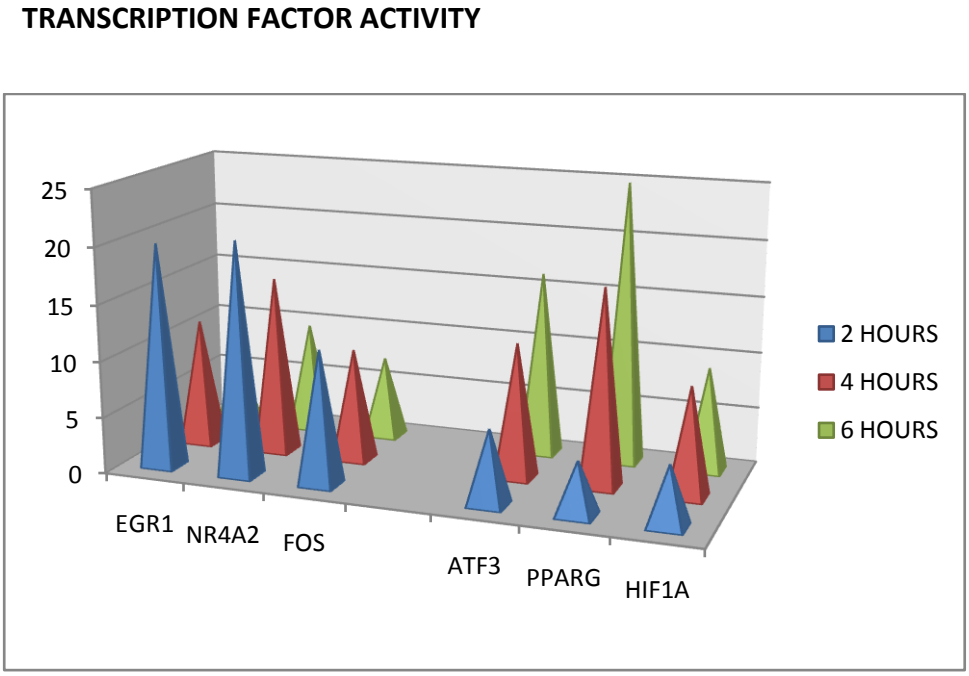


Figure 7.11: Changes in gene expression of transcription factor activity during thrombus formation. Figures are fold change in gene expression, and data is mean fold change measured in samples from 6 normal, healthy donors.

7.4. Discussion

Under normal haemostatic conditions within the vasculature, peripheral blood cells flow without generating a thrombotic event. However, following injury to the vessel wall, platelets, leukocytes and RBCs are rapidly recruited and incorporated into a fibrin mesh allowing formation of a thrombus at the injury site. Therefore, a thrombus contains multiple cell types, and the findings of this study are the combination of changes in gene expression from the different types of cells. These will be considered in more detail below.

7.4.1. Role of platelets in thrombus formation

Platelets are anucleate, yet have been reported to contain an active spliceosome that can process pre-mRNA transcripts into mature mRNA transcripts following platelet activation and integrin engagement (Denis *et al*, 2005; Schwertz *et al*, 2006). In this current study, although not being included in the 'top hits' list, there was increased expression of mRNA transcripts for genes found only in platelets (Watkins *et al*, 2009), encoding proteins involved in the initial tethering, and aggregation of platelets (GPIBB, GPIBA, GP9, VWF, and ITGA2B). *In vivo*, exposure to components of the disrupted vessel wall, *eg* collagen, results in platelet adherence and activation. The initial tethering of platelets is mediated by GPIb-V-IX complex (which is encoded by 4 different genes (alpha subunit of GPIb, beta subunit of GPIb, GPIX and GPV), and this binds to endothelial VWF. In the model of thrombus formation used in this study there are no endothelial components, therefore the formation of thrombi is not collagen-driven, and platelet GPIb requires either an alternative ligand, or an alternative source of VWF, *eg* from the platelet, itself. Here we see increased expression of VWF mRNA

within the early thrombus. Interestingly, studies employing a murine thrombosis model using ferric chloride have demonstrated that mice lacking platelet VWF are still able to form small, non-occlusive thrombi (Denis *et al*, 1998; Ni *et al*, 2000), however thrombus formation is significantly impaired in the absence of GPIb (Bergmeier *et al*, 2006). Therefore, GPIb plays a crucial role in early thrombus formation, and here we see increased mRNA expression for both subunits of the GPIb receptor. Other studies have shown that GPIb can initiate adhesion by interacting with other ligands including thrombospondin-1 (THBS1) under high shear flow conditions (Jurk *et al*, 2003). Therefore it is possible that in this study, THBS1 is upregulated to facilitate essential GPIb interaction. Following formation of the initial platelet monolayer, additional platelets are recruited into the growing haemostatic plug to stabilise it, and prevent premature disaggregation. This process requires integrin αIIbβ3, a ligand for fibrinogen, VWF, and fibronectin. This study found increased expression of ITGA2B, one subunit of the αIIbβ3 integrin complex, and also integrin ITGB5, a receptor for fibronectin. In addition to its role as an anchoring ligand, αIIbβ3 also plays a key role in intracellular signalling, as mice lacking the β3 integrin show lack of platelet aggregation, reduced clot retraction, reduced fibrinogen uptake into platelets, and have prolonged bleeding time (Petrich *et al*, 2007). Therefore, this study demonstrates that during the early stages of thrombus formation, there is increased expression of platelet-specific genes, encoding proteins involved in the initial tethering, and aggregation of platelets.

