For my patients, who have taught me so much
Declaration

This thesis is based on original research carried out by the author at the Haemostasis, Thrombosis and Vascular Biology Unit at the University Department of Medicine, City Hospital, Birmingham, under the supervision of Professor Gregory Y.H. Lip (University of Birmingham) and the mentorship of Professor Leong L. Ng (University of Leicester).
Acknowledgements

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Last but not least, I would like to thank the individuals who agreed to take part in the studies described in this thesis.
Publications Arising From This Thesis

**Chapter 1.**


**Chapter 2.**

**Chapter 4.**


**Chapter 5.**
Tan KT, Tayebjee MH, MacFadyen RJ, Lip GY, Blann AD. Elevated platelet microplatelets in stable coronary heart disease are unrelated to disease severity or to indices of inflammation. Platelets 2005; 16: 368-71


**Chapter 6.**
Tan KT, Tayebjee MH, Lim HS, Lip GY. Clinically apparent atherosclerotic disease in diabetes is associated with an increase in platelet microparticle levels. Diabet Med (In Press)

Tan KT, Tayebjee MH, Lim HS, Lip GY. Symptomatic diabetic macrovascular disease is associated with an increase in PMP Levels. (Abstract accepted for presentation at the European Society of Cardiology 2005, Stockholm)
Chapter 7.

Chapter 8.
Table of Contents

1. Platelets, Blood Vessels and Atherosclerosis
   1.1 Introduction 11
   1.2 Effects of Platelets on the Vessel Wall 12
   1.3 Effects of the Vessel Wall on Platelets 31
   1.4 Platelet Activation in Atherosclerosis 35

2. Methods for the Study of Platelet Activation
   2.1 Introduction 37
   2.2 Platelet Structure 37
   2.3 Platelet Activation 40
   2.4 Methods for Determining in vivo Platelet Activation 44
   2.5 Conclusion 53

3. Plan of Investigation
   3.1 Background 55
   3.2 Hypotheses 55
   3.3 Plan of Investigation 56
   3.4 Summary 57

4. Platelet Activation and the Severity of Peripheral Artery Disease
   4.1 Introduction 59
   4.2 Subjects and Methods 60
   4.3 Results 64
   4.4 Discussion 67

5. Platelet Microparticles and Coronary Heart Disease
   5.1 Introduction 70
   5.2 Methods 71
   5.3 Results 73
   5.4 Discussion 75

6. Type II Diabetes, Atherosclerosis and Platelet Microparticles
   6.1 Introduction 76
   6.2 Methods 78
   6.3 Results 80
   6.4 Discussion 83

7. Peripheral Arterial Disease, Angioplasty and CD40L
   7.1 Introduction 87
   7.2 Methods 88
   7.3 Results 90
   7.4 Discussion 95
8. Platelet Activation in Stable Coronary Heart Disease

8.1 Introduction 100
8.2 Methods 101
8.3 Results 104
8.4 Discussion 107

9. Conclusion
9.1 Summary of Findings 110
9.2 Future Studies 114
9.3 Conclusion 115

Appendix 117
References 118
List of Tables

Chapter 1.
Table 1.1 Some studies of P-selectin in human atherosclerotic disease
Table 1.2 Studies of sCD40L levels in atherosclerotic disease
Table 1.3 Some human studies of PMPs in atherosclerosis and associated conditions.
Table 1.4 Summary of effects of PMPs on coagulation
Table 1.5 Effects of platelet microparticles on the vessel wall

Chapter 2.
Table 2.1 The contents of platelet granules.

Chapter 4.
Table 4.1 Clinical, demographic and routine laboratory indices
Table 4.2 Platelet markers in case and controls
Table 4.3 Correlations between platelet markers

Chapter 5.
Table 5.1 Subject baseline characteristics
Table 5.2 Comparison of patients with coronary artery disease and healthy controls

Chapter 6.
Table 6.1 Baseline characteristics of the subjects
Table 6.2 Platelet indices in diabetes and controls

Chapter 7.
Table 7.1 Demographic and risk factor characteristics of the subjects
Table 7.2 Platelet markers in the three groups
Table 7.3 Correlations between platelet markers
Table 7.4 Baseline characteristics and effect of angioplasty on the subgroup of 11 patients who underwent angioplasty

Chapter 8.
Table 8.1 Baseline characteristics of patients and controls
Table 8.2 Platelet indices in patients with coronary artery disease and controls
Table 8.3 Collateralization scores and plasma markers
Table 8.4 Comparison of platelet indices between the first and fourth quartiles of the angiographic severity of coronary heart disease as measured by Coronary Atheroma Score
List of Figures

Chapter 1.
Figure 1.1 Platelet microparticles and coagulation
Figure 1.2 Platelet microparticles and the vessel wall

Chapter 2.
Figure 2.1 Platelet structure
Figure 2.2 Platelet adhesion.
Figure 2.3 Platelet activation
Figure 2.4 Diagrammatic representation of a simple flow cytometer
Figure 2.5 Flow cytometric detection of activated platelets
Figure 2.6 The platelet and platelet microparticle (PMP) population with flow cytometry.
Figure 2.7 A sample of platelet poor plasma labelled with anti-CD42-FITC and anti-CD61-PerCP mixed with red latex beads

Chapter 4.
Figure 4.2 Iliac angiogram showing diffuse atherosclerotic disease

Chapter 7.
Figure 7.1 Peripheral artery angioplasty is often used to treat critical ischaemia
Figure 7.2 CD40L levels pre- and post-angioplasty
Chapter 1. Platelets, Blood Vessels, and Atherosclerosis

1.1 Introduction

The role of platelets in the thrombotic complications of atherosclerosis, such as stroke and myocardial infarction, has been known for the past few decades. Classically, thrombotic disorders can be classified into those due to platelet aggregates, and those that are due to fibrin deposition. Platelets are predominantly involved in thrombosis affecting the arterial system, forming the so-called ‘white thrombus’. Therefore, it is not surprising that the use of anti-platelet agents such as aspirin and clopidogrel has a beneficial effect on mortality and morbidity in patients with atherosclerotic disease (Antithrombotic Trialists' Collaboration 2002).

More recently, it has been realised that the thrombotic and inflammatory pathways are closely related. Activation of one pathway may precipitate the other. For example, platelets, which have classically been associated with thrombosis, may release various substances which can elicit an inflammatory response (Ross R 1999). The pro-inflammatory function of platelets has probably evolved as part of the natural defence against parasitic infection, including infection by Trypanosoma cruzi, Plasmodium, and various microfilariae (Cesbron JY et al. 1987; Peyron F et al. 1989; Umekita LF, Piazza RM, & Mota I 1994).

However, too much of a good thing can have deleterious effects. In developed countries, where the rates of parasitic infection are low, the pro-inflammatory effects of platelets may actually contribute to human disease. Indeed, as atherosclerosis is now considered to be an inflammatory disorder, it is not surprising that activated
platelets may also contribute to the initiation and progression of atherosclerotic disease (Massberg S et al. 2002).

1.2 Effects of platelets on the vessel wall

Platelets, Adhesion molecules and Atherosclerosis

In the quiescent platelet, platelet adhesion molecules are stored inside the cell, present in an inactive form on the cell surface and/or do not have the right ligand to bind to.

Glycoprotein Ib/IX/V (GP Ib/IX/V)

GP Ib/IX/V is a member of the integrin family that is present on the platelet surface membrane (Hynes RO 1992; Savage B, Almus-Jacobs F, & Ruggeri ZM 1998; Shen Y et al. 2000). As its name suggests, GP Ib/IX/V is composed of three subunits, GP Ib, GP IX, and GP V. GP Ib is composed of two subunits, α and β. GP Ib associates with GPIX in a 1:1 stoichiometric ratio. GP V associates with the GPIb/IX complex in a 1:2 ratio. GP Ib/IX/V can bind to von Willebrand factor (vWF), an interaction which is mediated by the GP Ib/IX component of the molecule and can occur under high shear stress. When the endothelium lining the healthy blood vessel is damaged, vWF binds to the underlying subendothelium, thus providing a bridge to bind the platelet via GP Ib/IX/V. The initial binding of GP Ib/IX/V to vWF is transient and reversible. The interaction, however, slows the passage of the platelet in the blood vessel and allows the more permanent binding of the platelet to the subendothelium via Glycoprotein IIb/IIIa (GPIIb/IIIa).

GP Ib/IX/V may also mediate the binding of the platelet to dysfunctional endothelium, in conditions such as hypercholesterolaemia (Massberg S et al. 2002). In a mouse model of atherosclerosis, blockade of the adhesion of activated platelets to
the vessel wall using an anti-murine anti-Iba antibody attenuated the formation of the
atherosclerotic plaque (Massberg S et al. 2002). These findings suggest that GP
Ib/IX/V may be of great importance in the pathogenesis of atherosclerosis.

**Glycoprotein IIb/IIIa (GPIIb/IIIa)**

GPIIb/IIIa, also known as αIIbβ3, is another member of the integrin family and is the
most abundant platelet surface protein (Hynes RO 1992; Wagner CL et al. 1996). The
molecule is also found in the platelet α-granule membrane, which fuses with the cell
surface membrane when the platelet is activated, thus increasing the ligand binding
capacity of the cell. GPIIb/IIIa is a heterodimer formed by an alpha subunit (GPIIb)
and a beta subunit (GPIIIa).

In unstimulated platelets, GPIIb/IIIa does not bind any plasma proteins. However,
when the platelet is activated, GPIIb/IIIa undergoes a conformational change that
allows it to bind proteins containing the Arg-Gly-Asp (RGD) sequence, such as vWF
and fibrinogen, a process known as ‘inside-out’ signalling. The presence of multiple
RGD sites on fibrinogen and vWF allows these molecules to act as ‘crosslinks’ for
platelet aggregation, a process central to the formation of the ‘platelet plug’. In
addition, GPIIb/IIIa binding to fibrinogen is able to mediate various changes to
platelet physiology, a process known as ‘outside-in’ signalling. This process includes
stabilisation of the platelet aggregate/clot retraction and stimulation of α–granule
release (Peerschke EI 1995). Outside-in signalling also promotes the procoagulant
activity of platelets and its subsequent release into the bloodstream as ‘platelet
microparticles’ (Fox JE, Reynolds CC, & Austin CD 1990; Sims PJ et al. 1989).
The central role of GPIIb/IIIa in the thrombotic process has led to the development of pharmacological agents directed against this molecule. Indeed, these drugs are already in widespread clinical use and have been shown to be effective in reducing adverse outcomes in patients presenting with non-ST-elevation myocardial infarction (Cairns JA et al. 2001).

**P-selectin**

P-selectin, also referred to as CD62P, is a member of the selectin family (Frenette PS et al. 1995; Massberg S et al. 1998). The molecule is normally found in the α-granules of platelets and the Weibel-Palade bodies of endothelial cells (Merten M & Thiagarajan P 2004). The density of P-selectin expression in platelets is ten times higher than that in endothelial cells (Merten M & Thiagarajan P 2004). Platelet P-selectin is not normally expressed on the surface membrane but is translocated there on platelet activation (Berman CL et al. 1986), where it is cleaved to form soluble P-selectin (sP-selectin).

Platelet P-selectin interacts with the P-selectin Glycoprotein Ligand-1 (PSGL-1) found abundantly on leukocytes and promotes the adhesion of leukocytes to platelets and vice versa (Larsen E et al. 1989; McEver RP & Cummings RD 1997). The importance of this interaction lies in the fact that platelets which are immobilised on the vessel wall (for example, after vessel wall injury) can promote leukocyte recruitment to the site. Conversely, leukocyte adherent to areas of inflammation may enhance the immobilisation of platelets to that site, thus promoting thrombosis. Therefore, platelet P-selectin may provide one of the links between inflammation and thrombosis, a relationship that is pathophysiologically important in many

P-selectin can mediate the adhesion of activated platelets to monocytes, an event which may promote the translocation of inflammatory cells into the atherosclerotic plaque (Huo Y et al. 2003). This may be the mechanism behind the observation that infusion of activated platelets into the bloodstream can promote atherogenesis, at least in mice (Huo Y et al. 2003). Indeed, this effect was not seen when platelets from P-selectin -/- mice were used.

Similar to platelet P-selectin, endothelial P-selectin is rapidly expressed on the plasma membrane when the cell is stimulated (Naka Y et al. 1996). Endothelial P-selectin also promotes white-cell rolling on the vessel wall (Tan KT, Lip GY, & Blann AD 2003). In addition, endothelial (but not platelet) membrane P-selectin mediates rolling of both activated and non-activated platelets on the endothelium (Frenette PS et al 1995). It is possible that this mechanism may account for the accumulation of platelets in areas of post-ischemic reperfusion, which may lead to local ischaemia (Massberg S et al. 1998).

Interestingly, the identity of the major P-selectin ligand on platelets is believed to be GP Ibα (Massberg S et al 1998). Therefore, it is possible that the attenuation of atherosclerosis by GPIbα antagonism in Apo E -/- mice observed by Massberg et al may be due to inhibition of P-selectin-GP Ib/IX/V interaction (Massberg S et al. 2002).
Based on the findings above, a large amount of effort has been put into developing P-selectin antagonists. Indeed, experiments into animal models of atherosclerosis have shown that P-selectin antagonism can reduce neo-intima formation post balloon angioplasty and may have beneficial antithrombotic effects (Silver MJ et al. 1995; Theoret JF et al. 2001).

In contrast to the large amount of data available on the physiology of membrane associated P-selectin, not much is known about the role of sP-selectin in vivo, although its excess could result in a procoagulant state in the mouse (Andre P et al. 2000). sP-selectin is derived from both platelets and endothelium (Burger PC & Wagner DD 2003; Tan KT & Lip GY 2003a). However, it is believed that the majority of sP-selectin in humans is derived from platelets rather than endothelium (Fijnheer R et al. 2005). Indeed, it has been shown that the plasma level of sP-selectin can be correlated to that of beta-thromboglobulin, a marker of platelet activation, but not to markers of endothelial activation (Blann AD et al. 1997). It can therefore be concluded that sP-selectin, like its membrane-bound counterpart, can be used as a marker of platelet activation in health and disease. Some of the studies on P-selectin in atherosclerotic disease are summarised in table 1.1 below.

Interestingly, aspirin has no effect on either platelet surface P-selectin expression or sP-selectin levels (Blann AD et al. 2001; Pernerstorfer T et al. 1998). The explanation behind this observation is unclear, although Pernerstorfer et al have suggested that the expression of P-selectin may be less influenced by the prostaglandin pathway than other mechanisms (Pernerstorfer T et al. 1998)
<table>
<thead>
<tr>
<th>Findings</th>
<th>Number of patients</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherosclerotic stroke is associated with an increase in platelet P-selectin expression</td>
<td>72</td>
<td>(Zeller JA, Tschoepe D, &amp; Kessler C 1999)</td>
</tr>
<tr>
<td>sP-selectin levels associated with risk of developing adverse cardiovascular events in healthy women</td>
<td>115</td>
<td>(Ridker PM, Buring JE, Rifai N 2001)</td>
</tr>
<tr>
<td>Type II Diabetes is associated with an increase in both platelet P-selectin and sP-selectin levels</td>
<td>43</td>
<td>(Nomura S et al. 1998)</td>
</tr>
<tr>
<td>sP-selectin levels associated with vascular damage in hypertension</td>
<td>21 essential hypertensives and 21 renovascular hypertension</td>
<td>(Verhaar MC et al. 1998)</td>
</tr>
<tr>
<td>sP-selectin is raised in stable coronary heart disease and peripheral vascular disease</td>
<td>41</td>
<td>(Blann AD et al. 1995)</td>
</tr>
<tr>
<td>sP-selectin is raised in unstable angina</td>
<td>12 patients with unstable angina</td>
<td>(Ikeda H et al. 1995)</td>
</tr>
<tr>
<td>sP-selectin is raised in myocardial infarction</td>
<td>9 patients with acute myocardial infarction</td>
<td>(Ikeda H et al. 1994)</td>
</tr>
<tr>
<td>Platelet P-selectin expression is associated with atherosclerotic load</td>
<td>517 patients with diabetes, hypertension or hyperlipidaemia</td>
<td>(Koyama H et al. 2003)</td>
</tr>
<tr>
<td>Acute myocardial infarction is associated with increased platelet P-selectin expression</td>
<td>90</td>
<td>(Ault KA et al. 1999)</td>
</tr>
</tbody>
</table>

Table 1.1 Some studies of P-selectin in human atherosclerotic disease
Platelets, Cytokines, and Atherosclerosis

As discussed above, platelets can adhere to the vessel wall in many pathological conditions. This close association allows the platelet to release inflammatory mediators, such as CD40 Ligand (also known as CD40L or CD154), RANTES, and IL-1β, in close proximity to the vessel wall.

RANTES (Regulated on Activation, Normal T Expressed and Secreted)
Platelets secrete the chemokine RANTES on activation (Kameyoshi Y et al. 1992). Endothelial cells activated by inflammatory stimuli are capable of taking up RANTES released by platelets (Von Hundelshausen P et al. 2001). The presence of immobilised RANTES on the endothelium promotes the arrest of monocytes on the inflamed endothelium, an important step in atherogenesis as the conversion of macrophages into foam cells in the subendothelium is central to atheroma formation (Ross R 1999; Von Hundelshausen P et al. 2001).

The inhibition of RANTES could potentially be exploited to attenuate atherosclerosis. For example, Met-RANTES, a competitive antagonist of RANTES, has been shown to inhibit binding of monocytes to the activated endothelium (Proudfoot AE et al. 1996; Von Hundelshausen P et al. 2001).

Interleukin-1 (IL-1β)
Activated platelets have been found to secrete IL-1β (Hawrylowicz CM, Howells GL, & Feldmann M 1991). IL-1β promotes the endothelial expression of various pro-inflammatory adhesion molecules, such as Intercellular Adhesion Molecule-1 (ICAM-1), P-selectin and vascular cell adhesion molecule-1 (VCAM-1) (Hawrylowicz CM, Howells GL, & Feldmann M 1991; Tan KT, Lip GY, & Blann AD 2003). In addition,
IL-1β is a strong chemoattractant for leukocytes, thus attracting white cells to the area of inflammation. The presence of IL-1β in the vessel wall may also induce the production of other cytokines by vascular smooth muscle cells and monocytes, a process which can further perpetuate vascular inflammation (Loppnow H et al. 1998).

**CD40 Ligand (CD40L)**

CD40L, a member of the tumour necrosis factor superfamily, is produced primarily by platelets (Phipps RP 2000). The molecule is expressed on the surface membrane when the platelet is activated. The interaction of CD40L with its corresponding receptor (CD40) results in the production of various other pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and IL-1β by leukocytes and vascular endothelium (Phipps RP 2000). In addition, activated CD40 can stimulate the release of reactive oxygen species, which may contribute to endothelial dysfunction (Urbich C et al. 2002). Evidence for the involvement of the CD40-CD40L axis in atherogenesis comes from the finding that although CD40L expression cannot be found in healthy human arterial tissues, it can be detected in atheromatous plaques (Mach F et al. 1997). In addition, the administration of an antibody directed against CD40L attenuated atheroma formation in a mouse model of atherosclerosis, with a significant reduction in plaque size and decreased infiltration by monocytes (Mach F et al. 1998). Indeed, the CD40L knockout mouse has decreased atherosclerotic plaque formation (Lutgens E et al. 1999).

