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The Role of Inflammation and Platelet Activation in the Adverse Cardiovascular Outcomes of Patients undergoing Surgery for Critical Limb Ischaemia

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To Professor J. S. Burdess

DECLARATION

This thesis represents research undertaken in the Centre for Cardiovascular Sciences, University of Edinburgh and the Departments of Vascular and Orthopaedic Surgery, Royal Infirmary of Edinburgh.

A British Heart Foundation Clinical PhD fellowship (FS/05/038), and Research Grants from the European Society of Vascular Surgery and the Royal College of Surgeons of Edinburgh sponsored these studies. I was personally involved in all of the study designs, patient recruitment, sample processing and data analysis presented in the thesis.

Chapters 3, 4, and 5 have been published in peer-reviewed journals. I have copyright permission for inclusion of the printed journal manuscripts within this thesis. The thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged. All studies were undertaken in accordance with the regulations of the Lothian Research Ethics Committee and with the Declaration of Helsinki of the World Medical Association. The written informed consent of each subject or patient was obtained before entry into the study.

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ABSTRACT

Increased platelet activation and inflammation play a key role in atherothrombosis. Patients with peripheral arterial disease are at increased risk of adverse cardiovascular events, particularly at the time of surgery. We postulated that the increase in peri-operative cardiovascular events is mediated by increased platelet activation and inflammation. We hypothesized that peri-operative dual anti-platelet therapy would improve biomarkers of atherothrombosis without causing unacceptable bleeding in patients undergoing surgery for critical limb ischaemia (CLI).

Prior to interventional study, I validated a sensitive flow cytometric technique for the reproducible assessment of *in vivo* platelet activation in patients with peripheral arterial disease. Thirty patients with stable claudication, attended on two occasions to permit within-day and between-day comparisons. A variety of platelet activation markers were compared to the gold standard of platelet–monocyte aggregation. Platelet-monocyte aggregation demonstrated comparable within-day (mean difference ± co-efficient of reproducibility; 0.9±15.4%) and between-day reproducibility (2.0±12.4%). Platelet-monocyte aggregates correlated well with other platelet activation markers (P selectin r=0.30; Platelet CD40L r=0.41; Platelet microparticles r=0.27; $P \leq 0.026$) and monocyte activation markers (monocyte CD40 r=0.27;monocyte CD11b r=0.47; $P \leq 0.026$).

In a cross sectional study, I demonstrated that resting *in vivo* platelet activation and inflammation was increased in patients with CLI in comparison to healthy controls, patients with stable claudication and those undergoing treatment for acute coronary syndromes. In addition, platelet activation and inflammation throughout the peri-operative period was markedly increased in CLI patients compared with non-vascular patients undergoing arthroplasty, and exceeded the rise attributable to the stress of surgery itself.

In a prospective double-blind randomised controlled trial, 108 patients undergoing infra-inguinal revascularisation or amputation for CLI were maintained on aspirin (75 mg daily) and randomised to clopidogrel (600 mg prior to surgery, and 75 mg daily for 3 days; n=50) or matched placebo (n=58). Peri-operative *in vivo* platelet activation and inflammation, cardiac-Troponin I (c-TnI) release and bleeding outcomes were recorded. Clopidogrel reduced markers of platelet activation and inflammation before surgery and throughout the post-operative period. Overall, there were 18 troponin-positive events (16.7%), with half of the troponin rises (9) occurring prior to surgery. Patients with post-operative elevations in c-Tn I had significantly greater levels of pre-operative platelet-monocyte aggregation, monocyte CD40, IL-6 and hsCRP. However, despite reducing platelet and inflammatory markers, clopidogrel did not have a direct effect on peri-operative c-Tn I. There was no increase in major life-threatening or minor bleeding, although blood transfusions and wound haematomas were significantly increased.

Using sensitive and validated methodologies, I have provided a detailed examination of *in vivo* platelet activation and inflammation in high-risk vascular surgical patients. This approach has provided the first objective assessment of the risks and benefits of intensive peri-operative anti-platelet therapy in this patient group. Dual anti-platelet therapy reduced biomarkers of atherothrombosis without causing unacceptable bleeding. However, large-scale clinical trials would be required to confirm whether these reductions translate into improvements in clinical outcome.

CONTENTS

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CHAPTER 1

INTRODUCTION – PERIPHERAL ARTERIAL DISEASE AND ATHEROTHROMBOSIS

1.1 OVERVIEW

Patients with critical limb ischaemia are at particularly high risk of serious cardiovascular events, especially when undergoing surgery. Despite this risk, there has been little study of platelet activation and inflammation during this high-risk period. Increased platelet activation and systemic inflammation are central to the propagation of intravascular thrombus and subsequent clinical sequelae. We propose to investigate whether, in patients undergoing surgery for critical limb ischaemia, increased markers of systemic inflammation and platelet activation are associated with cardiac troponin-I (cTnI) and highly sensitive C-reactive protein (hsCRP) release. Although effective in the treatment of acute coronary syndromes, combined anti-platelet therapy with clopidogrel and aspirin is not routinely used in the peri-operative period because of the potential for bleeding complications. However, there is currently no level-one evidence in this population to compare the relative risks of adverse bleeding with improvements in cardiovascular outcome. We therefore propose to establish whether combined aspirin and clopidogrel therapy will reduce peri-operative cardiac troponin in addition to markers of systemic inflammation and platelet activation. This may provide the rationale for a therapeutic trial of intensive anti-platelet therapy in patients undergoing surgery for critical limb ischaemia.

1.2 PERIPHERAL ARTERIAL DISEASE AND CARDIOVASCULAR RISK

Peripheral arterial disease affects nearly 30 million people in Western Europe and North America. It encompasses a spectrum of atherosclerotic disease, ranging from asymptomatic disease and stable intermittent claudication to critical limb ischaemia. Progression of the disease requiring surgical intervention (reconstruction, angioplasty or amputation) occurs in 5-10% [Khan S *et al*, 2007]. However, this is relatively benign in comparison to overall cardiovascular mortality. Cardiovascular risk increases with the severity of disease, with progressive reductions in ankle brachial pressure index being an independent predictor of cardiovascular outcome [Lee AJ *et al*, 2004; O'Hare AM *et al*, 2004]. In up to three-quarters of cases, patients have co-existent coronary artery disease and a 3-fold increased risk of cardiovascular events and death compared to the general population [Criqui MH *et al*, 1992]. Approximately one third will die within 2 years of presentation and two-thirds within 5 years as a result of myocardial infarction or stroke [Dormandy J *et al*, 1999; Leng GC *et al*, 1996].

These patients are particularly at risk during the peri-operative period. Myocardial infarction is the commonest life-threatening complication of major vascular surgery with a reported peri-operative incidence ranging from 8 to 40% depending on the diagnostic criteria [Hobbs SD *et al* 2005; Kim LJ *et al* 2002; Landesburg G *et al* 2003]. This is comparable to the cardiovascular risk seen in patients with acute coronary syndromes (ACS): 30-day death and re-infarction rate of 8-20% [Bertrand ME *et al* 2002]. The "Guidelines for Peri-operative Cardiovascular Evaluation for Noncardiac Surgery" from the American College of Cardiology and American Heart Association categorise peripheral vascular operations as high risk procedures with a >5% risk of non-fatal myocardial infarction or cardiac death. In a recent large observational study (n=5,460), patients with peripheral arterial disease scheduled for

open vascular surgery had a worse prognosis (2.4-fold increase in cardiovascular morbidity) than matched patients with severe myocardial ischaemia referred for percutaneous coronary intervention [Welten GM *et al*, 2008]. Interestingly the occurrence of peri-operative cardiac complications following vascular surgery was associated with long-term cardiac death. There is therefore a clear unmet need to reduce cardiovascular events in patients with peripheral arterial disease, especially in the peri-operative period.

Despite increased cardiovascular risk, this patient population has attracted relatively little attention as a study group for the investigation of atherothrombosis. In addition, in spite of attempts to raise awareness of peripheral arterial disease as an important marker of cardiovascular risk, patients are often poorly provided with evidence-based therapies such as anti-platelet and lipid lowering therapies [Cassar K *et al*, 2003; Burns P *et al* 2003; Hirsch AT *et al*, 2001; Khan S *et al*, 2007]. The reasons for this are unclear, but appear to be related to a lack of awareness amongst health professionals of the severity of the disease. Patients with peripheral arterial disease may potentially benefit from risk-stratification and appropriately tailored medical regimes, as occurs in patients with coronary atherosclerotic disease.

1.3 ATHEROTHROMBOSIS

Emerging evidence has demonstrated atherosclerosis as a dynamic inflammatory process of which endothelial dysfunction is one of the earliest features [Ross, 1999]. Endothelial activation results in the up-regulation of leukocyte adhesion proteins and initiates local vascular inflammation. The release of growth factors and pro-inflammatory cytokines results in leukocyte and monocyte recruitment, induction of atheroma formation and further arterial damage. Indeed, interventions to reduce mononuclear cell recruitment to vessels have been demonstrated to protect animals from atherosclerosis [Johnson RC *et al*, 1997; Gu L *et al*, 1998; Boring L *et al*, 1998; Cybulsky *et al*, 2001].

Plaque expansion and disruption can lead to atherothrombosis and subsequent acute coronary syndromes, including myocardial infarction [Blum and Miller, 1996; Ross, 1999]. Areas of endothelial denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are usually sub-clinical [Davies M, 2000]. It is the dynamic regulation of intravascular thrombus formation that is crucial in determining the resolution and clinical outcome from a complex atherosclerotic plaque. Indeed, over 80% of coronary atherosclerotic lesions causing >50% luminal stenosis have evidence of previous healed plaque disruption with incorporation of old thrombus within the atherosclerotic lesion [Mann J *et al*, 1999].

1.3.1What converts a stable plaque into an unstable lesion prone to rupture?

The structural morphology, cellular composition, and biological activity of coronary plaques appear to be closely linked. Indeed, it is has been demonstrated that plaque instability correlates more with biological activity and cellular composition than with angiographical findings of stenosis. Exogenous factors (e.g. mechanical stress, vasomotor tone, infection,

blood viscosity, coagulability) further modify such interaction, making the final outcome even less predictable.

In the presence of platelet activation or marked vascular inflammation, microthrombi on the surface of atherosclerotic plaques may propagate, ultimately leading to arterial occlusion and myocardial infarction [Rosenburg RD *et al*, 1999]. Thus increases in platelet activation or inflammatory mediators may reflect plaque instability and provide information on the intracoronary dynamics of thrombosis and inflammation. Given this pathophysiological basis for atherothrombosis, markers of platelet activation and vascular inflammation have been extensively studied. There is emerging evidence to suggest that some markers may provide a more sensitive reflection of *in vivo* platelet activation and vascular inflammation than others. In addition, these markers have been shown to correlate with clinical risk and therefore, potentially provide a link between plaque pathophysiology and clinical outcome.

1.4 ASSESSMENT OF ATHEROTHROMBOTIC RISK

1.4.1 Cardiac Troponin I (cTnI)

Cardiac troponin I (cTnI) is a contractile protein that is released into the circulation after myocardial cell injury. Unlike creatine kinase and its MB isoenzyme (CK-MB), cTnI is not found in skeletal muscle and is therefore highly sensitive and specific for myocardial necrosis. Levels of cTnI have been demonstrated to predict increased risk of mortality and reinfarction in patients presenting with an acute coronary syndrome, and as such have been used to stratify clinical treatment [Galvani M *et al*, 1997; Antman EM *et al*, 1996; Luscher MS *et al*, 1997, Hamm CW *et al*, 1997]. Indeed, even small increases in cardiac troponins are associated with worse outcomes [Morrow DA *et al*, 2001; Reed MJ *et al*, 2012]. Studies have demonstrated correlation of levels of platelet activation markers with troponin release in patients with acute coronary syndromes [Zhang SZ *et al*, 2007], with markers such as platelet-monocyte aggregates predicting troponin rise after percutaneous coronary intervention [Ray MJ *et al*, 2005].

Although myocardial ischaemia commonly complicates vascular surgery, early detection of clinically meaningful ischaemia remains a challenge in the peri-operative setting [Raby KE *et al*, 1992]. During surgery, cTnI is reported to be more specific for myocardial necrosis than CK-MB. Several studies have now demonstrated that even at low cut-off levels, post-operative cTnI is an independent predictor of both short and long term mortality after major vascular surgery [Andrews A *et al*, 2001; Kim LJ *et al*, 2002; Landesberg G *et al*, 2003]. However, it has yet to be shown whether intervention aimed at reducing platelet activation and peri-operative Troponin release can improve clinical outcome.

1.4.2 *in vivo* Platelet Activation

Although markers of myocyte necrosis are invaluable diagnostic tools for patients with acute coronary syndromes, and are routinely used for risk stratification, only 22% to 50% of patients with unstable angina have positive troponin (I or T) tests [Hamm CW *et al*, 1997; Heeschen C *et al*, 1999; Morrow DA *et al*, 2000]. Many patients with troponin-negative acute coronary syndromes who have vulnerable coronary plaques remain at high risk for future ischemic events. Serum cardiac markers reflect evidence of myocardial necrosis, but they provide no information about the patho-physiological state within the coronary artery. In contrast, levels of *in vivo* circulating activated platelets may reflect plaque disruption and thrombosis.

A variety of techniques have been proposed for the assessment of platelet activation including platelet aggregometry, point of care devices, flow cytometric assessment of platelet surface antigens, and soluble plasma markers of platelet activation: all have advantages and disadvantages [Michelson AD**,** 2009]. Historically considered the gold standard, platelet aggregometry requires the preparation of platelet-rich plasma and a high sample volume. Centrifugation and washing procedures may produce cell loss and artefactually activate platelets. Many of the point-of-care systems assess *ex vivo* platelet aggregation to various exogenous agonists. Although more labour intensive, flow cytometry is emerging as the new sensitive gold standard with measurement of surface expression of platelet antigens providing an assessment of *in vivo* platelet activation. It requires only a small sample volume, is performed on whole blood, and allows analysis of platelets in their physiological milieu.

One of the most commonly studied markers of platelet activation is the α-granule membrane protein, P-selectin, that is present only on the surface of activated degranulated platelets. However, *in vivo* degranulated platelets rapidly lose their surface P-selectin, but continue to

circulate and function [Michelson AD *et al*, 1996]. In contrast, P-selectin-positive platelets very rapidly bind to leucocytes (mainly monocytes) via their constitutively expressed counter receptor, P-selectin glycoprotein ligand-1 (PSGL-1) to form platelet-monocyte aggregates (PMAs) [Michelsen AD *et al*, 2001].

Adherence of platelets to monocytes has been shown to regulate various monocyte actions. The binding of thrombin-stimulated platelets induces monocyte cytokine expression [Neumann F-J *et al*, 1997]. Platelets supply cholesterol to monocytes that may then mature into lipid-laden macrophages characteristic of atherosclerosis. In addition, platelet surface P-selectin induces the expression of tissue factor on monocytes [Celi A *et al*, 1994] and promotes fibrin deposition [McEver RP *et al*, 2002] within a growing thrombus at sites of vascular injury. Furthermore, platelet-monocyte aggregation promotes monocyte adhesion to activated endothelium and the growth of atherosclerotic lesions in $ApoE^{-/-}$ mice [Huo Y *et al*, 2003].

Increased levels of PMAs have been detected in smokers, patients with diabetes and coronary artery disease [Harding SA *et al*, 2004; Sarma J *et al*, 2002]. Indeed, increased levels have been shown to correlate with severity of acute coronary syndrome and be predictive of re-current thrombosis following percutaneous coronary intervention [Gurbel PA *et al*, 2005]. Thus, PMAs may not only contribute to the initiation and progression of atherosclerosis, but act as surrogate markers of clinical risk. Therefore, detection of circulating PMAs may be a more robust marker of *in vivo* platelet activation than platelet surface P-selectin.

1.4.3 Inflammatory markers

It is increasing recognised that inflammatory mechanisms play a central role in the pathogenesis of atherosclerosis and its complications [Ross R. 1999]. Many inflammatory mediators have been investigated but two of the most extensively studied include the CD40/CD40L complex and C-Reactive Protein (CRP).

CD40/CD40L

The interaction between transmembrane glycoprotein receptor CD40 and its counterpart CD40 ligand (CD40L) has been shown to play an integral part in the inflammatory pathways of the vascular system. The CD40/CD40L system is expressed in human atheroma and on a range of atheroma-associated cells including endothelial cells, smooth muscle cells, monocytes and platelets [Mach F *et al*, 1997; Schonbeck U *et al*, 2001]. Binding of the CD40 receptor mediates an array of pro-inflammatory effects including the expression of cytokines, chemokines, adhesion molecules, matrix metalloproteinases, and growth factors [Schonbeck U *et al*, 2001]. These activities are crucial to the process of atherogenesis and promote plaque instability. Indeed, disruption of CD40/CD40L interactions in hypercholesterolemic mice retards the initiation and progression of atherosclerotic lesions [Mach F *et al*, 1998].

CD40L induces tissue factor expression on endothelial cells and monocytes [Lindmark E *et al*, 2000], and contains a RGD integrin recognition sequence allowing it to bind to glycoprotein IIb/IIIa, activate platelets, and stabilize arterial thrombi [Andre P *et al*, 2002]. Thus CD40L has the potential to mediate both pro-inflammatory and pro-thrombotic activities within the vasculature.

Consistent with these activities, elevation of sCD40L is increased in patients with diabetes mellitus [Harding SA *et al*, 2004] and cigarette smokers [Harding SA *et al*, 2004], and is

associated with an increased risk of subsequent cardiovascular events in both healthy women [Schonbeck U *et al*, 2001] and patients presenting with acute coronary syndromes [Heeschen C *et al*, 2003]. CD40L has also been shown to correlate with PMAs and often parallels elevations in C-reactive protein [Niccoli G *et al*, 2008], which is, in turn, associated with acute coronary syndromes.

Highly sensitive C-reactive Protein (hs-CRP)

It has been widely demonstrated that plasma markers of inflammation are predictive of cardiovascular risk [Blake GJ *et al*, 2001]. Of these potential markers, C-reactive protein (CRP) has been the most extensively studied. Produced in the liver in response to interleukin (IL)-6, CRP is an acute phase reactant.

Numerous large-scale epidemiological studies among apparently healthy men and women have found that CRP is a strong independent predictor of future cardiovascular risk [Kuller LH *et al*, 1996; Ridker PM *et al*, 1997; Tracy RP *et al*, 1997; Ridker PM *et al*, 1998; Koenig W *et al*, 1999; Ridker PM *et al*, 2000; Ridker PM *et al*, 2001]. CAPTURE trial investigators found that, although only troponin T was predictive in the initial 72-h period, both CRP and troponin T were independent predictors of risk at six months [Heeschen C *et al*, 2000], while the FRISC II investigators reported that the risk associated with elevated CRP levels at the time of index event continues to increase for several years [Lindahl B *et al*, 2000]. In each of these studies, the predictive value of CRP was independent of, and additive to, troponin.

Recent publications suggest that an elevated CRP may also be an independent predictor of adverse cardiac events following vascular surgery [Rossi E *et al*, 2002; Owens CD *et al*, 2007]. A recent meta-analysis of prospective studies, demonstrated that patients undergoing vascular surgery with a pre-operative CRP > 3 mg/L were at significantly increased risk of long term

mortality and cardiac morbidity. There was no association with early or immediate-term adverse events [Padayachee L *et al*, 2009].

Initially thought of as a downstream bystander marker of vascular inflammation, recent data suggest that CRP may also play an active role in atherogenesis. C-reactive protein opsonization of low-density lipoprotein (LDL) mediates LDL uptake by macrophages [Zwaka TP *et al*, 2001], and CRP also stimulates monocyte release of pro-inflammatory cytokines such as IL-1b, IL-6, and tumour necrosis factor-alpha [Ballou SP *et al*, 1992]. Furthermore, CRP mediates monocyte chemotactic protein-1 induction in endothelial cells [Pasceri V *et al*, 2001] and causes expression of intercellular adhesion molecule-1 and vascular cellular adhesion molecule-1 by endothelial cells [Pasceri V *et al*, 2000].

Statin therapy may have powerful anti-inflammatory effects, and in recent clinical studies, statin therapy has been shown to lower CRP levels, an effect that is independent of lipid lowering [Ridker PM *et al*, 2001; Albert M *et al*, 2001; Jialal I *et al*, 2001]. In CARE trial population, patients with persistent low-grade vascular inflammation, as evidenced by high CRP and serum amyloid A levels, were at increased risk of recurrent events. Randomization to pravastatin therapy prevented 54% of recurrent events among those with persistent inflammation, compared with 25% among those without [Ridker PM *et al*, 1998]. The effect of aspirin on CRP levels is controversial [Ikonomidis I *et al*, 1999; Feldman M *et al*, 2001], but the benefit of aspirin therapy in preventing future MI appears to be greatest among those with elevated CRP levels [Ridker PM *et al*, 1997]. In addition, data from the FRISC-II study suggest that the benefits of an early invasive approach may be greatest among those with evidence of a heightened inflammatory response [Lindmark E *et al*, 2001]. Prospective randomized studies are required to test these hypotheses directly, but individuals with evidence

of heightened inflammation may benefit most from an aggressive modification of lifestyle and an intensification of proven preventive therapies such as antiplatelet and lipid-lowering agents.

1.5 PERIPHERAL ARTERIAL DISEASE, PLATELET ACTIVATION AND INFLAMMATION

Despite suffering significant cardiovascular risk, there has been relatively little investigation of platelet activation and inflammation in high-risk vascular surgical patients in comparison to patients with coronary atherosclerosis.

Several studies have assessed baseline platelet activation in patients with peripheral atherosclerosis. Although smaller studies have reported some conflicting results [Galt SW *et al*, 1991], larger series have demonstrated that activation is greater than in healthy controls, with a progressive increase in activation with increasing severity of disease [Cassar K *et al*, 2003, Robless PA *et al* 2003, Rajagopalan S *et al*, 2007].

Other than a few early, small size studies, utilising a variety of platelet and coagulation factors as outcome measures [McDaniel MD *et al*, 1984; Reininger CB *et al*, 1994], most data regarding peri-operative platelet activation in the vascular patient come from 2 UK centres.
Studies from Aberdeen have investigated platelet activation in patients with lifestyle-limiting claudication undergoing aorto-iliac or fem-pop angioplasty [Cassar K *et al*, 2005]; patients undergoing major vascular surgery (arterial re-vascularisation for sub-clinical ischaemia and infra-renal aortic aneurysm repair) [Rajagopalan S *et al*, 2007], and a small group (n=22) of patients undergoing surgery for critical limb ischaemia [Collins P *et al*, 2006]. These studies all showed an increase in post-procedural markers of platelet activation, with the greatest change

occurring in samples stimulated with agonists ex vivo as opposed to resting levels. However, the patient populations were heterogeneous in terms of underlying disease and severity. Platelet activation was assessed by flow cytometric and aggregometry measurements of both resting and *ex vivo* agonist stimulated samples. As discussed earlier, the process of aggregometry and agonist stimulation can cause artificial changes to platelet marker levels, and may not provide the most sensitive reflection of circulating activated platelets *in vivo*. In addition, assessment of resting platelet P- selectin may be inaccurate due to shedding of the molecule from the platelet surface.

Rajagopalan S *et al* demonstrated that post-operative platelet aggregation to arachodonic acid was significantly increased in those patients who developed a post-operative elevation in cardiac Troponin I. There was no other relationship between other markers of platelet activation and post-operative cardiac Troponin I.

Researchers in Leicester have studied platelet activation in patients undergoing carotid endarterectomy. They have demonstrated that elevated pre-operative levels of platelet activation are related to increases in post-operative transcranial emboli [Hayes PD *et al*, 1999 and 2003 and Payne DA *et al*, 2004]. They also measured platelet activation at 8 points throughout the pre-, intra- and post-operative period and demonstrated a significant rise in activation with the infusion of heparin [Webster SE *et al*, 2004]. Following heparin infusion, platelet activation levels fell but remained above baseline for 24 hours post surgery. Platelet activation was assessed using aggregometry or flow cytometric measurements of resting and agonist stimulated samples.

These studies support the concept that platelet activation is increased in the peri-operative period and may account for the high incidence of peri-operative cardiovascular events in

vascular patients. However the heterogeneity of patients studied and variety of *ex vivo* platelet markers utilisied make it difficult to specifically examine what occurs *in vivo* in patients with the highest risk of atherothrombosis. Patients undergoing peripheral angioplasty for claudication or carotid endarterectomy for carotid atherosclerosis could be expected to have a different profile of platelet activation and clinical risk compared to those with the greater disease burden and symptoms of critical limb ischaemia undergoing open surgery.

Coronary atherosclerosis has a spectrum of clinical presentations which have been shown to carry different degrees of cardiovascular risk. Risk-stratified treatment algorithms have subsequently been devised. Patients with peripheral arterial disease could benefit from similar targeted medical therapies, appropriately tailored to clinical risk. Although different anti-platelet regimes have been studied in peripheral angioplasty and carotid endarterectomy [Cassar K *et al*, 2005; Payne DA *et al* 2004] it would be logical to identify those with the greatest atherothrombotic risk and who potentially stand to gain the most from intensive anti-platelet therapy.

1.6 PHYSIOLOGICAL RESPONSE TO SURGERY AND PERI-OPERATIVE MYOCARDIAL INJURY

The physiological response to injury encompasses a wide range of endocrinological, metabolic, immunological and haematological effects, all of which have an impact on the vasculature.
The initial phase includes a pro-inflammatory immune response. This is characterized by pro-inflammatory cytokine release, microcirculatory disturbance, and injury-induced activation of the coagulation cascade.

1.6.1 Acute-phase response

Pro inflammatory cytokine production in the intraoperative and early postoperative period is initiated by macrophages and monocytes at the initial site of injury as part of the acute-phase response [Baumann H *et al*, 1994]. These cytokines include tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), which are primarily responsible for the non-hepatic manifestations of the acute-phase response, including fever and tachycardia. In turn, TNF-α and IL-1β stimulate the production and release of other cytokines, including IL-6 [Baigrie RJ *et al*, 1992; Desborough JP, 2000].

Interleukin 6 primarily regulates the hepatic component of the acute-phase response resulting in the generation of acute-phase proteins, including C-reactive protein [Baumann H *et al*, 1994]. Acute phase proteins act as inflammatory mediators, anti-proteinases and scavengers in tissue repair.

Concentrations of circulatory cytokines are normally low and often undetectable. Within 30–60 minutes after the start of surgery, Interleukin- 6 concentration increases after 2–4 hours.

Studies demonstrate that levels of IL-6 are proportional to degree of tissue trauma or duration of surgery, with lower levels of IL-6 being observed after laparoscopic surgery in comparison to open [Kloosterman T *et al*, 1994; Mokart D *et al*, 2002; Shenkin A *et al*, 1989; Ueo H *et al*, 1994]. A study by Cruickshank AM *et al*, 1990, demonstrated higher levels of IL-6 in patients undergoing abdominal aortic and colorectal surgery than in those undergoing hip replacement despite similar operating times. Additionally, both elevated peri-operative CRP and IL-6 levels correlate with subsequent development of postoperative complications [Choileain NN *et al*, 2006]

1.6.2 Vascular Dysfunction

The local microcirculatory inflammatory response to surgery is characterized by a pronounced leukocyte accumulation and adherence to the endothelial lining of blood vessels. [Yamauchi J *et al*, 1999]. In animal studies, mobilisation of bowel mesentery during laparotomy results in a marked increase of venular leukocyte accumulation secondary to enhanced rolling and adhesion interactions [Fiebig E *et al*, 1991].

Several studies have indicated that adhesion molecules also appear to be involved in the inflammatory response to trauma. Major surgery elevates serum levels of P-selectin, E-selectin, and vascular cell adhesion molecules as well as increasing expression of leukocytic CD11a and CD11b, and endothelial intercellular adhesion molecule 1 [Shijo H *et al*, 1998; Klava A *et al*, 1997; Sendt W *et al*, 1999]. During the initial phase of adherence, selectins on leukocytes (L-selectin), endothelial cells (E-selectin), and platelets (P-selectin) interact to produce leukocyte "rolling". Subsequently, up- regulation of leukocyte integrins can be observed after surgical injury [Seekamp A *et al*, 1998] Combined with the capillary leakage caused by proinflammatory cytokine release and increased nitric oxide production, the interaction of these adhesion molecules leads to a stable cell to cell contact with

polymorphonuclear cell attachment, resulting in microcirculatory obstruction and failure of transcapillary exchange.

The physiological response to vascular injury also induces a hypercoaguble state in order to achieve haemostasis. Sympathetic vessel constriction occurs and circulating platelets adhere to the vessel wall. Activated platelets aggregate and activation of the coagulation proteins produce fibrin to form a stable plug.

1.6.3 Peri-operative cardiac complications following surgery

Peri-operative myocardial infarction (PMI) is the most common cardiovascular complication following non-cardiac surgery and is one of the most important predictors of short- and long-term morbidity and mortality [Priebe HJ *et al*, 2005].

The pathophysiology of peri-operative MI is highly debated. As discussed above, the physiological response to surgery induces large changes in sympathetic tone, cardiovascular performance, coagulation and inflammatory response. Although supply demand mismatch (e.g due to hypotension or tachycardia) has long been thought to explain many peri-operative MIs, there is pathological and angiographic evidence to suggest that acute plaque disruption and haemorrhage is often the underlying culprit (as occurs in acute coronary syndromes out-with the peri-operative period) [Cohen MC *et al*, 1999; Dawood MA *et al*, 1996].

There are several features of the stress response to trauma that will increase plaque instability and chance of rupture. Most ischaemic episodes tend to start at the end of surgery and during emergence from anaesthesia. [Landesberg G *et al*, 2001]. This period is characterized by increases in heart rate, arterial pressure, sympathetic tone, and pro-coagulant activity. Increased sympathetic tone can result in increases in arterial pressure, heart rate, contractility, coronary

vasomotor tone, and coronary vascular shear stress. This, in turn, may trigger coronary vasospasm, plaque disruption, and coronary thrombosis. Surgery-induced pro-coagulant and anti- fibrinolytic activity combined with enhanced inflammatory cell may trigger coronary artery thrombosis during low-flow conditions in the presence of underlying stable coronary atherosclerosis even in the absence of acute plaque disruption.

1.6.4 Surgical stress in patients with underlying vascular disease

If the physiological response to surgical trauma increases a patient's risk of vascular thrombosis, what happens when there is also underlying vascular dysfunction, as in patients with peripheral arterial disease? A large proportion of these patients have pre-existing coronary disease, so the increase in vasomotor tone, shear stress and pro-thrombotic state induced by surgery could pre-dispose to coronary plaque rupture. In addition, patients with peripheral arterial disease also have a baseline elevation in platelet activation and inflammation. It could be hypothesized that this baseline pro-thrombotic state combined with the stress response to surgery accounts for the particularly high incidence of peri-operative cardiovascular events suffered by these patients.

1.7 ANTI-PLATELET THERAPY

Despite advances in the understanding of atherosclerotic disease, anti-platelet therapies remain integral in both the prevention and treatment of atherothrombosis.

Paradoxically, despite the epidemiological and prognostic importance of peripheral arterial disease, relatively few clinical trials with cardiovascular protective agents have been carried out specifically in these patients. In particular, anti-platelet therapy is used in peripheral arterial disease based essentially on meta-analyses, extrapolation of results from trials in other conditions, or subgroup analyses of large clinical trials enrolling patients with various clinical manifestations of atherothrombosis, and not on results of clinical trials specifically designed in this clinical condition. The role of aspirin in the secondary prevention of cardiovascular events in patients with peripheral arterial disease has been established by the Antithrombotic Trialists' Collaboration with a reduction of 23% in the rate of death, myocardial infarction and stroke [Antithrombotic Trialists' Collaboration, 2002]. Prophylactic use of the thienopyridine clopidogrel has modest additional secondary preventative benefits in comparison to [CAPRIE investigators, 1996], or in combination with aspirin [CHARISMA Investigators, 2006]. Sub-group analysis of patients in the CAPRIE trial demonstrated that patients with peripheral arterial disease derived the greatest benefit from clopidogrel therapy in comparison to other patients at risk of ischaemic events (24% relative risk reduction in composite end point compared to an 8.7% reduction across all study groups). However the limitations of post-hoc sub-group analysis must be taken into consideration.