7.4.2. Regulation of coagulation

Activation of platelets results in exposure of phosphatidylserine (PS) on the outer platelet membrane, creating a negatively charged surface for the binding of coagulation

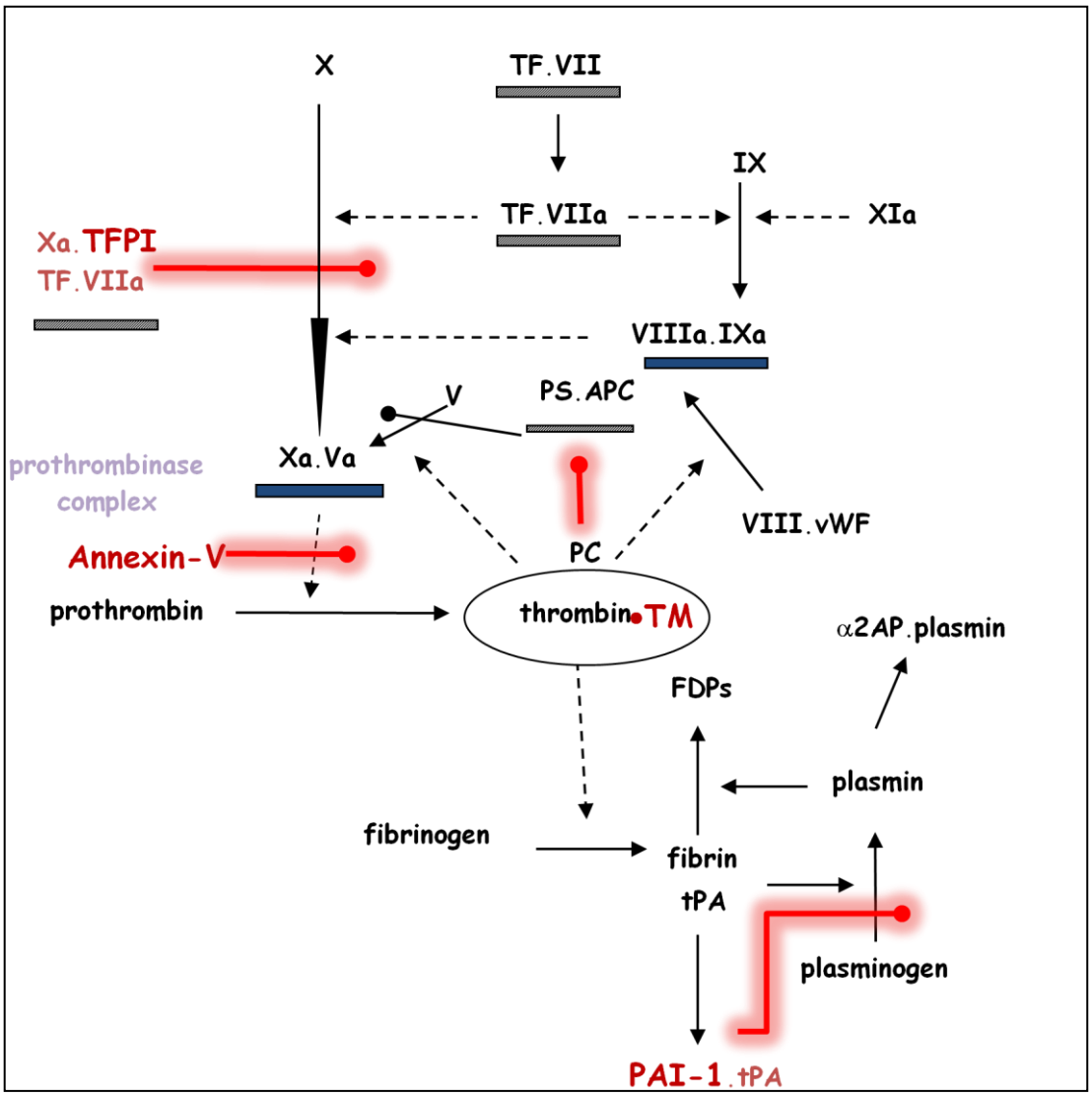
factors, eventually leading to the generation of thrombin, which in turn cleaves fibrinogen into fibrin. This process results in platelet-rich thrombi which are supported by a fibrin mesh. Studies carried out i*n vivo,* looking at thrombin generation within a growing thrombus, show that thrombin activity is distributed throughout the platelet-thrombus, and not just at the blood-thrombus interface, and that there is a definite increase, peak, and decline to thrombin activity (Baird *et al*, 2003). This could be due to a decrease in thrombin production or increase in inhibition of thrombin through increased levels of anticoagulant plasma proteins. At a molecular level, this could result from downregulation of prothrombotic genes including prothrombin itself, or upregulation of genes encoding anticoagulant proteins. Identification of the Illumina probe ID for F2 (prothrombin) revealed that there was only minimal signal of FI at all time points. However, analysis of inhibitors which act to inhibit thrombin generation demonstrated increased expression of several anticoagulant genes (TFPI, ANXA5, THBD, and PLAU). This suggests that the growth of the thrombus, driven by procoagulant factors, is being regulated at several stages, firstly by inhibition of TF due to increased expression of TFPI. Further regulation is gained by limiting the formation of the prothrombin complex through competitive binding of increased levels of Annexin-V, and increased inhibition of the Protein S complex, and finally, through increased control of thrombus lysis due to increased expression of Urokinase plasminogen activator (Figure 7.12).

7.4.3. Incorporation of white cells into a growing thrombus

Chemotaxis

Thrombin activity leads to further activation of platelets, inducing shape change and secretion of soluble mediators from platelet intracellular granules, leading to further

activation of platelets but also to recruitment of leukocytes, through release of chemokines. Infiltrating leukocytes then release further chemokines to attract various



- Inhibition of TF, initiator of coagulation
- Inhibition of Prothrombinase complex formation
- Inhibition of Protein C pathway
- Inhibition of fibrinolysis

Figure 7. 12: Regulation of coagulation genes within a thrombus

types of white blood cell to the growing thrombus environment. Early white cell recruitment to a thrombus mainly consists of neutrophils followed by monocytes, and finally a small number of eosinophils, basophils and lymphocytes are incorporated (Chandler *et al,* 1973). During the early stages of thrombus formation, there was significant upregulation of several chemokine and cytokine genes. This included IL-8, and CXCL1, encoding key proteins involved in neutrophil chemotaxis, and CCL7 and CCL2, which would preferentially attract monocytes. By 4 hours there was significant increased expression of CXCL2, known to be produced by both monocytes and neutrophils, which acts as a regulator of progenitor cell proliferation (Kollmar *et al*, 2006). Upregulation of CXCL5 would ensure further activation of neutrophils, and upregulation of CCL20, followed by CCL3 by 6 hours, would attract lymphocytes to the growing thrombus. Therefore, initiated by the release of chemoattractant soluble mediators from the intracellular granules of platelets forming the early part of the thrombus, and following the initial burst of thrombin generation (Celi *et al*, 2003), is the sequential gene induction of chemoattractants which allows further infiltration of neutrophils, monocytes, and finally lymphocytes. Once attracted to the injury site white cells may stay incorporated in the thrombus or, through a similar series of processes to the rolling, adherence and transmigration of leukocytes through inflamed endothelium, leukocytes are able to roll across the surface of aggregated platelets forming the initial plug, and transmigrate into the wound site (Wagner *et al,* 2008).