Interestingly, the mouse model of atherosclerosis suggests that inhibition of the CD40-CD40L axis can decrease plaque lipid content by up to 80%. This observation is supported by a recent study which showed that increased plasma levels of CD40L may be associated with evidence of intraplaque lipid, as detected by magnetic
resonance imaging (Blake GJ et al. 2003). As plaque lipid content may contribute to plaque instability and predispose to subsequent rupture, with consequent thrombosis, CD40L signalling may well play an important role in destabilising the atheromatous lesion. Indeed, CD40L binding has been shown to stimulate the production of various matrix metalloproteinases (MMP), such as MMP-2, MMP-9 and MMP-11 (Schonbeck U et al. 1997). The MMPs are enzymes that can break down the extracellular matrix in the atheromatous lesion, thus contributing to plaque rupture.

CD40L is also known to play a major role in thrombosis. Thrombus formed in the vasculature of the CD40L -/- mouse is highly unstable and has a tendency to rupture (Andre P et al. 2002a). Infusion of recombinant soluble CD40L (sCD40L) into these mice restored normal thrombus formation. The prothrombotic effect of CD40L appears to be mediated by its binding to GPIIbIIIa, resulting in ‘outside-in’ platelet activation.

Elevated sCD40L levels have been found to be associated with adverse cardiovascular events in humans (Novo S et al. 2005; Schonbeck U et al. 2001). Raised sCD40L levels may also be associated with an increased risk of death or recurrent non-fatal myocardial infarction in patients with acute coronary syndrome (CAPTURE Investigators 2003; Varo N et al. 2003a). In addition, pre-percutaneous transluminal coronary angioplasty levels of sCD40L are predictive of the risk of restenosis (Cipollone F et al. 2003). Some of the studies on CD40L levels in atherosclerotic disease are summarised in table 1.2 below.

CD40L is, therefore, an attractive target for drugs aimed at preventing cardiovascular events in patients with atherosclerosis. Indeed, the beneficial effect of interventions
aimed at reducing cardiovascular risk may partially be due to their effects on sCD40L levels (Lim HS, Blann AD, & Lip GY 2004; Varo N et al. 2003b). Monoclonal antibodies directed against CD40L are currently being evaluated for clinical use in patients with Systemic Lupus Erythematosus (SLE) (Toubi E & Shoenfeld Y 2004). Although they appear to be effective in the treatment of SLE nephritis, use of these monoclonal antibodies are paradoxically associated with an unexplained increase in thromboembolic events.

<table>
<thead>
<tr>
<th>Findings</th>
<th>Number of patients</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>sCD40L levels higher in healthy women who subsequently developed an adverse cardiovascular event</td>
<td>28263 women</td>
<td>(Schonbeck U et al. 2001)</td>
</tr>
<tr>
<td>sCD40L predicts cardiovascular risk in patients with asymptomatic carotid stenosis</td>
<td>42 patients</td>
<td>(Novo S et al. 2005)</td>
</tr>
<tr>
<td>Elevated sCD40L associated with increased risk of recurrent ACS and death</td>
<td></td>
<td>(1) 195 patients</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2) 1088 patients</td>
</tr>
<tr>
<td>sCD40L levels can predict risk of restenosis post percutaneous coronary interventional</td>
<td>70 patients</td>
<td>(Cipollone F et al. 2003)</td>
</tr>
<tr>
<td>sCD40L levels are raised in Type 2 Diabetes and may be modulated by treatment</td>
<td>(1) 97 patients</td>
<td>(1) (Lim HS, Blann AD, &amp; Lip GY 2004)</td>
</tr>
<tr>
<td></td>
<td>(2) 39</td>
<td>(2) (Marx N et al. 2003)</td>
</tr>
<tr>
<td>sCD40L levels are raised in hypercholesterolaemia and may be modulated by treatment</td>
<td>30 patients</td>
<td>(Sanguigni V et al. 2005)</td>
</tr>
<tr>
<td>sCD40L levels predict patients with features of high risk atherosclerotic lesions</td>
<td>46 patients</td>
<td>(Blake GJ et al. 2003)</td>
</tr>
</tbody>
</table>

Table 1.2 Studies of sCD40L levels in atherosclerotic disease
ACS Acute Coronary Syndrome
**Platelet Microparticles**

Platelet microparticles (PMPs), also known as platelet microvesicles, can be defined as particles measuring less than 1μm in diameter that bind monoclonal antibodies directed against platelet antigens (Tan KT & Lip GY 2003a). PMPs have been shown to be present in embolectomised thrombus (Siljander P, Carpen O, & Lassila R 1996), and raised PMP levels have been found in various atherosclerotic diseases, such as stroke (Lee YJ, Jy W, & Horstman L 1993), acute coronary syndromes (Bernal-Mizrachi L et al. 2003), and peripheral vascular disease (Zeiger F et al. 2000). Some of the clinical and experimental studies of PMPs in atherosclerosis are summarised in Table 1.3 (Bernal-Mizrachi L et al. 2003; Preston RA et al. 2003; Shouzu A et al. 2004; Zeiger F et al. 2000).

Vesicle production by activated platelets occurs by two distinct mechanisms: (i) the budding of the plasma membrane; and (ii) the exocytosis of exosomes (Heijnen HF et al. 1999). However, vesicles derived from exosomes are too small to be detected by flow cytometry and their physiological functions are unknown. PMPs formed by the budding of the plasma membrane are probably derived from the fracture of the pseudopods of activated platelets (Yano Y et al. 1994). It is believed that the formation of PMPs is due to the rearrangement of the platelet cytoskeleton, with local disruption of the cytoskeletal structure at the site of fracture. PMPs are believed to have many physiological effects, including effects on thrombosis, cell signalling and angiogenesis (Barry OP et al. 1998; English D, Garcia JG, & Brindley DN 2001; Tans G et al. 1991).
<table>
<thead>
<tr>
<th>Authors</th>
<th>Findings</th>
<th>Number of patients</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lee et al</td>
<td>Patients with cerebrovascular disease have increased PMP levels.</td>
<td>71 patients</td>
<td>(Lee YJ, Jy W, &amp; Horstman L 1993)</td>
</tr>
<tr>
<td>Zeiger F et al.</td>
<td>Patients with peripheral vascular disease have elevated PMP levels.</td>
<td>50 patients</td>
<td>(Zeiger F et al. 2000)</td>
</tr>
<tr>
<td>Bernal-Mizrachi L et al</td>
<td>Patients with ACS have increased PMP levels.</td>
<td>64 patients</td>
<td>(Bernal-Mizrachi L et al. 2003)</td>
</tr>
<tr>
<td>Shouzu A et al</td>
<td>PMPs increased in diabetic patients and decreased by administration of ticlopidine.</td>
<td>73 patients</td>
<td>(Shouzu A et al. 2004)</td>
</tr>
<tr>
<td>Preston R et al.</td>
<td>Hypertension associated with elevated PMP levels</td>
<td>24 patients with uncontrolled hypertension, 19 mildly hypertensive patients</td>
<td>(Preston RA et al. 2003)</td>
</tr>
</tbody>
</table>

Table 1.3 Some human studies of PMPs in atherosclerosis and associated conditions.
PMPs Platelet microparticles; ACS Acute Coronary Syndromes

Figure 1.1 shows how PMPs can be related to the coagulation pathway. Of note, PMPs are important carriers of platelet derived tissue factor (TF) (Muller I et al. 2003). TF in quiescent platelets is usually sequestered in the membrane and matrix of α-granules, and in the open canalicular system of the cell (Muller I et al. 2003). Upon cellular activation, the TF is expressed on the platelet surface and shed along with PMPs. Indeed, up to two-thirds of plasma TF activity is carried on microvesicles. It is also believed that the P-selectin mediated adhesion of PMPs and platelets to neutrophils is required for the TF to become functionally active. However, the exact mechanism for the activation of platelet-derived TF by neutrophils remain unclear, although reactive oxygen species and serine proteases produced by the polymorphs have been implicated in the process (Muller I et al. 2003).
In addition to their possible role in initiating thrombosis, PMPs may also propagate the coagulation pathway (Table 1.4). The outer leaflet of the plasma membrane of quiescent platelets is rich in choline phospholipids (PL), such as sphingomyelin and phosphatidylcholine, which do not promote thrombosis (Zwaal RF & Schroit AJ 1997). Amino PLs, such as phosphatidylethanolamine and phosphatidylserine (PS), which are strongly procoagulant, are sequestered within the inner leaflet of the plasma membrane. In the quiescent platelet, asymmetry of the platelet membrane is maintained by inward transport of amino PLs and outward transport of choline PLs by 'flippase' and 'floppase' respectively. When the platelet is activated, there is an increase in intracellular calcium, which is believed to lead to the activation of 'scramblase', which scrambles amino PLs and choline PLs, leading to the loss of membrane asymmetry. There is also inhibition of 'flippase' activity, but this alone is
insufficient to result in the rapid loss of membrane asymmetry. As a result, the surface of shed PMPs is also rich in amino PL. The importance of the scrambling of phospholipids is illustrated by the fact that defects in the molecule(s) responsible for this reaction are believed to result in a bleeding disorder known as the Scott Syndrome (Nurden AT 2005).

Amino PLs, particularly PS, associate with factors VIIIa and IXa in the presence of calcium ions, thus acting as a catalytic surface for the conversion of factor X to Xa (Zwaal RF & Schroit AJ 1997). Likewise, in the prothrombinase complex, binding of the amino PL to factors Va and Xa promotes the conversion of prothrombin to thrombin. By virtue of their large total surface area, PMPs are efficient at binding coagulation factors Va and VIIIa and thus promoting thrombosis. Indeed, it has been shown that PMPs can be found in association with fibrin in both an in vitro model of thrombosis as well as in pathological specimens of embolectomised thromboemboli (Siljander P, Carpen O, & Lassila R 1996).

In addition to their possible role in promoting thrombosis, PMPs may also play a part in the inhibition of fibrinolysis (Podor TJ et al. 2002). Plasminogen Activator Inhibitor-1 (PAI-1) is an inhibitor of the tissue- and urokinase-type plasminogen activators responsible for fibrinolysis (Horrevoets AJ 2004). PAI-1 is stored in the α-granules of quiescent platelets (Booth NA et al. 1988). Some of the PAI-1 is bound to vitronectin, an interaction that contributes to maintaining PAI-1 in its active configuration (Salonen EM et al. 1989). When the platelet is activated, the contents of the α-granule are released, and additionally, vimentin, which is a component of the platelet cytoskeleton, is exposed. Vimentin can bind the PAI-1/vitronectin complex. Subsequent budding of PMPs from the platelet membrane results in the presence of
PAI-1 bound to the membrane vesicles. Therefore, in view of the above findings, PMPs may contribute to a generalised 'prothrombotic' and 'anti-fibrinolytic' state away from the primary site of thrombosis, by virtue of their ability to circulate in the bloodstream.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Mediator</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiation of thrombosis</td>
<td>TF</td>
<td>(Muller I et al. 2003)</td>
</tr>
<tr>
<td>Propagation of thrombus</td>
<td>Amino-PL</td>
<td>(Zwaal RF &amp; Schroit AJ 1997; Munnix IC et al. 2003)</td>
</tr>
<tr>
<td>Platelet recruitment</td>
<td>GPIIb/IIIa</td>
<td>(Gawaz M et al. 1997)</td>
</tr>
<tr>
<td>Inhibition of thrombosis (as a negative feedback mechanism)</td>
<td>Amino-PL</td>
<td>(Tans G et al. 1991)</td>
</tr>
</tbody>
</table>

Table 1.4 Summary of effects of PMPs on coagulation.
TF Tissue Factor, Amino-PL Amino Phospholipid, GPIIb/IIIa Glycoprotein IIb/IIIa, PAI-1 Plasminogen Activator Inhibitor-1

Amino PLs can also bind activated protein C, a molecule which deactivates factor Va (Tans G et al. 1991). Indeed, Tans et al (1991) showed an in vitro increase in the rate of activated protein C mediated inactivation of factor Va following platelet activation, with PMPs contributing to around 25% of the anticoagulant effect. It is likely that the anticoagulant activity of PMPs may act as a negative feedback mechanism to prevent the uncontrolled propagation of the coagulation pathway. However, the relative importance of the in vivo anticoagulant effect of PMPs is still unclear.
PMPs and the vessel wall

PMPs may also have important effects on the vessel wall, as shown in Figure 1.2. Indeed, PMPs have been shown to adhere to both subendothelium and activated endothelial cells, interactions which are believed to be mediated by GPIIb/IIIa present on PMPs (Gawaz M et al. 1997; Merten M et al. 1999). The adhesion of PMPs to the vessel wall may affect the natural history of atherosclerosis by promoting; (1) local thrombus formation and; (2) atheroma formation.

PMPs bound to the vessel wall can recruit activated platelets to the area of endothelial injury, in the presence of fibrinogen, through a GPIIb/IIIa dependent mechanism (Merten M et al. 1999). Interestingly, in vitro work has suggested that PMPs can actually promote platelet activation (Barry OP et al. 1997). Therefore, it is possible that microparticles adherent to a particular site in the vasculature can precipitate local thrombosis. Indeed, this scenario may be of great clinical significance as PMPs have been shown to adhere to the site of peripheral angioplasty in animal models (Merten M et al. 1999). This observation suggests that patients with critical limb ischaemia may potentially benefit from GPIIb/IIIa inhibition prior to balloon angioplasty, in a manner analogous to the use of GPIIb/IIIa inhibition in patients with acute coronary syndromes prior to coronary angioplasty. However, to date, there are no large clinical trials on this subject.
Platelet microparticles (PMPs) may also have a direct effect in promoting atherosclerosis. They have been shown to enhance the adhesion of leukocytes to endothelial cells (Barry OP et al. 1998). Exposure of endothelial cells to PMPs results in the upregulation of ICAM-1, which mediates the firm adhesion of leukocytes to the endothelium, prior to their subsequent migration into the vessel wall, as well as induce the production of the pro-inflammatory cytokines IL-1β, IL-8 and IL-6 (Barry OP et al. 1998; Nomura S et al. 2001; Ross R 1999). It is believed that at least some of these effects are mediated by the arachidonic acid content of PMPs via a protein kinase C dependent pathway (Barry OP et al. 1998).

PMPs may also play a part in atherogenesis by inducing the proliferation of vascular smooth muscle cells (Weber A, Koppen HO, & Schror K 2000). The exact
mechanism behind this observation is not known, but it appears to be mediated by pre-formed heat-labile protein(s) in the PMP. The effects of PMPs on the vessel wall are summarised in table 1.5

The role of platelet surface P-selectin in promoting atherosclerotic disease is well established (Blann AD, Nadar SK, & Lip GY 2003; Tan KT & Lip GY 2003a). There is a growing body of evidence that the presence of P-selectin in PMPs may also be important in atherogenesis (Heijnen HF et al. 1999). Forlow et al have shown that P-selectin on PMPs may mediate leukocyte-leukocyte adhesion through a P-selectin glycoprotein-1 (PSGL-1) dependent mechanism in vitro (Forlow SB, McEver RP, & Nollert MU 2000). This action may enhance leukocyte recruitment into the atherosclerotic lesion by promoting the tethering of flowing leukocytes to those already rolling on the endothelial surface. It is also plausible that P-selectin present in PMPs immobilised on the vessel wall may directly promote leukocyte recruitment through PSGL-1, although this has not been demonstrated experimentally.

In addition, P-selectin mediated binding of PMPs to neutrophils results in activation of the polymorphonuclear cells, with an increase in CD11b expression and enhanced phagocytosis (Jy W et al. 1995). This observation may well be important in acute thrombotic stroke, which is associated with an elevation in blood levels of PMPs (Lee YJ, Jy W, & Horstman L 1993), and where the post-infarction plugging of the microcirculation by neutrophils may be responsible for extension of the stroke (Tan KT, Lip GY, & Blann AD 2003).
Effect Mediator Reference

<table>
<thead>
<tr>
<th>Effect</th>
<th>Mediator</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Adhesion</td>
<td>GPIIb/IIIa</td>
<td>(Gawaz M et al. 1997)</td>
</tr>
<tr>
<td>Platelet Activation</td>
<td>? Arachidonic acid</td>
<td>(Barry OP et al. 1997)</td>
</tr>
<tr>
<td>Endothelial Dysfunction</td>
<td>? Arachidonic acid</td>
<td>(Barry OP et al. 1997)</td>
</tr>
</tbody>
</table>

Table 1.5 Effects of platelet microparticles on the vessel wall

PMPs and Angiogenesis

PMPs may promote angiogenesis (English D, Garcia JG, & Brindley DN 2001; Kim HK et al. 2004), which is another pathophysiological process intimately related to atherosclerosis. It has been known for some time that bioactive lipids, such as sphingosine-1-phosphate (SPP), are present on the platelet surface membrane (English D, Garcia JG, & Brindley DN 2001). Indeed, SPP is the ligand for a G coupled receptor, Endothelial Differentiation Gene-1 (EDG-1, also known as SPP1), which is present on endothelial cells. The exposure of endothelial cells to SPP induced cellular proliferation, migration and tube formation, events which are crucial in angiogenesis. In addition, EDG-1 may contribute to vascular maturation by promoting the association of vascular smooth muscle cells to the endothelium and enhancing vascular integrity by decreasing vascular permeability. Further evidence on the role of lipid growth factors present on PMPs in the angiogenic process was provided by Kim et al (Kim HK et al. 2004), who showed that the angiogenic ability of PMPs was reduced by treatment with activated charcoal, a procedure known to remove bioactive lipids. Heat treatment of PMPs to denature microparticle proteins did not affect
angiogenic activity, suggesting that this activity was not due to the presence of angiogenic proteins, such as Vascular Endothelial Growth Factor (VEGF).

The exact clinical relevance of the angiogenic effect of PMPs remains to be elucidated. However, neovascularization within the atheromatous plaque may have deleterious effects on the vasculature. It has been suggested that enhanced neovessel formation in the atheromatous plaque may increase the surface area available for the entry of leukocytes into the lesion (O'Brien K et al. 1993). In addition, it is possible that the increased surface area can facilitate plaque expansion by enhancing nutrient delivery to the lesion. Indeed, inhibition of angiogenesis in a mouse model of atherosclerosis may decrease the progression of atherosclerosis (Moulton KS et al. 1999).

1.3 Effects of the Vessel Wall on Platelets

The exact cause of platelet activation in atherosclerosis is unclear. In the normal blood vessel, inappropriate platelet activation is prevented by a combination of blood flow and the antithrombotic action of the endothelium. Any pathological insult, such as smoking, high blood glucose, high cholesterol or abnormal flow dynamics, may cause the endothelium to change from an antithrombotic surface to a prothrombotic one. In addition, these pathological insults can lead to platelet activation, which can cause further endothelial damage/dysfunction.

The Prostanoids

The prostanoids, so called because they were first isolated from the prostate gland, are a group of molecules derived from the action of the enzyme cyclooxygenase (COX) on arachidonic acid (Fitzpatrick FA & Soberman R 2001). COX, which has two
isoforms, COX-1 and COX-2, converts arachidonic acid to the cyclic endoperoxide, prostaglandin H\textsubscript{2} (PGH\textsubscript{2}). COX-1, often referred to as the ‘constitutive’ form of COX, is expressed in most cell types in the body, including platelets, whilst COX-2, referred to as the inducible form of COX, is absent in the anucleate platelet (Patrignani P et al. 1999).

In the endothelial cell, PGH\textsubscript{2} is converted by the enzyme prostacyclin synthetase to prostacyclin (PGI\textsubscript{2}) (Smith WL 1989). PGI\textsubscript{2} acts to inhibit platelet activation, causes vasodilation and has a potent antiproliferative effect (Dusting GJ & MacDonald PS 1990; Feinstein MB et al. 1983). In contrast, PGH\textsubscript{2} is converted to thromboxane A\textsubscript{2} (TXA\textsubscript{2}) by the action of thromboxane synthase in the platelet. TXA\textsubscript{2} can cause platelet activation, leading to shape change, aggregation and secretion (Takahara K et al. 1990). In addition, it causes vasoconstriction and may promote smooth muscle proliferation, a process important in atherogenesis (Halushka PV, Mais DE, & Saussy DL 1987; Sachinidis A et al. 1995).