Although vascular patients are at high risk of cardiovascular events, bleeding concerns are a major disincentive for the investigation of peri-operative intensive anti-platelet regimes, and perhaps underlie the paucity of such data. Currently, vascular surgeons continue aspirin

throughout the surgical period, but other anti-platelet agents are discontinued. Most published reports of anti-platelet use in peripheral vascular surgery are observational [Smout J *et al*, 2003; Assadian A *et al*, 2005] although randomised-controlled trials of dual anti-platelet therapy have been performed in patients undergoing carotid endarterectomy [Payne PD *et al*, 2004] and peripheral angioplasty [Cassar K *et al*, 2005]. These studies reported reductions in surrogate markers of risk with no increases in major bleeding complications, although bleeding time was increased. However, there have been no randomised-controlled trials of dual anti-platelet therapy in open surgery for critical limb ischaemia, where the potential for both peri-operative bleeding and cardiac complications is greater.

Most surgical data on the peri-operative bleeding risks associated with anti-platelet agents, comes from patients undergoing coronary artery bypass grafting. Several small scale trials and observational studies of combination therapy have produced conflicting evidence, with either no difference [Cannon CP *et al*, 2005; Karabulut H *et al*, 2004; Carpino PA *et al*, 2001] or an increased risk of bleeding and bleeding related complications [Chu MWA *et al*, 2004; Hongo RH *et al*, 2002; Kapetanakis EI *et al*, 2005] being reported. These studies have been confounded by lack of randomization, small sample size and variation in defined end points. In addition, often no distinction is made between patients undergoing CABG in the setting of an ACS (where pre-surgical anti-platelet therapy is an essential part of medical therapy) and those undergoing CABG for stable coronary vascular disease, when intensive anti-platelet regimes are discontinued prior to surgery. In addition to discrepancies in cardiovascular risk, given the differences in the extent of platelet inhibition, the bleeding risk could be expected to differ greatly between these two patient populations.

Currently, patients presenting with acute coronary syndromes (ACS) frequently undergo early invasive investigation with a view to urgent coronary revascularisation (FRISC, RITA, TACTICS, CRUSADE, GRACE). Up to 14% of these patients currently undergo CABG during the index hospitalisation, leading to a significant increase overall, in the number of patients undergoing CABG in the urgent setting [Largerqvist B *et al***,** 2006**;** Fox KA *et al***,** 2005**,** Cannon CP *et al***,** 1998]**.** Current guidelines on the management of patients with non-ST elevation ACS recommend that both aspirin and clopidogrel be administered on hospital admission and continued for 9 to 12 months (Braunwald E *et al*, 2002; Bassand JP *et al*, 2007]. To date, although the surgical patients were not prospectively randomised, the CURE trial (Clopidogrel in Unstable angina to prevent Recurrent ischaemic Events) provides the most robust data for anti-platelet therapy in patients with ACS in whom CABG is performed [Fox KA *et al*, 2004]. Subgroup analysis of the 2072 patients undergoing CABG, revealed an overall relative reduction in adverse events similar to the entire study population (16.2% Placebo, 14.5% Clopidogrel, RR 0.89 for all CABG subjects, compared to 11.4% Placebo, 9.3% Clopidogrel, RR 0.80 for all CURE patients). The CURE trial reported an *overall* 1% absolute excess of major bleeding complications with the additional use of clopidogrel when compared to placebo in patients with ACS. There was no significant increase in life-threatening bleeding. In the subgroup undergoing CABG, there was no excess of major (RR 1.27, P=0.095 ns) or life-threatening bleeding (RR=1.24, P=0.2 ns). However, only a small number of patients continued dual anti-platelet therapy within 5 days of surgery. Despite these limitations, the trial proposed that the potential cardiovascular benefits of dual anti-platelet therapy may outweigh the risks of bleeding in the high-risk surgical patient.

Whether these outcomes can be applied to patients undergoing surgery for peripheral vascular disease remains to be demonstrated.

1.8 SUMMARY

Despite suffering significant cardiovascular morbidity, patients with peripheral arterial disease are poorly provided with cardio-protective therapies. There is clearly an unmet need to establish improved treatment for these patients, but the potential for both benefit and hazard associated with anti-platelet therapies during surgery requires careful consideration. There has been little investigation of the role of *in vivo* platelet activation and inflammation in contributing to the high incidence of peri-operative adverse cardiovascular events. An appreciation of the pathophysiology of atherothrombosis has guided the diagnosis, classification and management of coronary atherosclerotic disease. However, it is still unclear how inhibition of platelet function could be utilised appropriately to prevent peri-operative cardiovascular complications in patients with peripheral arterial disease.

1.9 AIMS AND HYPOTHESES

The principle aim of this thesis is to explore *in vivo* platelet activation and inflammation during the period of greatest risk for those patients with severe peripheral vascular disease – the peri-operative period. Specifically, we propose to investigate the effects of additional clopidogrel therapy in patients undergoing infra-inguinal revascularisation or amputation for critical limb ischaemia. We aim to demonstrate reproducible methodology for the assessment of *in vivo* platelet activation, and demonstrate that these markers are significantly elevated in our study population in comparison to other high-risk groups. We will determine whether additional clopidogrel has beneficial effects on surrogate markers of cardiovascular risk during the peri-operative period. In addition, this study will provide the first objective evidence of bleeding risk associated with peri-operative dual anti-platelet therapy in this population.

This proof of concept study could inform the design of larger scale clinical trials, which could subsequently shape clinical practice.

We hypothesise that in patients undergoing surgical intervention for criticial limb ischaemia:

1. Flow cytomteric assessment of platelet-monocyte aggregation in whole blood, provides a reproducible assessment of *in vivo* platelet activation, which correlates well with other markers of platelet activation and inflammation, [Chapter 3].

2. Markers of inflammation and platelet activation will be increased in comparison to healthy controls, patients with stable claudication, patients with acute coronary syndromes and those undergoing non-vascular surgery, [Chapter 4].

3. Markers of inflammation and platelet activation will be increased in patients who subsequently develop a post-operative rise in cardiac Troponin, [Chapters 5 and 6].

4. Additional thienopyridine (clopidogrel) therapy will reduce markers of inflammation and platelet activation, and peri-operative cardiac troponin release, [Chapters 5 and 6].

CHAPTER 2

METHODOLOGY – ASSESSMENT OF *IN VIVO* PLATELET ACTIVATION AND INFLAMMMATION IN PATIENTS WITH PERIPHERAL ARTERIAL DISEASE

2.1 INTRODUCTION

This thesis comprises a series of studies examining markers of platelet activation and inflammation in patients with peripheral arterial disease. The rationale behind these investigations was the concept that platelet hyperactivity and elevated inflammation is associated with an increased risk of atherothrombosis and could therefore account for the high incidence of peri-operative cardiovascular events in this patient group. However, despite increasing knowledge regarding the role of platelets in a number of medical disorders, there is no consensus on the ideal measure of platelet activation. In the introduction we discussed why some platelet markers provide a more sensitive reflection of *in vivo* platelet activation. What follows includes an overview of the methodology of platelet activation assessment, with an in-depth discussion of flow cytometric analysis and the protocols utilised in our studies. Detailed descriptions of the assays used in the assessment of inflammatory proteins (hsCRP), cardiac Troponin I, and serum platelet activation markers (soluble P-selectin and CD40L) are also provided. Clinical details of the individual study designs and patient selection criteria can be found in the methods sections of subsequent chapters.

2.2 TECHNIQUES FOR THE ASSESSMENT OF PLATELET ACTIVATION

Platelet activation comprises a change in platelet shape, platelet aggregation and release of platelet constituents. Platelet activation may therefore be quantified by factors such as change in shape, tendency to aggregate, and also by measuring blood or urine levels of relevant platelet metabolic products. However, in contrast to coagulation assessment, where screening tests (e.g APTT and PT) are cheap, quick and widely standardised, platelet function testing varies widely. Tests examine different aspects of platelet function and are poorly standardised. Different laboratories often use different protocols for sample preparation, different panels of agonists, often at different concentration ranges, and different types of assay utilising different control ranges. In addition, each technique has associated methodological issues that can affect the accuracy of the end measurement. There is a wide variation in the methodology utilised to assess platelet function within the literature, which makes it extremely difficult to compare and interpret results across studies. This is critical in quantifying the effect of intervention. A full appreciation of the techniques employed in a study is therefore imperative to understanding outcomes.

Platelet Aggregation

Historically considered the gold standard, platelet aggregometry uses turbidometric or impedance methods to assess platelet function. Spontaneous platelet aggregation or aggregation in response to agonists such as ADP or thrombin in platelet rich plasma (PRP) or whole blood can be assessed.

The turbidometric procedure is based on the detection of light transmission through a suspension of PRP before or after addition of agonists, while platelets are kept in motion by a stirring system (Light Transmission Aggregometry, LTA). The increase in light transmission is related to the extent of aggregation. The advantage of this method is that it is widely used and relatively simple to perform and interpret. However, these are *in vitro* studies, which may not reflect platelet activation *in vivo*. In addition, the preparation of platelet rich plasma and a high sample volume is required. Centrifugation and washing procedures may produce cell loss and artefactually activate platelets.

Whole blood aggregometry by the impedance method can also be performed. Whole blood is anticoagulated with citrate and constantly stirred. After the insertion of an electrode, impedance changes are recorded before and after addition of agonist. The detection of platelet aggregation is obtained in a millieu closer to that of platelets *in vivo*, however the process is slower and has been demonstrated to have poorer reproducibility.

Serum platelet activation markers

Substances stored in platelet granules and only released upon activation (such as P-selectin) can be detected in the serum by enzyme-linked immunosorbent assay **(**ELISA) or radioimmunoassay methods.

In brief, the fundamental principle of the ELISA is that the target analyte (the antigen) is recognised with high specificity by antibodies. ELISAs are plate-based assays designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

The use of such techniques allows serum samples to be stored for bulk and convenient processing. In addition, ELISA's have a level of sensitivity and robustness and allow easy visualization of results.

Although, stages in the assay such as anticoagulation of serum with heparin or citrate can artefactually affect results, the main limitations depend on the choice of marker. For example, Beta-thromboglobulin, β-TG, is artefactually elevated in renal insufficiency, whilst soluble P-selectin and CD40L demonstrate poor correlation with cell-bound measurements of the proteins [Gurbel PA *et al*, 2000; Ahn ER *et al*, 2004].

Point of care devices

Several bedside monitors have been developed for the rapid assessment of platelet function in whole blood (e.g platelet function analyser, PFA-100; rapid platelet function assay-RPFA; plateletworks, VerifyNow (Accumetrics) P2Y12, Table. 2.1). In general, they assess *ex vivo* platelet aggregation in response to agonists. Although relatively quick and easy to use, there is a lack of standardisation and validation with other more established methods. They do not provide a representation of *in vivo* platelet activation, are mostly utilised to assess efficacy of

anti-platelet therapy, and as such only represent a single aspect of platelet activation and function.

However, the European Society of Cardiology (ESC) has incorporated a recommendation under class IIb (B) to consider platelet function testing. The guidelines also include a second recommendation under class IIb (B) that addresses modifying treatment strategies based on platelet reactivity measurements in selected cases [Patti G *et al*, 2008].

However, the accuracy of point-of-care assays is still unclear. The VerifyNow-$P2Y_{12}$ test was designed to overcome the limitations of conventional optical platelet aggregation assays. It is a rapid test that uses ADP to stimulate platelets in the presence of prostaglandin (PG) E_1, which inhibits activation downstream of a second ADP receptor $P2Y_1$, thus making the assay more sensitive to the activity of $P2Y_{12}$. The test can be performed directly in the catheterization laboratory prior to percutaneous coronary intervention (PCI).

Early prospective, observational studies suggested that the VerifyNow P2Y12 assay could provide prognostic information in clopidogrel-treated patients undergoing PCI [Patti G *et al*, 2008; Price MJ *et al*, 2008; Marcucci R *et al*, 2009].

Some authors reported good correlation between the VerifyNow P2Y12 assay and ADP-induced platelet aggregation by LTA [Paniccia R et al, 2007; Malinin A et al, 2006]. However, more recent studies report poorer concordance. The dynamic range appears narrower than that of LTA; therefore, the assay may not be able to discriminate between very strong or between very weak levels of P2Y12 receptor inhibition [Jakubowski JA *et al*, 2008]. In addition, the percent inhibition reported by the device as a surrogate for the degree of P2Y12-mediated inhibition without a baseline pre-clopidogrel sample maybe inaccurate compared with the actual change before and after clopidogrel exposure.

Table 2.1. Platelet Function Assays for the Effect of Thienopyridines

Assay	Components	Measurement	Assay Limitations	Clinical Data
PFA-100	Analyzer device, disposable cartridge	Time to occlusion of aperture in collagen/ADP-coated membrane under high shear stress conditions	Cannot distinguish thienopyridine effect	No
Plateletworks	ICHOR cell counter, EDTA tube, ADP tube	Difference in single platelet counts after stimulation with ADP vs baseline	Results highly dependent on time between sample collection and testing	No
VerifyNow P2Y12	Instrument, disposable cartridge	Agglutination of fibrinogen-coated beads by platelets in the presence of ADP and PGE_1	Potential inaccuracy of surrogate measurement of percent inhibition without baseline preclopidogrel sample	Yes
TEG platelet mapping	Instrument, computer interface, software, reagents, disposable pin and cup	Platelet contribution to clot strength with ADP stimulation	Method requires substantial manual pipetting of several reagents and blood sample	Yes
TEG indicates thrombelastography.				

Flow Cytomtery

Although more labour intensive, flow cytometry is emerging as the new sensitive gold standard. It requires only a small sample volume (around 2μL), is performed on the more physiological milieu of whole blood, and the minimal manipulation of samples prevents artifactual in vitro platelet activation and loss of sub-populations. Flow cytomtery can be used to assess a variety of platelet abnormalities and functions. With the use of fluorescent labelled anti-bodies platelet activation can be measured by detection of activation dependent molecules on the platelet surface (e.g P-selectin, GP IIb-IIIa), platelet surface bound proteins (such as fibrinogen), platelet-monocyte aggregates and pro-coagulant platelet-derived microparticles. In addition, with the exogenous addition of agonists, the reactivity of circulating platelets can also be determined.

Disadvantages include the expensive equipment required and the complicated sample preparation. *Ex vivo* platelet activation can still occur, especially if there is a delay between blood draw and processing. Flow cytometry also only assesses circulating platelets. If activated platelets are rapidly cleared from the circulation or are adherent to blood vessel walls or extracorporeal circuits, artefactually low measurements are reported. However, it remains a highly sensitive method for assessment of in vivo platelet activation in comparison to other techniques.

2.3 FLOW CYTOMETRIC ASSESSMENT OF PLATELET ACTIVATION

2.3.1 General principles of flow cytometry

Flow cytometry utilises the principles of light scattering and light emission from fluorochrome molecules to provide rapid analysis of multiple characteristics of single cells (in the size range 0.5μm to 40 μm).

Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by right-angle scatter) can resolve different cell populations. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labelled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to there resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths but different emission wavelengths, allows several cell properties to be measured simultaneously.

In the flow cytometer, cells in suspension are drawn into a stream created by a surrounding sheath of isotonic fluid that creates laminar flow, forcing the cells to pass individually through an interrogation point. At this point, a beam of monochromic light, usually from a laser, intersects the cells. Emitted light is given off in all directions and is collected via optics that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands. The light signals are detected by photomultiplier tubes and are digitized for computer analysis, Figure 2.1.

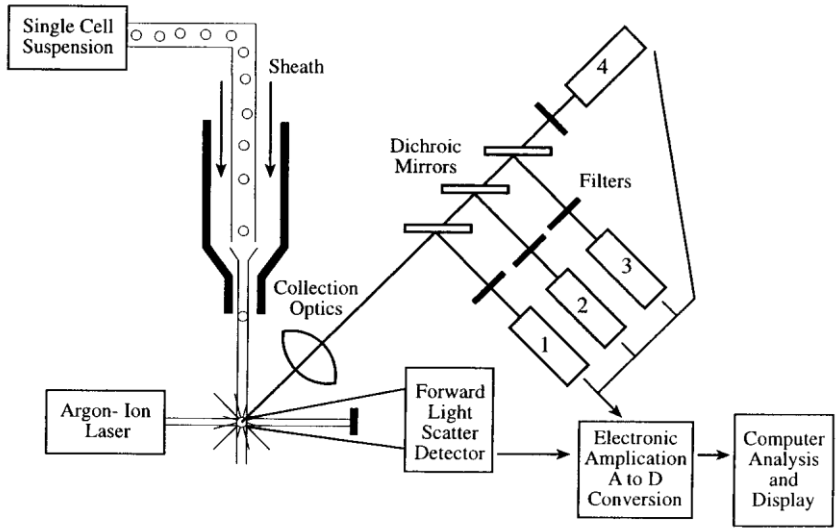


Figure 2.1

Hydrodynamic focusing in flow cytometry

The different light signals are converted into electrical pulses, which are processed by a series of linear and log amplifiers. Logarithmic amplification is often used to measure fluorescence in cells. After the different pulses are amplified they are then processed by an analog to digital converter, which allows for events to be plotted on a graphical scale (one-parameter or two-parameter histograms).

A one-parameter histogram, is a graph of cell count on the y-axis and the measurement parameter (voltage generated at a certain wavelength) on the x-axis, Figure 2.2. A two-parameter histogram is a graph representing two measurement parameters (e.g voltages generated at 2 wavelengths by 2 fluorochromes) on the x and y axes and cell count height on a density gradient. This is like a topographical map, Figure 2.3 [Givan AL *et al*, 1992; Brown M *et al*, 2000]

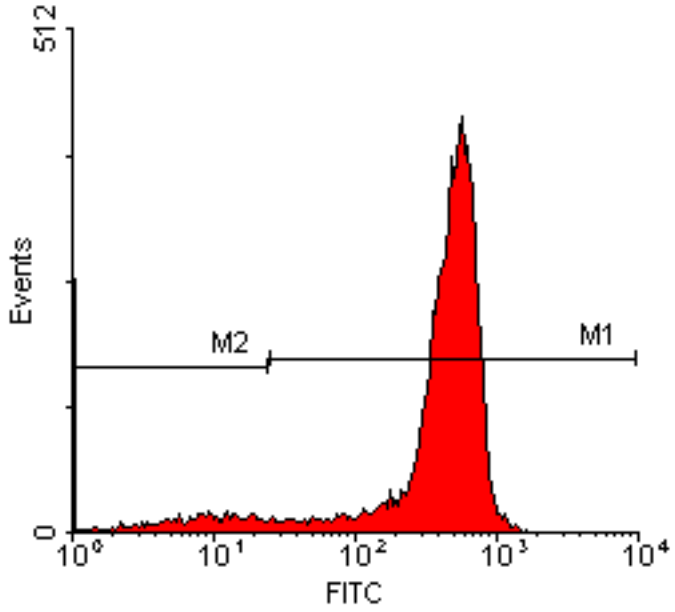


Figure 2.2 One-parameter histogram

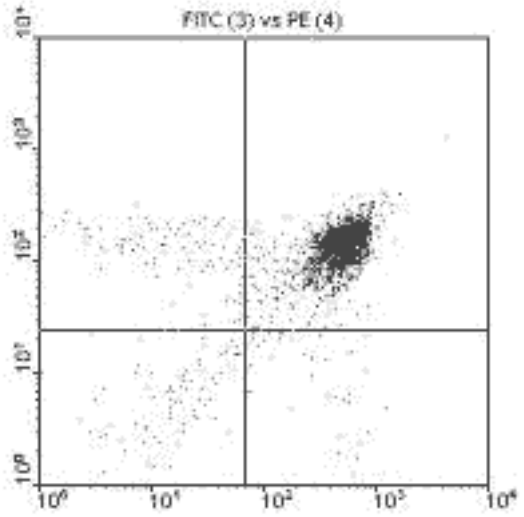


Figure 2.3 Two-parameter histogram

2.3.2 Sample preparation for flow cytometry

Clinical studies that utilise washed platelets or platelet-rich plasma for flow cytometry, are, like other assays of platelet function, potentially susceptible to artifactual in vitro platelet activation as a result of the obligatory separation procedures. However, flow cytometry can be performed on whole blood and therefore improve the sensitivity of the technique [Shattil SJ *et al*, 1987]. The general stages of sample preparation for whole blood flow cytometry are depicted in Figure 2.4.

Blood sampling

The technique of blood drawing is important as it can result in artefactual *in vitro* activation of platelets. For this reason, in our studies, blood was drawn by venepuncture of a large anticubital vein using a 19-gauge needle. Care was taken to ensure a smooth blood draw without venous stasis, and avoid artefactual activation by turbulent flow through a cannula. Our centre has previously reported that our technique of sampling results in reproducible measurements in healthy volunteers [Harding S.A *et al*, 2007].

Anticoagulation

If platelets are aggregated, the amount of antigen per platelet cannot be determined. Various measures to reduce platelet aggregation include smooth sample technique, reduced agitation of sample, prompt processing and anti-coagulation, dilution and fixation. The anticoagulant is often buffered sodium citrate, however, our centre has previously shown platelet-monocyte aggregation to be increased by heparin and reduced by sodium citrate and EDTA [Harding S.A, et al, 2007]. In our studies, samples for the assessment of *in vivo* platelet activation were collected into tubes containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK, Cambridge Biosciences).

Dilution

The purpose of the dilution stage is to minimise the formation of platelet aggregates. This is important as flow cytometry measures the amount of fluorescence per individual particle, irrespective of whether the particle is a single platelet or an aggregate of an unknown number of platelets. Further aggregation can be minimized by a smooth blood draw through a large gauge needle, avoidance of washing steps and avoidance of delays in procedure.

Fixation

Fixation is typically with 1% paraformaldehyde. The purpose of fixation is to prevent artifactual *in vitro* platelet activation. Once blood is drawn, in vitro platelet activation is time-dependent, so a greater length of time until sample analysis can lead to an increase in measures of platelet activation [Michelson AD *et al*, 1996]. Fixation is therefore advantageous if there is no immediate access to a flow cytometer. Antibody labelling can be performed before or after fixation, however the binding of activation dependent antibodies to fixed platelets is often decreased in comparison to unfixed platelets [Michelson AD *et al*, 1996]. In our studies, all samples were processed immediately, with immunolabelling of whole blood within 5 min of collection and prior to fixation.

Antibody labelling

Different epitopes reflect different aspects of platelet activation. Therefore use of different labelled antibodies may distinguish specific activation profiles. Monoclonal antibodies are preferable to polyclonal antibodies in whole blood flow cytometry as they are more reliable at saturating all specific epitopes and result in less non-specific binding. Platelet-specific antibodies are available from several commercial sources already conjugated to fluorochromes such as fluorescein isothiocyanate (FITC) or phycoerythrin (PE). This eliminates the need for

an additional conjugation step in sample preparation requiring additional antibodies, that may result in increased background fluorescence and decreased sensitivity.

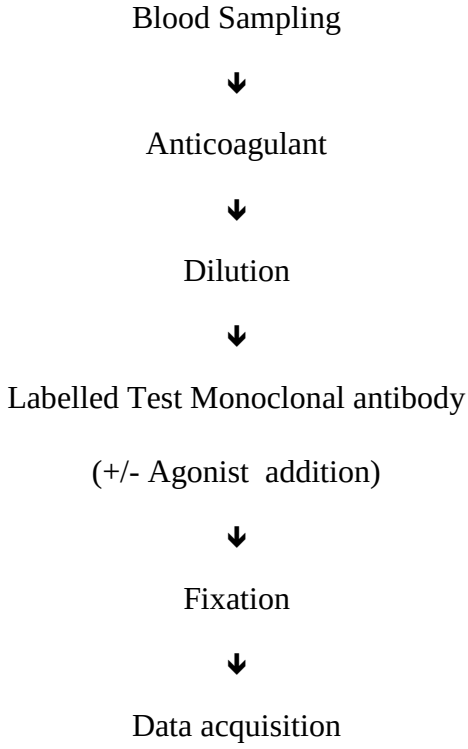
Platelets can be detected in whole blood by light scatter only (i.e identified by size and shape). However, sometimes, some particles falling within the light scatter gate for platelets may not bind any platelet-specific monoclonal antibody. Therefore a 2 colour/ 2 antibody technique can be used – one antibody to identify the platelet as a platelet and another to quantify the glycoprotein of interest.

The saturating concentration of each antibody for platelet binding must also be determined. This is usually between 1 and 20 μg/mL. Increasing concentrations of antibody should be titred to establish the minimal concentration of antibody required to produce saturation of cell binding sites. This is done to optimize the separation of positive and negative cell populations. For many surface and intracellular markers, the positive peak will not be clearly distinct from the negative peak, so titrating antibodies can significantly improve this. If the antibody concentration is below saturation, this will appear as very dim staining at low antibody concentrations.

In addition, control antibodies to examine non-specific binding can also be used. During staining, conjugated antibodies can bind to cells that would not specifically bind the antibody – i.e do not actually express the antigen of interest. In order to assess this level of background binding, isotype control antibodies of the same species class as the primary antibody and conjugated to the same fluorochrome, should be used. Ideally their specific saturation concentration should also be determined. The use of isotype controls can be used to set gates during flow cytometric acquisition.

Figure 2.4

General stages of sample preparation for whole blood flow cytometry



2.4 FLOW CYTOMETRIC PROTOCOL

We utilised the following stages in our flow cytometric assessment of platelet and monocyte activation markers

Blood collection and anticoagulation

Blood was drawn by venepuncture of a large anticubital vein using a 19-gauge needle. Care was taken to ensure a smooth blood draw without venous stasis, and avoid artefactual activation by turbulent flow through a cannula. Samples were all processed immediately.

Blood samples for assessment of platelet-monocyte aggregates, platelet expression of P-selectin and monocyte CD40 and 11b expression, were collected into tubes containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK, Cambridge Biosciences). Our centre has previously shown platelet-monocyte aggregation to be increased by heparin and reduced by sodium citrate and EDTA [Harding S.A *et al*, 2007]. Flow cytometric measurement of platelet microparticles was performed on platelet poor plasma (PPP). Blood was collected into 10 mL sodium citrate tubes and PPP prepared by centrifugation at 2000 *g* at 4 ^{0}C for 10 min and confirmed by a platelet count of $<10^{9}$/L / (dilution with autologous plasma as required). Blood for the assay assessment of platelet microparticles and soluble P-selectin and CD40L was collected into tubes containing sodium citrate.

Immunolabelling, fixation and flow cytometry

Flow cytometric measurements of platelet-monocyte aggregation, platelet surface expression of P-selectin, and monocyte CD40 and 11b expression were performed as described previously [Sarma J *et al*, 2002; Harding SA *et al*, 2004].

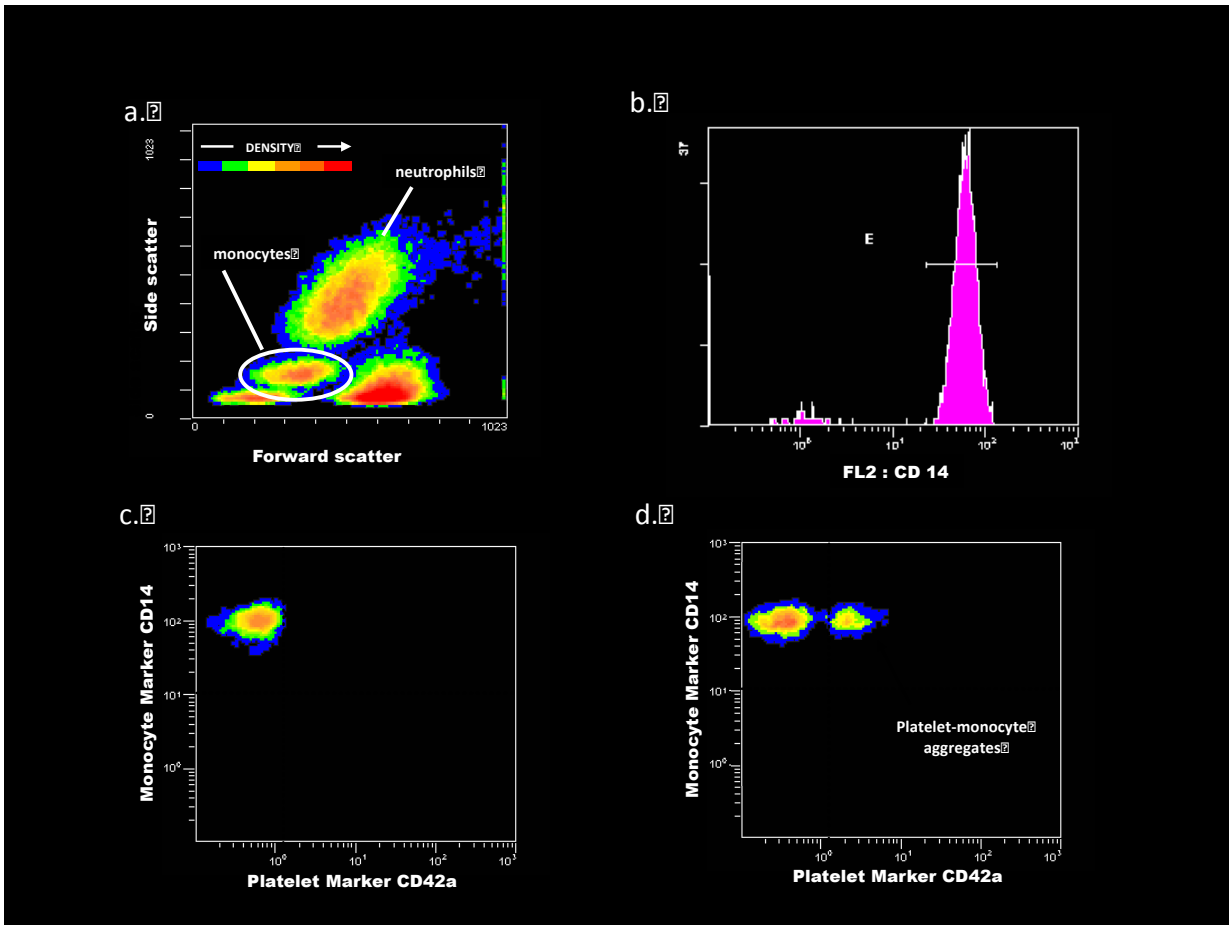
Immunolabelling was performed in whole blood within 5 min of collection. Directly conjugated monoclonal antibodies were obtained from DakoCytomation (Cambridge, UK) and Serotec (Oxford, UK).

In order to assess platelet-monocyte aggregates, 60 µL of blood were incubated for 15 min with a FITC-conjugated anti-CD42a monoclonal antibody (GRP-P, platelet marker) and a PE-conjugated anti-CD14 monoclonal antibody (Tuk-4, monocyte marker) before fixation and erythrocyte lysis with 500 µL of FACSLyse solution. Samples were processed using a BeckmanCoulter flow cytometer and at least 2,500 cell events were analysed by EXPO32 software. Platelet-monocyte aggregates were detected by gating for cells that were positive for both CD14 PE and CD42a FITC (Figure 2.5).

Platelet surface expression of P-selectin was assessed by gating for cells that were positive for both FITC-conjugated anti-CD42a monoclonal antibody, (platelet marker), and PE conjugated anti-CD62P monoclonal antibody (TRAP 1, IgG1). Isotype controls were used to reduce error from non-specific binding.