Apoptosis

Whereas the majority of cells attracted to the site of injury become incorporated into the thrombus and may remain there, some may no longer be required once they have carried out a specific function. Analysis of genes involved in cellular apoptosis

pathways showed that there was an equal induction of pro- and anti-apoptotic genes, whereas pro-apoptotic genes were strongly downregulated. On closer analysis, 6 of the pro-apoptotic genes that were upregulated were strongly associated with apoptosis of T-cells including STK17B, which possibly enhances T-cell apoptosis by stimulation of IL-2 synthesis, and PHLDA1, which regulates T-cell receptor/CD3-dependent induction of CD95/Fas and subsequent activation induced cell death (Oberg *et al*, 2004). The other T-cell related apoptosis genes were TNFRSF21, which is a TNFR-related death receptor; FOXO3A, which triggers apoptosis in the absence of certain survival factors; and GPR65, also known as T-cell death-associated protein 8; and there was downregulation of LCK, a T-cell specific protein-tyrosine kinase that is associated with T cell differentiation and survival (Zamoyska *et al*, 2003; Lin *et al*, 2004; Turrel-Davin *et al*, 2009). Therefore, it is possible that a subset of T-lymphocytes are drawn into the thrombus for a specific function, but then undergo apoptosis when no longer required, this is supported by the significant downregulation of expression of CD2 and CD6, two T-cell markers of differentiation. 3 apoptosis-related genes were strongly associated with monocyte survival, including SERPINB2, PROK2, and CCL2 (Dickinson *et al*, 1995; Roca *et al*, 2009; Duan *et al*, 2009).

7.4.4. The function of leukocytes in the thrombus

So what is the role of the white cell in the thrombus? The infiltration of neutrophils in the early stages of thrombus growth and presence of IL-8 may play a key role in the structural organisation of the forming thrombus (Wakefield *et al*, 1999; Henke *et al*, 2001). In support of this was the finding that gene expression of IL-8 was strongly upregulated by 2 hours and remained elevated during the period of thrombus formation

in this study. Electron microscope studies have shown that monocytes firstly appear around the edge of the thrombus and then become evenly distributed throughout (McGuiness *et al*, 2001). This may position them for phagocytosis of damaged ECM material, and removing debris of unwanted cells, eventually creating spaces between fibres and increasing permeability for the action of angiogenic factors, and fibrinolytic factors such as neutrophil urokinase (Moir *et al*, 2002). Throughout the period of thrombus formation studied, there was a steady and sustained upregulation of genes such as TSP1, PLAUR, THBD, and fibronectin receptors, mediating cell-ECM adhesion, which may eventually facilitate remodelling of the ECM to promote vascularisation. Delivery of ECM components may also occur via upregulation of laminin (LAMB3), and collagen gene COL7A1. Angiogenic effects may be further induced through induction of BMP6, a TGF-B family member which acts via the SMAD pathway. Therefore, recruitment of white cells into the thrombus allows delivery of chemokines, MMPs and proteins involved in ECM turnover, angiogenic proteins, as well as phagocytosis of unwanted cellular and matrix debris from the wound site.

7.4.5. Role of red blood cells within a thrombus

Although leukocytes and platelets are often the main focus of thrombus formation, erythrocytes or red blood cells also play a role, by dynamically enhancing the interaction of platelets with the vascular surface, thus facilitating their recruitment into the early thrombus (Reimers *et al*, 1984). Within the thrombus itself, computational studies have shown that RBCs affect the shape of the thrombus, making the aggregate spread more horizontally (Mori *et al*, 2008), and alter the structure, inducing a more

uniform arrangement of fibrin fibres and increasing elastoviscosity (Gersch *et al*, 2009). Although mature erythrocytes are anucleate, during the early stages of red cell development the basophilic erythroblast contains a large transcriptionally active nucleus that can process transcription of globin mRNAs. As the early erythrocyte develops into a reticulocyte, the nuclear chromatin condenses and eventually the nucleus is extruded from the cell, before the reticulocyte is released from the bone marrow into the circulation. Due to the long half-life of globin RNAs, through the action of RNA stabilising proteins (Weiss *et al*, 1994), reticulocytes can synthesise globin proteins for 2 – 3 days following release into the circulation. During the latter part of the differentiation process there is also synthesis of cytoskeletal proteins, ion exchange proteins and glycophorin A. The reticulocyte then undergoes a final maturation stage during which organelles, membranes and RNAs are cleared leaving the mature erythrocyte to circulate for around 120 days. Therefore in this study it would be expected that there may be small amounts of globin RNA detected, and this is confirmed with the presence of beta-type globin chain genes HBD, HBQ, and alpha chain (HBA1) and beta chain (HBB) genes of haemoglogin. Other genes upregulated that may support red cell function include ERAF, encoding alpha-haemoglobin stabilising protein, numerous ferritin genes, allowing delivery and storage of iron, and EPB41L3, (erythrocyte membrane protein band 4.1), a key protein for maintenance of cell shape. There is increased expression of HMOX1, which is involved in haemoglobin degradation, and clearance of red cells (Maines, 1988). Following on from studies revealing the presence of an active platelet transcriptome (Bugert *et al*, 2003; Gnatenko *et al*, 2003; Denis *et al*, 2005), a recent paper by Kabanova *et al*, (2009), suggests that RBCs may also have the cellular machinery for nucleus-dependent

protein synthesis, and contain a transcriptome of ~500 genes. It has been suggested that red blood cells may also support thrombin generation (Peyrou *et al*, 1999), however it is not clear whether this is due to intact RBCs or to RBC MPs (Horne *et al*, 2006).