It is, therefore, obvious that PGI\textsubscript{2} promotes an antithrombotic state, whereas TXA\textsubscript{2} facilitates thrombosis. In the healthy individual, the PGI\textsubscript{2} pathway predominates. However, the local balance of PGI\textsubscript{2} versus TXA\textsubscript{2} can be altered in various disease states, with important pathophysiological consequences. Certainly, platelet TXA\textsubscript{2} production is elevated in various conditions associated with atherosclerosis, such as hypertension, hypercholesterolaemia, smoking, diabetes mellitus and unstable angina (Davi G et al. 1997; Fitzgerald DJ et al. 1986). Thus, the use of aspirin, a non-selective COX inhibitor, in atherosclerotic disease may be particularly useful in preventing the morbidity and mortality associated with platelet activation (Antithrombotic Trialists' Collaboration 2002). More recently, the use of picotamide, a combined thromboxane
synthase inhibitor and thromboxane antagonist which has a potent inhibitory effect on platelet aggregation, has been shown to reduce atherosclerotic plaque progression in diabetic patients (Cocozza M et al. 1995). In addition, the DAVID Study suggests that picotamide may be more effective than aspirin at preventing cardiovascular death in Type 2 diabetic patients with atherosclerosis (DAVID Study Group 2004).

*Purines and platelets*

Nucleotides are important regulators of platelet function. Adenosine Diphosphate (ADP), which is released by activated platelets and damaged cells, especially erythrocytes, is a potent mediator of platelet activation. Exposure of platelets to extracellular ADP results in shape change, GPIIb/IIIa activation, TXA2 synthesis and the secretion of platelet granular contents (Gachet C et al. 1995; Jin J, Daniel JL, & Kunapuli SP 1998).

Two major ADP receptors have been cloned in humans; P2Y1 and P2Y12 (Oury C et al. 2001). P2Y1 and P2Y12 are both G-protein-coupled receptors. Agonist binding to the P2Y12 receptor leads to inhibition of adenylyl cyclase through Gi, whereas P2Y1 mobilises intracellular calcium stores through Gq. A third nucleotide receptor, the ligand-gated ion channel P2X1, was originally described as being an ADP receptor as well (Mahaut-Smith MP et al 2000). However, it is now known that the major physiological ligand for P2X1 is ATP and that earlier experiments were flawed as a result of the presence of contaminating ATP within commercial preparations of ADP (Mahaut-Smith MP et al 2000).

An inhibitor of P2Y12, administered as the prodrug clopidogrel, is already in widespread clinical use. Clopidogrel is converted by the liver to form the active
compound (Savi P et al. 2000), which irreversibly inhibits the P2Y12 receptor. Indeed, clopidogrel has been shown to be useful in the treatment and prevention of the thrombotic complications of atherosclerosis, such as ischaemic stroke and myocardial infarction (Bhatt DL et al. 2000).

*In vivo*, intravascular ADP is rapidly broken down to Adenosine Monophosphate (AMP) by the endothelial enzyme ecto-ATPDase, another mechanism by which the endothelium regulates platelet function (Marcus AJ et al. 1997). AMP formed by the ecto-ATPDase is then broken down into adenosine, which has antiplatelet effects (Marcus AJ et al. 2001). Endothelial ATPDase (CD39) activity is impaired by oxidative stress, the presence of pro-inflammatory cytokines and direct vascular injury, conditions often found in both acute and chronic atherosclerotic disease (Gangadharan SP et al. 2001; Robson SC et al. 1997).

**Nitric Oxide**

The Nobel Prize winning work by Furchgott and Zawadzki demonstrated that endothelial cells are capable of releasing a potent vasodilator. This agent has since been identified as nitric oxide (NO) (Feelisch M et al. 1994), produced by nitric oxide synthase (NOS) from L-arginine (Palmer RM, Ashton DS, & Moncada S 1998). There are three isoforms of NOS; (1) endothelial NOS (eNOS); (2) neuronal NOS (nNOS) and; (3) inducible NOS (iNOS). As its name suggests, eNOS is the predominant isoform of the enzyme in the endothelium (Huang PL et al. 1995). Inhibition of eNOS causes an increase in vascular resistance due to vascular smooth muscle cell contraction, with a resulting rise in blood pressure (Stamler JS et al. 1994).
NO has important antiplatelet activity. Inhibition of NOS can reduce bleeding time, which is a clinical measure of platelet function (Simon DI et al. 1996). Indeed, mice that lack a functional copy of the eNOS gene have decreased bleeding times and have platelets that are more easily activated (Freedman JE et al. 1999). NO may also reduce platelet aggregation, at least in vitro (Kader KN et al. 2000).

Impairment of NO activity is found in many vascular diseases associated with atherosclerosis, including hypercholesterolaemia, hypertension and diabetes, thus contributing to the endothelial dysfunction observed in these conditions (Cardillo C et al. 1998; Saenz de Tejada I et al. 1989; Zeiher AM et al. 1993). Therefore, it is interesting to note that NO production is actually increased in animal models of atherosclerosis (Minor RL et al. 1990). This apparent paradox can be explained by the observation that there is often increased oxidative stress in many vascular diseases, such as diabetes, hypertension and hypercholesterolaemia (Mehta JL et al. 1994; Ohara Y, Peterson TE, & Harrison DG 2003). Reactive oxygen species, such as superoxide, can destroy NO, thus leading to a net loss of endothelium-derived relaxing factor activity (Goldstein S & Czapski G 1995).

1.4 Platelet Activation in Atherosclerosis

It is obvious from the discussion above that platelets play a vital role in all stages of atherosclerosis. In addition, atherosclerosis and associated vascular risk factors can be a cause of platelet activation. The main aim of my thesis is to study platelet activation in patients with atherosclerosis and its possible relationship to disease severity and the risk factors associated with atherosclerotic disease. In addition, my thesis will explore the effect of peripheral artery intervention on the release of platelet-derived
inflammatory mediators. It is hoped that the result would provide some insight into
the progression of the disease.
Chapter 2. Methods for the Study of Platelet Activation

2.1 Introduction

The main physiological role of the platelet is to maintain vascular integrity at sites of injury. Therefore, the platelet has evolved to be activated by various stimuli which can be either cause or consequence of vascular injury. Platelet activation plays an important role in a wide range of pathological conditions. Therefore, there has been considerable interest in developing assays measuring in vivo platelet activation. These assays are generally based on changes in platelet structure and function on activation.

2.2 Platelet structure

White (White JG 1971) has conveniently divided the platelet structure into three distinct regions based on their functional and biochemical activities; (a) the peripheral zone; (b) the sol-gel zone; and (c) the organelles. The peripheral zone is composed of the internal and external membrane systems and their associated structures. The outer surface of the resting platelet has a corrugated appearance on electron microscopy (White JG & Krumweide M 1990). This zone is composed of the glycocalyx, the unit membrane, and the submembrane system.

The glycocalyx is the outermost layer of the platelet and is rich in glycoproteins (White JG 1971). These glycoproteins are responsible for cell adhesion and signal transduction. In the quiescent platelet, they include GPIIb/IIIa, GPIb/IX/V, P-selectin and the receptors for ADP and thrombin (Hynes RO 1992; Oury C et al. 2001; Vu T-KH et al. 1991).
The unit membrane forms the physical barrier between the intracellular contents of the platelet and the extracellular environment. The membrane is composed of a lipid bilayer, with asymmetrical distribution of the constituent lipids in the two layers (Zwaal RF & Schroit AJ 1997). Protein molecules, such as the sodium/potassium pump, are imbedded in the lipid bilayer. Invagination of the unit membrane forms tortuous channels within the platelet, which are in communication with the extracellular environment, termed the open canalicular system (OCS) (White JG 1968). The submembrane system is made of a filamentous system that is believed to interact with platelet microtubules to maintain the shape of the platelet, contribute to the formation of pseudopods, and aid platelet shape change (White JG 1969).

The sol-gel zone ('Cytoplasm') is composed of a gel containing colloids (e.g. proteins and carbohydrates) and dissolved ions embedded in a mesh of 'fibres' (White JG 1971). The fibres are composed of microtubules and microfilaments, which act to maintain platelet shape and to mediate shape change during platelet activation. Platelet organelles include mitochondria, dense bodies, α-granules, and lysosomes (White JG 1971). The latter three granules secrete their contents extracellularly when the platelet is activated. There are 5-6 dense granules (δ-granule) per platelet (Nishibori M et al. 1993; White JG 1998). δ-granules are around 150nm in diameter and their contents include serotonin, ADP, ATP, and calcium. Their membranes also contain GPIIb/IIIa, GPIb/IX/V, granulophysin (CD63), and P-selectin. α-granules measure 200-400nm in diameter (Harrison P & Cramer EM 1993), and contain various molecules which mediate thrombosis and inflammation. Their contents include beta-thromboglobulin, vWF, fibrinogen, VEGF and various protease inhibitors. Proteins associated with their membranes include P-selectin and GPIIb/IIIa.
Lysosomes (\(\lambda\)-granules) measure between 175-250nm in diameter and contain enzymes which break down the extracellular matrix (Ciferri S et al. 2000). The lysosomal membrane contains lysosomal associated membrane proteins (LAMPS), such as CD63, LAMP-1 and LAMP2. Table 2.1 lists the membrane proteins and contents of all three types of secretory bodies. Figure 2.1 below shows a simplified representation of a platelet.

<table>
<thead>
<tr>
<th>Granule</th>
<th>Membrane Protein</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dense Granules</td>
<td>GPIb, GPIIb/IIIa, CD63, LAMP-2, P-selectin</td>
<td>ATP, ADP, serotonin, calcium, magnesium, pyrophosphate, GTP, GDP, histamine</td>
</tr>
<tr>
<td>(\alpha)-Granules</td>
<td>P-selectin, GPIIb/IIIa, GPIb-IX, PECAM (CD31), vitronectin receptor, GPIV</td>
<td>Beta-thromboglobulin, platelet factor-4, serglycin, fibronectin, vitronectin, vWF, thrombospondin, fibrinogen, Factor V, Factor VIII, Factor XI, Factor XIII, kininogens, plasminogen, protein S, PDGF, TGF(\beta), VEGF, interleukin 1(\beta), (\alpha)2-macroglobulin, (\alpha)2-antitrypsin, albumin</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>CD63, LAMP1, LAMP2</td>
<td>Cathepsin D, Cathepsin E, Carboxypeptidase, Prolinecarboxypeptidase, Collagenase, Acid Phosphatase, Arylsulfatase, Heparinase, (\beta)-glucuronidase, (\beta)-galactosidase, (\beta)-glycrophosphatase, (\alpha)-D-glucosidase, (\beta)-D-glucosidase, (\alpha)-L-fucosidase, (\beta)-D-fucosidase, (\alpha)-L-arabinosidase, (\alpha)-D-mannosidase, (\beta)-N-acetyl-glucosaminidase</td>
</tr>
</tbody>
</table>

**Table 2.1 The contents of platelet granules.**
PECAM Platelet endothelial cell adhesion molecule; PDGF Platelet derived growth factor, TGF Transforming growth factor, GTP Guanosine Triphosphate; GDP Guanosine Diphosphate
2.3 Platelet Activation

On activation, the normal platelet undergoes a series of characteristic changes. These include adhesion, shape change, membrane budding, aggregation, release of granular contents and thromboxane synthesis.

Platelet Adhesion

The initial adhesion of the platelet to the injured vessel wall is through an interaction between GPIb/IX/V and vWF adherent to the blood vessel (Hynes RO 1992; Savage B, Almus-Jacobs F, & Ruggeri ZM 1998). This interaction enables the platelet to roll over the vessel wall under conditions of high shear stress, thus slowing down its
passage in the blood vessel. The loss of platelet kinetic energy enables firm adhesion of the platelet to the vessel wall through more permanent mechanisms, which requires the presence of GPIIb/IIIa, the collagen receptors $\alpha 2\beta 1$ and GPVI (Arai M et al. 1995; Kehrel B et al. 1998), and fibronectin GPIC/IIa (Beumer S et al. 2000).

Adhesion of the platelet to the site of injury enables the formation of a ‘platelet plug’ to prevent exsanguination and provide a catalytic surface for coagulation to take place (Figure 2.2).

Platelet surface GPIb/IX/V expression can be modulated by platelet activation. The complex is translocated into the OCS on platelet activation (Michelson AD et al. 1996). In addition, the action of thrombin can cleave a large portion of GPV from the complex, with the formation of soluble GPV (sGPV) (Michelson AD et al. 1996).

![Figure 2.2 Platelet Adhesion.](image)

Platelets labelled with the vital dye Calcein AM are applied to a microscope slide coated with a non-confluent layer of endothelial cells. Note that the majority of platelet adhesion is on areas not covered by endothelium.
Platelet Shape Change and Membrane Budding

Once the platelet is activated, its normally discoid shape changes into a more rounded structure with blebs and pseudopods (Born GV 1970). The shape change is considered to be the first measurable physiological change after activation by platelet agonists. The change in shape is mediated by actin filaments associated with the platelet microtubules (Hartwig JH 1992). Platelet shape change increases the surface area available to facilitate coagulation. In addition, there is release of procoagulant PMPs.

Aggregation

When a platelet is activated by an agonist, a change in the surface configuration of GPIIb/IIIa can be brought about; a process termed ‘inside-out signalling’ (Hynes RO 1992). Activated GPIIb/IIIa can bind fibrinogen and vWF through the RGD sequences of these molecules. Therefore, these molecules can act as bridges between adjacent platelets.

Secretion

‘Outside-in’ signalling by binding of activated GPIIb/IIIa to fibrinogen and vWF can promote further platelet activation (Peerschke EJ 1995). This causes the secretion of lysosomal, α-granular and dense body contents. The fusion of the granular walls to the surface membrane also increases the expression of P-selectin, CD63, GPIIb/IIIa and PECAM on the platelet surface (Ciferri S et al. 2000; Harrison P & Cramer EM 1993; Nishibori M et al. 1993).
Thromboxane Synthesis

The activity of the enzyme phospholipase A$_2$ (PLA$_2$) is increased when the platelet is activated (Halenda SP et al. 1989). PLA$_2$ releases arachidonic acid (AA) from phospholipids such as phosphatidylcholine, phophatidylethanolamine and phosphatidylinositol (Arita H, Nakano T, & Hanasaki K 1989). As the conversion of AA to prostaglandin H$_2$ represents the rate limiting step in the production of thromboxane A$_2$ (TXA$_2$), any increase in the cellular concentration of AA will promote TXA$_2$ synthesis. The changes associated with platelet activation are summarised in figure 2.3 below.

Figure 2.3 Platelet Activation
2.4 Methods for determining in vivo platelet activation

As discussed above, platelet activation results in changes to its structure and function. Therefore, any method aimed at determining platelet activation should be able to detect these changes either directly or indirectly. The discussion below will focus on commonly used methods in determining platelet activation.

(a) Direct Methods of Studying Platelet Activation

In general, direct methods of observing platelet activation are particularly useful in monitoring rapid changes in platelet activation status. For example, these methods should be utilised in studies looking at changes in platelet activation status during vascular interventions. However, erroneous results may occur as a consequence of artefactual platelet activation, as most direct methods of studying platelet activation require blood collection. Indeed, it is possible that many of the direct methods of studying platelet activation measures the ‘reactivity’ of platelets to blood collection as well as the degree of platelet activation. Therefore, the collection of blood for direct methods of studying platelet activation requires immaculate attention to avoid stasis (for example, using a lightly applied tourniquet). The collection of blood into potent antiplatelet media (e.g. a citrate, theophylline, adenosine, dipyridamole(CTAD) medium) may also help reduce artefactual platelet activation (Kuhne T et al. 1995; Kim HK et al. 2002; Papp AC et al. 1989). Indeed, PMP levels measured in CTAD tubes immediately after blood collection did not differ significantly from those assayed one hour post-collection (Kim HK et al. 2002). In contrast, there is a doubling of PMP values at 1 hour post-collection in samples collected into conventional citrated tubes.
**Electron Microscopy**

Electron microscopy can be used to study platelet shape change and vesiculation (Ahnadi CE et al. 2003). In practice, this method is seldom utilised as it is costly and time-consuming.

**Flow Cytometry**

Flow cytometry is the most widely used direct method of studying platelet activation. Studies on the flow cytometer can be carried out using small volumes of blood. In addition, the assay can be completed within an hour of blood collection with minimal preparation. The availability of a large number of fluorescent monoclonal antibodies and reagents has enabled the study of a large number of platelet activation markers. The flow cytometer is also extremely sensitive, with the ability to detect as few as 1% of activated platelets (Kestin AS et al. 1993).

The basic principle of platelet flow cytometry is relatively simple. The platelet is labelled with a platelet specific antibody conjugated to a fluorescent probe, such as anti-CD42b-fluorescein (CD42b-FITC) or anti-CD61-peridinin-chlorophyll-protein (CD61-PerCP). At the same time, another fluorescent labelled antibody directed against a platelet antigen (or neoantigen) related to platelet activation is applied. Commonly used markers of platelet activation include those related to α-granules and lysosomal release, such as anti-CD62P-phycoerythrin (CD62P-PE) and anti-CD63-PE respectively, and those associated with neoantigen exposure, such as the PAC-1-FITC antibody which recognises the RGD binding site of activated GPIIb/IIIa (Schmitz G et al. 1998; Shattil SJ et al. 1985).
The labelled platelet suspension is then passed through a laser beam, which has a wavelength either identical or nearly-identical to that needed to excite the fluorescent molecule. Light emitted by each type of fluorescent molecule has a characteristic wavelength and is detected by a specific detector in the flow cytometer. In addition, the size of the particle can be judged by the forward scatter (FSC) of the laser and the ‘granularity’ can be determined by side scatter (SSC). The structure of a simple flow cytometer is shown diagrammatically in Figure 2.4.

**Figure 2.4** Diagrammatic representation of a simple flow cytometer (based on the BD Biosciences FACScan)

FL1 is the detector for green light, wavelength of 530nm (e.g. emitted by FITC); FL2 detects orange light, wavelength of 585nm (e.g. from PE); FL3 is for red light, wavelength 650nm (e.g. from PerCP). NB. Other flow cytometers may have different laser excitation wavelengths and/or different detector wavelength sensitivities.

The ‘strength’ of each wavelength is measured by mean fluorescent intensity (MFI).

In addition, the percentage of platelets expressing a particular antigen can be obtained.
The MFI gives an indication of the total amount of antigen present and is used when the antigen in question is present at all times on the platelet surface (i.e. when the percentage expression of that antigen is likely to be or near 100% at all times). This is the reason why MFI is used in the measurement of the thrombin mediated down-regulation of GP Ib/IX/V (Michelson AD et al. 1991). On the other hand, the ‘percentage of positive platelets’ is used for antigens which are not normally present on the cell surface (or present in a small number of platelets). This method is extremely sensitive in detecting antigen expression on platelet activation. The detection of CD62P and CD63 are based on this method (Figure 2.5).