To evaluate CD40 and CD11b on monocytes, blood was diluted 1:2 with PBS and incubated with the following monoclonal antibodies: anti-CD14:FITC (Serotec), anti-CD40:PE (Serotec), anti-CD11b:PE (Serotec) and appropriate isotype-matched controls for 20 min**.**

Figure 2.5 Analysis of platelet-monocyte aggregates



In this flow cytometric profile, whole peripheral blood has been analysed by light scatter properties, indicating cellular size and morphology, and showing a mixed leukocyte population (**2.5a**). By specifically staining for CD14, a surface marker of human monocytes, this distinct sub-population of leukocytes may be highlighted (**2.5b**). Further counter-staining using CD42a, a surface platelet marker (GP IX), allows us to distinguish those monocytes that have bound platelets to their surface, (**2.5d**).

2.5 ASSAYS OF INFLAMMATORY PROTEINS, TROPONIN AND PLASMA PLATELET ACTIVATION MARKERS

Highly Sensitive C-reactive Protein (hsCRP)

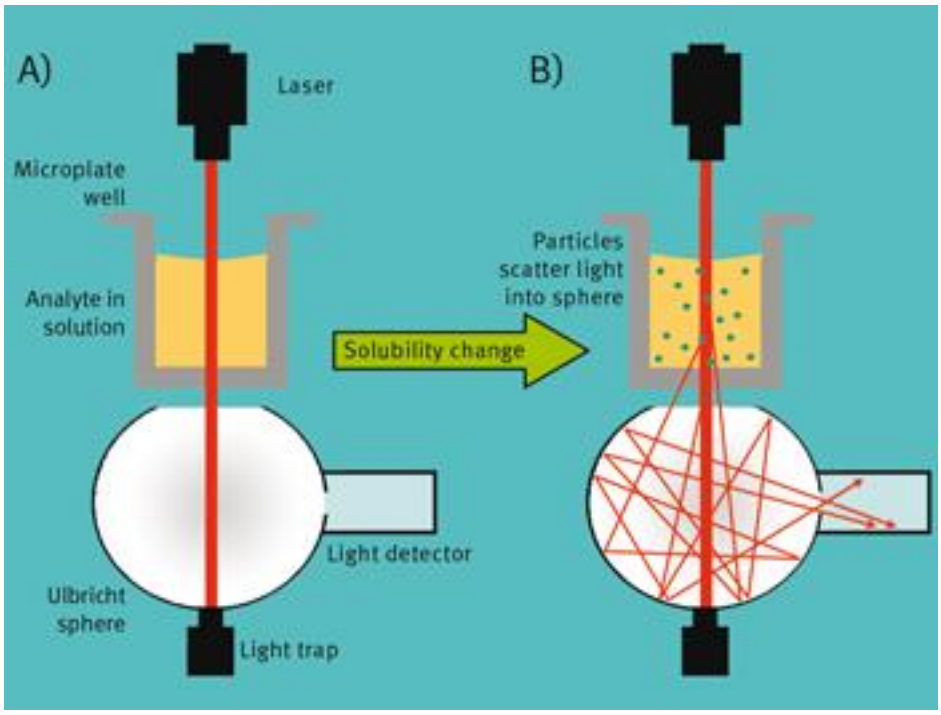
Serum hsCRP concentrations were determined with a validated highly sensitive assay (Department of Clinical Biochemistry; Fife NHS Trust, UK) using the method of particle-enhanced immunonephelometry (Behring BN II nephelometer, Dade Behring Inc).

Nephelometry is based on the principle that a dilute suspension of small particles will scatter light (usually a laser) passed through it rather than simply absorbing it. The amount of scatter is determined by collecting the light at an angle (usually at 30 and 90 degrees). In dilute solutions, where absorption and reflection are minimal, the intensity of the scattered light is a function of the concentration of scattering particles, Figure 2.6.

Antibody and the antigen are mixed in concentrations such that only small aggregates are formed that do not quickly settle to the bottom. The amount of light scatter is measured and compared to the amount of scatter from known mixtures. The amount of the unknown is then determined from a standard curve.

In our assay (Dade Behring Inc), particles consisting of a polystyrene core and a hydrophilic shell were used in order to link anti-CRP antibodies covalently. A dilute solution of the test sample was mixed with latex particles coated with mouse monoclonal anti-CRP antibodies. CRP present in the test sample formed an antigen-antibody complex with the latex particles. Light scattering was measured by a nephelometer after 6 min, and was proportional to the concentration of the analyte present in the sample.

Figure 2.6 Principle of particle-enhanced nephelometry



The Dade Behring method is one of the most commonly used in the field of acute cardiac care, enabling detection of CRP concentrations at the micro-inflammatory range. This assay has a detection limit of 0.18mg/L. Intra-assay and inter-assay coefficients of variability for hsCRP were 3.7% and 4.2% respectively. All assays were performed in duplicate and the mean value taken. Whole blood samples for the measurement of hsCRP were collected into citrated tubes, centrifuged and plasma collected at each time point. The serum was frozen and stored until the end of the trial when samples were processed en masse.

Myocardial Injury

Cardiac Troponin-I (cTnI) is a regulatory subunit of the troponin complex associated with the actin thin filament within muscle cells.1 cTnI, in conjunction with troponin-C and troponin-T, plays an integral role in the regulation of muscle contraction. These three distinct tissue specific isoforms of Troponin have been identified from skeletal and cardiac muscles. However, the cardiac isoform (cTnI) exhibits only 60% similarity with the skeletal muscle isoform. Clinical studies have demonstrated the release of cTnI into the blood stream within hours following myocardial infarction (MI) or ischemic damage. Elevated levels of cTnI (above the values established for non-MI specimens) are detectable in serum within 4 to 6 hours after the onset of chest pain, reach peak concentrations in approximately 8 to 28 hours, and remain elevated for 3 to 10 days following MI. Cardiac troponin I is the preferred biomarker for the detection of myocardial injury based on improved sensitivity and superior tissue-specificity compared to other available biomarkers of necrosis, including CK-MB, myoglobin, lactate dehydrogenase, and others.

The World Health Organization (WHO) criteria for defining myocardial infarction are the presence of two of the following three elements: ECG changes, serum cardiac enzyme changes, and prolonged chest pain. More recently, a Global Task Force with joint leadership among the European Society of Cardiology (ESC), the American College of Cardiology Foundation (ACCF), the American Heart Association (AHA), and the World Heart Federation (WHF) refined past criteria with a universal definition of myocardial infarction that also supports use of cardiac Troponin I as a preferred biomarker for myocardial injury. This has nearly absolute myocardial tissue specificity as well as high clinical sensitivity, thereby reflecting even microscopic zones of myocardial necrosis. Their universal definition of myocardial infarction is a typical rise and gradual fall of cardiac biomarkers (preferably troponin) with at least one

value above the 99th percentile of the upper reference limit (URL) together with evidence of myocardial ischemia with at least one of the following: ischemic symptoms, pathological Q waves on electrocardiogram (ECG), ischemic ECG changes, or imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.

In our studies a blinded independent cardiologist reviewed all clinical data and applied the universal definition of myocardial infarction [ESC/ACC/WHF, 2007]. An increased value for cardiac troponin I is defined as a measurement exceeding the 99th percentile of a normal reference population (URL = upper reference limit). The above-mentioned discriminatory percentile is designated as the decision level for the diagnosis of myocardial infarction, and must be determined for each specific assay with appropriate quality control. Optimal precision [coefficient of variation (CV)] at the 99th percentile URL for each assay should be defined as <10%. To establish the diagnosis of myocardial infarction, one elevated value above the decision level is required.

In our studies, the reference clinical biochemistry laboratory measured absolute plasma cardiac troponin I (cTnI) concentrations using the ARCHITECT Troponin I *STAT* assay (Abbott Diagnostics, Maidenhead, UK) using an autoanalyser. This is a sensitive cTnI assay, which has been shown to have a high diagnostic accuracy.

This assay is a two-step immunoassay to determine the presence of cTnI in human serum and plasma. In the first step, sample, assay diluent and anti-troponin-I antibody-coated paramagnetic microparticles are combined. Cardiac Troponin-I present in the sample binds to the anti-troponin-I coated microparticles. After incubation and wash, anti-troponin-I acridinium-labeled conjugate is added in the second step. Following another incubation and

wash, pre-trigger and trigger solutions are then added to the reaction mixture. The resulting chemi-luminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of cTnI in the sample and the RLUs detected by the ARCHITECT *i** System optics. The concentration of cTnI is read relative to a standard curve established with calibrators of known cTnI concentrations. Heterophilic antibodies in human serum can react with reagent immunoglobulins, causing anomalous values to be observed.

This assay has an analytical sensitivity of 0.009 ng/mL and a functional sensitivity of 0.032 ng/mL with a co-efficient of variation of <10%. This was the manufacturers guide, and was achieved by our host laboratory. The latter threshold was employed for the clinical case definition of myocardial infarction.

Analytical sensitivity is defined as the concentration at two standard deviations above the ARCHITECT *STAT* Troponin-I Calibrator A (0.00 ng/mL, 0.00 μg/L) grand mean and represents the lowest concentration of troponin that can be distinguished from zero (i.e the limit of detection). The ARCHITECT *STAT* Cardiac Troponin-I assay analytical sensitivity is ≤ 0.01 ng/mL (≤ 0.01 μg/L) at the 95% level of confidence.

Whole blood samples for the measurement of cardiac Troponin I were collected into citrated tubes, centrifuged and plasma collected at each time point. The plasma was frozen and stored until the end of the trial when samples were processed en masse using the same assay. Comprehensive evidence of cardiac injury was therefore blinded until the end of the trial.

Soluble P-selectin and CD40L

P-selectin is found in both platelets α-granules and on endothelial cells. P-selectin is not expressed on normal resting platelets. Platelet activation leads to a rapid release and surface expression of P-selectin. It then either remains attached to the platelet surface membrane or is released into the plasma. The majority of soluble P-selectin is thought to originate from platelet granules. Therefore increased plasma soluble P-selectin has been proposed as a marker of *in vivo* platelet activation. Elevated soluble P-selectin levels have been demonstrated in patients with stable, coronary disease [Atalar E *et al*, 2000] and in women who have subsequently developed cardiovascular events [Ridker PM *et al*, 2001]. However, although soluble P-selectin determination in plasma can be performed easily by a standard ELISA method, variations in anti-coagulants (heparin, EDTA, sodium citrate in different concentrations) and pipetting and washing techniques can influence the outcome level. In addition, groups have reported a lack of agreement between soluble levels of P-selectin and platelet bound P-selectin [Gurbel PA *et al*, 2000].

In our studies we used a solid phase sandwich ELISA from R and D systems to detect human soluble P-selectin (CD62P) in plasma. This had a lower detection limit of 0.5ng/mL, an assay range of 0.82-45.94ng/mL and a mean intra-assay co-efficient of variation of 5% and a mean inter-assay co-efficient of variation of 10%.

The soluble form of CD40 ligand (sCD40L) has emerged as a diagnostic and prognostic marker for cardiovascular events. Platelets are the main source of sCD40L, being responsible for >95% of circulating sCD40L levels [Heeschen C *et al*, 2003]. However, as platelets can shed CD40L upon activation, accurate measurement has proved challenging. Methodological issues regarding the measurement of circulating sCD40L levels in humans are thought to explain the variation in results in results between clinical studies. sCD40L concentrations are

much higher in serum than in plasma, because platelet CD40L is released during clotting [Ahn ER *et al*, 2004; Weber M *et al*. 2006]. Moreover, sCD40L increases during pre-analytical sample processing, suggesting that in vitro platelet activation may account for the release of sCD40L [Halldorsdottir AM *et al*, 2005].

We used a solid phase sandwich ELISA from BenderMed Systems, which detects human soluble CD40L in plasma. This has a lower detection limit of 10.1pg/mL; an assay range of 62.5-4000pg/mL and a mean intra-assay co-efficient of variation of <8% and a mean inter-assay co-efficient of variation of <10%.

Whole blood samples for the measurement of soluble P-selectin and CD40L were collected into EDTA tubes, centrifuged and plasma collected at each time point. The plasma was frozen and stored until the end of the trial when samples were processed en masse using the same assay.

Platelet-micro-particles

Quantification of PMPs is usually performed with flow cytometry. Flow cytometry requires fresh or fixed blood, and is unable to detect PMPs with a diameter less than 0.1 μm. Through collaboration with Michelsen AE *et al* at the Research Institute for Internal Medicine, Oslo, Norway we also sent frozen plasma samples to be assessed for platelet-microparticles utilizing their new time-resolved immunofluorometric assay. This quantifies the amount of PMP-located GPIIb antigen in detergent-treated platelet-free plasma (PPP) and has been shown to correlate well with flow cytometry. Details of this assay can be found in the methods section of Chapter three.

2.5 ETHICAL CONSIDERATIONS

Patients and subjects were recruited between June 2005 and February 2008, and gave written informed consent prior to study participation. All studies were undertaken in accordance with the regulations of the Lothian Research Ethics Committee (Eudract Number: 2005-000960-25, REC reference number: 05/S0501/41; Granted 29/04/05) and with the Declaration of Helsinki of the World Medical Association. The prospective double-blind randomised controlled trial was given Clinical Trial Authorisation by the Medicines and Healthcare products Regulatory Agency (UK), CTA number: 11449/0002/001-0001 (Granted 05/04/05). The study was registered on the International Standard Randomised Controlled Trial Number Register. International Standardised RCT: ISRCTN22305120.

Weblink: http://www.controlled-trials.com/ISRCTN22305120

2.6 SAMPLE SIZE CALCULATION

The primary end-point of our intervention study was platelet-monocyte aggregation. The sample size (n=50 per group) was based on our previous studies [Sarma J *et al*, 2002; Harding SA *et al*, 2004] and gave an 80% power of detecting an absolute difference of 4.8% in platelet-monocyte aggregates at a significance level of 5%. Mechanistic studies of dual anti-platelet therapy in patients with acute coronary syndromes have shown relative reductions in platelet markers of around 30% with additional clopidogrel therapy [Xiao Z *et al*, 2004]. Clinically, in patients with non–ST elevation MI, additional clopidogrel therapy leads to a risk reduction of 20% less adverse cardiovascular events [Fox KA *et al*, 2004]. Hence, there is evidence to suggest that even moderate reductions in platelet activation have clinical benefits.

This sample size also gave an 80% power of detecting a 3.6% difference in CD40 expression on monocytes, 3.7% in CD40L expression on platelets, and 0.11 ng/mL in plasma sCD40L concentrations, at a significance level of 5%. Secondary outcomes included plasma troponin concentration, and bleeding complications. This study was not powered to examine clinical end-points, which would require a much larger scale clinical trial.

2.7 DATA AND STATISTICAL ANALYSIS

The Bland Altman method was used to analyse the differences between paired measurements and to test the reproducibility of each measurement [Bland JM *et al*, 1986]. The co-efficient of reproducibility was calculated as 1.96 x the standard deviation of the differences between pairs of measurements in the same subjects. Correlation between variables was analysed using Pearson and Spearman's correlation coefficients as appropriate. Continuous variables were reported as mean ± SD. Analysis of variance with repeated measures, two-tailed Student's *t*-test and Chi-squared analysis were performed as appropriate using GraphPad Prism Version 4 (La Jolla, USA). Statistical significance was taken as a two-sided P value <0.05.

CHAPTER 3

PLATELET ACTIVATION IN PATIENTS WITH PERIPHERAL ARTERIAL DISEASE: REPRODUCIBILITY AND COMPARABILITY OF PLATELET MARKERS

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3.1 Summary

Many markers of platelet activation have been described but their reproducibility and comparability in patient populations are poorly defined. We sought to compare markers of platelet and monocyte activation with platelet-monocyte aggregates, a proposed gold standard of *in vivo* platelet activation, and assess their reproducibility in patients with peripheral arterial disease: a population with substantial platelet activation, inflammation and risk of thrombotic events.

Thirty patients with peripheral vascular disease attended on two occasions to permit within-day and between-day comparisons. *In vivo* platelet and monocyte activation were determined by flow-cytometric quantification of platelet-monocyte aggregation, platelet surface expression of P-selectin and CD40L, platelet-derived microparticles, and monocyte surface expression of CD40 and CD11b. Plasma concentrations of platelet-derived microparticles, soluble P-selectin and CD40L were measured by enzyme-linked immunosorbant assays.

Platelet-monocyte aggregation (36.7±7.86%), and platelet surface expression of P-selectin (5.8±1.65%) and CD40L (3.3±1.45%) demonstrated comparable within-day (mean difference±co-efficient of reproducibility; 0.9±15.4%, 0.21±1.65% and 0.2±2.8% respectively) and between-day reproducibility (2.0±12.4%, 0.10±2.25% and 0.9±6.4% respectively). Platelet-monocyte aggregates correlated well with other platelet ($r=0.30$-0.50, $P<0.02$) and monocyte ($r=0.27$-0.47, $P<0.03$) activation markers. Flow cytometric and assay quantified platelet-derived microparticles showed poorer reproducibility (co-efficient of reproducibility >40%).

In conclusion, in patients with peripheral arterial disease, measurements of platelet-monocyte aggregates have good reproducibility and consistently reflect other markers of platelet and monocyte activation.

3.2 Introduction

Atherothrombosis is the leading cause of mortality in the western world. Platelets play a major role in the inflammatory and thrombotic progression of atherosclerosis [Freedman JE *et al*, 2002, McGregor L *et al,* 2006]. Indeed, increased platelet activity is present in patients at increased risk of atherothrombotic events and predicts adverse cardiovascular events [Harding SA *et al,* 2004; Cassar K *et al*, 2003; Sarma J *et al*, 2002; Lee YJ *et al*, 1993; Bernal-Mizrachi L *et al*, 2003]. The measurement of platelet activity is therefore crucial to our understanding of the pathophysiology of atherothrombosis, the prediction of adverse cardiovascular events, and the development of novel therapeutic interventions.

Despite the development of several techniques, there is still no generally accepted ideal measure of platelet activation. A variety of methods exist, including platelet aggregometry, point of care devices, flow cytometric assessment of platelet surface antigens, and plasma markers of platelet activation: all have advantages and disadvantages [Michelson AD, 2009]. Historically considered the gold standard, platelet aggregometry requires the preparation of platelet rich plasma and a high sample volume. Centrifugation and washing procedures may produce cell loss and artefactually activate platelets. Many of the point-of-care systems assess *ex vivo* platelet aggregation to various exogenous agonists. Although more labour intensive, flow cytometry is emerging as the new sensitive gold standard with measurement of surface expression of platelet antigens providing an assessment of *in vivo* platelet activation. It requires only a small sample volume, is performed on whole blood, and allows analysis of platelets in their physiological milieu. One of the most commonly studied markers of platelet activation is the α-granule membrane protein, P-selectin, that is present only on the surface of activated degranulated platelets. However, *in vivo* degranulated platelets rapidly lose their surface P-selectin, but continue to circulate and function [Michelson AD, *et al*, 1996]. In

contrast, P-selectin-positive platelets very rapidly bind to leucocytes (mainly monocytes) via their constitutively expressed counter receptor, P-selectin glycoprotein ligand-1 (PSGL-1) [Sarma J *et al*, 2002]. Hence circulating platelet-monocyte aggregates provide a more sensitive measure of *in vivo* platelet activation [Michelson AD *et al*, 2001]. In addition platelet-monocyte aggregation may alter leucocyte function, causing monocyte arrest on the endothelium and potentially enhance the growth of atherosclerotic plaques [Huo Y *et al*, 2003].

Many methods and protocols for the measurement of platelet activation have been described [Tocchetti EV *et al*, 2001; Perez-Pujol S, 2007; Li N *et al*, 1997; Hagberg IA *et al*, 2000; Barnard MR *et al*, 2003]. Importantly, aspects of the techniques themselves can lead to artefactual platelet activation, thus altering the end result. However, there are few reports of test reproducibility. Even when studying the same platelet marker, the methodology and end unit of measurement may differ, making it extremely difficult to interpret and compare results. An appreciation of these issues is crucial for the meaningful interpretation of interventional studies, and the development of anti-platelet therapies.

In the present study we sought to assess the reproducibility of an established protocol [Harding SA *et al*, 2004; Sarma J *et al*, 2002] in measuring platelet-monocyte aggregation in a patient population expected to have elevated resting platelet activation. In addition, we wished to compare the reproducibility and correlation of other markers of platelet activation to a proposed gold standard of platelet-monocyte aggregation.

3.3 Methods

The study was performed with the approval of the local ethics committee, in accordance with the Declaration of Helsinki and the written informed consent of all participants.

Subjects

Peripheral venous blood was obtained from 30 patients with peripheral arterial disease. Inclusion criteria were (i) symptoms of claudication, without rest pain or ulceration, (ii) reduced ankle brachial pressure ratio, and (iii) evidence of arterial stenosis on Doppler scanning.

Study design

In order to assess reproducibility, four samples were taken from each subject – two were taken on the same day one hour apart (within-day reproducibility), and two were taken the following day at the same time points (between-day reproducibility).

Platelet activation was assessed by measuring percent platelet-monocyte aggregation, platelet expression of P-selectin and platelet-derived microparticles using flow cytometry. Platelet-derived microparticles, and plasma soluble P-selectin and CD40L concentrations were measured by enzyme-linked immunosorbant assays (ELISAs). Monocyte activation was measured via flow cytometric measurement of percent monocyte CD40 expression and mean fluorescent intensity of monocyte CD11b expression.

Blood Collection

Blood was drawn by venepuncture of a large anticubital vein using a 19-gauge needle. Care was taken to ensure a smooth blood draw without venous stasis. Samples were processed immediately. Blood samples for assessment of platelet-monocyte aggregates, platelet expression of P-selectin and monocyte CD40 and 11b expression, were collected into tubes containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK, Cambridge Biosciences). Flow cytometric measurement of platelet microparticles was performed on platelet poor plasma (PPP). Blood was collected into 10 mL sodium citrate tubes and PPP prepared by centrifugation at 2000 *g* at 4 ^{0}C for 10 min and confirmed by a platelet count of $<10^{9}$/L / (dilution with autologous plasma as required). Blood for the assay assessment of platelet microparticles and soluble P-selectin and CD40L was collected into tubes containing sodium citrate.

Immunolabelling and flow cytometry

Flow cytometric measurements of platelet-monocyte aggregation, platelet surface expression of P-selectin, and monocyte CD40 and 11b expression were performed as described previously [Harding SA *et al*, 2004; Sarma J *et al*, 2002]. Immunolabelling was performed in whole blood within 5 min of collection. Directly conjugated monoclonal antibodies were obtained from DakoCytomation (Cambridge, UK) and Serotec (Oxford, UK). In order to assess platelet-monocyte aggregates, 60 µL of blood were incubated for 15 min with a FITC-conjugated anti-CD42a monoclonal antibody (GRP-P, platelet marker) and a PE-conjugated anti-CD14 monoclonal antibody (Tuk-4, monocyte marker) before fixation and erythrocyte lysis with 500 µL of FACSLyse solution (Sarma J *et al*, 2002). Samples were processed using a BeckmanCoulter flow cytometer and at least 2,500 cell events were analysed by EXPO32 software. Platelet-monocyte aggregates were detected by gating for cells that were positive for

both CD14 PE and CD42a FITC. Platelet surface expression of P-selectin was assessed by gating for cells that were positive for both FITC-conjugated anti-CD42a monoclonal antibody (platelet marker) and PE conjugated anti-CD62P monoclonal antibody (TRAP 1, IgG1). Isotype controls were used to reduce error from non-specific binding. To evaluate CD40 and CD11b on monocytes, blood was diluted 1:2 with PBS and incubated with the following monoclonal antibodies: anti-CD14:FITC (Serotec), anti-CD40:PE (Serotec), anti-CD11b:PE (Serotec) and appropriate isotype-matched controls for 20 min**.**

Platelet microparticles were identified by both size and expression of platelet markers CD41 (GPIIb) and CD31 (GPIIa; PECAM). Aliquots (25 µL) of PPP were incubated for 30 min with a PE-conjugated anti-CD31 monoclonal antibody and a FITC-conjugated anti-CD41 monoclonal antibody (Serotec, Oxford, UK), before dilution with phosphate buffered saline to form a volume of 1 mL. Platelet microparticles were gated according to their size (events < 1.0 µm) by assessment of their forward light scatter. TruCOUNT beads of 1.0 µm (Becton Dickenson) of a known concentration were used to calculate the volume of sample analysed over 120 s at medium flow rate. This allowed the absolute number of platelet microparticles to be measured. Isotype controls were used to reduce error from non-specific binding. Platelet microparticles were detected by gating for events that were <1 µm in size (based on forward scatter) and positive for both CD31 and CD41.

ELISAs

Platelet-derived microparticles

Platelet-derived microparticles were assessed using a time-resolved immunofluorometric assay previously reported by Michelsen *et al* (Michelson AE *et al*, 2006). This method quantifies the amount of platelet-microparticle-located CD41 (GPIIb) antigen in detergent-treated platelet-free plasma (μg/L). In brief, PPP is filtered to remove any platelet micro-particle larger than 0.1 μm (Ultra-free-MC Filter Units, Millipore, Billerica, MA, USA). The GPIIb/GPIIIa complex (CD41/CD61) is then released from the microparticle membrane and solubilized by mixing 1 volume PPP and 1 volume Delfia Assay buffer containing 1% of the non-ionic detergent Igepal CA-630. Two different monoclonal antibodies to GPIIb (CD41) are used, one labelled with Europium (Diatec, Oslo, Norway), and the other conjugated with biotin (clone DD4.1, Southern Biotechnology, Birmingham, AL, USA). Samples (50 μL) of the solubilized GPIIb/IIIa were then added to a Delfia streptavidin-coated plate (Perkin-Elmer Life Sciences, Boston, MA, USA) and 150 μL of antibody mixture added. Following incubation for 2 hours at room temperature, the wells are washed and Delfia Enhancement solution (200 μL/well) added prior to measurement of time-resolved fluorescence in a Victor^{2}1420 (Perkin-Elmer Life Sciences, Boston, MA,USA).

Soluble plasma markers

Soluble human P-selectin and CD40L were assessed using ELISAs from RnD systems and Bender Med Systems respectively.

Statistical analysis

The Bland Altman method was used to analyse the differences between paired measurements and to test the reproducibility of each measurement (Bland JM *et al*, 1986). The co-efficient of reproducibility was calculated as 1.96 x the standard deviation of the differences between pairs of measurements in the same subjects. Statistical significance was assessed using a paired student's t-test. Correlation between variables was analysed using Pearson and Spearman's correlation coefficients as appropriate. The statistical package employed was GraphPad Prism, Version 4 (La Jolla, USA). Statistical significance was taken as a two-sided P value <0.05.

3.4 Results

All 30 patients were male, aged 58±4 years and had a mean ankle-brachial pressure index of 0.38±0.18. All patients were receiving maintenance aspirin (75-300 mg daily) and statin therapy, and were current smokers.

Reproducibility

Repeated measurements were similar for all markers used (Table 3.1) P>0.05. Flow cytometric measurements of per cent platelet-monocyte aggregation, and platelet surface expression of P-selectin and CD40L, showed good reproducibility (Table 3.1), with monocyte markers demonstrating a wider standard deviation (although statistically not significant). There was minimal difference when comparing within-day and between-day reproducibility although monocyte activation markers appeared to show better within-day reproducibility. Flow cytometric-derived measures of platelet-derived microparticles appeared to demonstrate better within-day and between-day reproducibility than immunoassay-derived measures, although both measurement techniques demonstrated poor co-efficients of reproducibility in comparison to the other markers.

Correlation Between Platelet Markers

Platelet-monocyte aggregates correlated with all activation markers suggesting that it represented a reliable measure of global activation (Table 3.2, Figure 3.1). Platelet surface expression of P-selectin showed the best correlation with platelet-monocyte aggregation (r = 0.50) although platelet CD40L and platelet-derived microparticles also showed statistically significant correlations. Monocyte activation markers also correlated with platelet-monocyte aggregation (monocyte CD11b P=0.0002; monocyte CD40; P=0.0026) but not with other

markers of platelet activation ($P>0.05$), suggesting that monocyte activation may also determine platelet-monocyte aggregate formation.

Immunoassay-derived measures of platelet activation, including plasma concentrations of soluble P-selectin and soluble CD40L, demonstrated poor or no correlations with flow cytometric-derived measures of platelet activation, except platelet-monocyte aggregates (Table 3.3). Indeed, when assessing the same variable, platelet-derived microparticles or P-selectin, there was only a weak correlation between the two measures, or none at all (Table 3.3).

Table 3.1

Within-day and between-day reproducibility of platelet activation markers.

A. Within Day Reproducibility

Variable	Mean	Mean Difference	SD of difference	Co-efficient of Reproducibility	P value
PMA (%)	36.7	0.9	7.86	15.4	0.53
Platelet P-selectin (%)	5.8	0.21	1.65	3.2	0.56
Platelet CD40L (%)	3.3	0.2	1.45	2.8	0.55
PMP: Flow (no./µL)	157.2	8.1	42.4	83.1	0.81
PMP: Assay (GPIIb/µg/L)	25.9	5.7	24.2	47.4	0.28
Monocyte CD40 (%)	69.5	0.2	7.2	14.1	0.88
Monocyte 11b (MFI)	47.4	3.7	11.9	11.4	0.16
Soluble P-selectin (ng/mL)	42.3	1.2	16.8	32.9	0.74
Soluble CD40L (ng/mL)	0.6	0.1	0.35	0.6	0.46

B. Between Day Reproducibility

Variable	Mean	Mean Difference	SD of difference	Co-efficient of Reproducibility	P value
PMA (%)	37.3	2.0	6.35	12.4	0.08
Platelet P-selectin (%)	5.9	0.1	1.15	2.25	0.81
Platelet CD40L (%)	4.2	0.9	3.25	6.37	0.28
PMP: Flow (no./µL)	161.9	7.2	39.9	78.2	0.43
PMP: Assay (GPIIb/µg/L)	58.4	0.9	27.65	54.2	0.84
Monocyte CD40 (%)	69.6	2.5	24.1	47.2	0.39
Monocyte 11b (MFI)	49.9	0.8	11.45	22.4	0.68
Soluble P-selectin (ng/mL)	41.7	5.0	19.3	37.8	0.08
Soluble CD40L (ng/mL)	0.6	0.02	0.25	0.49	0.61

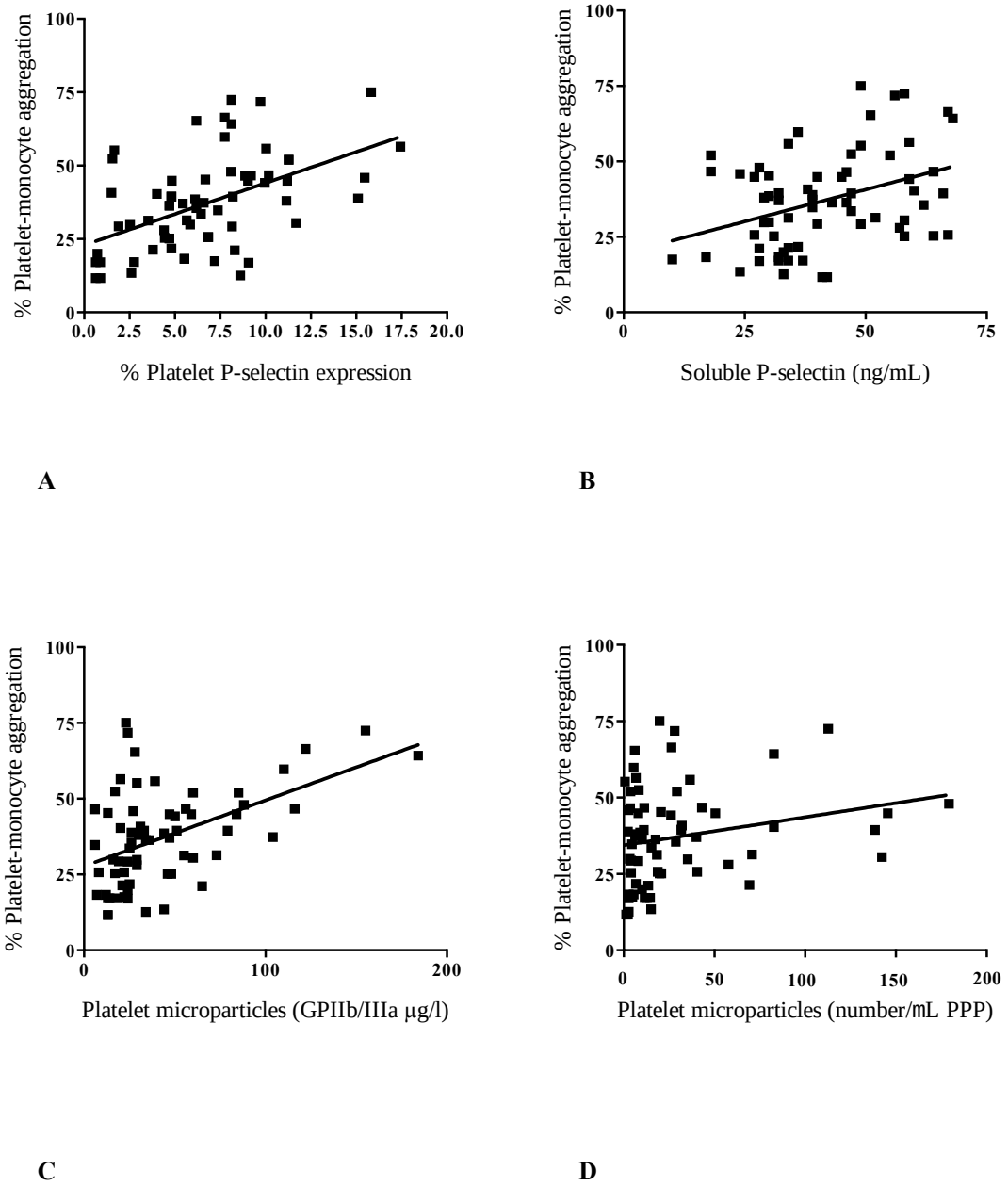
Table 3.2

Correlation of flow cytomteric-derived measures of platelet and monocyte activation.