7.4.6. Role of cellular microparticles within a thrombus

Platelets, leukocytes and red blood cells incorporated into thrombi generated in this study, are all capable of generating MPs that contain molecules and characteristics of their cell of origin. MPs have also been reported to carry RNA (Deregibus *et al*, 2007), therefore the contribution of cellular MPs to thrombus formation must be considered, as MP-mRNA could be included in the results of the array data. The actual role of these different cellular MPs is still under debate. A recent paper by Bernimoulin *et al*, (2009) suggests that monocytes can shed MPs with differential phenotype depending on the type of stimulus. This suggests that there may be a regulated mechanism of MP generation, and that each type of MP has a defined biological role. MPs may be a delivery system for getting TF into the thrombus for continued propagation of coagulation, and have been shown to support thrombin generation. Bidot *et al*, (2008), reported that patients with recurrent thrombosis had higher levels of platelet-MPs than healthy controls and elevated levels of thrombin generation. Interestingly, they found that whereas the number of Endothelial-MPs and Leukocyte-MPs correlated with lag-time, it was Platelet-MPs that correlated with amount of thrombin generation. In a proteomic study, platelet-MPs have been shown to contain a proteome of >500 proteins including chemokines and adhesion proteins (Garcia *et al*, 2005). Therefore MPs could also be a mechanism for stabilisation of the thrombus, eg via Mac-1 (Jy *et al*, 2005). A recent study found that thrombus weight correlated negatively with leukocyte-MPs, but

positively with platelet-MPs (Ramacciotti *et al,* 2009), suggesting that platelet-MPs play a key role in the propagation of thrombus size and growth.

7.4.7. Transcriptional regulation during thrombus formation

Upregulation of two immediate early genes, EGR1 and FOS, along with upregulation of nuclear hormone receptor NR4A2, may regulate cell chemotaxis, proliferation, differentiation, and adhesion during the early stages of thrombus formation. EGR1 has been shown to be rapidly induced within minutes of receiving signal of stimuli, and undergoes rapid decay, a pattern that is mirrored in the current data set. *In vitro* studies have shown EGR1 to induce inflammatory mediator TNF-α, adhesion molecules such as ICAM-1, and CD44, and induction of growth factors PDGF, BFGF, and TGF-β (Fahmy *et al*, 2003; Fu *et al*, 2003; Lucerna *et al*, 2003). Therefore EGR1 has the potential to induce many genes associated with vascular stress and tissue injury. *In vivo*, FOS forms a complex with the JUN/AP-1 transcription factor and has been shown to play an important role in signal transduction, cell proliferation and differentiation, in particular being involved in the formation and maintenance of the skeleton (Ruther *et al*, 1987). NR4A2 protein expression has been detected in the brain, and also has been reported in cells of T-cell, B-cell and fibroblast origin (Maruyama *et al,* 1998). NR4A2 has recently been associated with IL8 expression in inflammatory arthritis (Aherne *et al*, 2009), and also with atherogenesis (Bonta *et al*, 2006). In cardiovascular tissues isolated from mice, there was a significant induction of NR4A2 between 1 and 4 hours following B-adrenergic stimulation, supporting early expression (Myers *et al*, 2009). In the thrombus it may be involved in regulation of the early induction of IL8, leading to neutrophil chemotaxis and activation. Expression of all 3 of these transcription factors was gradually downregulated over the remaining period of thrombus formation.

In contrast, transcription factor genes ATF3, PPARγ, HIF1A, and EGR2 showed a continued increase in expression over the period of thrombus formation. ATF3 binds the cAMP response element (CRE), a sequence that is present in many viral and cellular promoters. It may repress transcription by stabilizing the binding of inhibitory cofactors at the promoter, therefore although ATF3 can be induced by inflammatory stimuli such as LPS, it appears to act as a negative regulator, limiting the inflammatory response through modulating NFκB (Gilchrist *et al*, 2006). Suganami *et al*, 2009 found that overexpression of ATF3 in macrophages resulted in the attenuation of activation of M1 macrophages (proinflammatory macrophages). Therefore in the thrombus, ATF3 may be involved in restriction of the inflammatory response, and possibly in regulation of the monocyte phenotype. PPARγ may also limit the inflammatory response. PPARγ is a receptor prominently expressed in adipose tissue and macrophages and binds small lipid-soluble molecules such as hypolipidemic drugs and fatty acids. Once activated by a ligand, the receptor binds to a promoter element in the gene for acyl-CoA oxidase and activates its transcription. It therefore controls the peroxisomal beta-oxidation pathway of fatty acids, and is a key regulator of adipocyte differentiation and glucose homeostasis. In macrophages PPAR-γ appears to be anti-atherogenic, inducing the removal of cholesterol (Akiyama *et al*, 2002; Babaev *et al*, 2005), and downregulates the inflammatory response (Chawla *et al*, 2001). Upregulation of the oxygen-sensitive subunit of HIF1A may regulate haematopoiesis in the bone marrow. Under hypoxic conditions, HIF1A activates the transcription of numerous genes including, erythropoietin, glucose transporters, glycolytic enzymes, vascular endothelial growth factor, endoglin, and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia (Forsythe *et al*, 1996; Tang *et al*, 2004;

Ceradini *et al*, 2004; Manalo *et al*, 2005). It has been demonstrated to play an essential role in embryonic vascularization, tumour angiogenesis and pathophysiology of ischemic disease, and may be involved in maintaining cellular phenotype (Krishnamurthy *et al*, 2004). EGR2 may also play a role in selection or survival of cellular subsets. In studies of T-lymphocytes, regulatory T-cells (CD4+ CD25- LAG3+), which maintain immunological self-tolerance and immune homeostasis, were reported to have high levels of Egr2 expression, which induces anergy (Zhu *et al*, 2008). Anergy is a tolerance mechanism that renders T-cells inactive following antigen encounter and remain in a hyporesponsive state (Schwartz; 2003). EGR2-deletion studies in mice showed that positive selection mechanisms of CD4 and CD8 lineages was impaired, mainly due to increased apoptosis due to increased expression of Bcl-2 (Lazarevic *et al*, 2009). Therefore whereas gene induction of key transcription factors during the early stages of thrombus formation appear to be regulating cell proliferation, chemotaxis, and cell adhesion, transcriptional regulation during the later stages of thrombus formation appear to focus on maintenance of cellular phenotypes and the negative regulation of the inflammatory response.