Figure 2.5. Flow cytometric detection of activated platelets. FL1-H and CD62 PE represent platelet binding to anti-CD42-FITC and anti-CD62P-PE respectively. The diagram on the left shows a typical dot-plot for a sample containing non-activated platelets while the one on the right shows significant platelet activation. Note the increased expression of CD62P in activated platelets.
Apart from its ability to detect changes in antigen expression during platelet activation, the flow cytometer could be used to detect PMPs and platelet-leukocyte aggregates (Furman MI et al. 1998; Tan KT et al. 2004). Platelet-leukocyte aggregates are formed by the interaction between surface P-selectin expressing platelets and leukocytes (Furman MI et al. 1998). They are detected by labelling the sample with a platelet specific antibody (e.g. anti-GPIIb/IIIa) and a leukocyte-specific antibody (e.g. CD11b). Platelet-leukocyte aggregates are defined by size and positive binding to both monoclonal antibodies and can be expressed as a percentage of total leukocytes.

Due to their small size, PMPs cannot be detected by conventional light microscopy, and most studies on PMPs have been carried out using flow cytometry. Indeed, in view of their small size, appropriate labelling of PMPs using fluorescent monoclonal antibodies is required for their detection. Common monoclonal antibodies used in the flow cytometric detection of PMPs include anti-CD42b (GPIb), anti-CD41 (GPIIb) and anti-CD61 (GPIIIa). The monoclonal antibody-labelled sample is then mixed with a known concentration of latex beads of a particular size to both enable the calculation of the concentration of the PMPs and to differentiate the PMPs from platelets.

In my studies, I used a dual labelling method whereby the PMPs were labelled with anti-CD42b and anti-CD61 conjugated with fluorescein (FITC) and Peridinin-Chlorophyll-Protein (PerCP) respectively. FITC and PerCP have emission wavelengths corresponding to the green and red parts of the light spectrum respectively. Latex beads of 1μm in diameter with an emission wavelength in the red part of the spectrum were then added to the sample (Figures 2.6 and 2.7). Subsequent flow cytometric analysis of the sample allowed quantitative measurement of the plasma PMP concentration. Other methods which have been used to study PMPs
include direct electron microscopy (Siljander P, Carpen O, & Lassila R 1996) and less ideally, Enzyme-Linked Immunosorbent Assay (ELISA) (Nomura S et al. 2003).

Figure 2.6 The platelet and platelet microparticle (PMP) population with flow cytometry.
The arrow represents the population of latex beads with a mean diameter of 1μm. The arrowhead represents the PMP and platelet population. The test sample has been labelled with CD-42-FITC and CD61-PerCP and the suspension has been gated using the FL-3 (red) detector. The 'cut-off size' for PMP is taken as the mean diameter of the latex beads, represented by the vertical line through the platelet population. SSC Side Scatter (A measure of the lucency of the particle) FSC Forward Scatter (represents size) consistent with the platelet and PMP population.
Figure 2.7 A sample of platelet poor plasma labelled with anti-CD42-FITC and anti-CD61-PerCP mixed with red latex beads.

FL1-H and FL-3 represents the magnitude of green and red fluorescence respectively of the particulate sample. The arrowhead indicates the platelet and PMP population while the arrow indicates the latex beads. Note that only the platelet/PMP population bound anti-CD42-FITC, as indicated by the green fluorescence. The PMP count can be deduced by the combination of data on size and fluorescence from figures 2.6 and 2.7 respectively.
Enzyme-linked Immunosorbent Assay (ELISA) of Platelet Granular Contents

Measurement of the plasma levels of β-thromboglobulin (βTG) and platelet factor 4 (PF4) are the two most well-established markers in this category. PF4 is taken up by endothelial cells and is considered to be a less reliable marker of platelet activation than βTG (Kaplan KL & Owen J 1981). However, as βTG is excreted by the kidneys, its levels may be erroneously elevated in renal impairment (Lane DA et al. 1984). It has also been suggested that measurement of urinary βTG may be a more reliable of platelet activation than measurement of its plasma level.

Soluble Glycoprotein V (sGpV)

As mentioned above, sGPV is cleaved from the GPIb/IX/V complex by the action of thrombin. As the GpIb/IX/V complex is present abundantly on the platelet surface membrane, sGpV levels may reflect the activity of thrombin on platelets (Ravanat C et al. 2000). The plasma concentration of sGPV has been found to be raised in atherosclerotic disease states such as coronary heart disease and peripheral artery disease (Blann AD et al. 2001).

(b) Indirect Methods of Studying Platelet Activation

These refer to the measurement of modified metabolites of activated platelets in either plasma or urine. In general, as these metabolites are modified in vivo prior to assay, these assays are not as sensitive to dynamic changes in platelet activation as the direct methods of assessing platelet activation and may be more useful as measurements of 'baseline' platelet activation. However, they do not tend to be as easily affected by artefactual platelet activation during sample collection.
**P-selectin**

It is believed that P-selectin expressed on the surface of the activated platelet can be cleaved or shed into the circulation to form sP-selectin (Fox JE 1994). The poor correlation between sP-selectin and βTG suggests that both molecules measure different aspects of platelet activation, with the former being a ‘baseline measurement’ and the latter ‘acute changes’ (Blann AD & Lip GY 1997). Further evidence to suggest the insensitivity of sP-selectin to acute changes in platelet activation status comes from a study in which the platelet activation status of patients undergoing diagnostic angiography were measured (Blann AD, Adams R, & Ashleigh R 2001). It was found that there was only a significant rise in sP-selectin a day after the procedure.

However, the lack of sensitivity of sP-selectin to acute changes in platelet activation status does not mean that it is of no use in the study of platelet activation status in human disease. In contrast, plasma concentrations of sP-selectin have been found to be elevated in association with diabetes, hypercholesterolaemia, and hypertension (Blann AD et al. 2003). Raised sP-selectin may also be associated with adverse cardiovascular outcomes in peripheral vascular disease and coronary heart disease (Blann AD et al. 2003).

**Urinary Thromboxane A\textsubscript{2} metabolites**

TXA\textsubscript{2} has a very short half life in the circulation and is rapidly transformed into the inactive TXB\textsubscript{2} (Fitzgerald GA, Pedersen AK, & Patrono C 1983). TXB\textsubscript{2} is further converted to 2,3-dinor-thromboxane B\textsubscript{2} (2,3TXB\textsubscript{2}) and 11-dehydrothromboxane B\textsubscript{2}(11TXB\textsubscript{2}). These metabolites are excreted in the urine and could be measured to give an indication of *in vivo* platelet activation. They have the added advantage of not
requiring venepuncture. However, TXA₂ is also produced by monocytes and the measurement of its urinary metabolites may not be specific for platelet activation (Mene P et al. 1998).

2.5 Conclusion

With the realisation that activated platelets may play a role in the defence against parasitic infection, progression of atherosclerosis, and inflammatory bowel disease, there is an increasing number of studies investigating platelet activation status in disease states (Tan KT & Lip GY 2003a). Indeed, the importance of the study of platelet activation is likely to increase even further with the introduction of the newer antithrombotic drugs, such as the GPIIb/IIIa inhibitors and the direct thrombin inhibitors.

Unfortunately, at present, there is no consensus on the best method of measuring platelet activation. Indeed, even the use of the flow cytometer to detect platelet activation is subject to great technical variation between various laboratories, despite the presence of a consensus document on platelet flow cytometry (Schmitz G et al. 1998). There is also a lot of variation amongst 'baseline' values of platelet activation. It is particularly important that the results from one study could be compared to that of another. Indeed, standardisation of the various measurements of platelet activation would facilitate the use of meta-analyses to combine the results of the often underpowered small studies on platelet activation.

An ideal study of platelet activation should include at least one of each of the indirect and direct methodologies. This is to ensure adequate detection of both acute and
chronic platelet activation. Apart from that, the choice of test should be tailored towards the population of patient to be studied.
Chapter 3. Plan of Investigation

3.1 Background

There is evidence that atherosclerosis is associated with excessive platelet activation, although it is often difficult to establish if this represents cause or effect (Blann AD et al 1995; Fitzgerald DJ et al. 1986; Furman MI et al. 1995). In addition, studies of platelet activation are complicated by the absence of a ‘gold standard’ of assessment of platelet activation. Moreover, it may be argued that many clinical studies are disadvantaged by the fact that many patients with vascular disease are on antiplatelet therapy or other drugs, such as angiotensin converting enzyme inhibitors and statins, which may influence platelet function. However, as most of these drugs work against platelet activation, their use cannot account for the increased platelet activation observed in patients with atherosclerosis. In addition, platelets from patients with coronary atherosclerosis also have increased reactivity when stimulated by various platelet agonists in vitro (Furman MI et al. 1998).

3.2 Hypotheses

(1) Platelet activation in patients with stable coronary heart disease increases with the angiographic severity of disease (Chapters 5 & 8).

(2) Patients presenting with the acute manifestations of atherosclerosis have a greater degree of platelet activation than patients with stable disease (Chapters 4 & 7).

(3) Peripheral artery intervention results in the release of sCD40L by platelets (Chapter 7).

(4) Platelet microparticle levels are higher in patients with Type 2 Diabetes who develop symptomatic macrovascular disease (Chapter 6).
3.3 Plan of Investigation

Source of blood:

I recruited; (1) patients undergoing elective coronary angiography and/or angioplasty at City Hospital, Birmingham. All patients undergoing the procedure were invited to take part in the study; (2) patients attending the City Hospital vascular clinic for lower limb ischaemia; (3) patients admitted to the vascular unit for critical limb ischaemia; and (4) patients attending the local diabetic clinic. Age- and sex-matched healthy controls were recruited from members of hospital staff, visitors to the hospital and those undergoing elective cataract or hernia operations. A detailed clinical history was obtained from all the subjects, along with their blood pressure. Approximately 20 ml of blood was collected from each subject. Additional details on patient recruitment can be found in the individual data chapters. Exclusion criteria include the presence of acute or chronic infection, presence of significant valvular disease, active cancer, and a history of connective tissue disease.

Flow cytometry.

Platelet Flow Cytometry

For my studies, I decided to use the well validated measurements of CD62P and CD63 for the detection of platelet activation. CD42b was used as the platelet marker. Although other flow cytometric markers of platelet activation (e.g. PAC-1) were available, I decided against using these as they were unlikely to add any new information to that available. In addition, use of additional flow cytometric tests can lead to prolongation of the time required for sample processing, resulting in artefactual platelet activation. The methodology is described in Chapter 4.
**Platelet Microparticle Detection**

The small size of platelet microparticles (PMPs) precludes their detection by flow cytometry in the absence of fluorescent monoclonal antibodies. The flow cytometric detection of PMPs can be either based on their direct detection in platelet poor plasma or in a suspension of previously extracted PMP. In my studies, I decided to use direct flow cytometry of platelet-poor plasma, similar to the methodology utilised by Preston et al (2003), as prior extraction of PMPs may introduce a degree of bias into the study as well as delay sample processing. I labelled PMPs with anti-CD42b and anti-CD61 prior to flow cytometry. The exact methodology is described in Chapter 4.

**ELISA**

Blood for ELISA-based studies was collected into citrated media and centrifuged at 1500g for 20 minutes. The resulting supernatant was snap frozen at -70°C within an hour of blood collection. The frozen plasma was thawed immediately before the assay. Baseline platelet activation status was determined using ELISA to detect soluble P-selectin in plasma samples. In addition, the plasma level of sCD40L was determined to assess the release of this pro-inflammatory molecule by platelets. sGPV, which is cleaved from the platelet surface by the action of thrombin, was used as the direct method of measuring platelet activation in patients with stable coronary heart disease.

**3.4 Summary**

My thesis assesses the degree of platelet activation in atherosclerotic disease, using both flow cytometry and plasma markers. It will attempt to provide a link between the laboratory markers of platelet activation and the clinical and angiographic features of
the disease. In addition, it will study the effect of peripheral artery angioplasty on markers of platelet activation.
Chapter 4. Platelet Activation and the Severity of Peripheral Artery Disease

4.1 Introduction

Atherosclerosis is a common condition in developed countries and is one of the most important causes of morbidity and mortality in the western hemisphere (Figure 4.1). The commonest complication of atherosclerotic disease is arterial thromboembolism. In addition, excess platelet activation in this disease may well play a part in the clinical progression of this condition.

Figure 4.1 Iliac angiogram showing diffuse atherosclerotic disease
An increase in plasma PMP levels has been described in patients with peripheral artery disease (PAD) (Zeiger F et al. 2000), but the relationship of PMPs to other markers of platelet activation and to disease severity is unknown. I therefore hypothesised that increased numbers of PMPs can be found in PAD, with the highest in those with the most severe clinical disease. I also aimed to determine if PMPs are comparable to the more conventional markers of platelet activation, such as sP-selectin and the platelet membrane expression of CD62P and CD63. My hypotheses were tested in a case-control study of patients with PAD versus healthy controls. For the purpose of clarity, although CD62P and P-selectin represent the same molecule, in this study, unless otherwise stated, ‘CD62P’ is used to refer to the platelet membrane-bound version of the molecule and ‘sP-selectin’ refers to the soluble version of the entity.

4.2 Subjects and Methods

Subjects

Patients were recruited from both the vascular outpatient clinic and the vascular inpatient facilities at City Hospital, Birmingham. The inclusion criteria for these patients include a clinical history suggestive of the disease and an ankle-brachial pressure index (ABPI) <0.8. Patients were recruited into two classes: those who had mild-to-moderate disease with simple intermittent claudication (IC), and those who suffered from critical limb ischaemia (CLI) with clear rest pain.

Exclusion criteria for the study include refusal of consent, renal failure, the presence of co-existent inflammatory disease (e.g. rheumatoid arthritis), recent heparin therapy, a history of cancer, the presence of active infection, and heart failure of at least New York Heart Association (NYHA) Grade II severity. Age- and sex-matched healthy
controls were recruited from members of hospital staff, visitors to the hospital and those undergoing elective cataract or hernia operations. A detailed clinical history was obtained from all the subjects, along with their blood pressure. Approximately 20 ml of blood was collected from each subject. Blood was collected prior to the administration of heparin in patients with critical ischaemia. The baseline characteristics of the controls and patients are summarised in table 4.1. Approval was obtained from the Ethics Committee of the West Birmingham Health Authority and written informed consent from each subject was obtained in accordance with the Declaration of Helsinki.

**Methods**

Blood for platelet and PMP flow cytometry was collected according to the method described in the European Working Group on Clinical Cell Analysis Consensus Protocol on platelet flow cytometry (Schmitz G et al. 1998). The blood samples were collected into Diatube-H vacutainers (BD Biosciences, Oxford, United Kingdom), which contain the platelet inhibitors theophylline, adenosine, and dipyridamole, as well as citrate. All the blood samples collected were processed on the flow cytometer (FACScan, BD Biosciences, Oxford, United Kingdom) within an hour of venepuncture. Forward (size-dependent) (FSC) and 90° sideways scatter (SSC) were set at logarithmic gain. Mouse anti-keyhole limpet haemocyanin monoclonal antibodies conjugated to phycoerythrin were used as isotype negative controls to define non-specific binding. Monoclonal antibodies were obtained from BD Biosciences (Oxford, United Kingdom). Data acquisition and analysis were performed with CELLQuest software version 3.1 (BD Biosciences, Oxford, United Kingdom). Intra-assay and inter-assay coefficients of variation were <5% and <10% respectively.
For platelet studies, 100 µl of blood was diluted with 800 µl of phosphate buffered saline (PBS) (Sigma, Poole, United Kingdom). The antibodies used for flow cytometry were conjugated to fluorescein (FITC), phycoerythrin (PE), or peridinin-chlorophyll-protein (PerCP). For determination of % CD62P positivity, 15 µl of diluted blood was incubated with 6 µl each of anti-CD42b-FITC and anti-CD62P-PE. Similarly, a 15 µl aliquot of diluted blood was added to 6 µl each of anti-CD42b-FITC and anti-CD63-PE for determination of % CD63 positivity. All the monoclonal antibodies were obtained from BD Biosciences, Oxford, United Kingdom. The sample was then incubated in the dark for 30 minutes. The labelled platelet sample was diluted with 800 µl of PBS immediately before flow cytometry. Platelet events were acquired on a threshold set for the FL-1 detector. Therefore, all events with a green fluorescence were collected. A region (R1) corresponding to the platelet population was then defined in the FSC vs SSC dot-plot. 10,000 events were collected in R1. A dot-plot of FL-1 (representing CD42b) vs. FL-2 (CD62P or CD63) was then plotted for all events in the R1 region. Results are expressed as the percentage of events positive for CD62P or CD63 in terms of the total number of CD42b positive events in the appropriate gate.

For PMP determination, platelet-poor plasma (PPP) was obtained from whole blood by centrifugation at 1500g for 20 minutes. 100 µl of PPP was incubated for 30 minutes in the dark with 10 µl each of anti-CD61 PerCP and anti-CD42b-FITC. 600 µl of PBS and 2 µl of a known concentration of red latex beads (Sigma, Poole, United Kingdom) measuring 1.01 µm in diameter were added to the sample immediately prior to flow cytometry. PMPs were defined as particles measuring less than the mean diameter of the latex beads and show positive binding to both anti-CD61 and anti-CD42b.
Plasma sP-selectin was determined by enzyme-linked immunosorbent assay (ELISA) on citrated plasma samples using commercial reagents (R&D Systems, Abingdon, UK). Intra-assay and inter-assay coefficients of variation were <5% and <10% respectively.

**Power calculations and statistical methods**

Expecting PMPs to follow a non-normal distribution, I modelled a data set with a median of 1.00 unit and an inter-quartile range of 0.67 to 2.00 in healthy controls.

I hypothesised that levels of PMPs will be approximately double in patients whose disease was mild-moderate (i.e. in IC, median = 2.00, IQR 1.4 – 4.0), and three times higher in patients with more severe disease (i.e. in CLI, median = 3.00, IQR = 2.1 – 6.0). After log transformation for power calculation this equates to a mean and standard deviation of 0.48/0.7, 0.55/0.7 and 0.59/0.7 (i.e. a minimum difference of 0.57 of a standard deviation) for the three groups respectively. To achieve this at p<0.001 overall and an alpha of p<0.05 with a 1-beta of 0.8 between groups, 21 subjects per group are required.

Continuous data were subjected to the Anderson-Darling test to determine their distribution. Non-normally distributed data, presented as median and inter-quartile range (IQR), were analysed by the Kruskal-Wallis test. Normally distributed data are presented as mean and standard deviation (SD) and analysed by one-way analysis of variance. Categorical data were analysed by the χ² test. Differences between multiple groups of subjects were analysed by Analysis of Variance (ANOVA) and Tukey’s post hoc test (data which were non-normally distributed were log transformed). Data
were correlated according to Spearman’s method. All analyses and power calculations were performed using Minitab 13 (Minitab Inc, State College, PA, USA).

4.3 Results

The two patient groups and the healthy controls were matched for age, sex, smoking and platelet count (Table 4.1). Total cholesterol was lowest in patients with CLI. Patients with IC had higher systolic blood pressure (SBP) than the healthy controls. Glycated haemoglobin (HbA1c) was higher, and High-Density Lipoprotein (HDL) lower, in both patient groups compared to the controls, but were not significantly different between patient groups.