VARIABLE	Platelet P-selectin (%)	Platelet CD40L (%)	PMP (/µL)	Monocyte CD40 (%)	Monocyte 11b (MFI)
PMA (%)	0.50 **P< 0.0001**	0.41 **P=0.0007**	0.27 **P=0.028**	0.27 **P=0.026**	0.47 **P=0.0002**
Platelet P-selectin (%)		0.52 **P< 0.0001**	0.08 P=0.11	0.04 P=0.095	0.24 P=0.07
Platelet CD40L (%)			0.03 P=0.88	0.00 P=0.96	0.42 **P=0.0013**
PMP (/µL)				0.00 P=1.0	0.08 P=0.98
Monocyte CD40 (%)					0.16 P=0.22

Pearson and Spearman correlation coefficient as appropriate

Figure 3.1



Comparison of percent platelet-monocyte aggregation *versus* (**A**) per cent platelet P-selectin expression (r=0.50 , P<0.0001), (**B**) plasma soluble P-selectin concentrations (ng/mL; r=0.37, P=0.002) (**C**) immunoassay-derived measures of platelet microparticles (µg/L; r =0.44, P=0.0002), and (**D**) flow cytometric-derived measures of platelet microparticles (number/µL; r=0.27, P=0.028).

Table 3.3

Comparison of Immunosorbant Assay-Derived and Flow Cytometric-Derived Markers of Platelet Activation

	Immunoassay			
Flow Cytometry		**PMP: Assay (GPIIb µg/L)**	**Soluble P-selectin**	**Soluble CD40L**
	PMA (%)	**r=0.44 P=0.0002**	**r=0.37 P=0.002**	**r=0.30 P=0.018**
	Platelet P-selectin (%)	**r=0.30 P=0.015**	r=0.05 P=0.69	r=0.04 P=0.73
	Platelet CD40L (%)	r=0.14 P=0.27	r=0.20 P=0.11	r=0.14 P=0.27
	PMP: Flow (no./µL)	**r=0.39 P=0.015**	r=0.03 P=0.41	r=0.08 P=0.19

Pearson or Spearman correlation coefficient as appropriate

3.5 Discussion

We have assessed the reproducibility and consistency of platelet activation markers in a patient population with high baseline levels of platelet activation. We have shown that most measures of platelet activation in patients with peripheral arterial disease have good reproducibility both within and between days. Platelet-monocyte aggregates appear to consistently reflect other markers of platelet and monocyte activation. We suggest that platelet-monocyte aggregates are reproducible and provide the best measure of *in vivo* platelet activation for clinical study.

Several reports have demonstrated an association between increased platelet activation and adverse cardiovascular events [Harding SA *et al*, 2004; Cassar K *et al*, 2003; Sarma J *et al*, 2002; Lee YJ *et al*, 1993; Bernal-Mizrachi L *et al*, 2003]. Proof-of-concept studies hypothesise that reductions in platelet activation by anti-platelet regimes should correlate with improved clinical outcomes. It is therefore useful to quantify the degree by which antiplatelet medications reduce platelet activation markers. However, clinically meaningful interpretation of these studies must account for the normal variation in marker levels that occur within patient populations and that associated with measurement techniques. A variety of methods are currently employed for the assessment of platelet activation and the reproducibility of techniques is poorly reported. Certain processing stages in the assessment of platelet activation markers can contribute to artefactual activation. Differences in technique make it difficult to compare results across studies, even when examining the same subject population. In addition, units of measurement often vary (e.g % platelet expression, versus mean fluorescent intensity). Therefore, for a given patient population, the range of platelet activation levels often varies widely between studies**.** If platelet activation markers are to be utilised in guiding

interventional studies, readers must have a clear appreciation of the limits of the methological techniques, and how they compare against an accepted 'gold standard'.

In the present study, we utilised a two-colour whole blood technique incorporating erythrocyte lysis and fixation to quantify platelet-monocyte aggregates [Harding SA *et al*, 2004; Sarma J *et al*, 2002]. We have previously reported a number of methodological considerations in the preparation and processing of samples for flow-cytometric quantification of platelet-monocyte aggregates, and this technique has shown good reproducibility in healthy volunteers (co-efficient of variation, 7.8%) [Blann AD *et al*, 2005]. We collected whole blood through direct venepuncture as opposed to via a cannula to avoid turbulent flow and increased activation. We chose to anticoagulate blood with a direct thrombin inhibitor as opposed to heparin or citrate as these compounds have been shown to increase and decrease aggregates respectively. We also chose not to stimulate samples with agonists *ex vivo* as we wanted to assess resting circulating *in vivo* levels.

We appreciate that our procedures differed when it came to the detection of platelet microparticles (when platelet poor plasma had to be prepared from whole blood) and when assays were used instead of flow cytometry. It is inevitable that processing may differ with the detection of different markers, however, as long as the methodology is fully reported (with clarity over the stages known to affect outcomes e.g anti-coagulant agent) then the relationship between measurements can still be examined**.**

Recently there has been increasing evidence to suggest that the role of platelets in atherosclerosis may be mediated by the production of microparticles [Lee WJ *et al*, 2006]. Platelet-derived microparticles (diameter <1 μm and bearing platelet-derived surface antigens) are involved in several stages of atherosclerosis from coagulation (tissue factor release;

inhibition of fibrinolysis) [Harding SA *et al*, 2007; Tan KT *et al*, 2005] to direct effects on the blood vessel wall (platelet adhesion) and angiogenesis [Leroyer AS *et al*, 2007; Muller I *et al*, 2003]. In addition, increased platelet-derived microparticles have been demonstrated in individuals with a smoking habit, diabetes mellitus, cerebrovascular disease and acute coronary syndromesis [Lee YJ *et al*, 1993; Bernal-Mizrachi L *et al*, 2003]. A variety of methods have been proposed for the assessment of platelet-derived microparticles. However, platelet microparticles are heterogeneous with respect to both size and composition, and can carry a range of antigenic markers from their parent platelet. We decided to compare flow cytometric and immunoassay based methods of quantification: both techniques identifying the same platelet antigen (CD41, GPIIb). The preparation of platelet poor plasma and use of counting beads is well validated and the assay has been reported as demonstrating good sensitivity for *in vitro* generated platelet microparticles [Michelson AE *et al*, 2006]. Although reproducibility was good for other markers, our measures of platelet microparticles were less consistent. This is likely to reflect several factors. First, significant additional processing was required by these assays and this will inevitably increase variability. Second, the accuracy of flow cytometry is reduced when assessing particles <1 μm in size. Finally, our flow protocol identified microparticles that were positive for two platelet markers, CD41 (GPIIb) *and* CD31 (GPIIa, PECAM) whereas the immunoassay only identified microparticles positive for CD41 (GPIIb).

Although platelet P-selectin and CD40L demonstrated good reproducibility (co-efficients<7), platelet monocyte aggregates showed the best global correlation with other markers. Platelet-monocyte aggregates primarily form through the binding of P-selectin on the platelet surface to the leucocyte counter-receptor, P-selectin glycoprotein ligand-1 (PSGL-1), on monocytes. It is therefore not surprising that platelet expression of P-selectin shows good correlation with levels of platelet-monocyte aggregates. Platelet microparticles modulate monocyte interactions

with other cell types by increasing expression of adhesion molecules [Podor TJ *et al*, 2002; Salonen *et al*, 1989]. The correlatation between microparticles and platelet-monocyte aggregates could reflect this, although the relationship is less strong. It is interesting to note the lack of correlation between soluble P-selectin and platelet expression of the molecule ((r=0.05; P=0.69). This has been previously reported [Nomura S *et al*, 2001]. Dissociation between platelet expressed and soluble P-selectin suggests that the soluble form cannot serve as a surrogate marker of platelet activation.

Inflammation plays a central role in the pathogenesis of atherosclerosis. Platelet-monocyte aggregation is not only a sensitive measure of platelet activation but also has important pro-inflammatory consequences. Platelet-monocyte adhesion activates (NF)-κB; a transcriptional activator thought to be important in the regulation of CD40 gene expression (Kim HK *et al*, 2004). The correlation between platelet-monocyte aggregates and CD40 expression on monocytes may reflect this process. Induction of nuclear factor (NF)-κB also stimulates expression of Mac-1 or CD11b/CD18 [Barry OP *et al*, 1998]. This could be reflected in the excellent correlation between monocyte CD11b expression and platelet-monocyte aggregation (Table 3). Subsequent activation of CD11b is associated with neutrophil recruitment to sites of inflammation.

In conclusion, we have demonstrated that, in patients with peripheral arterial disease, our measurement of platelet and monocyte activation markers demonstrated good reproducibility. Platelet-monocyte aggregates consistently reflected other markers of platelet and monocyte activation and we propose them as a sensitive ‘gold standard’ of in vivo platelet activation. In contrast, measures of platelet-derived microparticles appeared less reproducible. We plan to employ these techniques in a study of different anti-platelet regimes in this patient population.

The co-efficients of reproducibility will allow us to interpret the true contribution of such drugs to reductions in platelet activation. With increasing interest in the manipulation of platelet and inflammatory mediators for therapeutic gain, an understanding of the reproducibility and comparability of techniques for *in vivo* assessment of platelet activation is imperative.

CHAPTER 4

PERI-OPERATIVE PLATELET AND MONOCYTE ACTIVATION IN PATIENTS WITH CRITICAL LIMB ISCHAEMIA

Published by **Burdess A**, A. F. Nimmo, N. Campbell, S. A. Harding, O. J. Garden, A. R. W. Dawson, D.E. Newby. Perioperative platelet and monocyte activation in patients with critical limb ischaemia. *J Vasc Surg*. 2010 Sep;**52**(3):697-703

4.1 Summary

Patients with critical limb ischaemia (CLI) have a high rate of adverse cardiovascular events, particularly when undergoing surgery. We sought to determine the effect of surgery and vascular disease on platelet and monocyte activation *in vivo* in man.

An observational, cross sectional study was performed at a tertiary referral hospital in the South East of Scotland. Platelet and monocyte activation were measured in whole blood, in patients with CLI scheduled for infra-inguinal bypass/amputation and compared with matched healthy controls, patients with chronic intermittent claudication, patients with acute myocardial infarction and those undergoing arthroplasty (n=30 per group). Platelet and monocyte activation were quantified using flow cytometric assessment of platelet-monocyte aggregation, platelet P-selectin expression, platelet-derived microparticles, and monocyte CD40 and CD11b expression respectively.

In comparison to those with intermittent claudication, patients with CLI had increased platelet-monocyte aggregates (41.7±12.2% *versus* 32.6±8.5% respectively), platelet microparticles (178.7±106.9 versus 116.9±53.4) and monocyte CD40 expression (70.0±12.2% versus 52.4±15.2%) ($P<0.001$ for all). Indeed, these levels were equivalent (P-selectin, 4.4±2.0 versus 4.9±2.2%; $P>.05$) or higher (platelet-monocyte aggregation, 41.7±12.2 versus 33.6±7.0%; $P<0.05$: platelet microparticles, 178.7±106.9 versus 114.4±55.0 /μL; $P<.05$) than patients with acute myocardial infarction. All platelet and monocyte activation markers remained elevated throughout the peri-operative period in patients with CLI ($P<.01$) but not those undergoing arthroplasty.

Patients undergoing surgery for CLI have the highest level of *in vivo* platelet and monocyte activation and these persist throughout the peri-operative period. Additional anti-platelet therapy may be of benefit in protecting vascular patients with more severe disease during this period of increased risk.

4.2 Introduction

Patients with peripheral arterial disease (PAD) are at increased risk of adverse cardiovascular events [Criqui MH *et al*, 1992]. This risk increases with the severity of disease, with progressive reductions in ankle brachial pressure index being an independent predictor of cardiovascular outcome [Lee AJ *et al*, 2004; O'Hare AM *et al*, 2004]. Cardiovascular risk factor management is therefore the first line treatment of patients with PAD. These patients are particularly at risk during the peri-operative period. Myocardial infarction is the commonest life-threatening complication of major vascular surgery with a reported peri-operative incidence ranging from 8 to 40% depending on the diagnostic criteria [Hobbs SD *et al*, 2005; Kim LJ *et al*, 2002; Landesburg G *et al*, 2003]. This is comparable to the cardiovascular risk seen in patients with acute coronary syndromes (ACS): 30-day death and re-infarction rate of 8-20% [Bertrand ME *et al*, 2002]. Despite this, patients with PAD often fail to receive evidence-based medical therapies. There is clearly an unmet need for improved risk-stratification of these patients with appropriately tailored intensive medical regimes.

Atherogenesis and its complications are complex processes involving both inflammatory and thrombotic mechanisms [Freedman JE *et al*, 2002; Urbich *et al*, 2004; Rizvi *et al*, 2008]. Monocyte adhesion to a damaged endothelium is a central step in the initiation and progression of atherosclerosis and appears to play a key role in plaque destabilisation. In contrast, platelets can adhere to atherosclerotic lesions in the presence or absence of an overlying endothelium [Freedman JE *et al*, 2002] and contribute to thrombus formation that is responsible for acute cardiovascular events. Hence, both platelet and monocyte activation, pre-dispose to plaque growth, instability, and rupture. Platelets and monocytes also directly interact to form platelet-monocyte aggregates that promote expression of vascular cell adhesion molecules, and increase

leucocyte adhesion to the inflamed endothelium [Neuman F *et al*, 1997]. In addition, the CD40 /CD40 ligand dyad (expressed on the surface of activated monocytes and platelets) is a major inflammatory trigger, promoting release of inflammatory cytokines, adhesion molecules and procoagulant activity [Schonbeck U *et al*, 2001]. Disruption of either CD40/CD40L or platelet-monocyte aggregation, leads to the retardation of atherosclerotic lesions in animal studies [Mach F *et al*, 1998; Huo Y *et al*, 2003].

Raised levels of platelet-monocyte aggregates and monocyte CD40 have been detected in smokers, patients with diabetes mellitus, acute coronary syndromes, and those at risk of re-thrombosis following PCI [Harding SA *et al*, 2004; Sarma J *et al*, 2002; Gurbel PA *et al*, 2005]. They are surrogate markers of clinical risk and are predictive of adverse cardiac events. Several studies have assessed platelet activation in peripheral atherosclersosis and demonstrated a progressive increase in activation with increasing severity of disease [Cassar K *et al*, 2003; Robless PA *et al*, 2003; Rajagopalan S *et al*, 2007]. However, there have been relatively few reports of platelet activation and cellular inflammation at the time of operation [Payne AD *et al*, 2004; Cassar K *et al*, 2005; Rajagopalan S *et al*, 2007], and none comparing PAD patients with other high risk populations.

We wished to investigate platelet and monocyte activation in patients with PAD specifically at the time of surgery and compare this to levels of activation in other high-risk populations. The high incidence of peri-operative adverse cardiovascular events in patients with severe PAD could be mediated by increased systemic inflammation and platelet activation. These patients may potentially benefit from risk-stratification and appropriately tailored medical regimes, as occurs in patients with coronary atherosclerotic disease. This exploratory study may support

the conduct of interventional projects aimed at reducing surrogate markers of clinical risk, prior to clinical trials of intensive anti-platelet and anti-inflammatory strategies.

4.3 Methods

This observational, cross-sectional study was performed with the approval of the local ethics committee, in accordance with the Declaration of Helsinki and the written informed consent of all participants.

Subjects

Subjects were recruited from 5 different groups (n=30 per group): (i) patients with non-thromboembolic critical limb ischaemia, (ii) patients with chronic intermittent claudication, (iii) patients with a non-ST segment elevation myocardial infarction, (iv) otherwise healthy patients undergoing hip or knee arthroplasty, and (v) healthy volunteers. (Table 4.1 Inclusion and exclusion criteria).

Patients with critical limb ischaemia (CLI), defined by the presence of rest pain and/or skin ulceration and a reduced ankle brachial pressure index (ABPI), who were scheduled to undergo infra-inguinal bypass or amputation were recruited from the surgical vascular unit. Patients who had symptoms of intermittent claudication with a reduced ABPI, were recruited from the out-patient claudication clinic. All 60 patients with peripheral arterial disease were receiving maintenance aspirin (75 mg daily) and statin therapy for at least 6 weeks prior to inclusion. We wished to examine platelet activation under the standard medical regime, therefore patients receiving clopidogrel or warfarin were excluded. (At present, the only evidence-based indication for dual anti-platelet therapy in PAD is co-existing history of recent (< 6months) coronary stenting or stroke).

Table 4.1 Inclusion and exclusion criteria

	Healthy Volunteers	Claudicants	Critical Limb Ischaemia	Non-ST elevation myocardial infarction	Arthroplasty
Inclusion Criteria	Age >50 years	Age >50 years Intermittent Claudication ABPI <1 and >0.2 Rutherford Baker II, III	Age >50 years Rest pain and/or skin ulceration or necrosis ABPI ≤ 0.2 Rutherford-Baker IV-VI	Age >50 years Cardiac chest pain plus ECG changes plus plasma troponin I >0.2μg/L	Age >50 years
Exclusion Criteria	PAD IHD, CVD Smoking Hypertension Diabetes Lipid lowering agents Anti-platelet therapy Anti-hypertensive Agents Warfarin	Dual anti-platelet therapy Warfarin	Dual anti-platelet therapy Warfarin	PAD	PAD IHD, CVD Smoking Hypertension Diabetes Lipid lowering agents Anti-platelet therapy Anti-hypertensive agents Warfarin

Patients presenting to the coronary care unit and diagnosed with a non-ST elevation myocardial infarction (chest pain with electrocardiographic (ECG) changes and elevated plasma troponin I concentration (>0.2 μg/L)) were recruited.

We wished to examine the contribution of surgical stress (without underlying PAD) to *in vivo* platelet activation. Following discussion with a panel of consultant vascular surgeons and anaesthetists it was felt knee or hip arthroplasty represented surgery of a *similar magnitude* to peripheral bypass or amputation, and was likely to be performed in patients of a comparable age. Patients undergoing *elective* arthroplasty over the age of 50 were approached. In order to limit the effect of atherosclerosis on platelet activation, patients with a history of diabetes, hypertension, ischaemic heart disease or stroke, smoking or anti-platelet, anti-hypertensive or statin use, were excluded. Patients undergoing arthroplasty did not receive peri-operative heparin according to the unit policy.

Sequential healthy subjects over the age of 50 were recruited.

Blood Sampling

Single baseline samples were taken from all 150 subjects. In patients with non-ST segment elevation myocardial infarction, samples were taken within 24 hours of hospitalisation and after the initiation of dual anti-platelet medication with aspirin and clopidogrel. In order to compare peri-operative platelet and monocyte activation, three blood samples were taken from subjects undergoing vascular or orthopaedic surgery: pre-operatively, immediately post-operatively and on the day after the operation.

Blood was drawn by venepuncture of a large anticubital vein using a 19-gauge needle. Care was taken to ensure a smooth blood draw without venous stasis. Samples were processed immediately. Blood for assessment of platelet-monocyte aggregates, platelet expression of P-

selectin, and monocyte CD40 and CD11b, was collected into tubes containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK, Cambridge Biosciences). Blood for the assessment of platelet microparticles was collected into sodium citrate. Platelet poor plasma (PPP) was then prepared by centrifugation at 2000 *g* at 4 ^{0}C for 10 min and confirmed by a platelet count of $<10^{7}$/L.

Assessment of in vivo platelet activation

Flow cytometric measurements of platelet-monocyte aggregates and platelet surface expression of P-selectin were performed as described previously [Harding SA *et al*, 2004] Immunolabelling was performed in whole blood within 5 min of collection. Directly conjugated monoclonal antibodies, were obtained from DakoCytomation (Cambridge, UK) and Serotec (Oxford, UK). In order to assess platelet-monocyte aggregates, 60 µL of blood were incubated for 15 min with a FITC-conjugated anti-CD42a monoclonal antibody (GRP-P, platelet marker) and a PE-conjugated anti-CD14 monoclonal antibody (Tuk-4, monocyte marker) before fixation and erythrocyte lysis with 500 µL of FACSLyse solution (Sarma J *et al*,2002). Samples were processed using a BeckmanCoulter flow cytometer and at least 2,500 cell events were analysed by EXPO32 software. Platelet-monocyte aggregates were detected by gating for cells that were positive for both CD14 PE and CD42a FITC. Platelet surface expression of P-selectin was assessed by gating for cells that were positive for both FITC-conjugated anti-CD42a monoclonal antibody, (platelet marker) and PE conjugated anti-CD62P monoclonal antibody (TRAP 1, IgG1). Isotype controls were used to reduce error from non-specific binding.

Platelet microparticles were identified by both size and expression of platelet markers CD41 (GPIIb) and CD31 (GPIIIa; PECAM). Aliquots (25 µL) of PPP were incubated for 30 min with a PE-conjugated anti-CD31 monoclonal antibody and a FITC-conjugated anti-CD41 monoclonal antibody (Serotec, Oxford, UK), before dilution with phosphate buffered saline to form a volume of 1 mL. Platelet microparticles were gated according to their size (events <1.0 µm) by assessment of their forward light scatter. TruCOUNT beads of 1.0 µm (Becton Dickenson) of a known concentration were used to calculate the volume of sample analysed over 120 s at medium flow rate. This allowed the absolute number of platelet microparticles to

be measured. Isotype controls were used to reduce error from non-specific binding. Platelet microparticles were detected by gating for events that were <1 μm in size (based on forward scatter) and positive for both CD31 and CD41.

Assessment of in vivo monocyte activation

Monocyte activation was assessed via flow cytometric measurement of per cent monocyte CD40 expression and mean fluorescent intensity (MFI) of monocyte CD11b expression, as described previously (Harding SA *et al*, 2004). Immunolabelling was performed in whole blood within 5 min of collection. To evaluate CD40 and CD11b on monocytes, blood was diluted 1:2 with PBS and incubated with the following monoclonal antibodies: anti-CD14: FITC (Serotec), anti-CD40:PE (Serotec), anti-CD11b:PE (Serotec) and appropriate isotype-matched controls for 20 min, before fixation and erythrocyte lysis with 500 μL of FACSLyse solution. Monocytes were identified by gating for CD14-positive cells.

Statistical analysis

Data are shown as scatter plots or mean±SD. Data were analysed by analysis of variance, chi-squared and Bonferroni post-hoc tests where appropriate using GraphPad Prism Version 4 (La Jolla, USA). Statistical significance was taken as a two-sided P value <0.05.

4.4 Results

Subjects were predominantly middle-aged men, with groups having similar distribution of ages and no significant difference in mean age (Table 4.2). In keeping with their clinical presentation, patients with peripheral arterial and coronary heart disease had a range of cardiovascular risk factors and medications that were not present in the healthy volunteers or patients undergoing orthopaedic surgery (Table 4.2). Of the 30 patients with CLI, 18 underwent femoral-popliteal bypass and 12 underwent below knee amputations. We deliberately chose not to recruit patients with CLI undergoing angioplasty. We specifically wanted to assess platelet activation peri-operatively where the changes in such variables could be assumed to be greater than when undergoing a less invasive endovascular procedure. Seventeen patients had dry gangrene; none had wet gangrene.

Table 4.2 Subject Demographics

Variable	Healthy Volunteers (n=30)	Intermittent Claudication (n=30)	Critical Limb Ischaemia (n=30)	Acute Coronary Syndromes (n=30)	Arthroplasty (n=30)
Mean age	59±3	60±4	68±2	58±2	57±4
Male Sex	16 (53%)	22 (73%)	23 (77%)	20 (67%)	17 (57%)
CV RISK FACTORS n (%)					
Hypertension	0	18 (60%)	24 (80%)	20 (67%)	0
Diabetes	0	7 (23%)	10 (33%)	8 (27%)	0
CAD	0	10 (33%)	16 (53%)	13 (43%)	0
Current smoker	0	17 (57%)	16 (53%)	16 (53%)	0
MEDICATIONS n (%)					
Aspirin	0	30 (100%)	30 (100%)	30 (100%)	0
Clopidogrel	0	0	0	30 (100%)	0
Statin therapy	0	30 (100%)	30 (100%)	24 (80%)	0
ACE inhibitor	0	3 (10%)	12 (40%)	21 (70%)	0
B-blocker	0	2 (6.7%)	6 (20%)	16 (53%)	0

Baseline Platelet Activation

Platelet activation markers were lowest in healthy volunteers and patients scheduled for arthroplasty (Figure 4.1). Baseline platelet-monocyte aggregation (41.7±12.2%) and platelet microparticles (178.7±106.9) were highest in patients with CLI compared to *all* other groups (Figure 4.1). Although patients with CLI had higher values of platelet P-selectin than healthy volunteers or those undergoing arthoplasty ($P<0.001$), there was no demonstrable difference between these patients and those with claudication or non-ST elevation myocardial infarction (4.4±2.0% *versus* 4.2±2.0% and 4.9±2.2% respectively; $P>0.05$; Figure 4.1).

Baseline Monocyte Activation

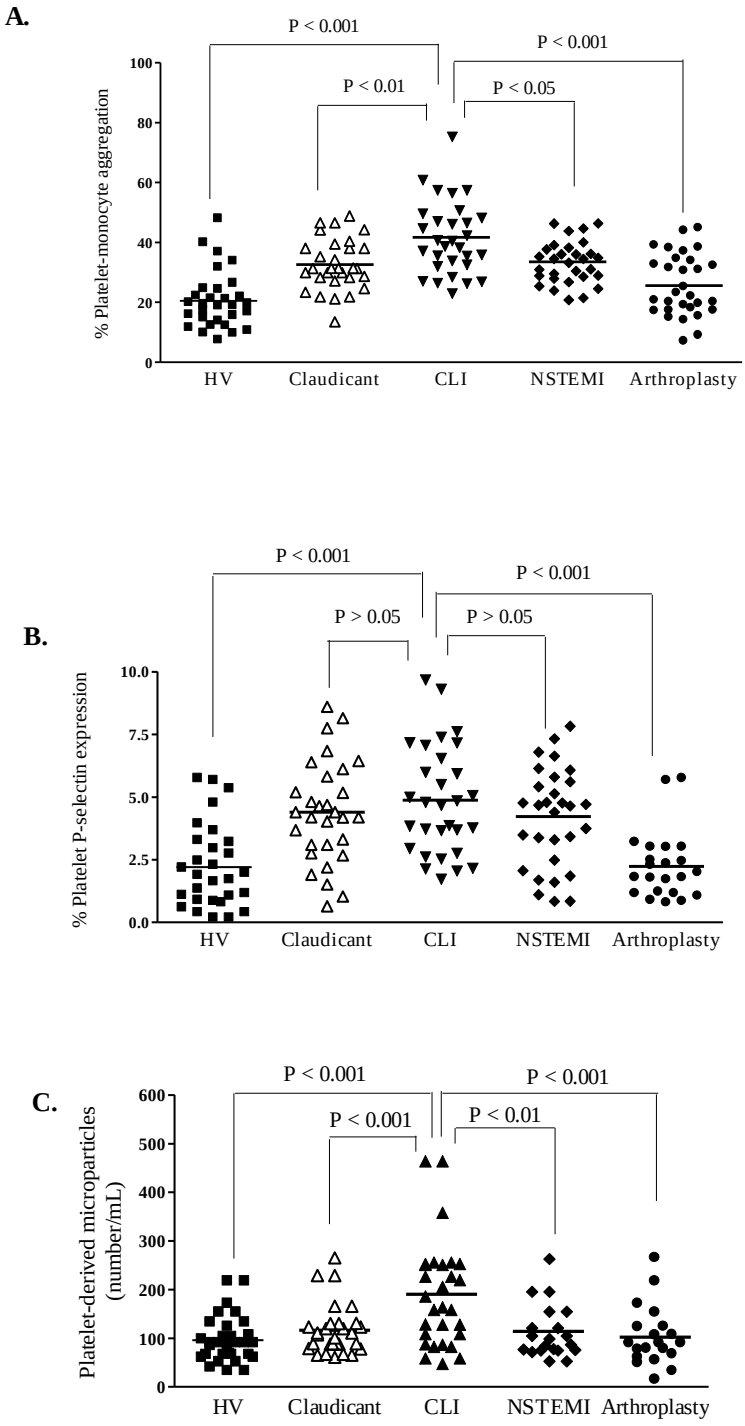
Monocyte activation markers were lowest in healthy volunteers and patients scheduled for arthroplasty (Figure 4.2). Baseline monocyte expression of CD40 (70±12.2%) was highest in patients with CLI compared to *all* other groups (Figure 4.2). Baseline monocyte CD11b was greatest in patients with CLI compared to all groups except those with claudication, where it was equivalent (56.6±18.3 *versus* 50.5±13.9 respectively, $P>0.05$; Figure 4.2)

Peri-operative Platelet and Monocyte activation

Throughout the peri-operative period, levels of *all* platelet and monocyte markers remained greater in patients with CLI in comparison to those undergoing arthroplasty (Figures 4.3 and 4.4; $P<0.0001$) Platelet and monocyte activation rose immediately post-operatively in patients undergoing joint arthroplasty ($P<0.05$) before falling on the first post-operative day (Figures 4.3 and 4.4). In contrast, platelet activation fell immediately after surgery in patients undergoing infra-inguinal revascularisation or amputation ($P<0.05$; Figure 4.3) whereas monocyte activation either remained unchanged (monocyte CD40, $P>0.05$) or rose on day one (monocyte CD11b, $P<0.05$; Figure 4.4).