7.4.8. Summary

This is the first study to investigate gene expression, and possible changes in patterns of gene expression, within a growing thrombus, with the finding that there is significant alteration in gene expression during the early stages of thrombus formation. The study covers a limited time period of 6 hours, and can therefore only report on the period where cells have recently been recruited from the periphery and incorporated into the thrombus. Further study is required to confirm whether gene expression continues to be modulated at later time points, *eg*, 24, 48, and 96 hours. A further limitation is that

although the Chandler loop system incorporates shear rate and flow, it is a closed system, and does not include endothelial components. Therefore, in this model system, thrombi are generated solely by an increase in calcium concentration which in turn triggers coagulation, therefore the formation of the thrombus in this study is thrombin-driven rather than collagen-driven. This also means that intercellular signalling and release of soluble mediators that would arise from cell-ECM interaction will not occur, and therefore when interpreting the gene expression data, it must be borne in mind that this study only partially reflects conditions *in vivo*. To include at least part of the endothelial component of thrombus formation, further studies of gene expression within a thrombus could be carried out using flow chambers where thrombi are allowed to form as blood is circulated over collagen-coated slides, or using thrombi taken from human arteries, or using an animal model where endothelial injury is induced by ferric chloride or laser.

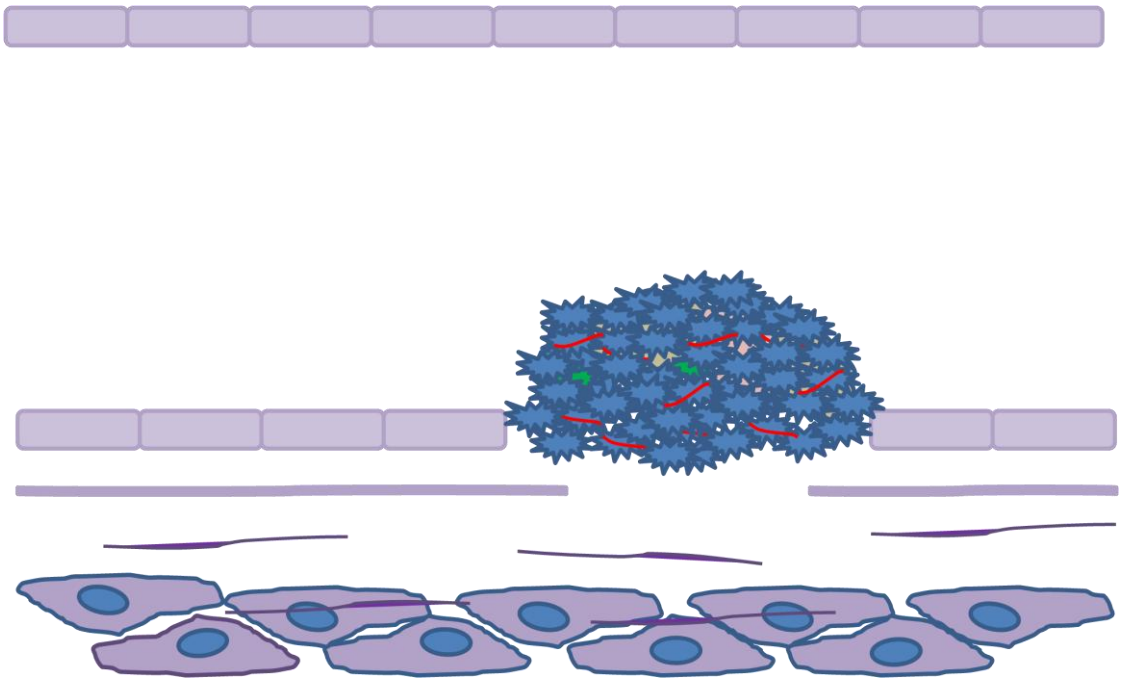
Therefore, the model of thrombus formation used in this study has focused on thrombi formed as a consequence of the cell-cell interaction of peripheral blood cells, and the activation of plasma coagulation and soluble mediators. The findings of this study support the results described in chapters 5 and 6, that platelets can drive leukocyte gene expression and modulate white cell phenotype from pro-inflammatory to pro-angiogenic. This study suggests that changes in gene expression, in particular in increased expression of IL-8, may play an important role in the structure and organisation of the forming thrombus. It also suggests that the procoagulant response that drives the growth of the thrombus may be regulated by changes in gene expression in cells that are within the thrombus, with the generation of thrombin kept in check due to increased expression of anticoagulant genes. Induction of TFPI would limit the

amount of TF available to drive the coagulation cascade, additionally, induction of Annexin-V and thrombomodulin may limit formation of the Prothrombinase complex, and increased urokinase plasminogen activator may further control thrombus resolution. These mechanisms of control of thrombus growth and structure may be transcriptionally regulated, with rapid induction and then decline of EGR1, FOS, and NR4A2 regulating genes involved in cell proliferation, chemotaxis and cell adhesion, and more gradual, and sustained induction of ATF3, PPARγ, and HIF1A being involved in the maintenance of cellular phenotype and the negative regulation of the inflammatory response. An illustration summarising the changes in gene expression during early thrombus formation is shown in Figure 7.13a – d. Following formation of the initial platelet plug and early establishment of the clot due to platelet activation and fibrin formation (figure a), by 2 hours (figure b), there is upregulation of genes within the thrombus, encoding chemokines to attract neutrophils and monocytes. There is low level regulation of thrombin generation due to upregulation of thrombomodulin, and upregulation of genes ensuring vasodilation (Prostaglandin-synthase-2 and adrenomedullin). Also key genes involved in determining the structure (IL-8), and stability of the initial clot (transglutaminase-2). Gene expression of these initial genes is transcriptionally regulated through expression of immediate early genes, Early Growth Response-1, FOS, and NR4A2. By 4 hours (figure c), expression of these three genes begins to decline, and is replaced by increased expression of ATF3, PPARγ, and HIF1A. There is further regulation of coagulation through induction of TFPI, and regulation of clot lysis (PAI-1). There is also increased expression of genes which facilitate ECM interaction, or are actual components of the tissue at the injury site (adhesion ligand, endoglin; ITGAV, a receptor for multiple ECM components; and