Plasma circulating PMPs were highest in patients with CLI, followed by IC, with the lowest levels in healthy controls (Table 4.2). The proportional rise and numbers in the patient groups (2.2 fold and 5.6 fold) exceeds the demand of the power calculation. The percentage of platelets positive for CD62P and the level of sP-selectin were both increased in both patient groups, but not between patient groups. CD63 expression was only higher in CLI compared to healthy controls.
<table>
<thead>
<tr>
<th></th>
<th>Critical Ischaemia (CLI) (n=23)</th>
<th>Intermittent Claudicants (IC) (n=36)</th>
<th>Healthy Controls (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66 ± 11</td>
<td>65 ± 9</td>
<td>66 ± 14</td>
<td>0.923</td>
</tr>
<tr>
<td>Males: Females</td>
<td>14:9</td>
<td>23:13</td>
<td>14:20</td>
<td>0.129</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>135 ± 28</td>
<td>145 ± 18</td>
<td>130 ± 13</td>
<td>0.007*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>74 ± 14</td>
<td>81 ± 8</td>
<td>75 ± 11</td>
<td>0.023</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>35</td>
<td>36</td>
<td>21</td>
<td>0.184</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>70</td>
<td>64</td>
<td>---------</td>
<td>0.653</td>
</tr>
<tr>
<td>Aspirin use (%)</td>
<td>70</td>
<td>56</td>
<td>---------</td>
<td>0.282</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.3 ± 0.9</td>
<td>5.0 ± 1.4</td>
<td>5.3 ± 0.9</td>
<td>0.004**</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>1.6 ± 0.3</td>
<td>0.001***</td>
</tr>
<tr>
<td>Platelet Count (x10^9/l)</td>
<td>309 ± 140</td>
<td>273 ± 77</td>
<td>256 ± 64</td>
<td>0.132</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>6.3 ± 1.6</td>
<td>6.3 ± 1.4</td>
<td>5.4 ± 0.5</td>
<td>0.01***</td>
</tr>
</tbody>
</table>

Table 4.1 Clinical, demographic and routine laboratory indices
Data are expressed as mean±SD. P Values by ANOVA except for the categories of sex, aspirin use and statin use [the latter two between patients only], which were analysed by the Chi-squared test. SBP = systolic blood pressure, DBP = diastolic blood pressure, HDL = high density lipoprotein cholesterol.

*P<0.05 (Tukey’s post-hoc test) between healthy controls and intermittent claudication group.

**P<0.05 between critical ischaemia and the remaining two groups. ***P<0.05 between healthy controls and both groups of patients. SBP Systolic Blood Pressure; DBP Diastolic Blood Pressure; HDL High Density Lipoprotein; HbA1C Glycated Haemoglobin
Critical Ischaemia (CLI) n=23 | Intermittent Claudication (IC) (n=36) | Healthy controls (n=30) | P value
---|---|---|---
PMP/ml (X10⁻⁷) | 7.13 (1.86-13.9) | 2.82 (1.50-4.38) | 1.26 (0.67-2.95) | <0.001*  
% CD62P +ve | 4.7 (2.6-12.5) | 7.5 (1.5-13.5) | 2.8 (1.7-5.5) | 0.005**  
% CD63 +ve | 13.0 (7.5-21.8) | 9.5 (4.8-12.7) | 7.0 (4.5-11.5) | 0.009***  
sP-selectin (ng/ml) | 62 ± 15 | 53 ± 12 | 41 ± 13 | <0.001**

Table 4.2 Platelet markers in cases and controls.
PMP = Platelet microparticles. PMP/ml, %CD62P+ve, and %CD63+ve are expressed as median (IQR). sP-selectin expressed as mean±SD. Overall P value by ANOVA. *P<0.05 (Tukey’s post-hoc test) between all three groups. **P<0.05 between the healthy controls and both patient groups but not between patient groups. ***P<0.05 between healthy controls and critical limb ischaemia.

Unsurprisingly, all platelet indices showed moderate correlations with each other (Table 4.3). On a posthoc subgroup analysis, there were no statistically significant differences in any platelet index in either patient group according to the use of aspirin (data not shown).

<table>
<thead>
<tr>
<th>PMPs</th>
<th>SP-selectin</th>
<th>% CD62 +ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD63 +ve</td>
<td>0.434</td>
<td>0.411</td>
</tr>
<tr>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>% CD62 +ve</td>
<td>0.337</td>
<td>0.307</td>
</tr>
<tr>
<td>&lt;0.001</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>sP-selectin</td>
<td>0.345</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 4.3 Correlations between platelet markers
The top and bottom numbers are correlation coefficient (r) and P value respectively. PMPs = platelet microparticles. % CD62 +ve and CD63 +ve = percentage of platelets expressing the particular antigen.
4.4 Discussion

The exact cause(s) of platelet activation in atherosclerotic disease is unclear, but can result in the release of procoagulant PMPs (Nomura S 2001; Tsiara S et al. 2003), which are active in promoting thrombosis and adversely influencing monocyte and endothelial function (Barry OP et al. 1998; Berckmans RJ et al. 2001; Boulanger CM et al. 2001; Nomura S et al. 2001).

Apart from PMPs, other estimators of platelet activation include the cell surface expression of CD62P and CD63, and the appearance of sP-selectin in plasma (Tsiara S et al. 2003; Zeiger F et al. 2000). Broadly speaking, the moderate correlations (correlation coefficient 0.3 to 0.5) between the indices of platelet activation are to be expected, although the strongest was between the two membrane markers.

Others have demonstrated increased numbers of PMPs in PAD (Zeiger F et al. 2000), and it has been shown that the degree of platelet activation, as defined by CD62P positivity, can be related to the atherosclerotic disease burden, as measured by intima-media thickness (Koyama H et al. 2003). Therefore, the graded relationship of PMPs to the clinical severity of PAD that I found probably reflects the increasing degree of platelet activation with disease severity. Indeed, this is supported by the correlations between PMP levels and the other markers of platelet activation. The correlation between sP-selectin and PMPs may be important epidemiologically as the former is established as a predictor of adverse cardiovascular events (Ridker PM, Buring JE, & Rifai N 2001). However, it is too early to say whether or not the measurement of plasma PMP levels is a better predictor of disease progression than sP-selectin.
Whatever the cause of platelet activation, the increased levels of PMPs in atherosclerosis is likely to have a significant role in the pathophysiology of the disease. The addition of PMPs to endothelial cells can promote the production of TXA\textsubscript{2}, which causes vasoconstriction and platelet activation (Pfister SL 2004). In addition, microparticles from patients with myocardial infarction can impair the endothelial production of nitric oxide, thus promoting endothelial dysfunction (Boulanger CM et al. 2001). Apart from that, in vitro stimulation of human umbilical vein endothelial cells by PMPs increased ICAM-1, IL-1, and IL-6 expression as well as promoting monocyte adhesion to the endothelial cells (Barry OP et al. 1998). These effects may promote vascular wall inflammation and the growth of the atheromatous plaque. Apart from their effects on the pathogenesis of the atherosclerotic plaque, PMPs are procoagulant (Siljander P, Carpen O, & Lassila R 1996; Tans G et al. 1991). Therefore, it is possible that PMPs can facilitate arterial thrombosis, thus resulting in the acute presentation of atherosclerotic disease, such as acute limb ischaemia and myocardial infarction. Finally, it should be noted that one of the mechanisms of action of anti-platelet and anti-lipid agents may be to reduce the number of circulating PMPs (Kagawa H et al. 1999; Kagawa H et al. 2001). Therefore, it is possible that future anti-platelet agents may be developed specifically to target the formation of PMPs.

This study is limited by its cross-sectional design, and can show associations rather than imply causality. In addition, the presence of confounding factors cannot be ruled out completely. For example, total cholesterol was lowest in patients with CLI, possibly because of a more rigorous control of lipid risk factors in these patients; although a more likely explanation for this finding may be the fact that cholesterol is often much lower in patients who are unwell.
In conclusion, there is a graded increase in PMP levels with the symptomatic severity of PAD. It is uncertain if this relationship is a cause or effect of atherosclerosis. However, this finding is likely to have clinical implications as PMPs have the potential to influence the progression of atheroma as well as promote thrombosis.
Chapter 5. Platelet Microparticles and Coronary Heart Disease

5.1 Introduction

Thrombosis and inflammation at the site of atheromatous plaque formation are key mechanisms in the pathogenesis of coronary artery disease. As platelet activation may represent an important mediator of both of these processes, accurate and reliable estimation of this activation is desirable. However, defining the role of platelet activation is complex, and several different methods of assessment, such as the quantification of platelet microparticles (PMPs) are available (Schmitz G et al. 1998; Tsiara S et al. 2003). PMPs are defined as membrane vesicles that are less than 1 μm in diameter and show positive binding to monoclonal antibodies directed against antigens present on the platelet surface membrane, such as CD42b and CD61. With the ability to bind coagulation factors Va and VIIIa, PMPs not only reflect platelet activation but may also contribute to the activation of the coagulation pathway and hence thrombogenesis (Nomura S 2001; Tans G et al. 1991). Increased numbers of PMPs have been described in atherosclerotic diseases, such as peripheral artery disease and the acute coronary syndromes, and it is thus possible that PMPs may be a useful surrogate marker of the pro-thrombotic state found in these conditions (Singh N et al. 1995; Zeiger F et al. 2000).

There is currently little data relating levels of PMPs with the imaging findings of stable chronic coronary artery disease (CAD). I therefore hypothesised that elevated PMPs in stable CAD are related to the angiographically-measured degree of coronary atheroma and stenosis, and to levels of systemic inflammation (as defined by high
sensitivity C-reactive protein (hsCRP)). I also compared PMPs to alternative markers of platelet activation, such as CD62P, CD63 and sP-selectin.

5.2 Methods

Patients attending the Day Case Unit at City Hospital, Birmingham for elective diagnostic coronary angiography or percutaneous coronary angioplasty were invited to participate in this study. Ethical approval for patient recruitment was obtained from the Ethics Committee of the West Birmingham Health Authority and written informed consent from each subject was obtained in accordance with the Declaration of Helsinki. Exclusion criteria for the study include refusal of consent, chronic renal failure, the presence of co-existent inflammatory disease (e.g. rheumatoid arthritis), a medical history of past or current cancer, the presence of active infection, recent (within 6 months) acute coronary syndrome, previous coronary artery stenting/bypass, current heparin therapy and heart failure of at least NYHA Grade II severity.

Angiographic severity of coronary disease was defined independently by two operators and graded by two indices: a coronary atheroma score (CAS) and a coronary stenosis score (CSS), according to criteria of the Coronary Artery Surgery Study (CASS) (Gersh BJ et al. 1983). Blood samples were obtained from age- and sex-matched healthy controls recruited from healthy hospital staff, spouses, friends and relatives of patients, and among patients attending for routine hernia repair or cataract extraction procedures. A detailed clinical history was obtained from all subjects, along with their blood pressure. The baseline characteristics of my study subjects are shown in table 5.1 below.
Flow cytometry and ELISA

The flow cytometric and ELISA-based techniques were performed as described in Chapter 4 (see page 60). Intra-assay and inter-assay coefficients of variation were <5% and <10% respectively.

Laboratory analyses

Lipoproteins, hsCRP, HbA1c, platelet count and glucose were estimated by the routine service of the Hospital Pathology Laboratory.

Power calculation and data analysis

I hypothesised an increase in PMPs in CAD compared to healthy controls of half of a standard deviation in logged data. For $p<0.05$ at $1-\beta = 0.8$, a minimum of 32 subjects per group are required. I also hypothesised that PMPs would correlate with the angiographic scores with a correlation coefficient of $\geq 0.4$. To achieve this at $p<0.05$ and $1-\beta = 0.85$, 53 data points were required (Machin D & Campbell M 1987). Continuous data were subjected to the Anderson-Darling test to determine their distribution. Non-normally distributed data, presented as median and inter-quartile range (IQR), were analysed by the Mann-Whitney U test. Normally distributed data are presented as mean and standard deviation (SD) and analysed by the Student t test. Categorical data were analysed by the $\chi^2$ test. A multivariate regression analysis was performed to dissect possible influences on levels of PMPs. Data were correlated using Spearman’s rank method. The cross-sectional power calculation and all analyses were performed using Minitab 13 (Minitab Inc, State College, PA, USA).
5.3 Results

Numerous indices were abnormal in the CAD patients compared with the age and gender matched controls (Table 5.2). The patients (of whom 21 had diabetes) had higher systolic blood pressure, hsCRP, glucose and HbA\textsubscript{1c}, but lower total cholesterol (due to concomitant lipid-lowering therapy) and HDL-cholesterol.

<table>
<thead>
<tr>
<th>Clinical, routine laboratory and demographic data</th>
<th>Patients (n=54)</th>
<th>Controls (n=35)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62 ± 8</td>
<td>65 ± 14</td>
<td>0.261</td>
</tr>
<tr>
<td>Males (%)</td>
<td>53.7</td>
<td>40.0</td>
<td>0.206</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.8 ± 1.00</td>
<td>5.5 ± 0.9</td>
<td>0.002</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.3 (1.1-1.6)</td>
<td>1.5 (1.4-1.9)</td>
<td>0.002</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>137 ± 23</td>
<td>129 ± 12</td>
<td>0.037</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>76 ± 14</td>
<td>76 ± 11</td>
<td>0.943</td>
</tr>
<tr>
<td>Smokers (yes/no)</td>
<td>8/46</td>
<td>7/28</td>
<td>0.523</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.8 (4.7-10.5)</td>
<td>4.4 (3.9-4.8)</td>
<td>0.007</td>
</tr>
<tr>
<td>HbA\textsubscript{1c} (%)</td>
<td>7.4 ± 2.1</td>
<td>5.4 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>3.55 (1.48-7.64)</td>
<td>1.74 (0.67-3.33)</td>
<td>0.0167</td>
</tr>
<tr>
<td>CSS</td>
<td>0.465 (0.035-1.250)</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>CAS</td>
<td>0.515 (0.100-1.151)</td>
<td>---------------</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 Subject baseline characteristics
CSS = Coronary Stenosis Score, CAS = Coronary Atheroma Score. Normally distributed data are expressed as mean ± standard deviation and analysed with Student t-test. Non-normally distributed data are expressed as median (IQR) and analysed with Mann-Whitney U-test. Categorical data analysed by chi-squared testing.
<table>
<thead>
<tr>
<th>Platelet indices</th>
<th>Patients (n=54)</th>
<th>Controls (n=35)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Count (X10^9/l)</td>
<td>275 ± 96</td>
<td>267 ± 70</td>
<td>0.655</td>
</tr>
<tr>
<td>sP-selectin (ng/ml)</td>
<td>61 ± 13</td>
<td>40 ± 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD62+ve (%)</td>
<td>8.4 (2.5-14.6)</td>
<td>2.8 (1.7-5.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>CD63+ve (%)</td>
<td>11.5 (6.4-17.4)</td>
<td>7.5 (4.3-11.5)</td>
<td>0.0088</td>
</tr>
<tr>
<td>PMP (X10^5/ml)</td>
<td>2.80 (1.52-6.65)</td>
<td>1.42 (0.82-2.94)</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

Table 5.2 Comparison of patients with coronary artery disease and healthy controls

Normally distributed data are expressed as mean ± standard deviation and analysed with Student’s t-test. Non-normally distributed data are expressed as median (IQR) and analysed with Mann-Whitney U-test.

Significant Spearman correlations (r>0.4) in the 54 CAD patients were found associating hsCRP with sP-selectin (r=0.45, p=0.002) and CD62P positivity with CD63 positivity (r=0.44, p=0.001). PMP levels did not correlate with either CAS (r=0.066, p=0.64) or CSS (r=0.115, p=0.408). CD62P positivity did not correlate with CAS (r=-0.199, p=0.167) or CSS (r=-0.151, p=0.295). CD63 positivity also did not correlate with CAS (r=-0.153, p=0.289) or CSS (r=-0.262, p=0.066).

PMPs, CD62P positivity and CD63 positivity did not correlate with hsCRP, with correlation coefficients of 0.195 (p=0.190), -0.031 (p=0.845) and -0.081 (p=0.608) respectively. Despite adequate power, there were no significant associations between PMPs and any other clinical, demographic, routine laboratory or research index. In multivariate analysis of all the conventional population risk factors for atherosclerosis (age, gender, smoking, diabetes, systolic and diastolic blood pressure, total and HDL-cholesterol and triglycerides), only diabetes (P=0.028) was (weakly) independently associated with (log) PMPs, accounting for 8.7% of the variance. Together, all the risk factors only accounted for 29.2% of the observed variance in PMPs.
5.4 Discussion

In this study we have demonstrated, as in acute myocardial infarction and unstable angina (Gawaz M et al. 1996; Singh N et al. 1995), that PMPs are elevated in stable CAD. It is likely that elevated levels of PMPs in CAD can be linked to the generalised increase in platelet activation found in atherosclerotic disease (Lee YJ, Jy W, & Horstman L 1993; Zeiger F et al. 2000). Indeed, patients with CAD show increases in other markers of platelet activation when compared to healthy controls.

There is ample evidence that PMPs generated from activated platelets can have a variety of physiological and pathophysiological effects. PMPs are known to express tissue factor, which is responsible for the initiation of the extrinsic coagulation pathway. In addition, the PMP may act as a catalyst for the coagulation cascade by binding the various coagulation factors (Tans G et al. 1991). It is possible, therefore, that the finding of elevated PMPs in stable CAD patients may account, at least in part, for the pro-thrombotic state in this group of patients.
Chapter 6. Type II Diabetes, Atherosclerosis and Platelet Microparticles

6.1 Introduction

Type 2 Diabetes is an increasingly common problem in the developed world, a finding that is often attributed to the epidemic of obesity currently being experienced in western countries. In a landmark paper in 1927, Joslin found an increase in death due to atherosclerotic disease in patients with diabetes (Joslin EP 1927). Subsequently, it was found that this risk is particularly high in patients with ‘insulin resistant diabetes’, a group now classified as suffering from Type 2 Diabetes. This group of patients often has associated abnormalities such as dyslipidaemia, central obesity and hypertension, a constellation of clinical features often referred to as ‘Syndrome X’ (Reaven GM 1988).

Patients with Syndrome X are at great risk of developing atherosclerosis and thrombosis (Carr ME 2001). Indeed, the ‘fatal attraction’ in patients with Type 2 Diabetes of the risk of atherosclerosis to the hypercoagulable state means that 80% of all deaths from Type 2 Diabetes can be attributed to the thrombotic complications of atherosclerosis (Calles-Escandon J et al. 1999). This predilection for atherosclerosis and thrombosis in patients with Type 2 Diabetes is multifactorial. For example, abdominal fat cells, which are increased in central obesity, secrete IL-6 and TNF-α, factors which promote vessel wall inflammation (Byrne CD 1999). Increases in plasma glucose, insulin, and free fatty acids can have direct effects in promoting endothelial dysfunction, a phenomenon known to be atherogenic (Byrne CD 1999). In addition to their effects on atherogenesis, Type 2 Diabetes and insulin resistance are associated with a hypercoagulable state, with abnormalities in coagulation, vWF,
PAI-1 and fibrinogen levels (Gray RP, Patterson DL, & Yudkin JS 1993; Juhan-Vague I et al. 1993; Schmidt MI et al. 1999). In addition, there is increased platelet activity in these patients (Mandal S et al. 1993). Studies have also shown that there is an increase in the reactivity of platelets, as measured by PMP release, to stimulation by the calcium ionophore A23187 in patients with Type 2 Diabetes (Nomura S et al. 1995).

Platelet microparticles (PMPs) are defined as vesicles measuring less than 1 μm in diameter which show positive binding to antibodies directed against platelet surface antigens, such as Glycoprotein IIb/IIIa (CD41), or Glycoprotein Ib (CD42b). PMPs are thrombogenic, pro-inflammatory, and may be involved in angiogenesis. It has also been suggested that PMPs may contribute to atherogenesis in diabetic patients, although this has never been shown in vivo (Nomura S et al. 1995). If this hypothesis is found to be true, it is likely to be of great clinical interest as treatment with drugs such as ticlopidine (an ADP antagonist) can reduce the levels of PMPs in patients with Type 2 Diabetes (Nomura S et al. 2004a).