There was no statistically significant difference in markers according to type of surgery performed for CLI (bypass or amputation), and no difference in post-operative trend (sub-analysis not shown).

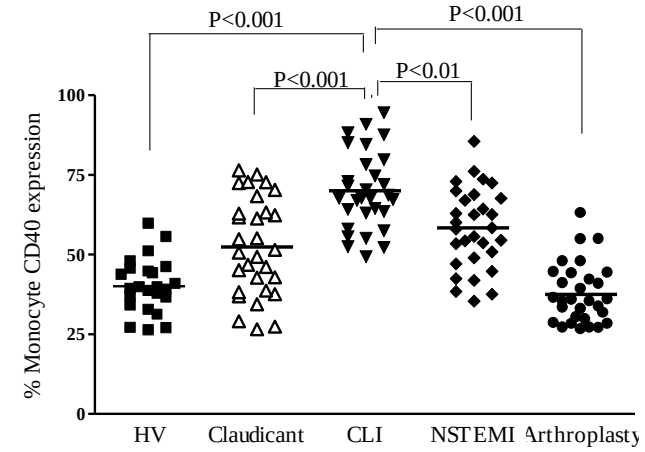
Figure 4.1 Resting *in vivo* platelet activation in different subject populations



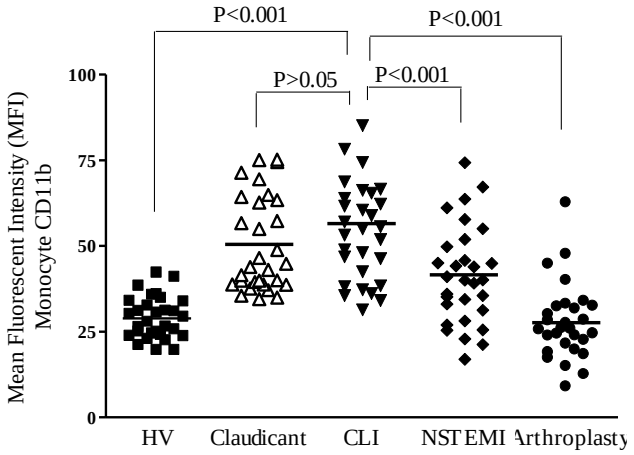
Scatter dot plots of **A**. % Platelet-monocyte aggregation **B.** % Platelet P-selectin expression **C.** Platelet-derived microparticles (/mL). ■ Healthy volunteers, Δ Claudicants ▼ Patients with Critical Limb Ischaemia (CLI) ♦ Patients with Non-ST Elevation Myocardial Infarction (NSTEMI) ● Patients undergoing arthroplasty. (n=30 per group), Horizontal lines represent population means. Analysis by one-way ANOVA with Bonferroni post-tests

Figure 4.2 Resting *in vivo* monocyte activation in different subject populations

A.

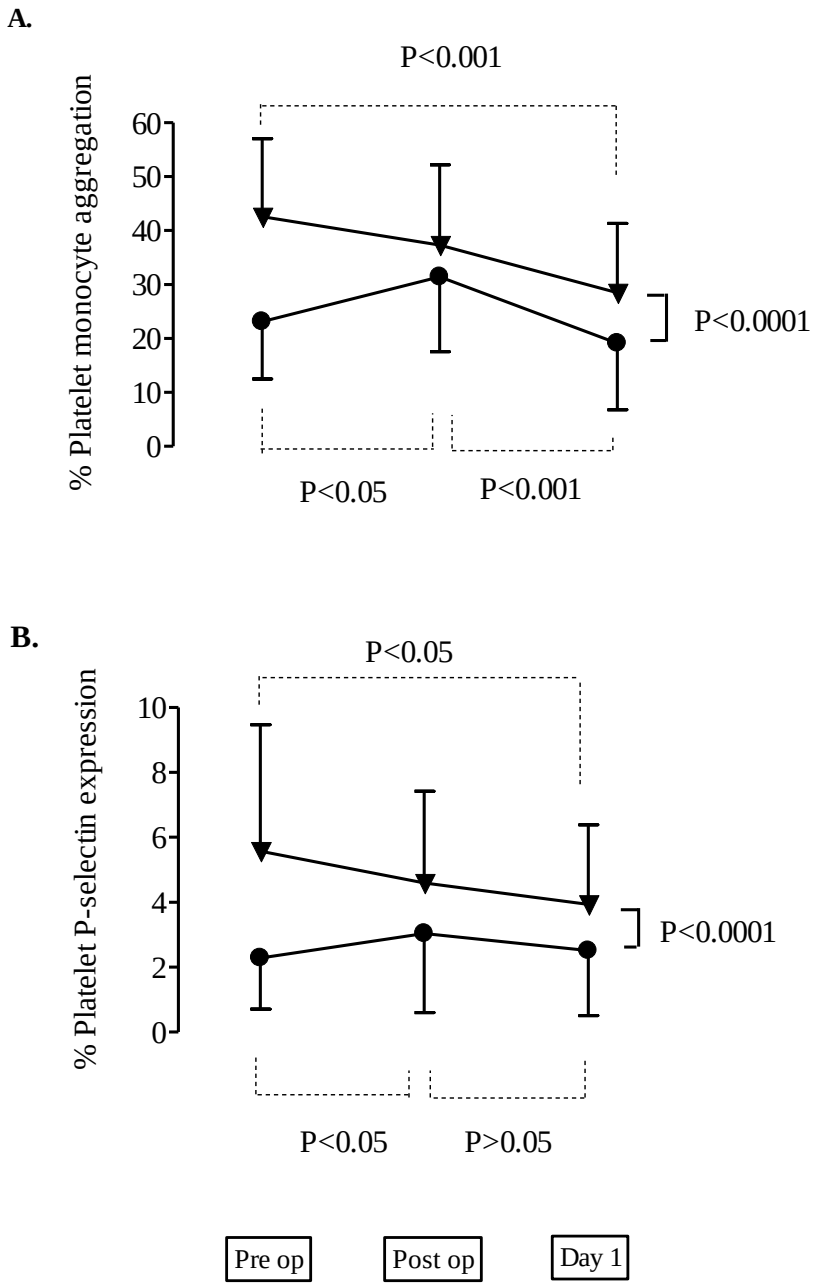


B.



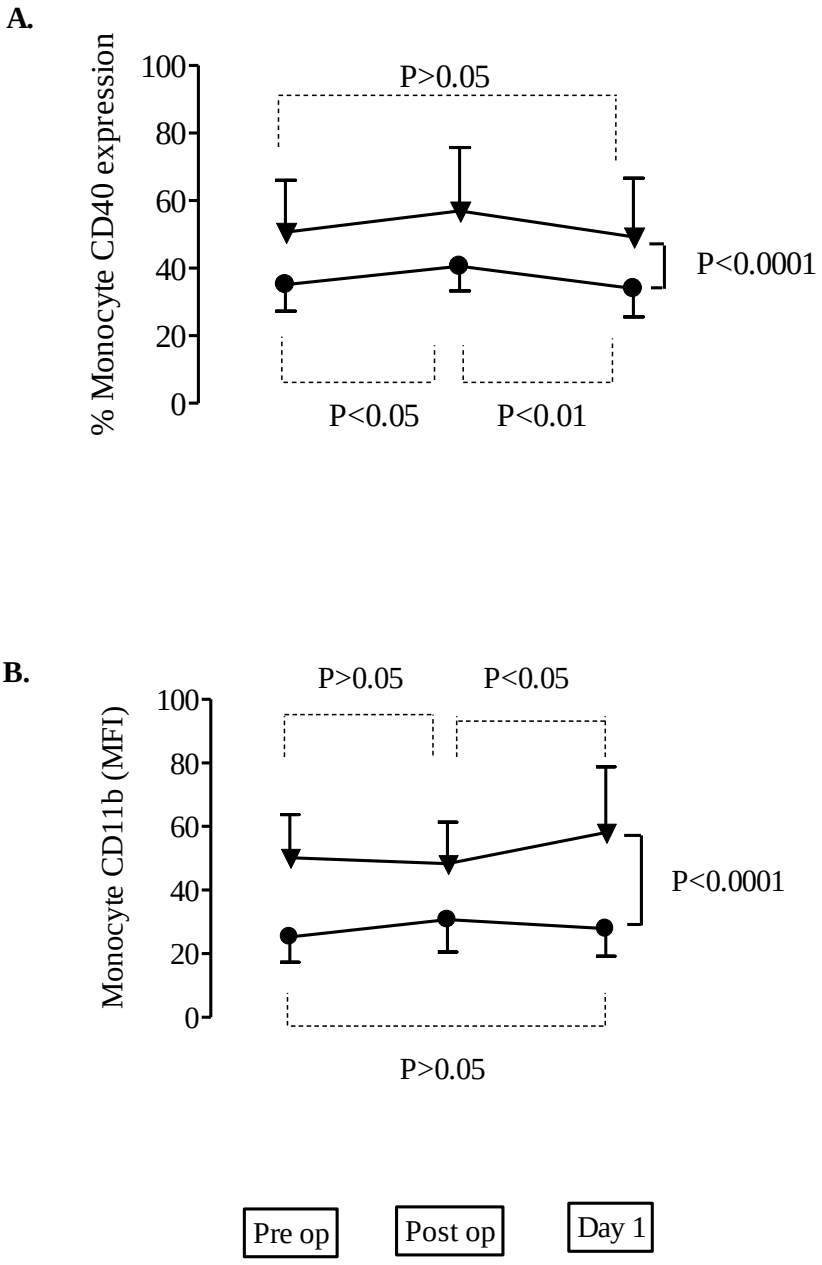
Scatter dot plots of **A**. % Monocyte CD40 expression **B.** Monocyte CD11b (Mean Fluorescent Intensity, MFI). ■ Healthy volunteers, Δ Claudicants ▼ Patients with Critical Limb Ischaemia (CLI) ♦ Patients with Non-ST Elevation Myocardial Infarction (NSTEMI) ● Patients undergoing arthroplasty. (n=30 per group) . Horizontal lines represent population means. Analysis by one-way ANOVA with Bonferroni post-tests

Figure 4.3 Peri-operative platelet activation in patients undergoing surgery for CLI (▼) and arthroplasty (●)



Platelet activation in patients undergoing infra-inguinal bypass or amputation was greater than in patients undergoing arthroplasty throughout the peri-operative period (P<0.0001). *In vivo* platelet activation **A**. % Platelet-monocyte aggregation (P<0.05) and **B.** % P-selectin expression (P<0.05), rose immediately post-operatively in patients undergoing arthroplasty but fell in those with critical limb ischaemia (P<0.001).
Mean±SD; analysis between patient groups by 2-way ANOVA with repeated measures; analysis between tme points for the same patient group by one way ANOVA with Bonferroni post-tests.

Figure 4.4 Peri-operative monocyte activation in patients undergoing surgery for CLI (▼) and arthroplasty (●)



Monocyte activation in patients undergoing infra-inguinal bypass or amputation was significantly greater than in patients undergoing arthroplasty throughout the whole peri-operative period (P<0.0001). **A**. % Monocyte CD40 expression tended to rise immediately post-operatively in orthopaedic patients before returning to baseline (P<0.05) B. There was no significant post-operative change in monocyte CD11b (MFI) in arthroplasty patients (P>0.05), but levels appeared to rise on day 1 post surgery in those with CLI (P<0.05).

4.5 Discussion

Consistent with previous studies [Cassar K *et al*, 2003; Robless PA *et al*, 2003; Rajagopalan S *et al*, 2007], we have demonstrated that platelet and monocyte activation is increased in patients with PAD. For the first time, we have shown that patients undergoing surgery for CLI have even greater levels of platelet and monocyte activation than patients being treated for acute myocardial infarction. In addition, peri-operative platelet and monocyte activation is markedly increased in these patients and exceeds the increase in platelet activation and inflammation, attributable to surgery itself. This study supports the need for an increased appreciation of the cardiovascular risk associated with this group of patients and an improvement in cardioprotective management, especially in the peri-operative period.

Peripheral arterial disease affects nearly 30 million people in Western Europe and North America. In up to three-quarters of cases, patients have co-existent coronary artery disease and a 3-fold increased risk of cardiovascular events and death [Sukhija R *et al*, 2004]. In spite of attempts to raise awareness of peripheral arterial disease as an important marker of cardiovascular risk, patients are often poorly provided with evidence-based therapies such as anti-platelet and lipid lowering therapies [Cassar K *et al*, 2003; Burns P *et al*, 2003; Hirsch AT *et al*, 2001; Khan S *et al*, 2007]. The reasons for this are unclear, but appear to be related to a lack of awareness amongst health professionals of the severity of the disease.

Our study demonstrated that patients with critical limb ischaemia have a greater elevation in systemic markers of platelet and monocyte activation than comparator groups. We acknowledge that the healthy volunteer subjects and patients undergoing arthroplasty did not receive anti-platelet agents or lipid lowering drugs which can affect platelet activation. However, patients with PAD have a high incidence of adverse cardiovascular events *despite*

existing medical therapy. Indeed, the elevations in platelet activation were seen despite the standard medical regime of aspirin and statin therapy and were even greater than those seen in patients with acute myocardial infarction. It may be that there is scope for further management of platelet activation in these patients.

Patients undergoing peripheral vascular surgery have a particularly high incidence of peri-operative cardiovascular events. It has been postulated that this is attributable to increased platelet and monocyte activity caused by the surgical process itself. Patients without cardiovascular risk factors undergoing arthroplasty (and therefore no reason for elevated baseline platelet activation) were recruited in order to assess the effect of surgery alone on platelet activation. In our study, throughout the peri-operative period, both platelet and monocyte activation markers were higher in patients undergoing vascular surgery compared to those undergoing arthroplasty. However, although patients undergoing arthroplasty sustained a post-operative rise in platelet markers, we were surprised to see that platelet activation *fell* immediately following surgery in those with CLI. Patients with PAD, especially CLI, have high *baseline* levels of activated platelets and monocytes. This is due to the underlying endothelial dysfunction and tissue ischaemia. We therefore propose that the lack of response following surgery in this particular group of patients could be due to the removal of thrombotic stimulus by amputation or revascularisation. These patients may benefit from increased platelet inhibition prior to surgery.

Cardiovascular disease is a critical public health issue. The prevalence of the disease and increased awareness of the cost-benefit associated with the management of cardiovascular risk, have led to the concept of potential screening programmes for vascular disease [Belch JJ *et al*, 2003]. In addition, recommendations are required for the most appropriate use of interventions

and therapies for patients with different manifestations of peripheral arterial disease. The use of imaging technologies or biomarkers could help risk-stratify patients and guide management. This study demonstrates that sensitive markers of *in vivo* platelet and monocyte activation – known to be predictors of clinical risk – are markedly elevated in patients with severe peripheral arterial disease at the time of surgery, despite current medical therapy. This is in line with reports of the increased cardiovascular risk associated with reducing ankle brachial pressure index [Criqui MH *et al*, 1992]. However, further work is required to demonstrate the link between platelet activation and adverse cardiovascular outcomes in this specific patient group. We are therefore conducting a trial of dual anti-platelet therapy (aspirin plus clopidogrel) versus aspirin alone, in patients undergoing surgery for CLI. The impact of intensive anti-platelet therapy on platelet markers and cardiac troponin and hsCRP will be noted. This may inform the design of larger scale clinical trials powered at examining clinical end-points in this population.

CHAPTER 5

RANDOMISED CONTROLLED TRIAL OF DUAL ANTI-PLATELET THERAPY IN PATIENTS UNDERGOING SURGERY FOR CRITICAL LIMB ISCHAEMIA

Published by **Burdess A**, Nimmo AF, Garden OJ, Murie JA, Dawson ARW, Fox KAA, Newby DE. Randomised Controlled Trial of Dual Anti-platelet Therapy in Patients Undergoing Surgery for Critical Limb Ischaemia. *Ann Surg*. 2010 Jul;**252**(1):37-42.

5.1 Summary

Patients with critical limb ischaemia have a peri-operative cardiovascular morbidity comparable to patients with acute coronary syndromes. We hypothesised that peri-operative dual anti-platelet therapy would improve biomarkers of atherothrombosis without causing unacceptable bleeding in patients undergoing surgery for critical limb ischaemia.

In a double-blind randomised controlled trial, 108 patients undergoing infra-inguinal revascularisation or amputation for critical limb ischaemia were maintained on aspirin (75 mg daily) and randomised to clopidogrel (600 mg prior to surgery, and 75 mg daily for 3 days; n=50) or matched placebo (n=58). Platelet activation and myocardial injury were assessed by flow-cytometry and plasma troponin concentrations respectively.

Clopidogrel reduced platelet-monocyte aggregation before surgery (38% to 30%; P=0.007). This was sustained in the post-operative period (P=0.0019). There were 18 troponin-positive events (8 (16.0%) clopidogrel *versus* 10 (17.2%) placebo; RR 0.93, 95% CI 0.39 to 2.17; P=0.86). Half of troponin-positive events occurred pre-operatively with clopidogrel causing a greater decline in troponin concentrations (P<0.001). There was no increase in major life-threatening bleeding (7 (14%) *versus* 6 (10%); RR 1.4, 95% CI 0.49 to 3.76; P=0.56) or minor bleeding (17 (34%) *versus* 12 (21%); RR 1.64, 95% CI 0.87 to 3.1; P=0.12), although blood transfusions were increased (28% *versus* 12.6%, RR 2.3 95% CI 1.0 to 5.29; P=0.037).

In patients with critical limb ischaemia, peri-operative dual anti-platelet therapy reduces biomarkers of atherothrombosis without causing unacceptable bleeding. Large-scale randomised controlled trials are needed to establish whether dual anti-platelet therapy improves clinical outcome in high-risk patients undergoing vascular surgery.

5.2 Introduction

Patients with peripheral arterial disease have an increased risk of adverse cardiovascular events [Criqui MH *et al*, 1992], particularly in the peri-operative period [Mangano DT *et al*, 1991]. Myocardial injury is the commonest life-threatening complication of vascular surgery with a reported incidence ranging from 8-40% [Hobbs SD *et al*, 2005; Kim LJ *et al*, 2002; Landesburg G *et al*, 2003]. This is comparable to the cardiovascular risk of patients presenting with an acute coronary syndrome: 30-day death and re-infarction rate of 8-20% [Bertrand *et al*, 2002].

In patients with peripheral arterial disease, prophylactic use of the thienopyridine clopidogrel has modest additional secondary preventative benefits in comparison to [CAPRIE, 1996], or in combination with [Bhatt DL *et al*, 2006], aspirin. Combination aspirin and clopidigrel therapy is of major benefit in reducing recurrent ischaemic events in patients with acute coronary syndromes [CURE Trial, 2001]. It is therefore reasonable to postulate that dual anti-platelet therapy may have particular benefits in patients undergoing vascular surgery. However, many clinicians would question the wisdom of dual anti-platelet therapy in the operative setting because of the risk of increased peri-operative bleeding. The CURE trial (Clopidogrel in Unstable angina to prevent Recurrent ischaemic Events) reported an *overall* relative risk reduction in cardiovascular death, myocardial infarction or stroke in patients undergoing coronary artery bypass grafting (CABG) following non-ST segment elevation myocardial infarction, without an increase in major life-threatening bleeding [Fox KA *et al*, 2004]. However, the surgical sub-group was not prospectively randomised and only a small proportion of patients received dual anti-platelet therapy within 5 days of operation. Despite these limitations, the trial proposed that the potential cardiovascular benefits of dual anti-platelet therapy may outweigh the risks of bleeding in the high-risk surgical patient.

Atherothrombosis is the major underlying cause of adverse cardiovascular events. Platelets play a key role in this process and are associated with both the inflammatory destabilisation of atherosclerotic plaques, and thrombus generation [Freedman JE *et al*, 2002; McGregor L *et al*, 2006]. Platelet activation is commonly assessed using aggregometry or detection of platelet surface P-selectin expression following *ex vivo* agonist stimulation. These approaches are artificial and may not truly reflect the status of *in vivo* platelet activation because of the potential for *in vitro* activation, and the rapid shedding of P-selectin from the platelet surface [Michelson AD *et al*, 1996]. Activated platelets are rapidly cleared from the circulation by monocytes and the quantification of platelet-monocyte aggregates is now emerging as the 'gold standard' assessment for *in vivo* platelet activation [Huo Y *et al*, 2003; Michelson *et al*, 2001]. We and others have demonstrated that platelet-monocyte aggregates are increased in those who smoke or have diabetes mellitus as well as in patients with peripheral arterial disease or an acute coronary syndrome [Harding SA *et al*, 2004; Cassar K *et al*, 2003; Samar J *et al*, 2002].

To date, there have been no studies to investigate the effects of dual anti-platelet therapy in patients undergoing surgery for critical limb ischaemia. Given the potential for both marked benefit and hazard, we embarked upon a proof-of-concept pilot randomised controlled trial. We hypothesised that combined peri-operative aspirin and clopidogrel therapy would improve biomarkers of atherothrombosis (platelet-monocyte aggregates and troponin release) without causing unacceptable bleeding, in patients undergoing surgery for critical limb ischaemia.

5.3 Methods

Subjects

Patients with critical limb ischaemia who were scheduled for infra-inguinal bypass, femoral endarterectomy or lower limb amputation under general anaesthesia were recruited into the study. Critical limb ischaemia was defined as the presence of rest pain or skin breakdown, resulting from arterial disease. Exclusion criteria included women of child bearing potential, non-atherosclerotic vascular disease, sudden acute limb ischaemia requiring emergency surgery, supra-inguinal or aortic surgery, history of acute coronary syndrome within 3 months, history of peptic ulcer disease, previous or current intracranial haemorrhage, bleeding diathesis, uncontrolled hypertension, or thrombocytopenia, planned epidural or spinal anesthesia, hypersensitivity or allergy to thienopyridines, and current warfarin or thienopyridine use.

Study Design

Patients were recruited between June 2005 and February 2008, and gave written informed consent prior to study participation. The study was approved by the local Research Ethics Committee, given Clinical Trial Authorisation by the Medicines and Healthcare products Regulatory Agency (UK), and was conducted in accordance with the Declaration of Helsinki and CONSORT guidelines [Moher *et al*, 2001; Altman DG *et al*, 2001].

This was a prospective single centre double-blind randomised controlled trial at a tertiary referral vascular surgical unit in the Royal Infirmary of Edinburgh, South-East Scotland, UK.

Treatment Allocation

Following recruitment, clopidogrel and matched placebo were assigned in identical packs by the pharmacy trials unit through allocation of sequentially numbered study medication packs that had been randomised using an independent computer-generated sequence. Patients

received 600 mg of clopidogrel or matched placebo 4-28 hours prior to surgery and received 75 mg of clopidogrel or matched placebo daily for 3 days after surgery. (Studies suggest that the incidence of peri-operative adverse cardiovascular events is greatest within the first 5 days of surgery [Mangano DT *et al*, 1991]. We therefore commenced therapy pre-operatively and continued maintenance levels into a short post-operative period. We only continued clopidogrel for 3 days post peratively not 5 days due to rationalisation of blood sampling. Therefore changes in platelet markers and troponin after this time will have been missed.) Patients undergoing bypass procedures received a single dose of 5000 IU of intravenous unfractioned heparin during surgery before arterial clamping. At the discretion of the clinical team, intravenous protamine was given only if excessive bleeding was felt to be present at the end of the operation. All patients received subcutaneous unfractionated heparin 5000 IU twice daily in the post-operative period and were maintained on aspirin (75 mg daily) throughout the study.

Biomarkers of Atherothrombosis

Platelet activation and inflammatory markers

Blood samples were taken before, and a minimum of 4 hours after, a loading dose of 600 mg of clopiogrel or matched placebo, immediately after the operation in the recovery room, and on day 1 after surgery. Flow cytometric measurements of platelet-monocyte aggregates and platelet surface expression of P-selectin were used as markers of *in vivo* platelet activation as described previously [Harding SA *et al*, 2004, 2007; Cassar *et al*, 2003, Sarma J *et al*, 2002]. Directly conjugated monoclonal antibodies were obtained from DakoCytomation (Cambridge, UK) and Serotec (Oxford, UK).

Myocardial Injury

The Reference Clinical Biochemistry Laboratory measured plasma troponin I concentrations using the ARCHITECT Troponin I *STAT* assay (Abbott Diagnostics, Maidenhead, UK) using an autoanalyser. This has an analytical sensitivity of 0.009 ng/mL and a functional sensitivity of 0.032 ng/mL with a co-efficient of variation of <10%. The latter threshold was employed for the clinical case definition of myocardial infarction (see below).

In-patient Clinical Outcomes

Acute Coronary Syndromes

Clinical symptoms, plasma troponin concentrations and electrocardiograms were recorded daily from the pre-operative day until day 3 post-surgery. A blinded independent cardiologist reviewed all clinical data and applied the universal definition of myocardial infarction [The Joint European Soc. of Cardiology, 2007].

Bleeding Complications

Bleeding events were defined as major (life-threatening or non-life threatening) and minor according to CURE criteria [Fox KA *et al*, 2004]. Post-operative blood transfusions were recommended according to Scottish Intercollegiate Guidelines Network (SIGN) criteria [Scottish Intercollegiate Guidelines Network, 2001]. Intra-operative blood loss, post-operative fall in haemoglobin, blood product transfusion, length of operation and length of hospital stay were recorded. Incidence of gastro-intestinal bleeding, persistent (>3 days) wound leak, haematoma or infection, were also documented.

Data and Statistical Analysis

An independent data monitoring committee performed an interim safety analysis of bleeding outcomes following recruitment of 50 patients and recommended continuation of the trial to completion. Following completion of trial recruitment, data collection and laboratory analyses, the data base was locked, treatment allocation unblinded and pre-specified analyses performed. The primary end-point was platelet-monocyte aggregation. The sample size (n=50 per group) was based on our previous studies [Harding SA *et al*, 2004; Sarma J *et al*, 2002] and gave an 80% power of detecting a 4.8% difference in platelet-monocyte aggregates at a significance level of 5%. Secondary outcomes included plasma troponin concentration, and rate of myocardial infarction and bleeding complications. Continuous variables are reported as mean±SD. Analysis of variance with repeated measures, two-tailed Student's *t*-test and Chi-squared analysis were performed as appropriate using GraphPad Prism Version 4 (La Jolla, USA). Statistical significance was taken as a two-sided P value <0.05.

5.4 Results

Of the 159 potentially eligible patients, 113 were randomised to trial medication (Figure 5.1). Of those who completed the study protocol, 58 received placebo and 50 received clopidogrel. There was no difference in baseline demographics between the two groups (Table 5.1).

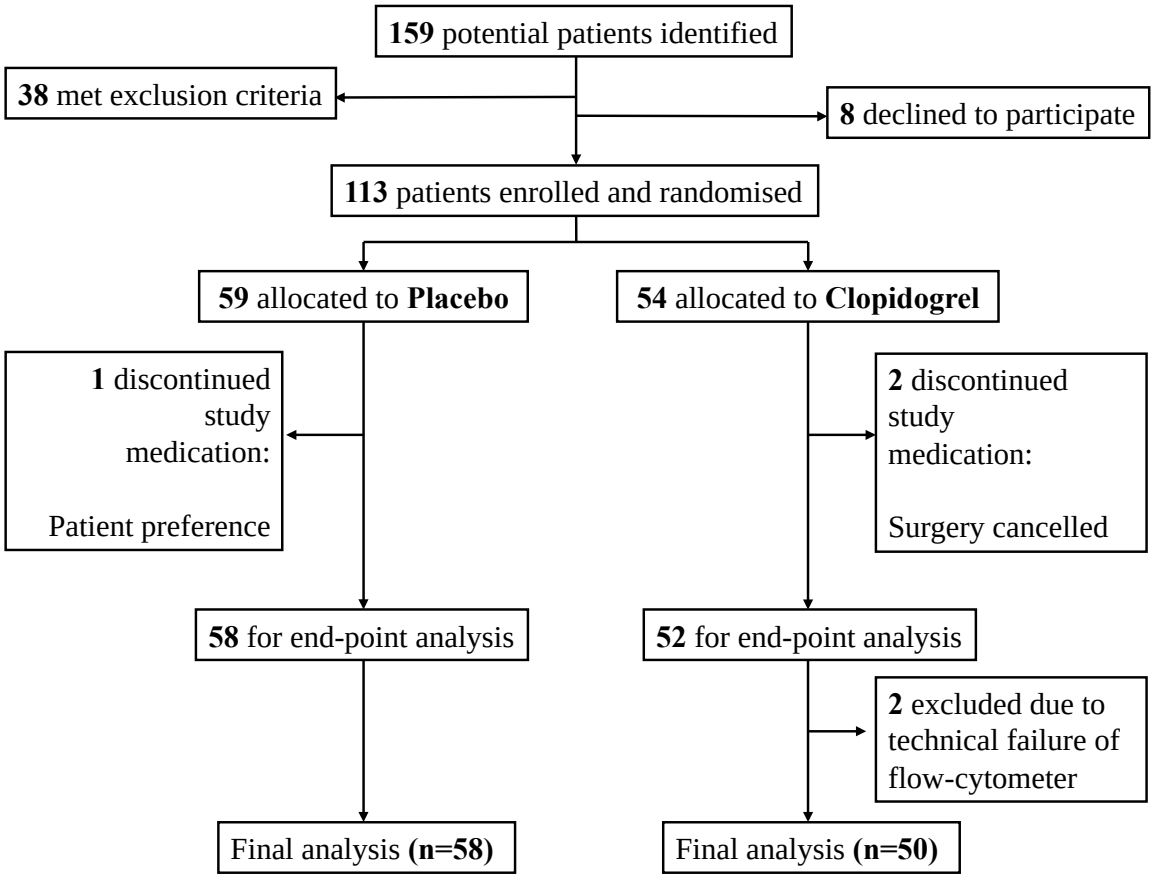


Figure 5.1 Trial profile

Table 5.1

Baseline characteristics of patients according to interventional group

Variable	Clopidogrel n=50	Placebo N=58	P value
Age (years)	68±2	68±2	0.83
Male Sex	39 (78)	45 (78)	0.96
CRITICAL LIMB ISCHAEMIA			
Ankle-Brachial Pressure Index <0.2	34 (68)	31 (53)	0.12
Skin Changes (ulcer/gangrene)	27 (54)	34 (59)	0.63
Rest Pain	42 (84)	49 (84)	1.00
OPERATION			
Bypass,	32	41	0.71
Amputation	14	14	0.71
Combined bypass and angioplasty	4	3	0.71
Lees Revised Cardiac Risk Index ≥ 3 (**42**)	40	43	0.47
CARDIAC RISK FACTORS			
Diabetes Mellitus	19 (38)	19 (33)	0.57
Hypertension	41 (82)	48 (83)	0.91
Hypercholesterolaemia	31 (62)	37 (64)	0.85
Family History of Ischaemic Heart Disease	5 (11)	8 (14)	0.55
Current Smoker	20 (40)	31 (53)	0.36

Serum Creatinine (µmol/L)	98±4	106±6	0.27
Ischaemic Heart Disease	20 (40)	31 (56)	0.16
Cerebrovascular Disease	11 (22)	15 (26)	0.64
DRUGS			
Aspirin	50 (100)	58 (100)	1.00
Statin	35 (70)	45 (78)	0.37
Beta-blockade	13 (26)	12 (21)	0.43
Angiotensin-converting Enzyme Inhibition	19 (38)	24 (41)	0.72

Variables reported as mean±SD or n (%) and analysed with un-paired *t*-test or Chi-squared analysis as appropriate

Biomarkers of Atherothrombosis

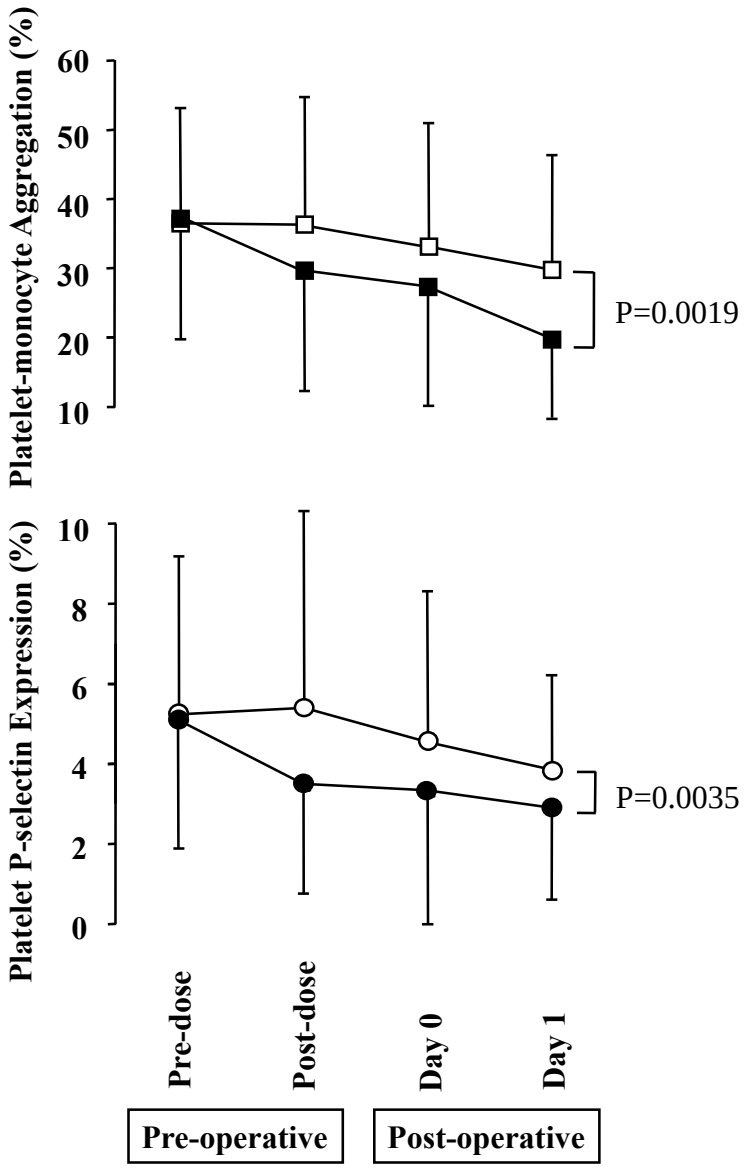
Platelet activation

In keeping with the patient population, baseline levels of platelet-monocyte aggregation were markedly elevated. There was no difference in baseline platelet-monocyte aggregates (P=0.80) and P-selectin expression (P=0.80) between the two groups. Platelet activation was unaffected by placebo (P=0.78) but clopidogrel (600 mg) caused a rapid reduction in platelet-monocyte aggregates (38±17% to 30±17%, P=0.007) and platelet P-selectin expression (4.9±2.7% to 2.8±1.6%, P<0.0001). In both groups, platelet activation declined within 24 hours of surgery (P≤0.005), but clopidogrel treatment was associated with greater reductions throughout the immediate post-operative period (P=0.0019; Figure 5.2). In order to assess pharmacological efficacy of the trial intervention, *ex vivo* platelet aggregation to 5 μM adenosine diphosphate was performed in a sub-group of trial participants (n=10 per group). This confirmed that clopidogrel inhibited adenosine diphosphate-induced aggregation (59±20% to 33±18%, P<0.0001) throughout the peri-operative period (P=0.0015; data not shown).