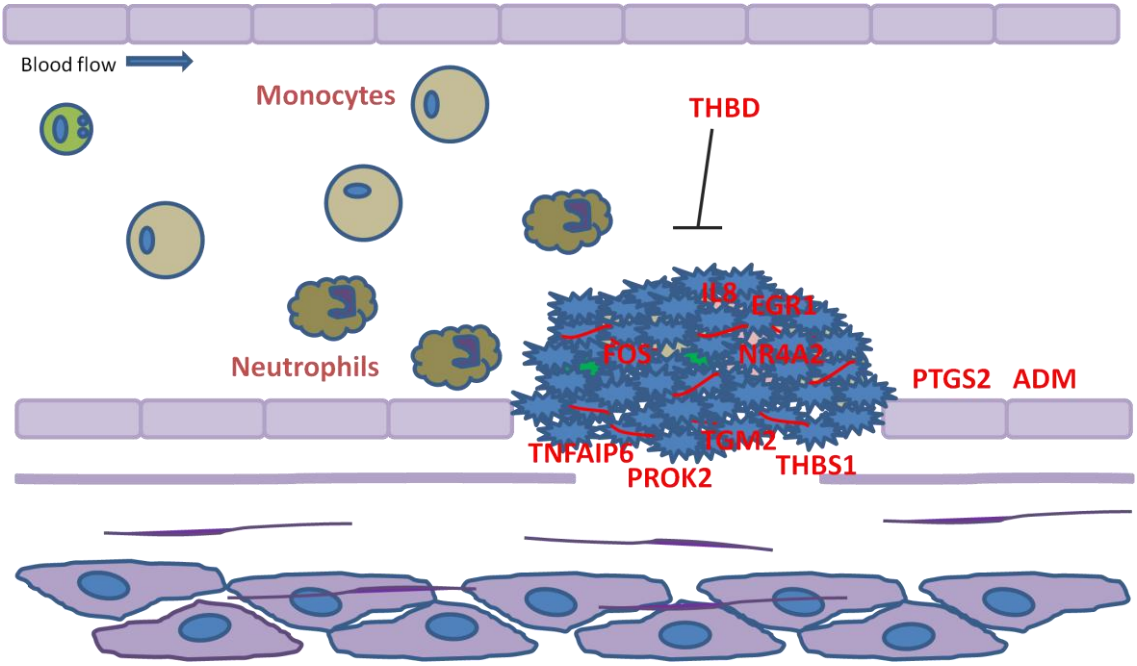
ECM components collagen, laminin, and osteopontin). Finally, by 6 hours (figure d), there is even further regulation of coagulation through increased expression of Annexin-V acting to regulate thrombin generation, and further increase in genes facilitating ECM interaction (ITGA5 and ITGB1 which are the α, and β subunits for the fibronectin receptor, and Activated Leukocyte Cell Adhesion Molecule, ALCAM).

Figure 7. 13: (a) Initial platelet thrombus. (b) 2 hour thrombus with infiltration of white cells and early gene expression