Apart from releasing PMPs, the activated platelet also expresses CD40L on its surface (Henn V et al. 1998). CD40L is a member of the TNF family and has potent pro-inflammatory properties. It therefore provides a link between thrombosis and inflammation, which may be another mechanism by which platelets influence atherosclerotic disease progression. Indeed, it has been shown that sCD40L levels are increased in patients with Type 2 Diabetes and can be reduced by intensive cardiovascular risk management (Lim HS, Blann AD, & Lip GY 2004). However, little is known about the association (if any) between sCD40L and PMPs.
The present cross-sectional study tests the hypothesis that PMPs are higher in patients with Type 2 Diabetes who also suffer from symptomatic atherosclerotic disease, when compared to patients with 'uncomplicated' Type 2 Diabetes. In addition, I hypothesised that PMP levels can be correlated with plasma levels of sCD40L as well as the various markers of platelet activation, including plasma sP-selectin levels and the platelet surface expression of CD62P and CD63.

6.2 Methods

Patients with Type 2 Diabetes were invited to participate in the study. The patients were divided into those who had clinically apparent atherosclerotic disease (e.g. stroke, ischaemic heart disease, and peripheral arterial disease; n=18) and those who did not (n=21). Exclusion criteria for the study include renal failure, the presence of co-existent inflammatory disease (e.g. rheumatoid arthritis), a medical history of cancer, the presence of active infection and heart failure of at least NYHA Grade II severity. None of the patients were on heparin. The duration of illness was not determined as Type 2 Diabetes can remain undetected for many years. The patients were compared to age- and sex-matched healthy controls, recruited from volunteers who were not taking any regular medication and had no clinical evidence of vascular, metabolic, neoplastic or inflammatory disease by careful clinical assessment and routine laboratory tests. Ethical approval for patient recruitment was obtained from the Ethics Committee of the West Birmingham Health Authority and written informed consent from each subject was obtained in accordance with the Declaration of Helsinki.

20 millilitres of blood was collected from each subject for flow cytometric and ELISA studies. Blood was also sent to the routine pathology laboratory for the determination
of lipid profile, fasting glucose, full blood count, glycated haemoglobin and biochemistry screen. Urinary albumin/creatinine excretion ratio and intima-media thickness was also quantified. The baseline characteristics of the patients are shown in Table 6.1.

**Flow cytometry**

The flow cytometric analysis of collected blood was carried out as described in Chapter 4 (see page 60).

**ELISA**

sP-selectin and sCD40L were determined by ELISA-based methods, from reagents obtained from R&D (Abingdon, United Kingdom). The intra- and inter-assay variations were <5% and <10% respectively.

**Power Calculation**

I am unaware of previous data comparing differences in PMPs levels in patients with Type 2 Diabetes with and without symptomatic cardiovascular disease. Thus, I based my power calculations on a study that compared PMP release from platelets derived from patients with diabetes and those from healthy controls (Nomura S et al. 1995), and hypothesised that the changes will be of similar magnitude. Therefore, for a difference of 1.1 SD, I needed a minimum of 18 individuals in each group to achieve a P value <0.05 and 1-β>0.8.

**Statistical Analyses**

Continuous data were analysed using the Anderson-Darling test to determine data distribution. All the markers of interest were not normally distributed, and thus, these
variables were logarithmically transformed for analysis. The difference in the means and SDs of these log-transformed data indicated that sufficient power was present to test the hypotheses in question. Parametric data are presented as mean ± standard deviation and analysed by ANOVA (3 groups) or Student unpaired t test (2 groups). Non-parametric data are expressed as median (interquartile range) and analysed using Kruskal-Wallis followed by Tukey's analysis after log-transformation. The correlations between platelet data and the clinical variables were determined by obtaining the Spearman correlation coefficient. All statistical analyses were done using Minitab (Minitab Inc, State College, PA).

6.3 Results

Platelet microparticles and markers of platelet activation

PMPs were lowest in controls and highest in patients with Type 2 Diabetes and clinically apparent atherosclerosis (Table 6.2). Surface markers of platelet activation, as measured by CD62P and CD63 positivities, were not significantly different between the three groups. Plasma levels of sP-selectin were identical between controls and patients with uncomplicated Type 2 Diabetes; however, both of these groups had significantly lower levels of sP-selectin when compared to patients with Type 2 Diabetes who also had clinically apparent atherosclerosis. Healthy controls had significantly lower sCD40L levels when compared to patients with Type 2 Diabetes; however, there were no differences in sCD40L levels between the two subgroups of patients with Type 2 diabetes.
### Table 6.1 Baseline Characteristics of Subjects.

SBP Systolic blood pressure; DBP Diastolic blood pressure; HbA1c Glycated haemoglobin; Trigs Triglyceride; HDL High Density Lipoprotein; BMI Body mass index; Alb/ Crea Ratio Albumin/Creatinine Excretion Ratio; IMT Intima-medial thickness.

Parametric data are presented as mean ± standard deviation and analysed by ANOVA (3 groups) or Student's unpaired t test (2 groups). Non-parametric data are expressed as median (interquartile range) and analysed using Kruskal-Wallis followed by Tukey's analysis after log-transformation. *There were no differences between the two groups of patients with diabetes.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=21)</th>
<th>Diabetes (n=21)</th>
<th>Diabetes with Atherosclerosis (n=18)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>67 ± 13</td>
<td>68 ± 6</td>
<td>71 ± 6</td>
<td>0.335</td>
</tr>
<tr>
<td><strong>Males (%)</strong></td>
<td>38.1</td>
<td>61.9</td>
<td>61.1</td>
<td>0.221</td>
</tr>
<tr>
<td><strong>Smokers (%)</strong></td>
<td>31.3</td>
<td>10</td>
<td>11.1</td>
<td>0.371</td>
</tr>
<tr>
<td><strong>SBP (mmHg)</strong></td>
<td>132 ± 14</td>
<td>136 ± 17</td>
<td>133 ± 16</td>
<td>0.655</td>
</tr>
<tr>
<td><strong>DBP (mmHg)</strong></td>
<td>77 ± 12</td>
<td>76 ± 8</td>
<td>70 ± 7</td>
<td>0.067</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>5.5 (5.2-5.8)</td>
<td>6.6 (6.0-7.4)</td>
<td>7.2 (6.8-7.8)</td>
<td>0.03*</td>
</tr>
<tr>
<td><strong>Cholesterol (mmol/l)</strong></td>
<td>5.4 ± 0.9</td>
<td>4.6 ± 0.9</td>
<td>4.0 ± 0.7</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Trigs (mmol/l)</strong></td>
<td>1.4 (1.0-1.8)</td>
<td>1.8 (1.2-2.7)</td>
<td>1.4 (0.7-2.1)</td>
<td>0.191</td>
</tr>
<tr>
<td><strong>HDL (mmol/l)</strong></td>
<td>1.5 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>Platelet Count (X10^9/l)</strong></td>
<td>269 ± 75</td>
<td>307 ± 123</td>
<td>295 ± 104</td>
<td>0.468</td>
</tr>
<tr>
<td><strong>Statin Use (%)</strong></td>
<td>---------------</td>
<td>76.2</td>
<td>88.9</td>
<td>0.303</td>
</tr>
<tr>
<td><strong>Aspirin Use (%)</strong></td>
<td>---------------</td>
<td>42.9</td>
<td>94.4</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>---------------</td>
<td>29.5 ± 3.6</td>
<td>25.1 ± 2.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Hip/Waist Ratio</strong></td>
<td>---------------</td>
<td>1.06 ± 0.05</td>
<td>1.05 ± 0.05</td>
<td>0.574</td>
</tr>
<tr>
<td><strong>Alb/Crea Ratio</strong></td>
<td>---------------</td>
<td>1.00</td>
<td>1.27</td>
<td>0.112</td>
</tr>
<tr>
<td><strong>IMT</strong></td>
<td>1.085</td>
<td>1.142</td>
<td>1.24</td>
<td>0.244</td>
</tr>
</tbody>
</table>
Table 6.2 Platelet indices in diabetes and controls
Data expressed as median(IQR) and analysed using Kruskal-Wallis followed by Tukey’s analysis after log-transformation. * P<0.05 between all three groups. ** P<0.05 between controls vs diabetes with atherosclerosis and diabetes vs diabetes with atherosclerosis only. *** P<0.05 between controls and patients but not between patient groups. PMP platelet microparticles

PMP levels correlated with %CD62P positivity (r=0.356, P=0.026) but not to %CD63 positivity (r=0.276, P=0.089), sCD40L (r=0.141, P=0.407), and sP-selectin (r=0.228, P=0.163). There was a strong correlation between %CD62 and %CD63 positivities (r=0.553, P<0.0001). sCD40L levels did not correlate with any of the markers of platelet activation.

Correlations between the various clinical variables

There were strong inverse correlations between the level of HDL-cholesterol and both CD62P and CD63 positivities (r=-0.399, P=0.012 and r=-0.390, P=0.014 respectively). No other marker of platelet activation correlated with any of the other measured clinical parameters (including HbA1c).
6.4 Discussion

This study provides support to the study by Nomura et al (1995), who found increased ex vivo platelet reactivity to calcium ionophore, as measured by PMP release, in platelets derived from patients with Type 2 Diabetes. Although elevation of plasma PMP levels has been shown to be associated with the presence of Type 2 Diabetes in patients with hyperlipidaemia but not in patients with hypertension (Nomura S et al. 2004b; Nomura S, Kanazawa S, & Fukuhara S 2003), these studies were primarily to study microparticle levels in patients with the latter conditions.

I believe that this study is one of the first in which PMPs have been found to be raised in patients with Type 2 Diabetes, in a study designed specifically for the condition.

My finding differs from that of Diamant et al (Diamant M et al. 2002) who found that PMP levels were similar in controls and patients with Type 2 Diabetes. One possible reason behind this discrepancy may lie in significant differences in sample preparation between Diamant et al and this study. For example, Diamant et al detected PMPs by first isolating the PMPs by sequential centrifugation before resuspending the pellet prior to flow cytometry; In contrast, I detected PMPs by direct flow cytometry of platelet poor plasma. At the present time, I would emphasise that there is no consensus on the best method for the flow cytometric detection of PMPs. Another possible explanation for this discrepancy in results may be the age difference between the patients in the two studies - the patients in the study by Diamant et al had a mean age of 54 years, while patients in my study were older, with a mean age of 68 years.

Another study by Sabatier et al also found that there were no differences in PMP levels between healthy controls and patients with Type 2 Diabetes (Sabatier F et al. 2002). They utilised a method whereby blood was collected into a citrated Vacutainer, spun down within two hours, and frozen until subsequent flow cytometric analysis. It
is possible their use of citrate as the anticoagulant and the delay in blood processing may result in erroneous measurement, as illustrated in the paper by Kim et al (Kim HK et al. 2002). Since the completion of my study, a paper has been published showing an association between raised PMP levels with diabetes (Nomura S et al. 2004a)

The current study confirms the hypothesis that the presence of symptomatic atherosclerosis is associated with higher plasma levels of PMPs in patients with Type 2 Diabetes. Interestingly, there were no differences between the IMT measurements of both groups of diabetic patients, suggesting that there were no differences in the atherosclerotic ‘loads’ of both groups of patients. As the measurement of IMT represents a well established index of atherosclerosis (Wendelhag I, Wiklund O, & Wikstrand J 1993), the difference in PMP levels between the two groups of patients with Type 2 Diabetes does not appear to be simply due to differences in atherosclerotic load. As IMT is not a measure of plaque instability, it is possible that the increase in PMPs may reflect the presence of an unstable plaque. Therefore, even though the 2 groups of patients had similar atherosclerotic loads, patients with raised PMPs may be at a higher risk of developing adverse cardiovascular events. The possibility arises that PMPs may be useful as a risk stratification tool in patients with Type 2 Diabetes.

The finding of a raised sP-selectin level in patients with both Type 2 Diabetes and symptomatic atherosclerosis provides support for data presented in Chapter 4 of this thesis, which showed an association between raised sP-selectin levels and the presence of symptomatic atherosclerotic disease. Indeed, my study provides confirmatory evidence that a raised sP-selectin level is associated with an increased
risk of developing adverse cardiovascular events (Ridker PM, Buring JE, & Rifai N
2001).

Interestingly, CD62P and CD63 were both inversely correlated with HDL levels. It is
well established that a low HDL level is associated with an increased risk of
developing adverse cardiovascular events (Gaziano JM et al. 1997; Huttunen JK et al.
1991). It has also been known for more than a decade that HDL may inhibit platelet
activation by promoting platelet nitric oxide synthase activity. Therefore, it is possible
that the cardioprotective effect of HDL and treatments aimed at raising HDL levels,
such as gemfibrozil, may in part be modulated by effects on platelet activation.

My results are in concordance with previous work (Lim HS, Blann AD, & Lip GY
2004), which showed that although patients with diabetes have much higher levels of
sCD40L than controls, the additional presence of symptomatic atherosclerosis does
not appear to further increase sCD40L levels. There was no correlation between
sCD40L and PMP levels, but this is perhaps not surprising given the lack of
correlation between sCD40L and the markers of platelet activation in patients with
Type 2 Diabetes (Lim HS, Blann AD, & Lip GY 2004).

Another area which merits further study is on whether pharmacological interventions
aimed at reducing PMPs would be of benefit in Type 2 Diabetes. The use of aspirin as
an antiplatelet agent to prevent atherothrombosis in patients with Type 2 Diabetes
with presumed/known atherosclerotic disease is now well established. The long-term
administration of antiplatelet agents to patients with diabetes may also attenuate the
progression of atheroma (Cocozza M et al. 1995; Kodama M et al. 2000). As certain
antiplatelet agents, such as ticlopidine, can significantly reduce the levels of PMPs in
diabetes (Nomura S et al. 2004a), it is possible that the beneficial effect of antiplatelet agents in diabetes may be due in part to a reduction in PMP levels. In addition, as some of the biological effects of PMPs are mediated by their production of thromboxane, it is possible that the beneficial effects of aspirin on cardiovascular morbidity and mortality in patients with atherosclerosis may in part be mediated by its effect on PMP prostaglandin synthesis (Pfister SL 2004). However, these hypotheses will need to be confirmed by further studies.
Chapter 7. Peripheral Arterial Disease, Angioplasty and CD40L

7.1 Introduction

CD40L is a member of the tumour necrosis factor family that is predominantly found in leukocytes and platelets (Henn V et al. 1998). The molecule is expressed on the platelet surface when the cell is activated and mediates various pro-thrombotic and pro-inflammatory reactions by binding to CD40 and GPIIb/IIIa (Schonbeck U & Libby P 2001; Tan KT & Lip GY 2003a). The cell surface expression of CD40L is followed within minutes by cleavage of the molecule, resulting in the formation of sCD40L (Henn V et al. 2001). sCD40L has structurally intact receptor binding sites, thus allowing it to bind to CD40 and GPIIb/IIIa (Henn V et al. 2001). Increased levels of sCD40L have been found in many atherosclerotic diseases, including the acute coronary syndromes (Aukrust P et al. 1999) and peripheral artery disease (Tsakiris DA et al. 2000). sCD40L has also been found to be increased by percutaneous coronary angioplasty (Cipollone et al. 2003; Aggarwal A et al. 2004). However, there are no data on the acute effects of peripheral artery angioplasty (Figure 7.1) on sCD40L levels. In addition, it is uncertain if sCD40L levels are affected by the clinical severity of peripheral artery disease. Furthermore, sCD40L has not been rigorously compared to other markers of platelet function.
I therefore hypothesized that: (a) sCD40L can be related to the clinical severity of PAD, and (b) sCD40L levels are rapidly increased by angioplasty of the lower limb vessels. In addition, I compared sCD40L with established markers of platelet activation. The hypotheses were tested in cross-sectional and interventional studies of patients with stable PAD.

7.2 Methods

Subjects

Patients attending the vascular outpatient clinic, the vascular inpatient facility as well as the interventional radiology unit at City Hospital, Birmingham were invited to
participate in the study. The inclusion criteria for these patients include a clinical history suggestive of the disease and an ankle-brachial pressure index (ABPI) measurement of less than 0.8. Patients were further classified by the presence of rest pain and, if present, were defined as having critical limb ischaemia. All patients with critical limb ischaemia subsequently underwent peripheral angiography as part of their routine clinical care. Exclusion criteria for the study include refusal of consent, renal failure, the presence of co-existent inflammatory disease (e.g. rheumatoid arthritis), a history of cancer, the presence of active infection, and heart failure of at least New York Heart Association (NYHA) Grade II severity. None of the patients were on heparin therapy when blood was first collected. Age- and sex-matched healthy controls were recruited from hospital staff, visitors and patients admitted for elective cataract and hernia operations. The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki.

Written evidence of informed consent was obtained from each participant. A full clinical history was taken from each subject. The baseline characteristics of the subjects are shown in table 7.1. A total of 11 patients with critical ischaemia later underwent peripheral balloon angioplasty. All of these patients were on heparin therapy prior to peripheral vascular catheterisation. Blood samples were taken immediately prior to peripheral balloon angioplasty, but after the administration of 3000-5000 units of heparin, and 10 minutes post angioplasty.

**Plasma markers**

sP-selectin and sCD40L were estimated in citrated plasma by ELISA (R&D Systems, Abingdon, United Kingdom). Intra- and inter-assay coefficients of variation were <5% and <10% respectively.
Flow cytometry

Platelet flow cytometry was carried out as described in Chapter 4 (see page 60).

Power calculation and statistical analysis

Previous work has reported median sCD40L levels of 30 pg/ml, 520 pg/ml and 700 pg/ml in 39 healthy controls, 56 patients with diabetes but free of overt cardiovascular disease, and 41 patients with diabetes with symptomatic macrovascular disease respectively (p<0.001) (Lim HS, Blann AD, & Lip GY 2004). I hypothesised a doubling of sCD40L in intermittent claudication and a four-fold increase in critical limb ischaemia. For the same degree of power (i.e. overall p<0.001, between groups alpha = p<0.05 and 1-beta = 0.8), I needed a minimum of 30 subjects per group.

Continuous data were subjected to the Anderson-Darling test to determine their distribution. Non-normally distributed data, presented as median and inter-quartile range (IQR), were analysed by the Kruskal-Wallis test. Differences between normally distributed data (presented as mean and standard deviation (SD)) were sought by analysis of variance. Differences between groups were sought by Tukey’s post-hoc test (after log transformation, if necessary). Categorical data were analysed by the χ² test. Wilcoxon Signed Ranked test was utilised to compare pre- and post-angioplasty changes. Correlations were determined using the Spearman rank method. Data were analysed by Minitab release 13 (Minitab Inc, State College, PA, USA).

7.3 Results

Baseline Characteristics

The three groups (healthy controls, and patients with intermittent claudication or critical limb ischaemia) were matched for age, sex, smoking and platelet count (Table
7.1). A history of stroke, ischaemic heart disease or Type 2 Diabetes was present in 5 (15%), 13 (39%) and 13 (39%) patients with intermittent claudication compared to 4 (13%), 10 (33%) and 9 (30%) of those with critical limb ischaemia. Similarly, prescribed drugs in the intermittent claudication and critical ischaemia groups were (respectively) aspirin 18 (54%) and 22 (73%); clopidogrel 3 (9%) and zero; beta blockers 7 (21%) and 5 (17%); calcium channel blockers 14 (42%) and 9 (30%); nitrates 5 (15%) and 3 (10%); diuretics 10 (33%) and 9 (30%); angiotensin converting enzyme inhibitors 8 (24%) and 7 (23%); angiotensin receptor blockers 7 (21%) and 2 (7%); statins 21 (64%) and 20 (67%) (no significant differences).