Myocardial Injury

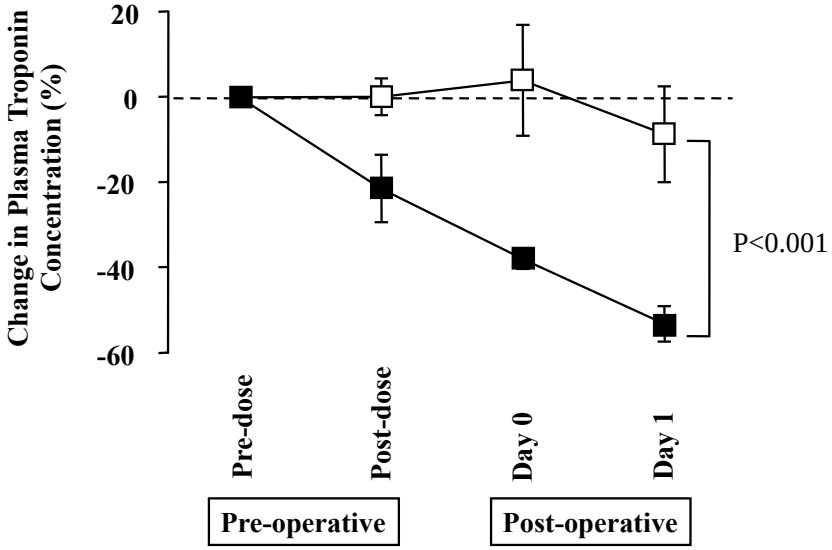
Of the 108 trial subjects, eighteen (16.7%) suffered an elevated plasma troponin concentration (>0.032 ng/mL): 8 (16.0%) received clopidogrel and 10 (17.2%) placebo (relative risk (RR) 0.93, 95% confidence intervals (CI) 0.40 to 2.17; P=0.86). Nine (8.3%) patients (4 clopidogrel and 5 placebo) had an elevation of plasma troponin concentration *prior* to surgery, and nine patients (4 clopidogrel and 5 placebo) suffered a post-operative rise in troponin. Of those nine patients who sustained a *pre*-operative troponin rise, plasma troponin concentrations fell with clopidogrel therapy but remained unchanged or increased in those on placebo (Figure 5.3). Patients with *post*-operative elevation in plasma troponin concentrations had greater platelet-monocyte aggregates (40±4% versus 30±2%; P=0.033).

Figure 5.2 Platelet-monocyte aggregates and platelet P-selectin expression over the peri-operative period in patients receiving placebo and clopidogrel .



Platelet-monocyte aggregates (squares; P<0.0001 for both groups) and platelet P-selectin expression (circles; P≤0.005 for both groups) fell in both groups following surgery. Clopidogrel (closed symbols) caused a greater reduction in both platelet-monocyte aggregates (P=0.0019) and platelet P-selectin expression (P=0.0035) compared to placebo (open symbols). Symbols represent mean±SD; analysis by 2-way ANOVA

Figure 5.3 Change in plasma troponin concentrations in patients with pre-operative troponin elevations on placebo (open squares) and clopidogrel (closed squares).



Symbols represent mean±SD. P<0.001; 2-way ANOVA, clopidogrel (n=4) *versus* placebo (n=5).

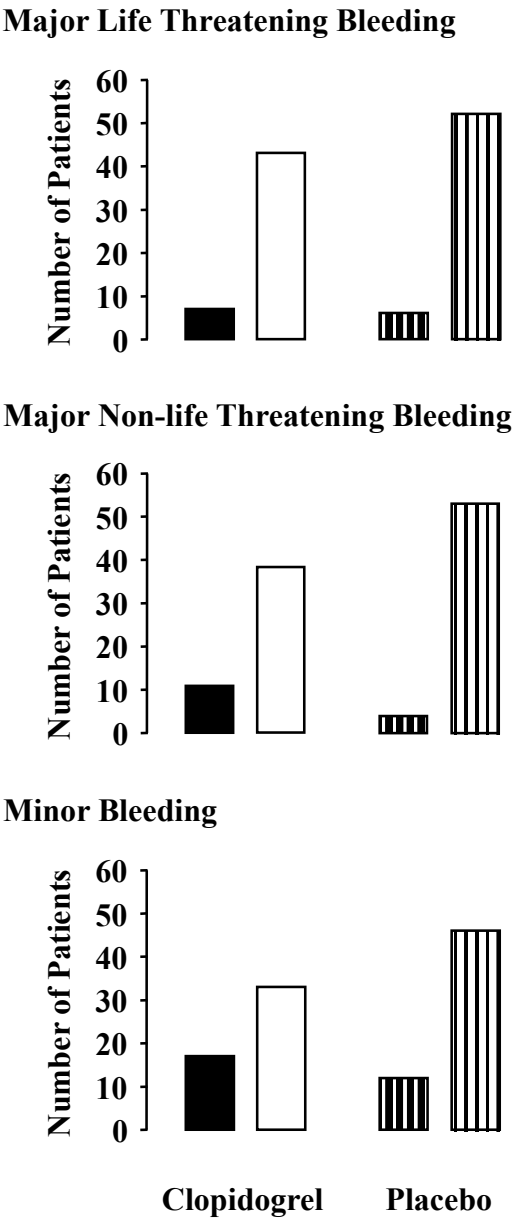
Clinical Outcomes

Of the 108 trial participants, seven patients (6.5%) sustained an acute myocardial infarction: 3 (6.0%) in the clopidogrel group and 4 (6.9%) in the placebo group (RR 0.87 95% CI 0.20 to 3.7, P=0.85). There were no in-patient deaths, intracranial haemorrhages or incidences of inotrope use. There was no difference in life-threatening major bleeding between treatment groups (7 (14%) clopidogrel and 6 (10%) placebo; RR 1.35, 95% CI 0.49 to 3.76; P=0.56), although those who received clopidogrel had an increased risk of major non-life-threatening bleeding (11 (22%) clopidogrel and 4 (7%) placebo, RR 3.19, 95% CI 1.1 to 9.4; P=0.024; Figure 5.4). Twenty (40%) patients receiving clopidogrel underwent red-cell transfusion compared to only 8 (14%) on placebo (P=0.0019). Restricting analyses to transfusions administered in accordance with the SIGN guidelines, there remained an increased transfusion rate in the clopidogrel group (14 (28%) clopidogrel and 7 (12%) placebo; RR 2.32, 95%CI 1.02 to 5.29; P=0.037). There was no difference in minor bleeding between the two groups (17 (34%) clopidogrel and 12 (21%) placebo; RR 1.64, 95% CI 0.87 to 3.10; P=0.12). Five patients suffered gastrointestinal bleeding (haematemesis or malaena); four of whom were receiving placebo. Two patients received intra-operative protamine – one in each intervention arm.

There were two re-operations for bleeding in the placebo group and one in the clopidogrel group. Although there was an increase in wound leak in those patients who received clopidogrel (13 (26%) versus 3 (5%); RR 5.03, 95% CI 1.52 to 16.6; P=0.0024), there was no difference in incidence of wound infection at 3 months (P=0.80). Clopidogrel therapy did not increase the length of operation (P=0.60) or hospital stay (P=0.72). Sub-group analysis of patients undergoing revascularisation compared to amputation revealed no significant differences in clopidogrel versus placebo in terms of peri-operative adverse cardiovascular

events or bleeding outcomes (major life threatening, major non-life threatening or minor). There were no incidences of early graft failure in either group.

Figure 5.4 Bleeding Outcomes



Major life-threatening (upper panel; RR 1.35, 95% CI 0.49 to 3.76, P=0.56), major non-life-threatening (middle panel; RR 3.19, 95% CI 1.08 to 9.4, P=0.024) and minor (RR 1.64 95% CI 0.87 to 3.1, P=0.12) bleeding (closed bars) in patients on clopidogrel (left-hand panels, non-stippled) and placebo (right-hand panels, stippled).
Chi-squared analysis.

5.5 Discussion

We have conducted the first double-blind randomised controlled trial of peri-operative dual anti-platelet therapy in patients undergoing surgery for critical limb ischaemia. We demonstrate improvements in biomarkers of platelet activation and myocardial injury without causing unacceptable bleeding complications. These data form the first objective assessment of the risks and benefits of peri-operative dual anti-platelet therapy in patients undergoing high-risk vascular procedures.

Peripheral arterial disease affects nearly 30 million people in Western Europe and North America. In up to three-quarters of cases, patients have co-existent coronary artery disease and a 3-fold increased risk of cardiovascular events and death [Hackman DG *et al*, 2007]. In a recent large observational study (n=5,460), patients with peripheral arterial disease scheduled for open vascular surgery had a worse prognosis (2.4-fold increase in cardiovascular morbidity) than matched patients with severe myocardial ischaemia referred for percutaneous coronary intervention [Welten GM *et al*, 2008]. Interestingly the occurrence of peri-operative cardiac complications following vascular surgery was associated with long-term cardiac death. There is therefore a clear unmet need to reduce cardiovascular events in patients with peripheral arterial disease, especially in the peri-operative period.

In agreement with previous studies [Mangano DT *et al*, 1991; Hobbs SD *et al*, 2005; Kim LJ *et al*, 2002; Landesburg G *et al*, 2003], we report a high incidence of peri-operative myocardial infarction (6.5%) and troponin elevation (16.7%) in patients undergoing surgery for critical limb ischaemia. We also found that post-operative elevations in troponin were associated with increased levels of post-operative platelet activation. However, we were surprised to find that

markers of platelet activation fell post-operatively (both in clopidogrel and placebo groups) and that half of the troponin-positive events occurred *before* surgery. This occurred in the absence of renal dysfunction. This suggests that rather than surgery being an additional thrombotic stimulus, removal of ischaemic tissue may actually reduce platelet activation in the majority of these patients. These findings highlight the prevalence of 'silent' pre-operative myocardial injury, and the important systemic pro-thrombotic consequences of critical limb ischaemia, as well as the need for pre-operative intervention.

This study aimed to explore a proof of concept. Pre-clinical studies of platelet activation in coronary patients have informed the design of large scale trials which have subsequently shaped clinical practice. These trials required several thousands of patients to demonstrate a small but significant clinical benefit from anti-platelet regimes. Despite suffering significant cardiovascular risk, there has been little study of platelet activation in high-risk vascular surgical patients. The potential for adverse bleeding would seem greater for patients undergoing open surgery under anti-platelet therapy. Consequently, any large-scale trial powered to examine clinical end points should be justified by 'pilot' data suggesting that some cardiovascular benefit could be achieved without excessive bleeding.

Through the use of surrogate biomarkers, we have demonstrated the potential benefits of dual anti-platelet therapy in the peri-operative period. Clopidogrel reduced sensitive markers of platelet activation (Figure 5.2) that are associated with increased clinical risk as well as the progression of atherothrombosis [Freedman JE *et al*, 2002; McGregor L *et al*, 2006; Michelson AD *et al*, 1996; Huo Y *et al*, 2003; Michelson AD *et al*, 2001; Harding SA *et al*, 2004; Furman MI *et al*, 2001; Hayes PD *et al*, 2003]. Although clopidogrel did not have an overall effect on the number of peri-operative troponin-positive events (8 clopidogrel *versus* 10 placebo;

P=0.86), half of the troponin elevations occurred pre-operatively and prior to the administration of the study medication. Subsequent institution of clopidogrel therapy was associated with a marked reduction in troponin concentrations (Figure 5.3). However, we readily acknowledge that our study was not primarily powered to assess cardiac troponin and clinical outcomes and whether these improvements in surrogate biomarkers translate into clinical benefit remains to be established.

All therapies have potential benefits and risks, and it is inevitable that anti-platelet therapies will be associated with increased bleeding complications. Although it may be regarded as potentially reversible, the importance of major bleeding must not be underestimated as it remains an independent predictor of adverse clinical outcome [John W *et al*, 2006; Moscucci M *et al*, 2003; Yang Xin *et al*, 2005]. Although vascular patients are at high risk of cardiovascular events, bleeding concerns are a major disincentive for the investigation of peri-operative intensive anti-platelet regimes, and perhaps underlie the paucity of such data. Most published reports are observational [Smout J *et al*, 2003; Assadian A *et al*, 2005] although randomised-controlled trials of dual anti-platelet therapy have been performed in patients undergoing carotid endarterectomy [Payne AD *et al*, 2004] and peripheral angioplasty [Cassar K *et al*, 2005], and report no major increase in bleeding complications. However, there have been no randomised-controlled trials of dual anti-platelet therapy in surgery for critical limb ischaemia, where the potential for both peri-operative bleeding and cardiac complications is greater. We have successfully delivered such a trial and confirmed that whilst bleeding is increased, there was no excess of life-threatening bleeds, re-operations or wound infections. Our results are consistent those reported by the CURE trial [Fox KA. *et al*, 2004] where those patients who continued clopidogrel therapy within 5 days of coronary artery bypass had a 2-fold increased relative risk of major bleeding. Arguably our pilot data suggests that the

bleeding risks of dual anti-platelet therapy in the peri-operative period are modest, and perhaps often over estimated.

Increasingly patients are undergoing surgery for critical limb ischaemia with a recent history of a cerebrovascular event, acute coronary syndrome or percutaneous coronary intervention, and will be receiving intensive anti-platelet regimes. Their peri-operative management must protect them from both co-existing pathology as well as the associated risks of surgery. Our study has shown that it is feasible to perform vascular surgery for critical limb ischaemia, under dual anti-platelet therapy with an acceptable bleeding profile. Although the absolute clinical benefits of such a regime need to be validated in a large-scale clinical trial, we believe that our study provides evidence for the beneficial role of peri-operative anti-platelet agents in protecting these patients against cardiovascular complications.

Patients receiving epidural or spinal anaesthesia were not recruited into the study due to the theoretical risk of epidural haematoma. We are aware that many vascular units aim for epidural or spinal anaesthesia, thus precluding many patients from peri-operative anti-platelet therapy. However, there is currently no level-one evidence for superior cardiovascular outcome with neuraxial blockade compared to general anaesthesia. The main benefit lies in reducing respiratory complications associated with abdominal surgery [Rodgers A *et al*, 2000]. Although many of the patients who were recruited smoked, none had significant chronic obstructive airway disease (based on lung function testing) and none were undergoing abdominal or emergency surgery. Use of both trial medication and general anaesthesia was therefore deemed appropriate.

Persistent platelet reactivity despite anti-platelet therapy (clopidogrel 'resistance') has been proposed as a risk factor for the recurrence of ischemic events following PCI. Recent mechanistic and clinical data suggest that higher loading and maintenance doses of clopidogrel may achieve a more rapid and greater degree of platelet inhibition that translates into improved clinical outcomes, but this is yet to be formally evaluated in an adequately powered randomized trial (CURRENT-OASIS 7, 2008). We administered a relatively high pre-operative loading dose of clopidogrel (600 mg) to ensure efficacy of the intervention during surgery, and in an attempt to overcome 'non-responders' to the drug. It was hypothesized that if additional peri-operative anti-platelet therapy was to be of any therapeutic advantage then this should be demonstrated with the largest degree of platelet inhibition. Previous studies have reported increased platelet activation, [Hayes PD *et al*, 2003; Collins P *et al*, 2006; Mohan IV *et al*, 2007; Samama CM *et al*, 2001; Rajagopalan S *et al*, 2007], and cardiovascular events occurring within the first 5 days following surgery. We therefore rationalised that clopidogrel therapy would be of most benefit when given pre-operatively and continued in the immediate post-operative period. It is possible that we could still achieve atherothrombotic protection with reduced bleeding complications by administering lower doses of clopidogrel and this requires further clarification. However, given the relatively high incidence of troponin-positive events before surgery, we would recommend initiation of therapy prior to surgery.

In conclusion, we have demonstrated that peri-operative dual anti-platelet therapy has beneficial effects on reducing biomarkers of atherothrombosis without increasing life-threatening bleeds in patients with critical limb ischaemia. We propose that large-scale randomised controlled trials are needed to establish whether dual anti-platelet therapy can improve clinical outcomes in high-risk patients undergoing vascular surgery.

CHAPTER 6

INFLAMMATORY BIOMARKERS AND DUAL ANTI-PLATELET THERAPY IN PATIENTS UNDERGOING SURGERY FOR CRITICAL LIMB ISCHAEMIA

6.1 Summary

Platelets play a key role in vascular inflammation through release of pro-inflammatory mediators and interactions with other inflammatory cells. We investigated the changes in inflammatory biomarkers during surgery for critical limb ischaemia and examined the effect of additional pre-operative clopidogrel therapy on peri-operative inflammation.

In a double-blind randomised controlled trial of pre-operative clopidogrel therapy in surgery for critical limb ischaemia, peri-operative serum markers of inflammation (hsCRP, IL-6, sCD40L) and expression of CD40/CD40L and CD11b on platelets and monocytes, were measured by sensitive assays and flow cytometry, respectively.

In contrast to *in vivo* platelet activation, levels of inflammatory markers rose in the post-operative period (monocyte CD40 P=0.018; IL-6 P<0.0001; hsCRP P<0.0001). Pre-operative clopidogrel therapy reduced monocyte CD40 expression (53.4±2.3% to 46.6±2.1%, P<0.0001); monocyte CD11b (53.6±3.4 MFI to 39.9±2.6 MFI, P<0.0001); platelet CD40L expression (14.4±0.9% to 8.5±0.78%, P<0.0001); soluble CD40L (1.3±0.3 ng/mL to 0.98±0.3ng/mL, P=0.029) and serum IL-6 (28.3±5.2pg/mL to 16.1±3.5pg.mL, P<0.0001), but had no significant effect on peri-operative hsCRP (P=0.208). Patients with peri-operative elevation in plasma troponin concentrations had greater pre-operative levels of monocyte CD40 expression, serum hsCRP and IL-6 (59.0±3.9% versus 50.1±1.4%, P=0.014; 53.0±20.3mg/mL versus 17.4±2.9mg/mL, P=0.002 and 55.6±22.6pg/mL versus 16.1±3.5pg/mL, P=0.0038, respectively).

In conclusion, pre-operative clopidogrel therapy had beneficial effects on cellular and serum markers of inflammation, although whether this was via a platelet mediated action or direct effect is unclear.

6.2 Introduction

Inflammation plays a central role in the pathogenesis of atherosclerosis and its complications [Lusis AJ, 2000]. In addition, elevations in inflammatory biomarkers have been demonstrated to be prognostic of clinical risk. Patients with peripheral arterial disease (PAD) have an increased incidence of adverse cardiovascular events compared to the healthy population, especially at the time of operation. Surgical stress is known to generate a pro-inflammatory state, yet there has been little investigation of its role in peri-operative cardiovascular events, especially in those with underlying vascular dysfunction.

CRP

C-reactive protein (CRP) is an acute phase protein produced by hepatocytes largely in response to interleukin-6 stimulation. A substantial body of evidence demonstrates that elevated CRP is predictive of atherothrombotic events. Large-scale studies of healthy subjects have found that CRP is a strong independent predictor of future cardiovascular risk [Kuller LH *et al*, 1996; Koenig W et al, 1999; Tracy RP *et al*, 1997; Ridker PM *et al*, 1997,1998, 2000]. In patients with acute coronary syndromes, elevated baseline levels of CRP predict increased adverse cardiovascular outcome at intervals ranging from 14 days [Morrow DA *et al*, TIMI IIa substudy, 1998] to several years [Lindahl B *et al*, FRISC study group, 2000]. Studies have also demonstrated baseline levels of CRP to be predictive of intermediate and long term adverse cardiovascular events following surgery for PAD [Padayachee L *et al*, 2009].

In addition, CRP may also play a pathogenic role in atherothrombosis. CRP promotes potent proatherogenic effects in endothelial cells, smooth muscle cells, platelets, and monocyte macrophages *in vitro* [Zwaka TP *et al*, 2001; Ballou SP *et al*, 1992; Pasceri V *et al*, 2000, 2001]. Griselli *et al*, 1999, demonstrated that administration of human CRP in experimental acute myocardial infarction reproducibly enhanced infarct size by ~40%. The JUPITER trial

also reported that in patients with high CRP and no other major risk factor, statin therapy resulted in a significant benefit in cardiovascular outcome [Ridker PM *et al*, 2008].

CD40/CD40L

A key inflammatory mediator involved in atherosclerosis is membrane glycoprotein receptor CD40 and its ligand CD40L [Schonbeck U et al, 2001]. CD40L stimulates the release of various inflammatory cytokines as well as the expression of adhesion molecules and matrix-degrading metalloproteinases [Urbich C *et al*, 2004]. In addition, CD40/CD40L interaction increases procoagulant activity by stimulating tissue factor expression [Slupsky JR *et al*, 1998]. In animal models, disruption of CD40/CD40L in ApoE$^{-/-}$ mice leads to reduction in size of atherosclerotic lesions [Mach F *et al*, 1997].

CD40L is carried within platelet granules and translocates to the cell surface on activation, with platelets providing 95% of soluble CD40L in the circulation. This has led to the suggestion that platelet CD40L may be the pivotal link between the processes of thrombosis, inflammation and atherosclerosis.

Consistent with these activities, elevations in the CD40/CD40L dyad have been associated with increased risk of cardiovascular events in healthy women, diabetics, smokers and patients with acute coronary syndromes [Schonbeck U *et al*, 2001; Harding SA *et al*, 2004; Harding SA *et al*, 2004; Heeschen C *et al*, 2003]. Several observational studies have also demonstrated that patients with PAD have higher levels of circulating inflammatory biomarkers compared to the healthy population [Wildman RP *et al*, 2005], with levels increasing with severity of disease [Mohler ER *et al*, 2003; Cassar K *et al*, 2005; Blann AD *et al*, 2005; Lee WJ *et al*, 2006; Nylænde M *et al*, 2006].

Anti-inflammatory action of anti-platelet agents

An increasing body of evidence suggests that inhibition of platelet function with anti-platelet agents can modulate inflammatory markers. During platelet activation, degranulation of intra-cellular granules occurs, releasing inflammatory chemokines, cytokines and growth factors. This leads to cellular adhesion, release of pro-inflammtory mediators and further vascular inflammation and thrombus propagation [Libby PM *et al*, 2002; Libby PM *et al*, 2006]. Thus, platelets play key roles in both thrombus formation and inflammation, highlighting the complex interplay between coagulation and inflammation in the pathogenesis of atherothrombosis. Anti-platelet agents have been demonstrated to reduce inflammatory markers, both in animal models and clinical studies [Ayral Y *et al*, 2007; Egan KM *et al*, 2005; Herbert JM *et al*, 1992; Molero L *et al*, 2005].

Clopidogrel in addition to aspirin, has been shown to reduce hsCRP and soluble CD40L in patients with stable coronary artery disease [Heitzer T *et al*, 2006; Azar RR *et al*, 2006] and in acute coronary syndromes and percutaneous coronary intervention [Vavuranakis M *et al*, 2006; Xiao Z *et al*, 2004; Vivekananthan DP et al, 2004; Quinn MJ *et al*, 2004; Saw J *et al*, 2008; Chew DP *et al*, 2001, Gottsauner-Wolf M *et al*, 2000]. However, a causal relationship between reduced inflammatory biomarkers and reduced clinical adverse events, remains to be clearly demonstrated.

There has been no investigation of anti-platelet agents and inflammation during the peri-operative period in patients with severe critical limb ischaemia. As part of a double-blind prospective randomised trial of pre-operative clopidogrel in addition to aspirin, we studied the changes in peri-operative inflammatory biomarkers and the effect of dual anti-platelet therapy upon them. We hypothesized that:

1. Inflammatory biomarkers would be increased in those patients who developed peri-operative myocardial injury.
2. Combined peri-operative aspirin and clopidogrel therapy would improve inflammatory biomarkers.

6.3 Methods

Study Design and Subjects

As previously outlined in Chapter 5, a prospective single centre double-blind randomised controlled trial was conducted at a tertiary referral vascular surgical unit in the Royal Infirmary of Edinburgh, South-East Scotland, UK. Patients with critical limb ischaemia who were scheduled for infra-inguinal bypass, femoral endarterectomy or lower limb amputation under general anaesthesia were recruited into the study.

In brief, following recruitment, patients received 600 mg of clopidogrel or matched placebo 4-28 hours prior to surgery and received 75 mg of clopidogrel or matched placebo daily for 3 days after surgery.

Assessment of inflammatory bio-markers

Blood samples were taken before, and a minimum of 4 hours after, a loading dose of 600 mg of clopiogrel or matched placebo, immediately after the operation in the recovery room, and on days 1 and 2 after surgery**.**

CD40/CD40L

Flow cytometric measurements of *monocyte* CD40 and CD11b expression were used as markers of *in vivo* monocyte activation as described previously [Harding SA *et al*, 2004]. Immunolabelling was performed in whole blood within 5 min of collection. To evaluate CD40 and CD11b on monocytes, blood was diluted 1:2 with PBS and incubated with the following monoclonal antibodies: anti-CD14:FITC (Serotec), anti-CD40:PE (Serotec), anti-CD11b:PE (Serotec) and appropriate isotype-matched controls for 20 min, before fixation and erythrocyte lysis with 500 µL of FACSLyse solution. Monocytes were identified by gating for CD14 positive cells.

In addition *platelet* CD40L and soluble CD40L were also assessed. Platelet membrane CD40L expression was measured using flow cytometry as previously described [Harding SA *et al*, 2004]. To evaluate CD40L on platelets, whole blood was diluted 1:10 with PBS and incubated with FITC-conjugated CD42a (Serotec) and PE-conjugated CD154 (eBioscience) and appropriate isotype-matched controls for 20 minutes, before cells were further diluted with 1% paraformaldehyde. CD40L positive platelets were identified by gating for CD42a and CD154 positive cells. Soluble CD40L was assessed using an ELISA from Bender Med Systems.

CRP

Serum CRP concentrations were determined with a validated highly sensitive assay (Department of Clinical Biochemistry; Fife NHS Trust, UK) using the method of particle-enhanced immunonephelometry (Behring BN II nephelometer, Dade Behring Inc.). This sensitive assay has a detection limit of 0.18mg/L. Intra-assay and inter-assay coefficients of variability for hs-CRP were 3.7% and 4.2% respectively. All assays were performed in duplicate and the mean value taken. Whole blood samples for the measurement of hsCRP were collected into citrated tubes, centrifuged and plasma collected at each time point. The serum was frozen and stored until the end of the trial when samples were processed en masse.

IL-6

Plasma interleukin- 6 (IL-6) concentrations were measured with commercially available ELISA (Quantikine, R&D systems). Intra-assay and inter-assay coefficients of variability were 4.2% and 6.4% respectively. Whole blood samples for the measurement of IL-6 were collected at each time point and plasma prepared, frozen and stored until the end of the trial when samples were processed en masse.

Myocardial Injury

The Reference Clinical Biochemistry Laboratory measured plasma troponin I (cTn I) concentrations using the ARCHITECT Troponin I *STAT* assay (Abbott Diagnostics, Maidenhead, UK) using an autoanalyser. This has an analytical sensitivity of 0.009 ng/mL and a functional sensitivity of 0.032 ng/mL with a co-efficient of variation of <10%. The latter threshold was employed for the clinical case definition of myocardial infarction [ESC/ACC/AHA/WHF, 2007].

Statistical Analysis

The overall study was primarily powered to examine differences in peri-operative platelet-activation. The sample size (n=50 per group), was based on power calculations derived from our previous studies [Sarma J *et al*, 2002; Harding SA *et al*, 2004], and were designed to give an 80% power of detecting a 3.6% difference in CD40 expression on monocytes, 3.7% in CD40L expression on platelets, and 0.11 ng/mL in plasma sCD40L concentrations, at a significance level of 5%. Continuous variables are reported as mean±SEM. Analysis of variance with repeated measures, two-tailed Student's *t*-test and Spearman or Pearson correlation analysis were performed as appropriate using GraphPad Prism Version 4 (La Jolla, USA). Statistical significance was taken as a two-sided P value <0.05.

6.4 Results

Of the 159 potentially eligible patients, 113 were randomised to trial medication (Figure 5.1, Chapter 5). Of those who completed the study protocol, 58 received placebo and 50 received clopidogrel. There was no difference in baseline demographics between the two groups (Table 5.1, Chapter 5).