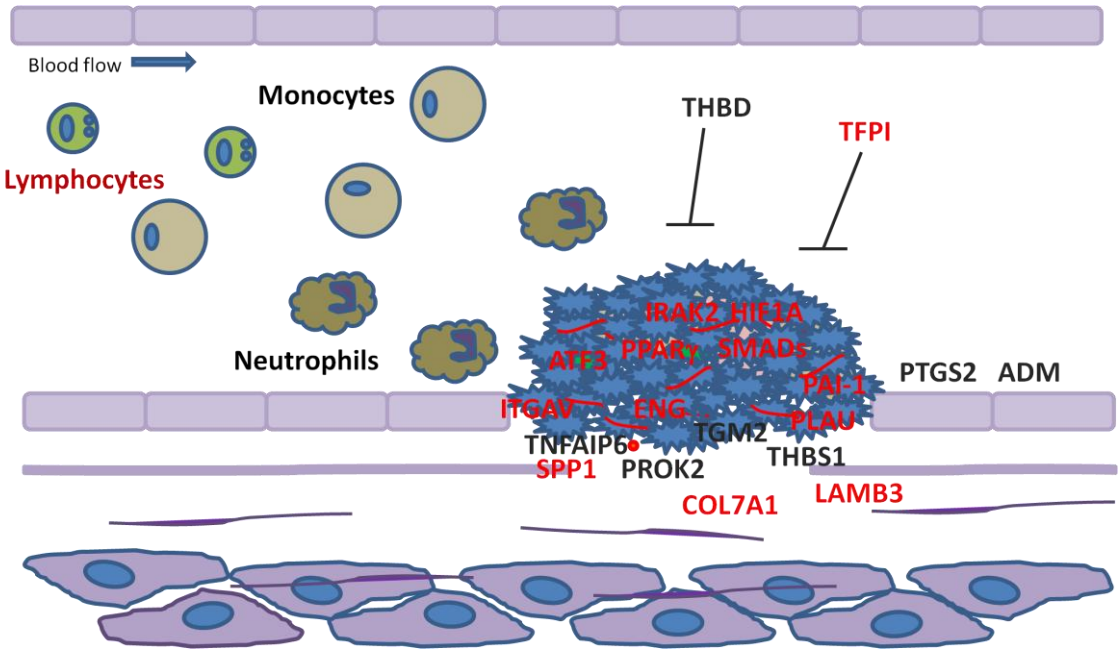
a) Initial thrombus



b) 2 hours



c) 4 hours



d) 6 hours

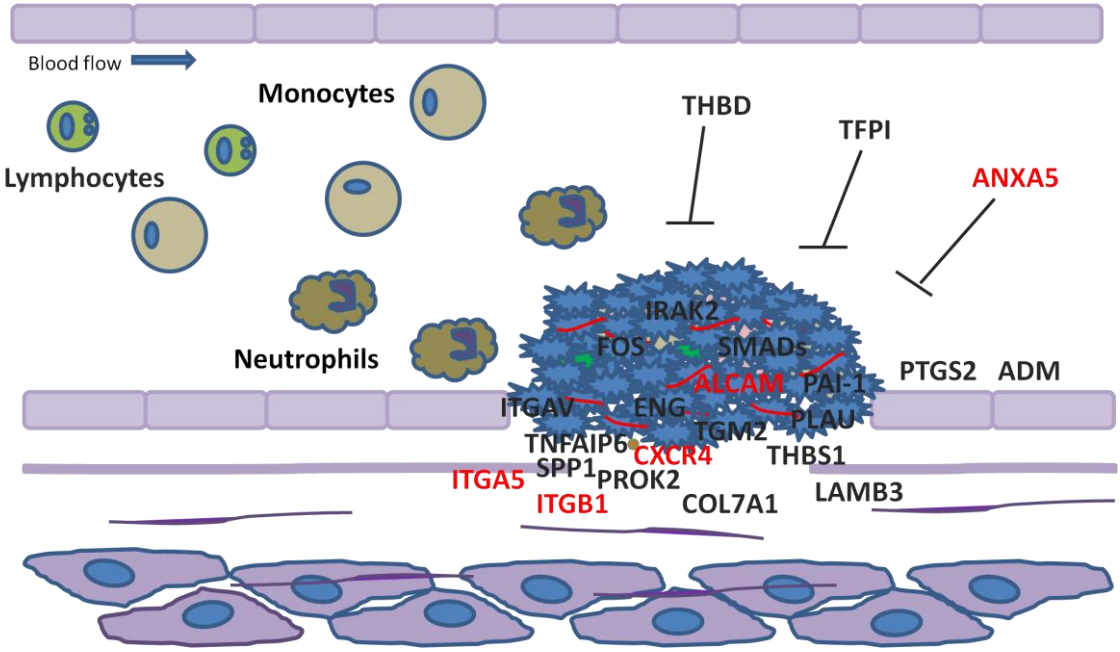


Figure 7. 13: (c) 4 hour thrombus with increased transcriptional regulation by PPARγ, and increased regulation of coagulation; (d) 6 hour thrombus with further regulation of coagulation, and increased facilitation of adhesion and ECM interaction.

The current study was carried out using blood obtained from healthy individuals, but the observation that gene expression within the growing thrombus may affect its structure, and perhaps whether it becomes occlusive *in vivo*, may account for some of the differences seen in clot structure and size in individuals with CAD (Carter *et al*, 2007; Wolberg *et al*, 2008). Therefore, further study could be made into gene expression, and also gene polymorphism, of some of the key genes identified here as playing a key role in thrombus formation, to see how these findings translate pathologically.

In summary:

- Gene expression is modulated during the process of thrombus formation. This has not previously been reported.
- Induction of expression of haemostasis genes which will increase the level of anti-coagulant proteins may be a mechanism for regulation of coagulation during thrombus formation.
- Two phases of gene expression of transcription factors were seen: EGR1 and FOS appearing first and rapidly declining, followed by later increased expression of PPARγ, ATF3, and HIF1A. This may regulate cell proliferation, chemotaxis and adhesion during the early period of thrombus formation, with later transcriptional regulation acting to maintain cellular phenotype and negatively regulate the inflammatory response.

Limitations:

- This study looks at the whole thrombus, and cannot investigate which individual cell-types are contributing expression of specific genes.
- This study was carried out only on healthy controls. Gene expression patterns may be different in individuals with CAD.
- This study studies thrombus formation during a limited 6 hour period. It may be interesting to study whether gene expression continues to be modified at later stages of thrombus formation, in particularly whether thrombus lysis is also regulated by gene expression within the thrombus.

Future Work:

- Validation of particular genes of interest, for example, transcription factors, by real-time PCR.

- Further studies of gene regulation at later time points during thrombus formation and lysis.
- Studies of gene expression during thrombus formation in blood collected from, for example, off-spring of healthy controls compared to that of off-spring with family history of premature MI.

CHAPTER 8: SUMMARY AND CONCLUSIONS

MI at an early age often occurs against a modest level of atherosclerosis. Therefore the haemostatic response may be a determining factor in putting these individuals at risk of developing an occlusive thrombus following rupture of a plaque in a coronary artery. Exploration of the endogenous haemostatic potential, through measurement of the ETP in plasma using the thrombin generation assay in a large group of premature MI patients and controls, demonstrated that the premature MI subjects were able to generate more thrombin. This hypercoagualable response was associated with elevated levels of several procoagulant factors, including TF, FX, FIX, FVIII, and FII, that was not compensated by increased levels of the main anticoagulant proteins, TFPI and ATIII. These effects were not due to the confounding effects of other demographic variables such as age, gender, smoking or BMI. Furthermore, higher ETP levels were seen in offspring with a strong family history of premature MI, suggesting an inherent, genetically determined element. Investigation of genetic polymorphisms in a small number of coagulation factors confirmed the association with MI in two SNPs (TF -1812T and FII G20210A), but did not account for the difference in haemostatic activity between the premature MI and control groups. This study of coagulation SNPs, and the number of donors in the study was relatively small therefore a more comprehensive genotyping study could be carried out, incorporating all known polymorphisms of the coagulation genes, in a much larger cohort or multiple cohorts, to establish the genetic role in producing a hypercoagulable phenotype (Smith *et al*, 2010). Since TF appeared to be a significant factor in driving the elevated thrombin-generating potential in the MI patients, further exploration was made of the regulation of TF within the blood

environment, and study of its specific inhibitor, TFPI. Since monocytes are a known source of TF in response to LPS (Brand *et al*, 1991; Guha *et al*, 2001), and also in response to direct platelet binding (Celi *et al*, 1994), these cells were chosen for further study.