<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls (n=36)</th>
<th>Intermittent Claudication (n=33)</th>
<th>Critical Limb Ischaemia (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66 ± 14</td>
<td>65 ± 9</td>
<td>69 ± 11</td>
<td>0.852</td>
</tr>
<tr>
<td>Males (%)</td>
<td>45</td>
<td>61</td>
<td>63</td>
<td>0.237</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>130 ± 12</td>
<td>145 ± 17</td>
<td>144 ± 35</td>
<td>0.014a</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76 ± 11</td>
<td>81 ± 6</td>
<td>71 ± 11</td>
<td>0.001b</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>19</td>
<td>33</td>
<td>37</td>
<td>0.124</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.6 ± 0.9</td>
<td>5.6 ± 0.8</td>
<td>6.6 ± 2.0</td>
<td>0.02c</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.5 ± 0.4</td>
<td>6.3 ± 1.4</td>
<td>6.1 ± 1.4</td>
<td>0.026d</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.4 ± 0.9</td>
<td>5.1 ± 1.4</td>
<td>4.4 ± 1.0</td>
<td>0.007e</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.5 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>1.3 ± 0.3</td>
<td>0.002f</td>
</tr>
<tr>
<td>Platelet Count</td>
<td>264 ± 70</td>
<td>277 ± 79</td>
<td>306 ± 148</td>
<td>0.280</td>
</tr>
<tr>
<td>White Cell Count</td>
<td>7.0 ± 1.7</td>
<td>8.4 ± 2.1</td>
<td>8.6 ± 3.8</td>
<td>0.048</td>
</tr>
</tbody>
</table>

Table 7.1 Demographic and risk factor characteristics of the subjects
Data are presented as percentage, mean and standard deviation or median and interquartile range. P value by chi-squared test, analysis of variance or the Kruskal-Wallis test. Sub-analyses by Tukey's post-hoc test. P <0.05 is regarded as significant. a difference between both disease groups and controls, not between disease groups, b between two disease groups, no difference to controls, c healthy controls versus critical limb ischaemia only, d healthy controls versus intermittent claudication.
Research indices

Plasma sCD40L levels were higher in patients with critical limb ischaemia and intermittent claudication when compared to healthy age-sex matched controls (Table 7.2) but there were no significant differences between levels in the patient groups. The percentage of platelets expressing CD62P and levels of sP-selectin followed the same pattern. The percentage of CD63 positivity was significantly different between controls and patients with critical limb ischaemia, but not with intermittent claudication.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=36)</th>
<th>Intermittent Claudication (n=33)</th>
<th>Critical Limb Ischaemia (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>%CD62P</td>
<td>2.6 (1.5-6.5)</td>
<td>8.5 (1.6-14.6)</td>
<td>5.8 (3.6-14.0)</td>
<td>0.002a</td>
</tr>
<tr>
<td>%CD63</td>
<td>6.9 (6.1-12.0)</td>
<td>10.0 (4.9-13.1)</td>
<td>12.2 (7.9-21.4)</td>
<td>0.031b</td>
</tr>
<tr>
<td>sP-selectin (ng/ml)</td>
<td>48 (41-60)</td>
<td>68 (50-76)</td>
<td>69 (64-82)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>sCD40L (pg/ml)</td>
<td>35 (28-55)</td>
<td>68 (28-333)</td>
<td>64 (34-282)</td>
<td>0.009a</td>
</tr>
</tbody>
</table>

Table 7.2 Platelet markers in the three groups
Data are presented as percentage, mean and standard deviation or median and inter-quartile range. P value by chi-squared test, analysis of variance or the Kruskal-Wallis test. Sub-analyses by Tukey’s post-hoc test. P < 0.05 is regarded as significant. *difference between both disease groups and controls, not between disease groups, **healthy controls versus critical limb ischaemia only

Spearman Correlations

Relationships between the platelet markers are presented in table 7.3. sP-selectin, %CD62P positivity and %CD63 positivity all correlated with each other. However, sCD40L failed to correlate with any platelet marker. Of the risk factors, only systolic blood pressure consistently correlated with %CD62P positivity (r=0.32, P=0.002), %CD63 positivity (r=0.271, P=0.008), sP-selectin (r=0.5, P<0.001) and sCD40L (r=0.27, P=0.007). %CD62P positive platelets also correlated with diastolic blood.
pressure ($r=0.3$, $P=0.004$), %CD63 positive platelets with HDL-cholesterol ($r=-0.3$, $P=0.005$), and sP selectin with both HbA1c ($r=0.264$, $P=0.014$) and HDL-cholesterol ($r=-0.273$, $P=0.009$). However, many of these relationships with weak correlations are likely to be pathogenetically spurious.

<table>
<thead>
<tr>
<th>%CD62P +ve</th>
<th>% CD63 +ve</th>
<th>sP-selectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD63 +ve</td>
<td>0.525</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>sP-selectin</td>
<td>0.314</td>
<td>0.444</td>
</tr>
<tr>
<td></td>
<td>P=0.002</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>sCD40L</td>
<td>0.053</td>
<td>0.097</td>
</tr>
<tr>
<td></td>
<td>P=0.613</td>
<td>P=0.350</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P=0.111</td>
</tr>
</tbody>
</table>

Table 7.3 Correlations between platelet markers
Data are presented as Spearman correlation coefficient and P value
<table>
<thead>
<tr>
<th></th>
<th>Pre-Angioplasty</th>
<th>Post-Angioplasty</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66 ± 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males (%)</td>
<td>73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>133 ± 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>73 ± 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.6 ± 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.2 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiplatelet Drug Use (%)</td>
<td>83%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma CD40L (pg/ml)</td>
<td>58 (8-189)</td>
<td>80 (28-485)</td>
<td>0.018</td>
</tr>
<tr>
<td>sP-selectin (ng/ml)</td>
<td>61 ± 13</td>
<td>54 ± 10</td>
<td>0.124</td>
</tr>
<tr>
<td>%CD62P Expression</td>
<td>6.7 (4.8-16.8)</td>
<td>7.8 (2.4-19.0)</td>
<td>0.450</td>
</tr>
<tr>
<td>%CD63 Expression</td>
<td>9.4 (8.1-20.5)</td>
<td>9.6 (7.6-15.3)</td>
<td>0.505</td>
</tr>
</tbody>
</table>

Table 7.4 Baseline characteristics and effect of angioplasty on the subgroup of 11 patients who underwent angioplasty.

Data are absolute number, percentage, mean and standard deviation or median and interquartile range.
P values by paired t test or Wilcoxon’s test (for normally and non-normally distributed data respectively).

**Effect of angioplasty**

Details of the 11 patients are presented in table 7.4. There was an increase in sCD40L levels post-angioplasty in 10 of the 11 patients (Figure 7.2). The other indices were unchanged.
Figure 7.2 sCD40L levels pre- and post-angioplasty. Note the increase in CD40L levels in 10 out of the 11 patients.

7.4 Discussion

The results confirm those of previous studies, in which patients with atherosclerosis show increased levels of sCD40L (Tsakiris DA et al. 2000). Indeed, it has been shown that sCD40L levels can be related to increased intraplaque lipids, as determined by Magnetic Resonance Imaging (Blake GJ et al. 2003). As intraplaque lipids may contribute to plaque instability and rupture, it is possible that sCD40L contributes towards a predisposition for the acute presentation of atherosclerotic disease. Apart from that, CD40L is found abundantly in rupture-prone atherosclerotic plaques (Lutgens E & Daemen MJ 2002). Therefore, it is perhaps surprising that sCD40L did
not show a graded increase with the clinical severity of peripheral artery disease. A possible explanation to this observation lies in the fact that the clinical severity of atherosclerotic disease may not correlate with total atherosclerotic load. However, more studies are required to study this paradox.

The primary source of sCD40L is unclear and although it may arise from many cell types (Schonbeck U & Libby P 2001), interest centres on the platelet (Henn V et al. 1998). As excess platelet activation is a feature of atherosclerosis (Tsiara S et al. 2003), then my finding of raised sCD40L is unsurprising. However, despite the hypothesis that the majority of sCD40L is platelet-derived, it failed to correlate with the surface expression of CD62P and CD63 and the plasma level of sP-selectin. These results are consistent with the findings of both Chapter 6 and a previous study (Lim HS, Blann AD, & Lip GY 2004), in which no correlation between sCD40L and sP-selectin could be found; however, Cipollone et al did correlate the two molecules in subjects with hypercholesterolaemia (Cipollone F et al. 2002). My data fails to support the hypothesis that the platelet is the major source of sCD40L and it is likely that other cells may contribute significantly to sCD40L levels. An alternative explanation would be that the pathway for the release of platelet-derived sCD40L differs from those of the more conventional markers of platelet activation.

The current study confirms the hypothesis that peripheral artery angioplasty increases sCD40L levels. Peripheral angioplasty is a traumatic procedure which essentially leads to controlled endothelial disruption. This may lead to the release of CD40L found within the plaque. Indeed, my data is consistent with that of a previous study, which showed that there is an increase in sCD40L ten minutes post coronary angioplasty (Aggarwal et al 2004). In addition, trauma to the vessel wall can lead to
local platelet and leukocyte activation, with consequent release of sCD40L. It is uncertain as to the length of time sCD40L remains elevated post-peripheral angioplasty. However, a previous study showed that sCD40L levels remain elevated for at least one day post-coronary angioplasty, with a return to baseline levels by the fifth day (Cipollone F et al 2003).

The release of sCD40L may have important pathophysiological consequences. sCD40L has been shown to inhibit endothelial cell migration, thereby delaying re-endothelialization of angioplasty-induced vascular damage (Cipollone F et al. 2003). sCD40L can also stimulate the release of various pro-inflammatory molecules, including monocyte chemoattractant protein-1 (MCP-1), TNF-α, and IL-1, by endothelial cells (Cipollone F et al. 2003; Henn V et al. 2001). In addition, sCD40L can induce the expression of various vascular adhesion molecules, such as E-selectin and VCAM-1 (Henn V et al. 2001; Tan KT & Lip GY 2003a). All of these processes can contribute to post-angioplasty restenosis by promoting the formation of neointima.

sCD40L also has important pro-thrombotic effects. As mentioned above, sCD40L can induce tissue factor expression. Apart from that, sCD40L can bind to platelet GPIIb/IIIa, a process which may lead to stabilization of platelet-rich thrombi under conditions of high shear stress (Andre P et al. 2002a). This pro-thrombotic effect can contribute to the dreaded complication of acute vessel closure due to thrombosis post-angioplasty.

It has been proposed that anti-CD40L therapy may be beneficial in atherosclerosis (Lutgens E & Daemen MJ 2002). Inhibition of CD40L activity in rodents by the use
of a monoclonal antibody can promote the formation of a more stable plaque phenotype, with less probability of plaque rupture (Lutgens E & Daemen MJ 2002). A humanised monoclonal antibody directed against CD40L has been developed (Davis JC et al. 2001); however, there are no data on its use in atherosclerotic disease.

Patients undergoing angioplasty are routinely administered a 3000-5000 unit dose of unfractionated heparin to prevent coagulation at the site of angioplasty. Paradoxically, unfractionated heparin has been shown to result in platelet activation, as measured by CD62P and PAC-1 expression (Xiao Z & Theroux P 1998; Aggarwal A, Sobel BE & Schneider DJ 2002; Cella G & Girolami A 1999). The role of heparin in platelet activation is incompletely understood. Unfractionated heparin may reduce thrombin-induced platelet activation (De Candia, De Cristofaro R & Landolfi R 1999). However, it has also been shown to promote the activation of platelets by the synthetic peptide TRAP (amino acid sequence SFLLRNPHDKYEPF) and ADP (Xiao Z & Theroux P 1998). As TRAP stimulates the platelet thrombin receptor, it may be hypothesised that unfractionated heparin has an indirect effect of inhibiting platelet activation by facilitating the deactivation of thrombin whilst directly promoting platelet activation.

A possible confounding factor of the interventional aspect of my study would be the concurrent use of heparin in patients with critical ischaemia undergoing angioplasty. I have tried to minimise this by ensuring that blood was collected only after the administration of heparin. Indeed, the indices of platelet activation such as CD62P, CD63 and P-selectin were not significantly different between pre- and post-angioplasty samples. Therefore, it is likely that the post-angioplasty increase in sCD40L level is directly related to the procedure itself.
In conclusion, the present study confirms that sCD40L is elevated in peripheral artery disease and that angioplasty can result in an acute elevation of sCD40L levels. The exact pathophysiological significance of these findings needs to be explored further in order for them to be translated into therapeutic gain.
8.1 Introduction

Coronary heart disease is the commonest cause of death in many developed countries. As with other atherosclerotic diseases, the commonest pathological change resulting in death or sickness is intra-arterial thrombosis. It is now well established that platelets play a central role in arterial thrombosis (Tan KT & Lip GY 2003b). Thrombus in atherosclerotic blood vessels is composed predominantly of platelet rich ‘white thrombus’. In addition, antiplatelet drugs like aspirin are the mainstay in the prevention and treatment of atherothrombotic conditions such as myocardial infarction and critical ischaemia (Antithrombotic Triallists' Collaboration 2002). The thrombotic function of platelets is largely mediated through GPIIb/IIIa and GPIb/IX/V (Andrews RK et al. 2003). GPIb/IX/V is believed to bind vWF, in a process that can lead to platelet activation (Schmugge M, Rand ML, & Freedman J 2003). On binding to vWF, GPIb/IX/V mediates platelet shape change, the release of platelet granules, and ‘inside-out’ activation of GPIIb/IIIa. Interestingly, GpV can be cleaved by thrombin to release a 69kd fragment, GPVfl (sGPV) (Azorsa D et al. 1999). It has been proposed that this molecule can be used to monitor the platelet activation status in thrombotic disease.

In addition to its role in promoting thrombosis, the platelet is now known to play a major role in vascular cell signalling. For example, activated platelets express CD40L and P-selectin, molecules which are pro-inflammatory and which may promote endothelial dysfunction (Tan KT & Lip GY 2003a). Endothelial dysfunction is
associated with raised plasma vWF levels (Felmeden DC et al. 2003). Indeed, vWF is believed to be a marker of endothelial dysfunction.

The platelet may also play a role in collateralisation and angiogenesis. Platelets are known to release vascular endothelial growth factor (VEGF) upon activation (Verheul HM et al. 1997). In addition, activated platelets release membrane microvesicles, which may mediate angiogenesis through lipid growth factors (Kim HK et al. 2004).

In the present study, I hypothesised that sCD40L, sP-selectin, and sGPV can be related to the angiographic severity of coronary heart disease and the plasma level of vWF. In addition, I determined if the degree of platelet activation can be related to the degree of coronary collateral vessel formation.

8.2 Methods

Patient Recruitment

Ethical approval for patient recruitment was obtained from the Ethics Committee of the West Birmingham Health Authority. Written informed consent from each subject was obtained in accordance with the Declaration of Helsinki. Exclusion criteria for the study include refusal of consent, renal failure, the presence of co-existent inflammatory disease (e.g. rheumatoid arthritis), a medical history of cancer, the presence of active infection, recent (within 6 months) acute coronary syndrome, previous coronary artery stenting/bypass, and heart failure of at least NYHA Grade II severity. None of the patients were on current heparin therapy.
Stable Coronary Heart Disease

Patients attending the Day Case Unit at City Hospital, Birmingham, for elective coronary angiography/angioplasty were invited to participate in this study. A total of 186 patients were recruited for the study. The presence of CAD was defined as the presence of coronary artery plaques, as detected by angiography. The baseline characteristics of the patients are summarised in table 8.1 below.

Healthy Controls

50 age- and sex-matched healthy controls were recruited from healthy volunteers (Table 8.1), as previously described (see page 70).

ELISA

Blood was collected prior to coronary angiography into citrated Vacutainers (BD Biosciences, Oxford, United Kingdom) and centrifuged at 1500g for 20 minutes within one hour of collection. The resulting platelet poor plasma was snap frozen at -70°C immediately after centrifugation and thawed immediately before testing. Plasma levels of vWF (Dako, Ely, United Kingdom), sP-selectin (R&D, Abingdon, United Kingdom), sGPV (Diagnostica Stago, Vesnieres, France), and sCD40L (R&D Systems, Abingdon, United Kingdom) were determined using ELISA-based assays. The intra- and inter-assay variations were <5% and <10% respectively.

Assessment of Coronary Artery Disease And Collateralisation

The severity of coronary artery disease, as measured by coronary artery stenosis score (CSS) and coronary atheroma score (CAS), was determined independently by two cardiologists blinded to the laboratory data, according to the criteria set out by the Coronary Artery Surgery Study (CASS) (Gersh BJ et al. 1983). Assessment of
collateralisation was performed according to the modified Rentrop and TIMI schemes (Tayebjee MH, Lip GY, & MacFadyen MH 2004).

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=186)</th>
<th>Controls (n=50)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.2±9.1</td>
<td>64.1±12.6</td>
<td>0.305</td>
</tr>
<tr>
<td>Men (%)</td>
<td>61</td>
<td>54</td>
<td>0.283</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>137.3±21.0</td>
<td>131.7±12.6</td>
<td>0.023</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>77.1±10.6</td>
<td>75.6±10.1</td>
<td>0.378</td>
</tr>
<tr>
<td>Diabetics mellitus (%)</td>
<td>30.1</td>
<td>0</td>
<td>--------</td>
</tr>
<tr>
<td>Systemic hypertension (%)</td>
<td>60.0</td>
<td>0</td>
<td>--------</td>
</tr>
<tr>
<td>Hypercholesterolaemia (%)</td>
<td>64.5</td>
<td>0</td>
<td>--------</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>11</td>
<td>14</td>
<td>0.530</td>
</tr>
<tr>
<td>Antiplatelet Use (%)</td>
<td>93.5</td>
<td>0</td>
<td>--------</td>
</tr>
<tr>
<td>Beta-Blocker Use (%)</td>
<td>63</td>
<td>0</td>
<td>--------</td>
</tr>
<tr>
<td>Calcium Channel Blocker Use (%)</td>
<td>40</td>
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<td>--------</td>
</tr>
<tr>
<td>Long-acting Nitrate Use (%)</td>
<td>40</td>
<td>0</td>
<td>--------</td>
</tr>
<tr>
<td>Nicorandil Use (%)</td>
<td>17</td>
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<td>--------</td>
</tr>
<tr>
<td>Diuretic Use (%)</td>
<td>21</td>
<td>0</td>
<td>--------</td>
</tr>
<tr>
<td>Angiotensin converting enzyme inhibitor or angiotensin receptor blocker use (%)</td>
<td>38</td>
<td>0</td>
<td>--------</td>
</tr>
<tr>
<td>Statin Use (%)</td>
<td>77</td>
<td>0</td>
<td>--------</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.4 (3.7-5.1)</td>
<td>5.2 (4.5-5.8)</td>
<td>0.0001</td>
</tr>
<tr>
<td>High Density Lipoprotein (mmol/l)</td>
<td>1.2 (1.0-1.4)</td>
<td>1.4 (1.2-1.7)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Platelets (X10⁹/l)</td>
<td>265 (217-319)</td>
<td>238 (197-298)</td>
<td>0.12</td>
</tr>
<tr>
<td>coronary atheroma score</td>
<td>1.00 (0.4-1.80)</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>coronary stenosis score</td>
<td>0.80 (0.21-1.48)</td>
<td>--------</td>
<td>--------</td>
</tr>
</tbody>
</table>

Table 8.1 Baseline characteristics of patients and controls
Normally distributed data are expressed as mean± SD and analysed with Student t-test. Non-normally distributed data are expressed as median (IQR) and analysed with Mann-Whitney U-test. Categorical data were analysed by chi-squared testing.
Statistical Analyses

It was hypothesised that a minimum difference of 0.5 of a standard deviation of log sP-selectin would be observed in a normally-distributed index amongst cases. To achieve this at $2p < 0.025$ and $1-beta = 0.85$, I required data from a minimum of 125 cases (ratio cases to control 3:1). I recruited in excess of this number for subgroup analyses. The Student t-test was used for normally distributed continuous data while the Mann-Whitney U test was used for non-normally distributed data.