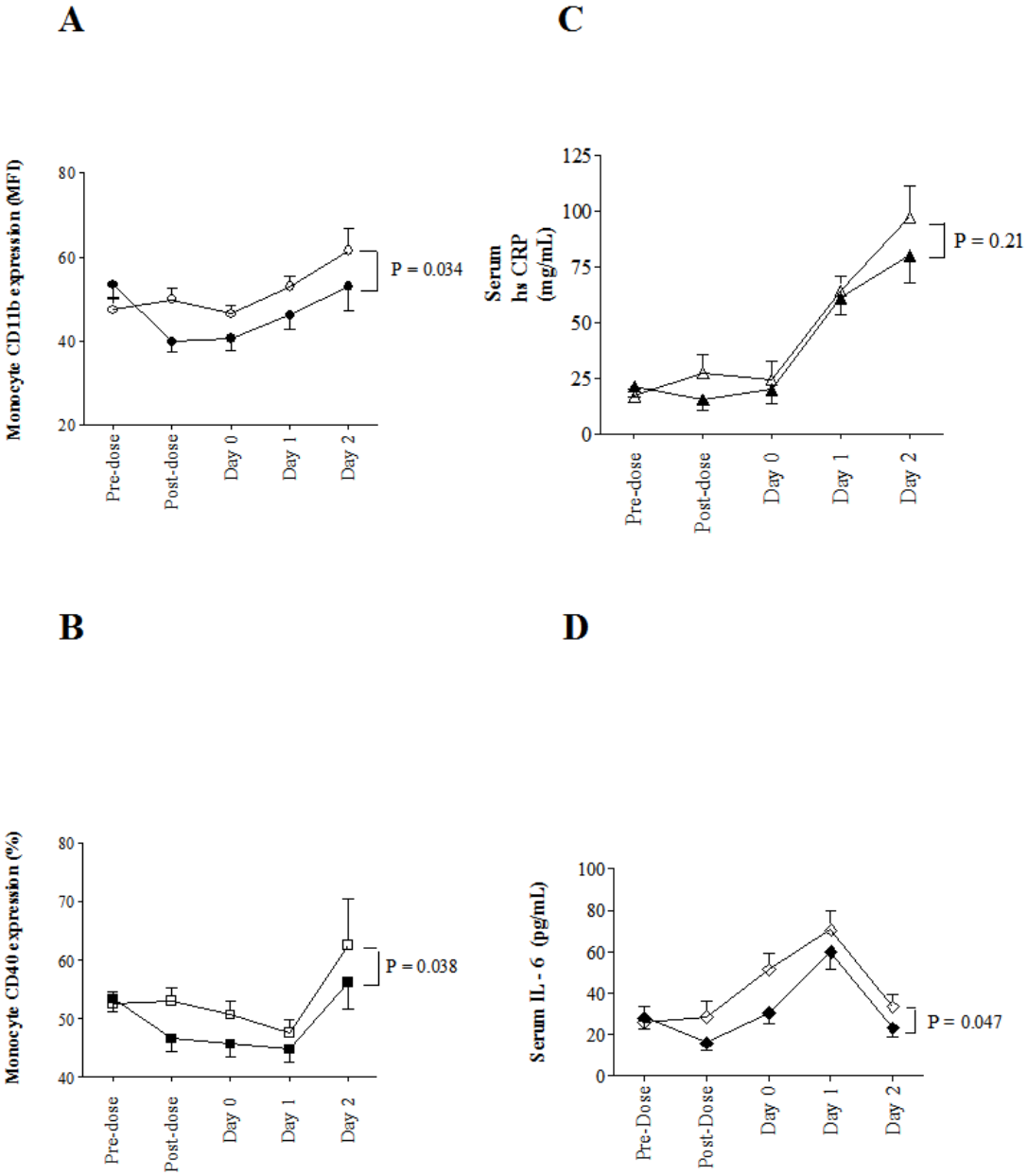
1. Post-operative inflammation and platelet activation

We have previously reported, in the same patient population, that markers of platelet activation fell following surgery in both placebo and clopidogrel groups (Figure 5.2, Chapter 5). In contrast, inflammatory markers rose in the post-operative period (monocyte CD40 P=0.018; IL-6 P<0.0001; hsCRP P<0.0001) Figure 6.1.

2. Dual anti-platelet agents and peri-operative inflammatory markers

Inflammatory markers were unaffected by placebo (P>0.05) but clopidogrel (600 mg) caused a rapid reduction in monocyte CD40 expression (53.4±2.3% to 46.6±2.1%, P<0.0001); monocyte CD11b (53.6±3.4 MFI to 39.9±2.6 MFI, P<0.0001); platelet CD40L expression (14.4±0.9% to 8.5±0.78%, P<0.0001); soluble CD40L (1.3±0.3 ng/mL to 0.98±0.3ng/mL, P=0.029) and serum IL-6 (28.3±5.2pg/mL to 16.1±3.5pg.mL, P<0.0001). Clopidogrel treatment was associated with greater reductions in monocyte CD40 and CD11b and IL-6 throughout the immediate post-operative period (P=0.038; P=0.034; P=0.047 respectively. Figure 6.1 A, B and D). Additional clopidogrel therapy had no statistically significant effect on peri-operative hsCRP (P=0.208. Figure 6.1C).

Figure 6.1 Dual anti-platelet therapy and peri-operative inflammation in patients undergoing surgery for CLI



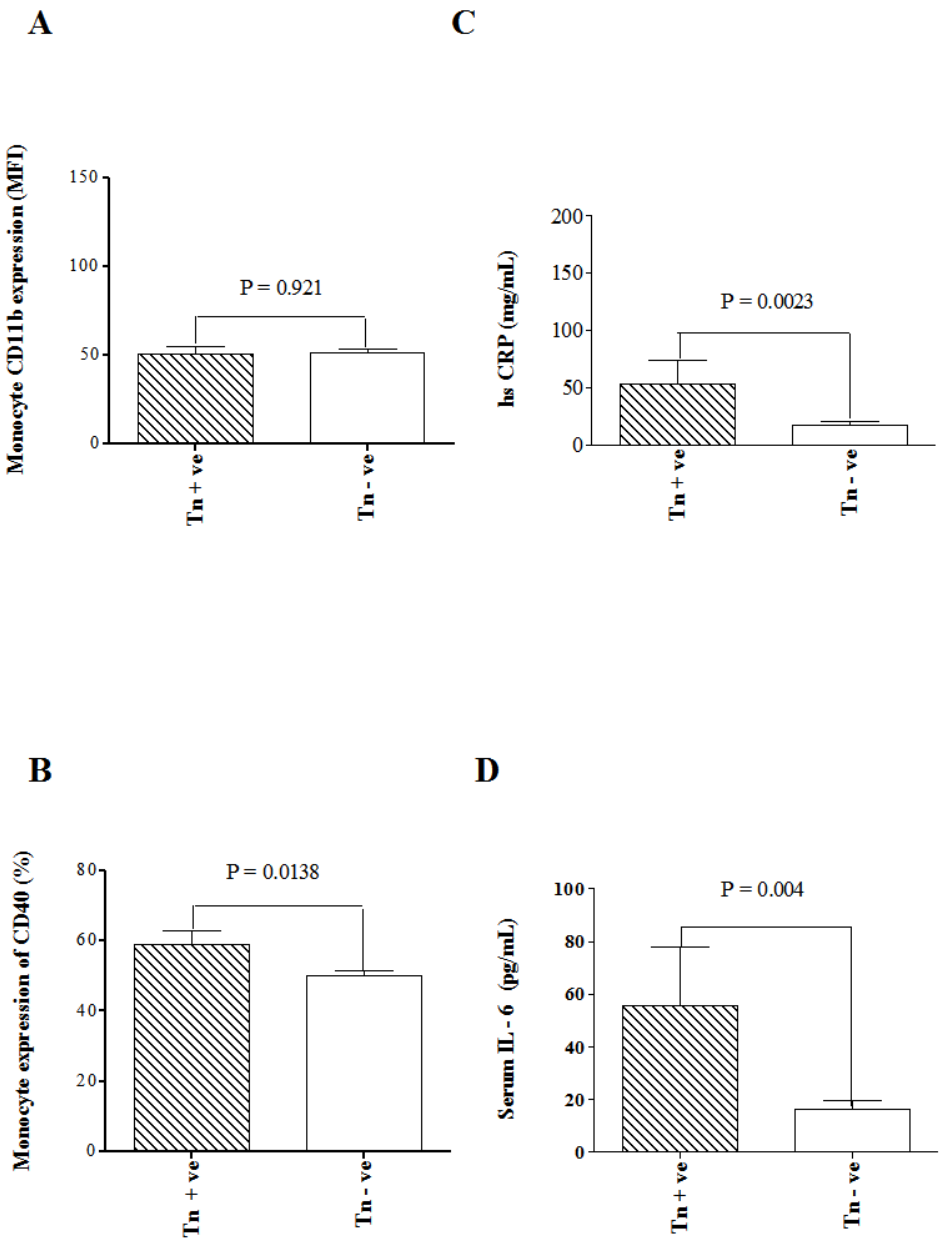
Monocyte CD11b (●), **B.** Monocyte CD40 expression (■), **C**. Serum hsCRP (▼) and **D.** Serum IL-6 (♦) rose in both groups on day 1 post-surgery (P=0.008; P=0.038; P<0.0001 and P= respectively). Clopidogrel (closed symbols) caused a greater reduction in monocyte CD11b expression (P=0.034); monocyte CD40 expression (P=0.038) and serum IL-6 levels (P=0.047) compared to placebo (open symbols), but had no overall effect on hsCRP levels (P=0.208). Symbols represent mean±SEM; analysis by 2-way ANOVA.

3. Inflammatory markers and myocardial injury

Of the 108 trial subjects, eighteen (16.7%) suffered an elevated plasma troponin concentration (>0.032 ng/mL): 8 (16.0%) received clopidogrel and 10 (17.2%) placebo (relative risk (RR) 0.93, 95% confidence intervals (CI) 0.40 to 2.17; P=0.86).

Patients with peri-operative elevation in plasma troponin concentrations had greater pre-operative levels of monocyte CD40 expression, serum hsCRP and IL-6 (59.0±3.9% versus 50.1±1.4%, P=0.014; 53.0±20.3mg/mL versus 17.4±2.9mg/mL, P=0.002 and 55.6±22.6pg/mL versus 16.1±3.5pg/mL, P=0.0038. Figure 6.2). Pre-operative monocyte CD11b, platelet CD40L or soluble CD40 levels were not increased in those patients suffering an increase in plasma Troponin concentration (cTn I) (P=0.92; P=0.62; P=0.4 respectively). Peri-operative monocyte CD40 and CD11b expression correlated with serum hsCRP (r=0.11, P=0.038 and r=0.11, P=0.046 respectively) but only hsCRP and IL-6 directly correlated with cTn I levels (r=0.29, P<0.0001 and r=0.21, P=0.0008 respectively).

Figure 6.2 Inflammatory biomarkers in patients with or without a peri-operative Troponin elevation.



Pre-operative levels of **A.** Monocyte CD11b, **B.** Monocyte CD40 expression, **C**. Serum hsCRP, **D.** Serum IL-6, in Troponin + ve (hached bars) and Troponin –ve (open bars) patients. Monocyte CD40 expression (59.0±3.9% versus 50.1±1.4%, P=0.014), hsCRP levels (53.0±20.3mg/mL versus 17.4±2.9mg/mL, P=0.002) and IL-6 levels (55.6±22.6pg/mL versus 16.1±3.5pg/mL, P=0.0038.) were significantly greater in patients suffering a peri-operative Troponin elevation. There was no difference in monocyte CD11b (P=0.92). Bars represent mean±SEM; analysis by un-paired t-test.

6.5 Discussion

We have previously demonstrated in accordance with other studies, that patients with severe peripheral arterial disease have increased baseline levels of inflammation in comparison to healthy controls, or those with claudication. In addition, their inflammatory profile is similar to those being treated for acute coronary syndromes (Chapter 4). In this study, surgery for critical limb ischaemia was associated with a rise in inflammation post-operatively. This is in contrast to a post-operative fall in platelet activation (Chapter 5). Additional pre-operative clopidogrel therapy had beneficial effects on cellular markers of inflammation and IL-6, although serum hsCRP, was not directly affected. Inflammatory markers correlated with peri-operative Troponin release, although whether this was the cause or effect of myocardial ischaemia is unclear.

Vascular inflammation is an underlying mechanism central to atherosclerotic disease. Data from animal and human model systems suggest that modulation of platelet function may also modulate expression of known inflammatory mediators, including CD40L and CRP. Therefore, anti-platelet therapy may reduce adverse cardiovascular outcomes, not only by reducing platelet activation and aggregation, but also by limiting inflammatory amplification pathways.

Post-operative changes in markers

We were surprised to find that in our patient population markers of platelet activation fell following surgery (Chapter 5) whilst inflammatory markers increased (Figure 6.1). It is generally accepted that the physiological response to trauma includes an increase in circulating inflammatory mediators and a pro-thrombotic state (Chapter 1). Inflammatory markers show a sharp rise in the setting of an acute coronary syndrome or PCI that lasts for 48-72 hours, and then returns to baseline [Liuzzo G *et al*, 1994; 1998]. It has been suggested that intra-arterial thrombus formation during ACS may cause the subsequent increase in inflammatory markers

by means of CD40L and sCD40L activity [Levi M *et al*, 2004; Ankrust P *et al*, 2004; Henn V *et al*, 1998]. Perhaps the disconnect between the post-operative fall in platelet activation and the later rise in inflammation seen in our studies, represents this pathogenic cycle of thrombosis and inflammation.

Effect of clopidogrel

Together with earlier data (Chapter 5), we have shown that additional clopidogrel therapy reduces both peri-operative platelet activation markers (platelet-monocyte aggregation (PMA), platelet P-selectin, and platelet CD40L), and markers of inflammation (soluble CD40L, monocyte CD40 and 11b and IL-6). This is in line with the anti-inflammatory effect of additional clopidogrel therapy in patients with acute coronary syndromes [Xiao Z *et al*, 2004] or undergoing PCI [Quinn MJ *et al*, 2004]. However, it is unclear whether the reduction in inflammatory markers, is due to a direct action of clopidogrel; as a result of platelet inhibition or due to prevention of thrombosis and ischaemia (and subsequent inflammation).

PMA correlates positively with CD40 and CD11b expression on monocytes (Chapter 3). Formation of platelet-monocyte aggregates is known to activate NF-B – a transcriptional activator involved in regulating CD40 gene expression. In addition, binding of platelets to leucocytes, leads to surface expression of B2 integrin Mac-1. Inhibition of platelet activation through the action of anti-platelet agents, with subsequent reduction of PMA formation may therefore reduce CD40 and CD11b expression on monocytes. Similar correlations between platelet and inflammatory markers, have been demonstrated in smokers and patients with acute coronary syndromes [Harding S *et al*, 2004; Zhang SZ *et al*, 2007]. Human platelets carry preformed CD40L molecules, which appear on the platelet surface after stimulation. Many studies support the hypothesis that platelet activation/aggregation, via CD40L and sCD40L activity, may induce a pro-inflammatory cytokine response [Henn V *et al*, 1998; Levi M *et al*,

2004; Merino A *et al*, 2012]. Since CD40/CD40L binding stimulates release of cytokines such as IL-6, inhibition of platelet activation and subsequent reduction in CD40 could be expected to reduce levels of this inflammatory protein.

We did not demonstrate any reduction in hsCRP levels with additional clopidogrel therapy. In contrast, larger scale studies in patients undergoing PCI have reported reductions in post-procedural hsCRP with combined pre-procedural clopidogrel and aspirin therapy [Vivekananthan DP *et al*, 2004; Chew DP *et al*, 2001, Gottsauner-Wolf M *et al*, 2000]. It may be that we were underpowered to demonstrate changes in CRP.

In other studies of patients with acute coronary syndromes, the reduction in serum hsCRP was not seen until after at least 7 days of additional clopidogrel treatment [Chen YG *et al*, 2006] with a shorter duration of treatment having no effect [Montalescot G *et al*, 2006]. Combination therapy in patients with stable coronary heart disease has also had a variable result on serum CRP [Azar RR *et al*, 2006; Heitzer T *et al*, 2006]. Our duration of clopidogrel treatment may have therefore been too short to produce any change in CRP (although we had given loading doses). Furthermore, our-post-operative sampling period may not have been long enough to reveal any impact from the drug.

Inflammatory markers and peri-operative Troponin

Inflammatory markers correlated with troponin levels. However, it is unclear whether this is cause or effect, as ischaemia itself can induce an inflammatory state. Dual anti-platelet therapy reduced monocyte CD40 and IL-6, which were associated with peri-operative Troponin release. However, there was no significant reduction in peri-operative Troponin with additional clopidogrel therapy. Despite 18 (16.7%) of the study population suffering a Troponin rise, our study was still underpowered to examine Troponin as a primary outcome measure.

In summary, although there is increasing understanding of the role of inflammatory mediators in atherothrombotic events, the complex pathogenesis of peri-operative atherothrombotic complications remains poorly understood. We have demonstrated that in patients with critical limb ischaemia, who already have a baseline elevation in inflammatory markers, the surgical process causes a further increase in inflammation. Inflammation was increased in those who developed peri-operative troponin rises. However, whether this was the cause of subsequent myocardial injury or an effect of it, is unclear. Although dual anti-platelet agents reduced peri-operative inflammation this was not translated into improved clinical outcome. It remains unclear whether clopidogrel causes a reduction in inflammatory markers through a direct action or via reduction in platelet activation and a subsequent dampened down-stream inflammatory response.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 SUMMARY OF THESIS FINDINGS

7.1.1 Reproducible assessment of *in vivo* platelet activation in patients with peripheral arterial disease.

7.1.2 Patients with critical limb ischaemia have a high-risk profile of platelet activation and inflammation

7.1.3 Peri-operative platelet activation and inflammation in surgery for critical limb ischaemia and impact of dual anti-platelet therapy.

7.1.4 Dual anti-platelet therapy and bleeding in surgery for CLI

7.2 FUTURE DIRECTIONS

7.2.1 Assessment of platelet activation and platelet reactivity

7.2.2 Variability in response to clopidogrel

7.2.3 Peri-operative c-Troponin I release

7.2.4 Limitations of current clinical trials of anti-platelet agents in PAD

7.2.5 Novel anti-platelet agents

7.1 SUMMARY OF THESIS FINDINGS

Patients with peripheral arterial disease (PAD) have a significant risk of cardiovascular morbidity and mortality, yet are poorly provided with targeted medical therapies.

Increased platelet activation and inflammation play key roles in the complex pathogenesis of atherothrombosis. Investigation of these mediators has helped guide the tailored anti-platelet regimes of patients with coronary disease, especially at times of invasive treatment. Although PAD patients are at greatest risk during the operative period, there is a paucity of data to guide cardio-protective management of the surgical PAD patient. In addition, perioperative platelet activation and inflammation has been incompletely characterised. Existing studies are confounded by heterogeneity of patient populations, methodological techniques and concern regarding the potential bleeding complications of peri-operative anti-platelet agents.

We aimed to focus attention on this high risk patient population and address previous study limitations with targeted investigation of a homogeneous patient group, clear methodology and a robust RCT study design for the investigation of peri-operative anti-platelet therapy.

Increased platelet activation and vascular inflammation are associated with atherothrombotic progression of atherosclerotic plaques [Rosenburg RD *et al*, 1999]. In addition, platelet activation and inflammatory markers are associated with elevations in cardiac Troponin [Zhang SZ *et al*; 2007] and adverse cardiovascular outcomes following PCI [Gurbel PA *et al*, 2005]. We hypothesised that *in vivo* platelet activation and inflammation would be increased following surgery for critical limb ischaemia, particularly in those developing peri-operative cardiac Troponin release. In this pilot study we aimed to demonstrate a beneficial effect of dual anti-platelet agents on these surrogate measures of cardiovascular outcome.

7.1.1 Reproducible assessment of *in vivo* platelet activation in patients with peripheral arterial disease.

Despite significant evidence for the key role of platelets in atherothrombosis, there is considerable variation in clinically useful tests of platelet function. This is partly due to the methodological issues associated with many of the techniques, and makes comparison and interpretation of different studies extremely difficult. We attempted to address this issue by deliberately employing the sensitive technique of whole blood flow cytometry to assess resting *in vivo* markers of platelet activation. Although this methodology is not without its limitations, we clearly discuss our reasoning behind its use and demonstrated repeatability of technique in our study population [Burdess *et al*, 2011]. Assessment of platelet-monocyte aggregation and platelet membrane P-selectin was our primary outcome measure in our interventional study and therefore validation of measurement technique was essential. We demonstrated acceptable within day and between day co-efficients of reproducibility and standard deviations for our variables (Within day PMA measurement: SD of difference 7.86%; co-efficient of reproducibility 15.4%. Between day: SD of difference 6.35%; co-efficient of reproducibility 12.4%). In addition, PMA showed the best correlation with a range of platelet activation markers, suggesting that it may provide the best measure of *in vivo* platelet activation for clinical study. Intervention with additional clopidogrel therapy caused a 21% reduction in PMA and a 43% reduction in platelet P-selectin [Burdess *et al*, 2010]. Given the variation in measurement attributable to technique, the reduction seen with interventional therapy can be assumed to be real.

7.1.2 Patients with critical limb ischaemia (CLI) have a high-risk profile of platelet activation and inflammation

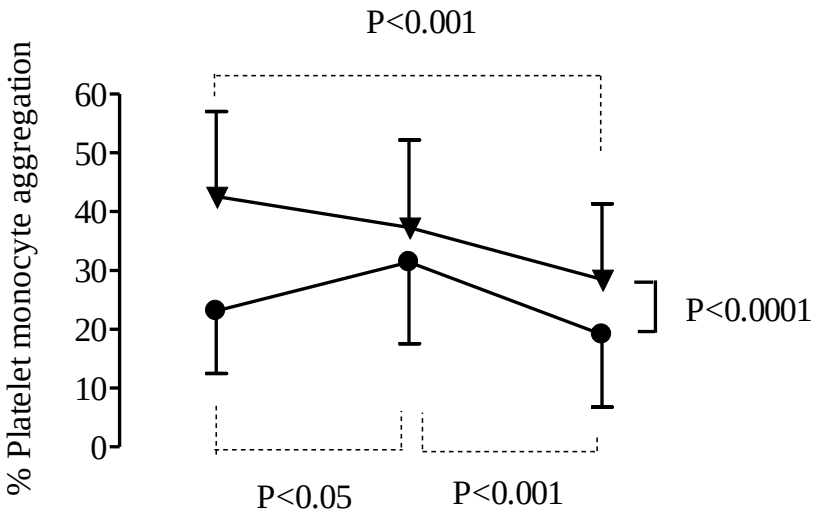
In a cross sectional study we demonstrated that resting *in vivo* platelet activation and cellular inflammation increase with severity of peripheral arterial disease. In addition, for the first time, we demonstrated that patients scheduled for surgery for critical limb ischaemia have levels of platelet activation comparable to those treated for an acute coronary syndrome [Burdess *et al*, 2011].

We also compared peri-operative changes in both resting platelet activation and platelet response to ADP in patients undergoing infra-inguinal surgery for CLI and patients undergoing joint replacement.

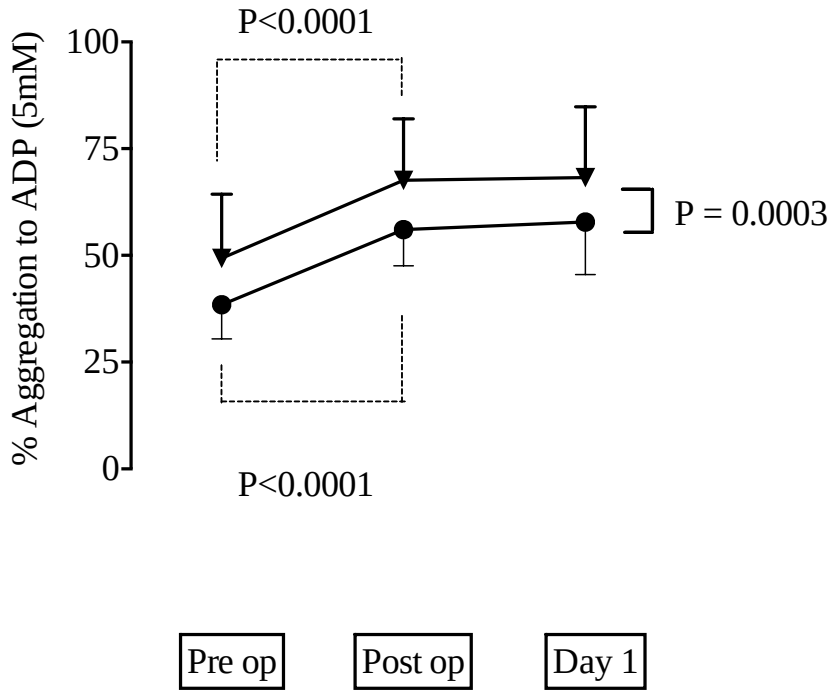
We wished to examine the contribution of surgical stress (without underlying PAD) to *in vivo* platelet activation. Following discussion with a panel of consultant vascular surgeons and anaesthetists it was felt that knee or hip arthroplasty represented surgery of a *similar magnitude* to peripheral bypass or amputation, and was likely to be performed in patients of a comparable age. Patients undergoing elective arthroplasty over the age of 50 were approached. In order to limit the effect of atherosclerosis on platelet activation, patients with a history of diabetes, hypertension, ischaemic heart disease or stroke, smoking or anti-platelet, anti-hypertensive or statin use, were excluded. Markers of both resting platelet activation (PMA and platelet P-selectin) and platelet response to ADP stimulation were significantly greater in CLI patients compared to those undergoing arthroplasty, throughout the peri-operative period. Although platelet response to ADP increased post-operatively in both patient groups**,** resting platelet activation fell in CLI patients but rose following arthroplasty (Figure 7.1). Potentially, this suggests that patients with CLI have elevated levels of baseline platelet activation that supercedes the increase in activation associated with surgery itself.

Figure 7.1 Peri-operative *in vivo* platelet activation and *ex vivo* reactivity in patients undergoing surgery for CLI and arthroplasty

A.



B.



A. *in vivo* platelet monocyte activation and **B**. *ex vivo* platelet aggregation to ADP in patients undergoing surgery for CLI **(▼)** and arthroplasty **(●)**

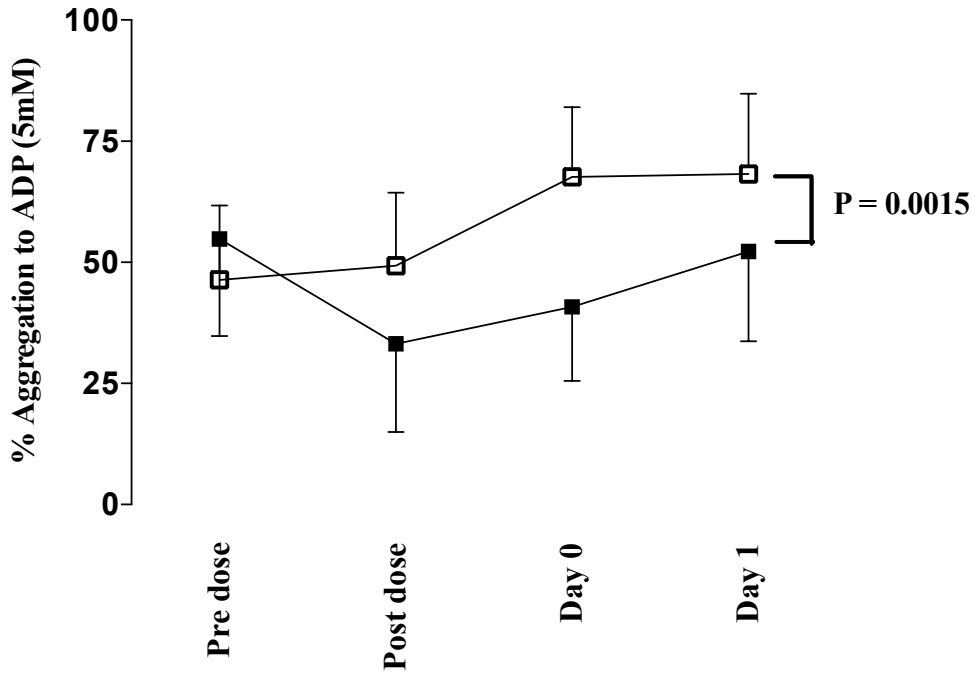
7.1.3 Peri-operative platelet activation and inflammation in surgery for critical limb ischaemia and impact of dual anti-platelet therapy.

For the first time, we demonstrated the changes in *in vivo* platelet activation and inflammation that occur in the immediate peri-operative period for this specific group of patients. To date, existing data comes from heterogenous surgical groups. These have encompassed patients with varying severities of peripheral arterial occlusive disease and aneurysmal disease or those undergoing carotid surgery or endovascular intervention.

Surprisingly, we demonstrated that platelet-monocyte aggregation and P-selectin expression fell following surgery, whilst markers of monocyte activation and inflammatory markers appeared to rise post-operatively. In a small sub-group of patients (n=20; 10 patients receiving placebo, 10 patients receiving clopidogrel), *ex vivo* platelet reactivity to ADP stimulation throughout the operative period was examined (Figure 7.2). This was primarily to assess efficacy of study medication. In accordance with other studies, platelet *reactivity* increased post-operatively. In those patients receiving clopidogrel, the % aggregation in response to ADP was significantly reduced throughout the peri-operative period (P=0.0015).

Additional pre-operative clopidogrel therapy significantly reduced peri-operative *in vivo* platelet activation, *ex vivo* reactivity to ADP, and inflammatory markers [Burdess *et al*, 2010]. Although additional clopidogrel treatment was not directly associated with a reduction in peri-operative c-Troponin I release, elevated markers of platelet activation and inflammation were related to c-Troponin I, suggesting that clopidogrel could have beneficial results on peri-operative cardiac injury through reduction of platelet and inflammatory mediators of atherothrombosis. However, we acknowledge that for patients undergoing surgery for critical limb ischaemia an improvement in clinical end-points with intensive anti-platelet therapy still requires validation in a large-scale clinical study.

Figure 7.2 Peri-operative *ex vivo* aggregation to ADP in patients undergoing surgery for CLI – effect of clopidogrel



Pre-operative loading with 600mg of clopidogrel (closed symbols) caused a greater reduction in ADP induced platelet aggregation compared to placebo (open symbols). Symbols represent mean±SD; analysis by 2-way ANOVA.

7.1.4 Dual anti-platelet therapy and bleeding in surgery for CLI

In conducting the first prospective, double–blind, randomised controlled trial of pre-operative dual anti-platelet therapy in surgery for critical limb ischaemia, our study provides the first robust, objective evidence for the potential bleeding outcomes associated with intensive anti-platelet therapy in this specific surgical population [Burdess *et al*, 2010]. In 108 patients undergoing infra-inguinal surgery for CLI, a 600mg loading dose of pre-operative clopidogrel in addition to 75mg of maintenance aspirin in 50 patients was associated with an increase in major non-life-threatening bleeding (transfusion of $\geq$2 but <4 units of red cells) and incidence of wound leak/haematoma. There were no statistically significant increases in minor or life-threatening major bleeding. Our results are consistent those reported by the CURE trial where those patients who continued clopidogrel therapy within 5 days of coronary artery bypass had a 2-fold increased relative risk of major bleeding [Fox KA *et al*, 2001].