Cellular activation and interaction are key components of maintaining the haemostatic balance. Study of platelet-monocyte interaction, and the ensuing changes in monocyte gene expression, demonstrated that whilst platelets are known to play a crucial role in maintaining haemostasis through formation of the initial platelet plug at the site of vascular injury, they also play an important role in modulating the monocyte genetic profile and phenotype to one that can facilitate wound healing. This is in contradiction to the widely-held view that platelets induce an inflammatory response in monocytes. Whereas it is known that platelet adhesion to monocytes results in induction of pro-inflammatory cytokines and TF gene expression, gene induction was at a much lower level than that seen in response to LPS. In contrast, platelet soluble mediators released from intracellular granules following platelet activation had a greater effect on monocyte gene expression, in particular a strong induction of TFPI. This establishes a possible mechanism for regulation of procoagulant activity in a haemostatic environment, either in the plasma due to secretion of soluble TFPI protein, or on the monocyte surface membrane. The finding that increased surface procoagulant activity is measurable in monocytes exposed to activated platelets, but not on the surface of LPS-treated monocytes may be due to encryption of TF, and suggests the possibility that within the wound site, platelets could possibly play a role in decryption of TF. This could be due to presence of surface phospholipid components on the platelet which has formed an aggregate with the TF-expressing monocyte, or due to release of PDI

(thought to play a role in regulation of TF encryption) from platelet granules, or could possibly be due to both mechanisms, and has implications for continued thrombin generation within a thrombus. Therefore, further studies could be carried out to study the possible relationship of release of platelet PDI, platelet-monocyte aggregation, platelet surface PS exposure, and monocyte TF de-encryption. Study is also required to establish the translation of monocyte TFPI mRNA, to determine its cellular location, and confirm its function as having anticoagulant activity. Identification of platelet soluble mediators responsible for induction of TFPI could be achieved through incubation of monocytes with various components of platelet secretome, such as recombinant fibronectin, growth factors (especially FGF2), and prothrombin, and establishing the transcriptional regulation of this pathway, with particular focus on KLF2, and PPARγ. Further study could also be carried out on monocyte expression of the different isoforms of TFPI, in particular in relation to cardiovascular disease, as study of a second cohort of premature MI subjects (BLOODOMICS cohort) demonstrated they had monocytes with higher gene expression of TF and lower levels of TFPI than monocytes from healthy controls, potentially having circulating monocytes with a more procoagulant phenotype, therefore further contributing to the hypercoagulable state already established due to the elevated endogenous thrombotic potential of the plasma.

Study of the two mechanisms of platelet-monocyte interaction, that of direct interaction through binding of platelet P-selectin and monocyte PSGL-1 or indirectly through monocyte interaction with platelet soluble mediators, on monocyte gene expression of TF and TFPI was expanded to a whole genome approach. The study again, emphasised that whereas platelet-monocyte aggregation induced significant changes in monocyte

gene expression, platelet soluble mediators have a substantial effect on modulating the monocyte genetic profile, inducing an EPC-like phenotype rather than inflammatory phenotype, and act to facilitate its incorporation into the thrombus, enabling the monocyte to play an important role in wound healing. Further study is needed to ascertain which soluble mediators are responsible for modulation of key monocyte genes, for example TFPI, and DUSP4 and 5, determining signalling pathways, transcriptional regulation, and the role of the translated proteins. The importance of gaining further understanding of major molecules involved in this process, also of the possible variation in secretome contents released in response to different agonists and concentration of agonists, is emphasized by the findings of the meta-analysis of array studies. This analysis found that there was a differential response to platelet soluble mediators in monocytes isolated from premature MI subjects, resulting in a phenotype that is more adhesive, proinflammatory, and procoagulant than that of monocytes isolated from healthy individuals. These findings support the earlier study examining monocyte expression of TF and TFPI in premature MI patients. In addition, these monocytes may have reduced expression of genes involved in intracellular trafficking of molecules, and also of genes that play a major role in clot structure.

These findings are compounded by the discovery that gene expression is still induced during the early stages of thrombus formation. Expression of platelet-specific genes involved in platelet aggregation and adhesion was supported by a co-ordinated induction of genes encoding regulators of coagulation, and chemokines and cytokines, inducing infiltration of white cells into the thrombus which amongst a number of roles, are involved in the structure and organisation of the thrombus. Induction of transcription factors demonstrated two phases of transcriptional regulation during

thrombus formation, with early transcription factors seeking to regulate cell proliferation, chemotaxis and adhesion, and later transcriptional regulation acting to maintain cellular phenotype and negatively regulate the inflammatory response. Further study is needed to ascertain whether gene regulation within the thrombus is still being further altered at later time points during thrombus formation and lysis. In addition, there have been several studies linking gene polymorphisms, *eg* IL8 to increased risk of cardiovascular disease (CVD). IL-8 has been shown to play an important role in determining the organisation of the thrombus. Therefore, the finding of active gene induction of IL8 within the thrombus further emphasizes how gene polymorphisms may translate into increased risk of CVD.

Taken together, these data can contribute to our current understanding of the important role played by platelets within the wound healing environment; where, in addition to formation of the initial haemostatic plug to maintain vascular integrity, and provision of a negatively-charged surface to support thrombin generation, platelets influence the plasticity of monocytes, modulating gene expression away from an pro-inflammatory phenotype, instead inducing a pro-angiogenic phenotype. This role may further expand into the thrombus. Whereas initiation of the thrombus is driven by plasma proteins and facilitated by the platelet surface, this study provides evidence that thrombus resolution may be driven by changes in gene expression within the thrombus that regulate the haemostatic response, thrombus growth, and facilitate wound healing. These findings could have implications for individuals at risk of plaque rupture, where variation in gene expression may affect not just the formation of an occlusive thrombus but also the rate of resolution. Additional studies to increase understanding of the regulation of

haemostasis and thrombosis at a cellular level may provide novel targets for treatment of thrombotic disorders.

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