Comparisons between the various patients with CAD with different degrees of collateralisation were performed using ANOVA or Kruskal-Wallis, followed by Tukey’s analysis. The Chi-square test was used to test hypotheses amongst categorical data. Correlations were performed using the Spearman rank method. A stepwise multiple regression analysis was performed to ascertain independent clinical predictors for the research parameters. Statistical analysis was performed using the MINITAB Statistical Software package (State College, PA, USA). A $P$ value of $<0.05$ was considered as statistically significant.

8.3 Results

Comparison between patients and controls

The results of the main cross-sectional study are summarized in table 8.2. Plasma levels of sGPV, sP-selectin, vWF, and sCD40L were all higher in patients when compared to healthy controls. Female patients had a slightly higher plasma level of sGPV, when compared to male patients (median of 36.0(IQR 24.8-55.3) ng/ml vs. 31.5(22.0-41.0) ng/ml, $P=0.033$).
Table 8.2 Platelet indices in patients with coronary artery disease and controls.
Normally distributed data are expressed as mean± SD and analysed with Student’s t-test. Non-normally distributed data are expressed as median (IQR) and analysed with Mann-Whitney U-test.

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=186)</th>
<th>Controls (n=50)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sP-selectin (ng/ml)</td>
<td>54±15</td>
<td>42±13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>sCD40L (pg/ml)</td>
<td>184 (51-672)</td>
<td>55 (33-133)</td>
<td>0.0001</td>
</tr>
<tr>
<td>sGPV (ng/ml)</td>
<td>32.0 (23-46)</td>
<td>23.0 (15-30)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>vWF (IU/dl)</td>
<td>159±32</td>
<td>139±41</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Relationship of plasma markers to disease severity

As expected, there was a very strong correlation between CSS and CAS (r=0.863, P<0.0001). There were no significant correlations between CAS and sGPV (r=-0.057, P=0.441), sP-selectin (r=0.048, P=0.517), vWF(r=0.042, P=0.57), or sCD40L levels(r=-0.097, P=0.186). Similarly, CSS did not correlate with sGPV(r=-0.090, P=0.221), sP-selectin (r=0.080, P=0.279), vWF(r=0.053, P=0.474), and sCD40L (r=-0.116, P=0.116). There were no significant correlations between the degree of collateralization and plasma levels of sCD40L, sP-selectin, vWF, and sGPV (Table 8.3). There were also no differences in the plasma levels of sCD40L, sP-selectin, vWF, and sGPV between patients in the first and fourth quartile of coronary atheroma score (Table 8.4).
Table 8.3 Collateralization scores and plasma markers

Normally distributed data are expressed as mean± SD and analysed with one-way ANOVA. Non-normally distributed data are expressed as median (IQR) and analysed with Kruskal-Wallis.

<table>
<thead>
<tr>
<th></th>
<th>0 (n=128)</th>
<th>1 (n=28)</th>
<th>2 (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sP-selectin (ng/ml)</td>
<td>54±15</td>
<td>54±15</td>
<td>57±15</td>
<td>0.665</td>
</tr>
<tr>
<td>CD40L (pg/ml)</td>
<td>263 (60-687)</td>
<td>86 (33-473)</td>
<td>99 (47-765)</td>
<td>0.206</td>
</tr>
<tr>
<td>sGPV (ng/ml)</td>
<td>33.5 (24.0-46.0)</td>
<td>31.0 (22-52.3)</td>
<td>30.0 (20.8-38.5)</td>
<td>0.431</td>
</tr>
<tr>
<td>vWF (IU/dl)</td>
<td>157±31</td>
<td>162±32</td>
<td>161±34</td>
<td>0.681</td>
</tr>
</tbody>
</table>

Table 8.4 Comparison of platelet indices between the first and fourth quartiles of the angiographic severity of coronary heart disease as measured by Coronary Atheroma Score

Normally distributed data are expressed as mean± SD and analysed with Student t-test. Non-normally distributed data are expressed as median (IQR) and analysed with Mann-Whitney U-test.

<table>
<thead>
<tr>
<th></th>
<th>Q1 (n=47)</th>
<th>Q4 (n=47)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>60.0±8.1</td>
<td>65.5±8.8</td>
<td>0.002</td>
</tr>
<tr>
<td>Sex (%Males)</td>
<td>42.6</td>
<td>20.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>139±24</td>
<td>139±20</td>
<td>0.968</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>77±11</td>
<td>78±1</td>
<td>0.677</td>
</tr>
<tr>
<td>Coronary Atheroma Score</td>
<td>0.17(0.06-0.27)</td>
<td>2.87(2.12-3.13)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>sP-selectin (ng/ml)</td>
<td>54±17</td>
<td>55±12</td>
<td>0.787</td>
</tr>
<tr>
<td>sCD40L (pg/ml)</td>
<td>231 (66-739)</td>
<td>112 (51-580)</td>
<td>0.320</td>
</tr>
<tr>
<td>sGPV (ng/ml)</td>
<td>36 (24-55)</td>
<td>32 (22-41)</td>
<td>0.265</td>
</tr>
<tr>
<td>vWF (IU/ml)</td>
<td>153±33</td>
<td>157±29</td>
<td>0.588</td>
</tr>
</tbody>
</table>

Correlation between sCD40L, sP-selectin, vWF, and GPV

There was a strong correlation between sGPV and sP-selectin (r=0.379, P<0.0001). In addition, there was a weak correlation between sCD40L and vWF (r=-0.222, P=0.002). On a stepwise multiple regression analysis, sex (P=0.009), sP-selectin (P<0.0001) and sCD40L (P=0.04) were independent predictors of sGPV levels.
Relationship between angiographic collateralization and sCD40L, sP-selectin, vWF, and sGPV

No relationships could be found between the degree of collateralization, as determined by coronary angiography and the plasma levels of sCD40L, sP-selectin, vWF, and sGPV (Table 8.3)

Drugs and Plasma Markers

Patients using beta-blockers had a lower level of sGPV, with a median of 30 (25-54) ng/ml, than those not on beta-blockers, median 37 (22-42) ng/ml (P=0.031). The use of aspirin did not influence the plasma levels of sCD40L (P=0.711), sGPV (P=0.601), vWF (P=0.485), and sP-selectin (P=0.634) [data not shown]

8.4 Discussion

The high degree of correlation between sGPV and sP-selectin suggests that both molecules are comparable as markers of platelet activation in stable coronary heart disease. Indeed, it has recently been suggested that sGPV may be useful as a clinical marker of platelet activation in thrombotic states (Azorsa D et al. 1999), a statement which holds equally true for sP-selectin. However, at present, there is no agreed 'gold standard' to monitor platelet activation in patients and a combination of direct- and indirect- tests of platelet activation is probably required to give a good picture of platelet activation in disease states.

Apart from acting as a marker of platelet activation, the clinical significance of a raised plasma level of sGPV is uncertain. Indeed, there is very little literature on the role of GPV in health and disease. Although abnormalities in the other components of
the GPIb/IX/V complex are associated with the clinical syndrome of Bernard-Soulier, there is no known disease state ascribed to abnormalities of GPV (Katsutani S, Fujimoto TT, & Noda M 1998). Apart from that, the physiological significance of the cleavage of sGPV from the GPIb/IX/V complex by thrombin is unknown.

P-selectin is found on both platelets and endothelium (Tan KT & Lip GY 2003a) and is an important mediator of vessel wall inflammation and atherosclerosis. Virtually all sP-selectin is derived from activated platelets. However, the physiological role of plasma sP-selectin is uncertain.

In contrast to sP-selectin and sGPV, sCD40L may play an important role in the pathophysiology of atherosclerosis. CD40L is a member of the tumour necrosis factor family and is found abundantly on platelets (Andre P et al. 2002b). Platelet-surface associated CD40L has been shown to elicit a pro-thrombotic and pro-inflammatory reaction in vascular endothelium and leukocytes, including inducing the surface expression of cell adhesion molecules, the release of pro-inflammatory cytokines, and the expression of tissue factor. sCD40L is also believed to have similar pathophysiological effects. Indeed, a raised sCD40L level is associated with an increase in cardiovascular risk and may be associated with a higher risk of restenosis post-angioplasty (Cipollone F et al. 2003; Schonbeck U et al. 2001).

The finding of higher levels of sGPV, vWF, and sP-selectin in the plasma of patients with stable ischaemic heart disease suggests there is increased platelet activation and endothelial dysfunction in these patients. As discussed in Chapter 7, the raised sCD40L level may either represent platelet activation or its release by other cells. Although these findings have been well described in previous studies, there is no
previous literature relating the plasma levels of these molecules to the angiographic severity of coronary heart disease (Blann AD et al. 2001; Tan KT & Lip GY 2003a). However, the lack of correlation between the angiographic severity of coronary heart disease and these plasma markers should not be taken to mean that platelet activation cannot be related to atherosclerotic load. Indeed, a study by Koyama et al has shown that platelet activation can be correlated to intima-media thickness (Koyama H et al. 2003). The apparent paradox can be explained by the fact that coronary arteries can have significant atherosclerosis but appear relatively normal on angiography due to vessel remodelling. Indeed, it has been shown that the presence of an unstable plaque in the arterial tree cannot be ruled out by the use of conventional X-ray angiography (Tan KT, Aw-Yeang HW, & Bakshi D 2005). Another possible explanation to our observation may be that the angiographic severity of coronary heart disease may not be a good indicator of atherosclerotic disease severity elsewhere in the vasculature, a factor which may be important in determining the thrombogenic abnormalities found in atherosclerosis.
Chapter 9. Conclusion

9.1 Summary of Findings

The aim of this thesis is to study platelet activation in atherosclerotic disease. I performed an extensive literature search (Chapters 1 and 2) to determine the extent of previous work as well as to give an insight into the physiological roles of the various markers of platelet activation. Although much work has been done on platelet activation in atherosclerosis, there is a lack of data relating platelet activation status to the clinical state of the patient. This data would be of interest to all clinicians involved in the care of the patient with atherosclerosis and I decided that this should form a major part of this thesis. I have also studied platelet activation status in relation to imaging findings in atherosclerotic disease as well as the effect of angioplasty on sCD40L levels.

The studies in this thesis provide confirmatory evidence on the presence of increased platelet activation in atherosclerosis, as shown by flow cytometric and ELISA-based techniques. In addition, the presence of Type 2 Diabetes is linked to enhanced platelet activation, an association which is even more pronounced in the presence of symptomatic atherosclerotic disease.

In Chapter 4, I found increased platelet activation in patients with peripheral artery disease. This finding is supported by data from Chapters 5, 7 and 8, which showed consistently that atherosclerosis is associated with increased platelet activation. In addition, there is a graded increase in PMP levels with the clinical severity of peripheral artery disease. This finding would be consistent with that of Chapter 6,
which showed that PMP levels are raised in diabetic patients with symptomatic atherosclerosis.

In Chapter 5, I found an increase in platelet activation, as measured by sP-selectin, CD62P and CD63 positivity and PMP levels, in stable coronary heart disease. Surprisingly, in view of previous work, none of the various markers of platelet activation showed a correlation with either CAS or CSS. Two possible explanations exist for this negative finding; (1) a lack of statistical power in this study; or (2) a true absence of correlation between the angiographic severity of coronary heart disease and platelet activation. As the much larger study in Chapter 8 also did not show a correlation between markers of platelet activation and either CAS or CASS, the latter is likely to be true.

In Chapter 6, I found a graded increase in PMP levels from healthy controls, through Type 2 diabetic patients without symptomatic atherosclerotic disease, to Type 2 diabetic patients with symptomatic atherosclerosis. Interestingly, the IMT measurements in the latter two groups were similar, suggesting that PMP levels are related to symptoms rather than atherosclerotic load per se. In view of these results and those from Chapter 4, it is reasonable to hypothesise that the flow cytometric measurement of PMPs may aid risk stratification in patients at risk of developing the acute complications of atherosclerosis.

In Chapter 7, I found that sCD40L levels were increased in patients with peripheral artery disease. This observation is consistent with the finding in Chapter 8, which showed that patients with coronary heart disease had raised sCD40L levels. In addition, percutaneous angioplasty of the lower limb arteries caused a rise in sCD40L
levels. This finding is consistent with that of Aggarwal et al (2004), who found that there is a rise in plasma CD40L within ten minutes of percutaneous coronary artery angioplasty. At present, it is uncertain as to the length of time in which sCD40L levels would remain elevated post peripheral angioplasty. However, it has been shown that sCD40L levels remain elevated for at least one day post coronary angioplasty, with a return to baseline levels by five days (Cipollone F et al 2003).

In Chapter 8, I found an increase in platelet activation, as measured by sP-selectin and sGPV, in patients with coronary heart disease. In addition, sCD40L levels were elevated in this group of patients. However, neither CSS nor CAS could be correlated with any of these soluble markers. In addition, comparison of platelet indices of patients in the first and fourth quartiles of the angiographic coronary artery disease severity as measured by CAS did not reveal any significant differences. As expected, there was a strong correlation between sP-selectin and sGPV levels. As platelets may be involved in collateralisation in atherosclerosis, I also attempted to correlate the various platelet indices to the degree of collateralisation found on angiography. However, this aspect also proved to be negative.

Michelson et al (1996) have shown that the surface expression of P-selectin of thrombin activated platelets is lost within 2 hours of their infusion into the bloodstream. In addition, platelets which have lost their P-selectin continue to circulate and function. However, it should be noted that the increase in platelet surface P-selectin expression is not reversible over time in vitro (Michelson AD & Furman MI 1999). Based on these results, my findings of increased platelet surface expression of CD62P and raised sP-selectin level in patients with atherosclerosis suggest that there must be a continuous source platelet activation in this population.
The finding that the degree of platelet activation is not related to the severity of coronary heart disease (Chapters 5 and 8), as determined by X-ray coronary angiography, while there is a graded increase in platelet activation with symptom severity (Chapters 4 and 6), may seem contradictory. However, these findings can be explained by the fact that coronary X-ray angiography may: (i) not be a good indicator of the presence of atherosclerotic disease elsewhere in the vascular tree; (ii) be relatively insensitive in detecting vulnerable plaques in the circulation; and (iii) be normal even with the presence of significant coronary atheroma due to the phenomenon of positive remodelling. Indeed, it has been shown that the degree of platelet activation in atherosclerosis can be correlated to the well-established marker of atherosclerotic load, intima-media thickness.

I also found confirmatory evidence that the plasma levels of the pro-inflammatory cytokine sCD40L are elevated in diabetes (Chapter 6), coronary heart disease (Chapter 8) and peripheral artery disease (Chapter 7). In view of the previous finding that an elevated sCD40L level is linked with adverse cardiovascular events, it is perhaps surprising to find that sCD40L could not be related to the clinical severity of disease in my studies. The explanation behind these contradictory findings is unclear. One possibility may lie in the fact that the previous work prospectively examined a cohort of patients whilst my studies had a case-control design. Thus, my studies would be unable to detect any event which occurred beyond the time-frame of the sample collection. Another explanation may be that the current studies are underpowered to detect significant differences in sCD40L levels.
9.2 Future Studies

Although my studies showed an increase in platelet activation with the clinical severity of atherosclerotic disease, these findings should be confirmed by large prospective cohort studies. I believe that the availability of prognostic information provided by such studies, such as the need for intervention, death or the development of adverse cardiovascular events, would be useful in the assessment of the role of platelet activation markers in clinical practice. In particular, I believe that there should be a larger study to look at the significance of the sCD40L rise post-peripheral angioplasty. It would be useful to see if the rise in sCD40L level post-angioplasty can be related to an increase in adverse events.

In addition, there should be studies on the effects of novel anti-platelet agents on these markers. An ideal example of a drug to be studied is picotamide, a combined thromboxane synthase inhibitor and thromboxane antagonist, which has recently been shown to be superior to aspirin in preventing both death and disease progression in patients with Type 2 Diabetes (DAVID Study Group 2004). Studies of this sort would not only allow further characterisation of the mechanism of action of the drug in question, but would also shed light on the various physiological pathways in the platelet. It would also be interesting to discover the physiological roles (if any) of GPV and sP-selectin, and the interactions between these molecules.

It has been shown that certain genetic polymorphisms can influence the degree of platelet activation in an individual with atherosclerotic disease. Indeed, this observation may partially account for variations in the thrombotic profiles observed between different ethnic groups. In addition, genetic polymorphisms may influence the response of platelets to pharmacological agents. It would therefore be of great
clinical importance to study the molecular biology of platelet activation in atherosclerosis.

Previous studies have suggested that some of the changes in platelet physiology in atherosclerotic disease can be attributed to the megakaryocyte in the bone marrow. With the advances in molecular biology seen in recent times (e.g. microarray technology), it has become possible to study the origin of these changes to the level of genetic transcription in the megakaryocyte. It is possible that the megakaryocyte could represent a potential target for pharmacological manipulation.

The studies in this thesis have investigated changes in platelet activation status in atherosclerotic disease. However, it needs to be emphasised that other factors also contribute to the pro-inflammatory and prothrombotic state in atherosclerosis. The vessel wall plays a very important role in atherosclerotic disease. As mentioned above, the role of X-ray angiography in the assessment of atherosclerotic disease is likely to be superseded by other modes of radiological investigation that offer more information to the nature of the atherosclerotic plaque, such as multislice computed tomography, magnetic resonance imaging and positron-emission tomography. It would therefore be useful to relate the indices of platelet activation and the information regarding the vessel wall provided by these imaging modalities with prognostic data in future clinical studies.

9.3 Conclusion

This thesis has advanced our understanding of platelet function in atherosclerotic vascular disease, by providing novel data on the indices of platelet activation in atherosclerotic disease, and relating this to the clinical and angiographic features of
the disease. In addition, it is the first study to show that peripheral artery angioplasty results in the release of the pro-inflammatory molecule, sCD40L. The studies in this thesis lend support to the clinical use of platelet markers in the risk stratification of patients with atherosclerosis.

In the future, it is likely that the combined use of blood tests, such as the ones described in this thesis, and appropriate imaging modalities will allow the in-depth analysis of the components of Virchow's triad, that is, blood stasis, abnormal vessel wall and abnormal blood constituents, which have proved so important in the pathogenesis of atherosclerotic disease. This approach to the clinical investigation of patients with atherosclerosis could lead to the identification of those at higher risk of developing adverse events and who may need aggressive intervention, leading to a better outcome for these individuals.
Appendix

I certify that I was involved in the planning of all the studies described in this thesis. I collaborated with Dr. MH Tayebjee in the collection of blood samples from patients with coronary heart disease (Chapters 5 and 8). All blood samples from patients with peripheral vascular disease were collected solely by me (Chapters 4 and 7), with the exception of the pre- and post-angioplasty samples, which were collected by Dr. I Davagnanam. I am indebted to Dr. HS Lim for the blood samples from patients with Type 2 Diabetes as well as for obtaining the IMT measurements on these patients (Chapter 6).

I single-handedly developed and carried out all the flow cytometric studies described in this thesis. The ELISAs were personally performed by me from commercially available reagents, with the exceptions of the sGPV and sCD40L assays, which were done in collaboration with Dr. MH Tayebjee. The assessments of collateralisation and severity of coronary heart disease were performed by Drs. MH Tayebjee and RJ MacFadyen.
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124


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128


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