Approximately two thirds of patients with peripheral arterial disease have co-existing coronary atherosclerosis, and 40% will have had a coronary intervention prior to vascular surgery. More than 90% of percutaneous coronary interventions involve the placement of intracoronary stents. This procedure requires long-term treatment with anti-platelet agents, which are mandatory for the re-endothelialization and success of the stent. Around 5% of patients who have undergone PCI will undergo non-cardiac surgery within the first year of stenting [Vicenzi MN *et al*, 2006]. Objective evidence is therefore required for the management of high-risk vascular patients undergoing surgery. Studies have demonstrated an increased incidence of adverse cardiac events in the peri-operative period, in patients undergoing non-cardiac surgery early after PCI and stenting [Kaluza GL *et al*, 2000; Wilson SH *et al*, 2003]. In addition, interruption of anti-platelet therapy is associated with an increase in major adverse cardiac events [Kaluza GL *et al*, 2000; McFadden EP *et al*, 2004]. Increasingly, the surgical team is confronted with

the decision of whether to continue anti-platelet therapy into the peri-operative period. Currently there is no protocol for the peri-operative use of anti-platelet therapies, and no prospective studies of different anti-platelet regimes during non-cardiac surgery. However, a recent review of the current data proposed that apart from low coronary risk situations, patients on anti-platelet drugs should continue their treatment throughout surgery, except when bleeding might occur in a closed space or massive blood loss may be expected [Chassot PG *et al*, 2007].

Although our work does not provide comprehensive evidence for the benefits associated with peri-operative dual anti-platelet therapy, it does provide the first objective evidence of the degree of bleeding that could be expected with use of these agents in the immediate peri-operative period. It therefore provides a valuable guide to the vascular team in the surgical preparation of these high-risk patients.

7.2 FUTURE DIRECTIONS

7.2.1 Assessment of platelet activation and platelet reactivity

We proposed that the high incidence of peri-operative adverse cardiovascular events associated with surgery for CLI was mediated by increased *in vivo* platelet activation and inflammation at the time of operation.

We demonstrated that *in vivo* platelet-monocyte aggregation and platelet P-selectin expression *fell* following surgery for CLI. This profile was the same for both amputation and bypass procedures. In contrast, when we assessed *ex vivo* platelet reactivity to ADP in a small sub-group of patients, we demonstrated a rise in platelet aggregation post-operatively (Figure 7.1).

Previous studies, which have reported post-procedural rises in platelet activation markers, have predominantly examined platelet responsiveness to exogenous agonists as opposed to resting activation states. However, where both measures of platelet activation have been studied (both *in vivo* and *ex vivo*), subtle differences in the peri-operative trend have been observed [Samama CM *et al*, 2001; Rajagopalan S *et al*, 2007]. Therefore these conflicting results could be due to differences in methodology.

In vivo platelet-monocyte aggregation and platelet P-selectin expression rose post-operatively in patients undergoing arthroplasty (Figure 7.1 A). Therefore it can be assumed that the stress of surgery alone can cause a rise in these markers. In those patients with pre-operative endothelial dysfunction (as in CLI), the baseline elevation in resting platelet activation may supersede the post-operative increase attributable to surgery.

Alternatively, this finding could be due to removal of thrombotic stimulus with amputation or revascularisation of ischaemic tissue, suggesting that surgery actually protects patients from thrombotic risk. The fact that 50% of Troponin positive events occurred pre-operatively could

suggest that patients with critical limb ischaemia are actually at highest risk of atherothrombotic events in the run up to surgery and are experiencing silent cardiac ischaemia. Sequestration of circulating activated platelets within amputated tissue or a new bypass conduit, may also mean that post-operative rises in platelet activation were not detected by flow cytometry. Flow-cytometric assessment of *in vivo* resting platelet-monocyte aggregation provides information on circulating activation states. If activated platelets are rapidly cleared or are adherent to blood vessel walls, flow cytometry may not detect evidence of platelet activation [Abrams CS *et al*, 1990; Kestin AS *et al*, 1993].

We would therefore propose to re-examine the changes in both peri-operative *in vivo* platelet activation and *ex vivo* reactivity in patients with critical limb ischaemia. In a non-interventional study in the same patient population we would assess both *in vivo* platelet monocyte aggregation and *ex vivo* reactivity to a panel of platelet agonists. We would expand our blood sampling, obtaining a further samples in the run up to surgery, in addition to extending the post-operative sampling period. This would provide a more detailed profile of *in vivo* platelet activation and *ex vivo* function during the peri-operative period. This would potentially provide a more specific time period of maximum risk for this patient group.

7.2.2 Variability in response to clopidogrel

It has been noted that clopidogrel's anti-platelet effect is not uniform in all patients. This concept of variability in response to clopidogrel or clopidogrel-resistance, currently has no clear definition. This is largely due to differences in the technique used to measure extent of clopidogrel induced anti-platelet effects (different assays, different agonist doses, different cut-off values). There is also evidence to suggest that subjects with a higher baseline (pre-

treatment) level of platelet reactivity, contribute to an apparent reduced clopidogrel induced anti-platelet effect [Gurbel PA *et al*; 2003].

This inter-patient variability in clopidogrel response is multifactorial. Extrinsic mechanisms reflect a reduced bioavailability of clopidogrel, such as drug-drug interactions affecting the transformation of clopidogrel into its active metabolite. Intrinsic mechanisms encompass genetic polymorphisms of the $P2Y_{12}$ receptor and P3A Cytochromes [Nguyen TA *et al*; 2005].

This individual variability in response to clopidogrel has been demonstrated to have significant clinical implications. Persistent platelet activity despite clopidogrel treatment has been linked with increased incidence of stent thrombosis and post-stent ischaemic events [CREST Study. Gurbel PA *et al*, 2005].

In an attempt to manage this phenomenon, several studies have focused on the loading dose of clopidogrel utilized in patients undergoing PCI. Clopidogrel-induced platelet inhibition is both dose and time dependent. Investigations have now shown that a 600mg loading dose achieves maximum platelet function inhibition after 2 hours [Montalescot G *et al*, 2006].

We deliberately chose a loading dose of 600 mg in our trial and all post-treatment bloods were taken at least four hours after ingestion of study drug. We postulated that if patients undergoing surgery for CLI were to achieve a cardio-protective benefit from intensive anti-platelet therapy, then ensuring a significant platelet inhibition in the majority of our trial subjects would provide the best opportunity for demonstrating this. However we acknowledge that although this high loading dose will have reduced the number of 'non-responders' it will not have eliminated inter-individual variability in response. Indeed, results from the GRAVITAS trial [2011] suggested that a treatment strategy of high dose clopidogrel for high residual platelet reactivity post PCI, does not improve outcome.

We did not specifically examine the variability in response to clopidogrel in the 50 patients receiving the trial medication. Nor did we explore any relationship between low response to clopidogrel and incidence of cTroponin I release in this small number of patients.

In subsequent studies, the bleeding risks associated with greater platelet inhibition must also be balanced against atherothrombotic protection, and other dosing regimes should ideally be assessed. It is clear from the evolving literature that clopidogrel-induced platelet inhibition is patient specific and the ideal anti-platelet regime will have to be tailored to a patient's baseline reactivity, in addition to the clinical scenario [Gravitas Group, 2011].

7.2.3 Peri-operative c-Troponin I release

Pre-clinical studies and mechanistic data have informed the design of large-scale clinical trials powered to demonstrate differences in clinical outcomes in patients suffering coronary atherothrombotic disease. Given the greater potential for bleeding in surgical patients receiving intensive anti-platelet therapies, it was with this aim that we conducted our proof-of concept study of peri-operative platelet activation and inflammation. Our study was primarily powered to examine differences in platelet activation markers between patients on clopidogrel and those on placebo. However, by utilising a sensitive assay for cardiac Troponin I we hoped to capture all incidences of cardiac injury.

Previous studies have shown that elevations in pre-procedural platelet activation or reactivity markers are associated with cardiac Troponin release following PCI [Zhang SZ *et al*, 2007; Ray MJ *et al*, 2005; Frossard M *et al*, 2004; Faraday N *et al*, 2004]. We specifically recruited only patients with severe PAD (CLI) and utilised a sensitive assay for cardiac-Troponin I, demonstrating 18 positive events in our study population (16.7%). However, equivalent sized studies of patients with acute coronary syndromes and PCI have displayed a greater incidence

of Troponin release, with a larger proportion of patients having higher levels of cardiac-Troponin. In addition, we only measured Troponin at 4 time points and could have therefore missed episodes of cardiac necrosis, especially since there is a delayed increase in circulating levels of cardiac troponins at the time of cardiac injury [Macrae AR *et al*, 2006].

Evidence indicates that even small rises in cTnI are associated with poor outcome [Galvani M *et al*, 1997; Morrow DA *et al*, 2001]. Newer, *highly* sensitive cTnI assays (hs-cTnI) are therefore being developed in order to measure very low levels of cardiac Troponin in an attempt to improve rapid diagnosis and treatment of patients [Reichlin T *et al*, 2009]. Although we used a sensitive troponin assay we were still underpowered to demonstrate a direct effect of clopidogrel on cardiac troponin release. It therefore remains to be conclusively demonstrated that reductions in platelet activation with dual anti-platelet therapy in patients undergoing surgery for CLI, translate into a reduction in cardiac injury.

7.2.4 Limitations of current clinical trials of anti-platelet agents in PAD

PAD is recognised as a serious cardiovascular disorder, yet despite its epidemiological and prognostic importance, relatively few clinical trials with cardioprotective agents have been conducted specifically in this group of patients. In particular, anti-platelet therapy is used in PAD based essentially on meta-anlayses [Antithrombotic Trialists' Collaboration; 2002], extrapolation of results from trials in other conditions [Gresele P *et al*; 1998], or subgroup analyses of large clinical trials enrolling patients with various manifestions of atherosclerotic disease. In coronary atherosclerotic disease there are an abundance of trials of various anti-platelet agents, in a variety of clinical scenarios (stable disease, acute coronary syndromes,

peri-PCI). In comparison, the most recent AHA guidelines for anti-platelet use in PAD can still only cite level A evidence for the prophylactic use of aspirin only (Figure 7.1).

Two large clinical trials that have contained a significant number of PAD patients include CAPRIE and CHARISMA. The CAPRIE trial compared clopidogrel versus aspirin in patients at high risk of atherosclerotic events (three pre-defined subgroups of patients with recent stroke, myocardial infarction, or PAD.) CHARISMA compared long-term aspirin with aspirin plus clopidogrel in patients with symptomatic and asymptomatic disease. In both studies, the PAD patients were heterogeneous in terms of disease severity and cardiovascular risk profile. Both trials have been criticised for the conclusions they made following post-hoc sub-group analysis of PAD patients [Gebel JM, 2007; Gresel P *et al*, 2009] and neither trial has changed the current therapeutic indications for PAD.

At the time of our study the CASPAR trial [Belch JJ *et al*, 2010] was embarking on a randomized, placebo-controlled trial of clopidogrel and aspirin in bypass surgery for peripheral arterial disease. In this multi-centre study, patients undergoing unilateral, below-knee bypass grafting for atherosclerotic peripheral arterial disease were randomised to clopidogrel or placebo 2-4 days after surgery and the primary efficacy endpoint was a composite of index-graft occlusion or revascularization, above-ankle amputation of the affected limb, or death. The primary safety endpoint was severe bleeding (GUSTO classification).

Overall, additional post-operative clopidogrel did not improve limb or systemic outcomes. Subgroup analysis suggested that combination therapy conferred benefit in patients receiving prosthetic grafts without significantly increasing major bleeding risk. Although cardiovascular out-comes were not a primary end-point, the trial did not show any benefit from combination therapy. However, additional clopidogrel was not commenced until after surgery and no

loading dose was given, therefore any peri-operative advantage of intensive anti-platelet therapy may have been missed.

Figure 7.2

ACC/AHA Guidelines for the Management of Patients With Peripheral Arterial Disease (Lower Extremity, Renal, Mesenteric, and Abdominal Aortic)

F. ANTIPLATELET AND ANTITHROMBOTIC DRUGS.
RECOMMENDATIONS
Class I
1. Antiplatelet therapy is indicated to reduce the risk of MI, stroke, or vascular death in individuals with atherosclerotic lower extremity PAD.
(Level of Evidence: A)
2. Aspirin, in daily doses of 75 to 325 mg, is recommended as safe and effective antiplatelet therapy to reduce the risk of MI, stroke, or vascular death in individuals with atherosclerotic lower extremity PAD.
(Level of Evidence: A)
3. Clopidogrel (75 mg per day) is recommended as an effective alternative antiplatelet therapy to aspirin to reduce the risk of MI, stroke, or vascular death in individuals with atherosclerotic lower extremity PAD.
(Level of Evidence: B)
Class III
1. Oral anticoagulation therapy with warfarin is not indicated to reduce the risk of adverse cardiovascular ischemic events in individuals with atherosclerotic lower extremity PAD.
(Level of Evidence: C)

ACC/AHA Guidelines for Cardiovascular Risk Reduction in Patients with peripheral arterial disease. Reproduced from Hirsch AT *et al*. 2006.

In the CURE trial, subgroup analysis of the 2072 ACS patients undergoing CABG, revealed an overall relative reduction in adverse events. However, this reduction was only significant for pre-surgical events in patients progressing to early CABG at the index hospitalisation. Post-operative clopidogrel use was not associated with a significant reduction in cardiovascular events, irrespective of the duration of time to CABG. However, the CURE trial examines anti-platelet therapy in surgical patients who are progressing to surgery *because* of cardiac ischaemia. It remains to be confirmed if PAD patients are most at risk from cardiovascular events before or after surgery.

In summary, no trial to date has risk stratified PAD patients and examined treatment options accordingly. None specifically address the cardiovascular risk associated with the peri-operative period. Although our studies do not provide conclusive evidence for a beneficial role of peri-operative dual anti-platelet therapy, they highlight the need for improved medical treatment of these patients with robust trials of cardio-protective therapies specifically organised to address the spectrum of PAD.

7.2.5 Novel anti-platelet agents

Arguably, the role of well known anti-platelet agents, aspirin and clopidogrel, has still not been fully determined in the management of specific cases of PAD. However, other anti-platelet agents also remain to be examined in the setting of PAD. Although anti-platelet therapy is highly effective at reducing atherothrombotic risk, patients continue to experience thrombotic events despite the use of agents such as aspirin and clopidogrel. Many of these events occur, in part, because of the inadequate response to these drugs [Barragan P *et al*, 2003; Gurbel PA *et al*, 2005; Hochholzer W *et al*, 2006; Bonello L *et al*, 2008]. This has prompted the pursuit of novel agents with aspirations of optimising anti-platelet therapy.

GP IIb/IIIa Inhibitors

The platelet surface membrane glycoprotein (GP) IIb/IIIa receptor, binds circulating fibrinogen or von Willebrand factor and crosslinks platelets in the final common pathway to platelet aggregation. Intravenous agents directed against this receptor include the chimeric monoclonal antibody fragment abciximab, the peptide inhibitor eptifibatide and nonpeptide mimetics tirofiban and lamifiban. Several large trials involving patients with UA/NSTEMI have shown that the GP IIb/IIIa inhibitors are of substantial benefit for patients at high risk, those undergoing PCI, or both [PRISM-PLUS investigators, 1998; Boersma E *et al*, 2002]. The 2007 ACC/AHA guidelines recommend that, for patients with UA/NSTEMI who will be treated initially according to an invasive strategy, either an intravenous GP IIb/IIIa inhibitor or clopidogrel should be added to aspirin and anticoagulant therapy (upstream) before diagnostic angiography is performed (class I recommendation). They also state that adding both agents is reasonable (class IIa recommendation) [Anderson JL *et al*, 2007]. Questions remain about their benefit in the setting of primary PCI, as well as in patients with acute coronary syndromes who do not undergo PCI.

One disadvantage of both aspirin and clopidogrel is the irreversible nature of platelet inhibition. One advantage of GP IIb/IIIa inhibitors is their shorter duration of action and potential reversibility. Despite this, these drugs have not been specifically examined in the peri-operative setting. Pooled analysis of the 82 patients in the EPILOG [1997] and EPISTENT [1998] trials undergoing CABG during hospitalization for ACS suggested a trend towards a reduction in ischemic endpoints in patients treated with abciximab (14% vs 24%, $P>0.2$). Almost two thirds of these patients were operated on within 6 hours of discontinuing abciximab. A similar benefit has been demonstrated with the shorter acting, reversible eptifibatide. In the PURSUIT trial [1998], 78 patients underwent emergency CABG within 2 hours of eptifibatide cessation. Peri-operative myocardial infarction was significantly reduced

in patients who received eptifibatide (46% vs 22%, $p < 0.05$). However, whether these findings apply to surgical PAD patients remains to demonstrated.

There remains a relative paucity of data examining the use of the GPIIb/IIIa antagonists during peripheral arterial interventions despite years of use by coronary interventionalists. The studies available are conducted in small patient groups, and mostly examine the role of GP IIb/IIIa antagonists during the treatment of acute peripheral arterial thrombosis to enhance thrombolysis, and to a lesser extent, in chronic lesions and critical limb ischemia. In a prospective randomized controlled trial, pre-procedural Abciximab therapy in addition to standard dose heparin and aspirin, was demonstrated to improve patency following endovascular revascularisation of long segment femoropopliteal occlusions [Dörffler-Melly J *et al*, 2005]. Risk of major bleeding (TIMI criteria) was not significantly increased, while access-site bleeding was significantly higher amoungst patients receiving abciximab. However, cardiovascular outcomes were not examined and the patient group contained both claudicants and those with CLI. The potential for both adverse cardiovascular outcomes and bleeding complications in endovascular procedures would also be expected to be less than that of open surgery.

Novel P2Y12 receptor antagonists

New P2Y12 antagonists such as prasugrel and ticagrelor, and the reversible non-thienopyridine drugs AZD6140 and Cangrelor, have been developed to provide a more rapid and consistent platelet inhibition, and overcome the non-reversibility of clopidogrel.

The TRITON-TIMI 38 trial [2006], demonstrated that prasugel therapy in patients with ACS undergoing PCI, was associated with significantly reduced rates of ischaemic events, including stent thrombosis, but with an increased risk of bleeding, in comparison to clopidogrel. In the PLATO trial [2009], patients with acute coronary syndromes with or without ST-segment

elevation, who were treated with ticagrelor as compared with clopidogrel, had a significantly reduced rate of death from vascular causes, myocardial infarction, or stroke but *without* an increase in the rate of overall major bleeding.

Whether the risk-benefit profile is favourable amoungst patients with PAD remains to be demonstrated.

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APPENDIX

PUBLICATIONS

Arising from thesis

- **Burdess A**, Michelsen AE, Brosstad F, Fox KAA, Newby DE, Nimmo AF.Platelet activation in patients with peripheral arterial disease: reproducibility and comparibility of markers. *Thromb Res*. 2012 Jan; **29**(1)50-5. [Appended]

- **Burdess A**, Nimmo AF, Garden OJ, Murie JA, Dawson ARW, Fox KAA, Newby DE. Letter to the Editor. *Ann Surg* 2011 Jul 26.

- **Burdess A**, A. F. Nimmo, N. Campbell, S. A. Harding, O. J. Garden, A. R. W. Dawson, D.E. Newby. Perioperative platelet and monocyte activation in patients with critical limb ischaemia. *J Vasc Surg*. 2010 Sep;**52**(3):697-703 [Appended]

- **Burdess A**, Nimmo AF, Garden OJ, Murie JA, Dawson ARW, Fox KAA, Newby DE. Randomised Controlled Trial of Dual Anti-platelet Therapy in Patients Undergoing Surgery for Critical Limb Ischaemia. *Ann Surg*. 2010 Jul;**252**(1):37-42. [Appended]

During research period

- Richards JM, Shaw CA, Lang NN, Williams MC, Semple SI, MacGillivray TJ, Gray C, Crawford JH, Alam SR, Atkinson AP, Forrest EK, Bienek C, Mills NL, **Burdess A**, Dhaliwal K, Simpson AJ, Wallace WA, Hill AT, Roddie PH, McKillop G, Connolly TA, Feuerstein GZ, Barclay GR, Turner ML, Newby DE. In vivo mononuclear cell tracking using superparamagnetic particles of iron oxide: feasibility and safety in humans. *Circ Cardiovasc Imaging*. 2012 Jul;**5**(4):509-17.

- Erridge C, **Burdess A**, Jackson AJ, Murray C, Riggio M, Lappin D, Milligan S, Spickett CM, Webb DJ. Vascular cell responsiveness to Toll-like receptor ligands in carotid atheroma. *Eur J Clin Invest*. 2008 Oct; **38**(10):713-20.

Editorial

- **Burdess A**, Newby DE. 'Harnessing the preconditioning phenomenon: does remote organ ischaemia provide the answer?' *Heart*. 2006 Oct; **92**(10):1367-8.2005 Sep 13.

Book Chapter

- **Burdess A**, Cruden N, Fox K.AA Antiplatelet therapy and coronary bypass surgery, risks and benefits. The American Heart Association, Blackwell Publishing

Randomized Controlled Trial of Dual Antiplatelet Therapy in Patients Undergoing Surgery for Critical Limb Ischemia

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Background and Objective: Patients with critical limb ischemia have a perioperative cardiovascular morbidity comparable to patients with acute coronary syndromes. We hypothesized that perioperative dual antiplatelet therapy would improve biomarkers of atherothrombosis without causing unacceptable bleeding in patients undergoing surgery for critical limb ischemia.

Methods: In a double-blind randomized controlled trial, 108 patients undergoing infrainguinal revascularization or amputation for critical limb ischemia were maintained on aspirin (75 mg daily) and randomized to clopidogrel (600 mg prior to surgery, and 75 mg daily for 3 days; n = 50) or matched placebo (n = 58). Platelet activation and myocardial injury were assessed by flow cytometry and plasma troponin concentrations, respectively.

Results: Clopidogrel reduced platelet-monocyte aggregation before surgery (38%–30%; $P = 0.007$). This was sustained in the postoperative period ($P = 0.0019$). There were 18 troponin-positive events (8 [16.0%] clopidogrel vs. 10 [17.2%] placebo; relative risk [RR]: 0.93, 95% confidence interval [CI]: 0.39–2.17; $P = 0.86$). Half of troponin-positive events occurred preoperatively with clopidogrel causing a greater decline in troponin concentrations ($P < 0.001$). There was no increase in major life-threatening bleeding (7 [14%] vs. 6 [10%]; RR: 1.4, 95% CI: 0.49–3.76; $P = 0.56$) or minor bleeding (17 [34%] vs. 12 [21%]; RR 1.64, 95% CI: 0.87–3.1; $P = 0.12$), although blood transfusions were increased (28% vs. 12.6%, RR: 2.3, 95% CI: 1.0–5.29; $P = 0.037$).

Conclusions: In patients with critical limb ischemia, perioperative dual antiplatelet therapy reduces biomarkers of atherothrombosis without causing unacceptable bleeding. Large-scale randomized controlled trials are needed to establish whether dual antiplatelet therapy improves clinical outcome in high-risk patients undergoing vascular surgery.

(*Ann Surg* 2010;252: 37–42)

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Supported by grants from the British Heart Foundation (FS/05/038); European Society of Vascular Surgery Research Awards; Royal College of Surgeons of Edinburgh Research Grants and an unrestricted educational award from Sanofi Aventis.

This study was registered on the ISRCT and EUDRA websites. International Standardised RCT: ISRCTN22305120. Weblink: http://www.controlled-trials.com/ISRCTN22305120.

Trial start date: June 1, 2005. Eudract Number: 2005–000960–25. CTA Number: 11449/0002/001–0001 (Granted 05/04/05). REC reference number: 05/S0501/41 (Granted 29/04/05).

There was no involvement of Sanofi Aventis in any aspect of the trial including protocol design, study conduct or data analysis.

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Patients with peripheral arterial disease have an increased risk of adverse cardiovascular events,[1] particularly in the perioperative period.[2] Myocardial injury is the commonest life-threatening complication of vascular surgery with a reported incidence ranging from 8% to 40%.[3–5] This is comparable to the cardiovascular risk of patients presenting with an acute coronary syndrome: 30-day death and reinfarction rate of 8% to 20%.[6]

In patients with peripheral arterial disease, prophylactic use of the thienopyridine clopidogrel has modest additional secondary preventative benefits in comparison to,[7] or in combination with,[8] aspirin. Combination aspirin and clopidigrel therapy is of major benefit in reducing recurrent ischemic events in patients with acute coronary syndromes.[9] It is therefore reasonable to postulate that dual antiplatelet therapy may have particular benefits in patients undergoing vascular surgery. However, many clinicians would question the wisdom of dual antiplatelet therapy in the operative setting because of the risk of increased peri-operative bleeding. The CURE (Clopidogrel in Unstable angina to prevent Recurrent ischemic Events) trial reported an overall relative risk reduction in cardiovascular death, myocardial infarction, or stroke in patients undergoing coronary artery bypass grafting following non-ST segment elevation myocardial infarction, without an increase in major life-threatening bleeding.[10] However, the surgical subgroup was not prospectively randomized and only a small proportion of patients received dual antiplatelet therapy within 5 days of operation. Despite these limitations, the trial proposed that the potential cardiovascular benefits of dual antiplatelet therapy may outweigh the risks of bleeding in the high-risk surgical patient.

Atherothrombosis is the major underlying cause of adverse cardiovascular events. Platelets play a key role in this process and are associated with both the inflammatory destabilization of atherosclerotic plaques, and thrombus generation.[11,12] Platelet activation is commonly assessed using aggregometry or detection of platelet surface P-selectin expression following ex vivo agonist stimulation. These approaches are artificial and may not truly reflect the status of in vivo platelet activation because of the potential for in vitro activation, and the rapid shedding of P-selectin from the platelet surface.[13] Activated platelets are rapidly cleared from the circulation by monocytes and the quantification of platelet-monocyte aggregates is now emerging as the "gold standard " assessment for in vivo platelet activation.[14,15] We and others have demonstrated that platelet-monocyte aggregates are increased in those who smoke or have diabetes mellitus as well as in patients with peripheral arterial disease or an acute coronary syndrome.[16–19]

To date, there have been no studies to investigate the effects of dual antiplatelet therapy in patients undergoing surgery for critical limb ischemia. Given the potential for both marked benefit and hazard, we embarked upon a proof-of-concept pilot randomized controlled trial. We hypothesized that combined perioperative aspirin and clopidogrel therapy would improve biomarkers of atherothrombosis (platelet-monocyte aggregates and troponin release) without causing unacceptable bleeding, in patients undergoing surgery for critical limb ischemia.

METHODS

Subjects

Patients with critical limb ischemia who were scheduled for infra-inguinal bypass, femoral endarterectomy or lower limb amputation under general anesthesia were recruited into the study. Critical limb ischemia was defined as the presence of rest pain or skin breakdown, resulting from arterial disease. Exclusion criteria included women of child bearing potential, nonatherosclerotic vascular disease, sudden acute limb ischemia requiring emergency surgery, supra-inguinal or aortic surgery, history of acute coronary syndrome within 3 months, history of peptic ulcer disease, previous or current intracranial hemorrhage, bleeding diathesis, uncontrolled hypertension, or thrombocytopenia, planned epidural or spinal anesthesia, hypersensitivity or allergy to thienopyridines, and current warfarin or thienopyridine use.

Study Design

Patients were recruited between June 2005 and February 2008, and gave written informed consent prior to study participation. The study was approved by the local Research Ethics Committee, given Clinical Trial Authorization by the Medicines and Healthcare products Regulatory Agency (United Kingdom), and was conducted in accordance with the Declaration of Helsinki and CONSORT guidelines.[20,21]

This was a prospective single center double-blind randomized controlled trial at a tertiary referral vascular surgical unit in the Royal Infirmary of Edinburgh, South-East Scotland, United Kingdom.

Treatment Allocation

Following recruitment, clopidogrel and matched placebo were assigned in identical packs by the pharmacy trials unit through allocation of sequentially numbered study medication packs that had been randomized using an independent computer-generated sequence. Patients received 600 mg of clopidogrel or matched placebo 4 to 28 hours prior to surgery and received 75 mg of clopidogrel or matched placebo daily for 3 days after surgery (Studies suggest that the incidence of peri-operative adverse cardiovascular events is greatest within the first 5 days of surgery.[2] We therefore commenced therapy preoperatively and continued maintenance levels into a short postoperative period.). Patients undergoing bypass procedures received a single dose of 5000 IU of intravenous unfractioned heparin during surgery before arterial clamping. At the discretion of the clinical team, intravenous protamine was given only if excessive bleeding was felt to be present at the end of the operation. All patients received subcutaneous unfractionated heparin 5000 IU twice daily in the postoperative period and were maintained on aspirin (75 mg daily) throughout the study.

Biomarkers of Atherothrombosis

Platelet Activation and Inflammatory Markers

Blood samples were taken before, and a minimum of 4 hours after, a loading dose of 600 mg of clopiogrel or matched placebo, immediately after the operation in the recovery room, and on day 1 after surgery. Flow cytometric measurements of platelet-monocyte aggregates and platelet surface expression of P-selectin were used as markers of in vivo platelet activation as described previously.[16,17,19,22] Directly conjugated monoclonal antibodies were obtained from DakoCytomation (Cambridge, United Kingdom) and Serotec (Oxford, United Kingdom).

Myocardial Injury

The Reference Clinical Biochemistry Laboratory measured plasma troponin I concentrations using the ARCHITECT Troponin I *STAT* assay (Abbott Diagnostics, Maidenhead, United Kingdom) using an autoanalyzer. This has an analytical sensitivity of 0.009 ng/mL and a functional sensitivity of 0.032 ng/mL with a coefficient of variation of $<10\%$. The latter threshold was employed for the clinical case definition of myocardial infarction (see below).

In-Patient Clinical Outcomes

Acute Coronary Syndromes

Clinical symptoms, plasma troponin concentrations and electrocardiograms were recorded daily from the preoperative day until day 3 postsurgery. A blinded independent cardiologist reviewed all clinical data and applied the universal definition of myocardial infarction.[23]

Bleeding Complications

Bleeding events were defined as major (life-threatening or nonlife threatening) and minor according to CURE criteria.[10] Postoperative blood transfusions were recommended according to Scottish Intercollegiate Guidelines Network (SIGN) criteria.[24] Intraoperative blood loss, postoperative fall in hemoglobin, blood product transfusion, length of operation and length of hospital stay were recorded. Incidence of gastro-intestinal bleeding, persistent (>3 days) wound leak, hematoma, or infection were documented.

Data and Statistical Analysis

An independent data monitoring committee performed an interim safety analysis of bleeding outcomes following recruitment of 50 patients and recommended continuation of the trial to completion. Following completion of trial recruitment, data collection, and laboratory analyses, the data base was locked, treatment allocation unblinded and prespecified analyses performed. The primary end-point was platelet-monocyte aggregation. The sample size ($n = 50$ per group) was based on our previous studies[16,17,19] and gave an 80% power of detecting a 4.8% difference in platelet-monocyte aggregates at a significance level of 5%. Secondary outcomes included plasma troponin concentration, and rate of myocardial infarction and bleeding complications. Continuous variables are reported as mean ± SD. Analysis of variance with repeated measures, 2-tailed Student t test and χ^2 analysis were performed as appropriate using GraphPad Prism Version 4 (La Jolla, US). Statistical significance was taken as a 2-sided P value <0.05.

Statement of Responsibility

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

Of the 159 potentially eligible patients, 113 were randomized to trial medication (Fig. 1). Of those who completed the study protocol, 58 received placebo and 50 received clopidogrel. There was no difference in baseline demographics between the 2 groups (Table 1; Appendix 1, Supplemental Digital Content 1, online only, available at: http://links.lww.com/SLA/A53).

Biomarkers of Atherothrombosis

Platelet Activation

In keeping with the patient population, baseline levels of platelet-monocyte aggregation were markedly elevated. There was no difference in baseline platelet-monocyte aggregates ($P = 0.80$) and P-selectin expression ($P = 0.80$) between the 2 groups. Platelet activation was unaffected by placebo ($P = 0.78$) but clopidogrel (600 mg) caused a rapid reduction in platelet-monocyte aggregates (38% ± 17%–30% ± 17%, $P = 0.007$) and platelet P-selectin expression (4.9% ± 2.7%–2.8% ± 1.6%, $P < 0.0001$). In both

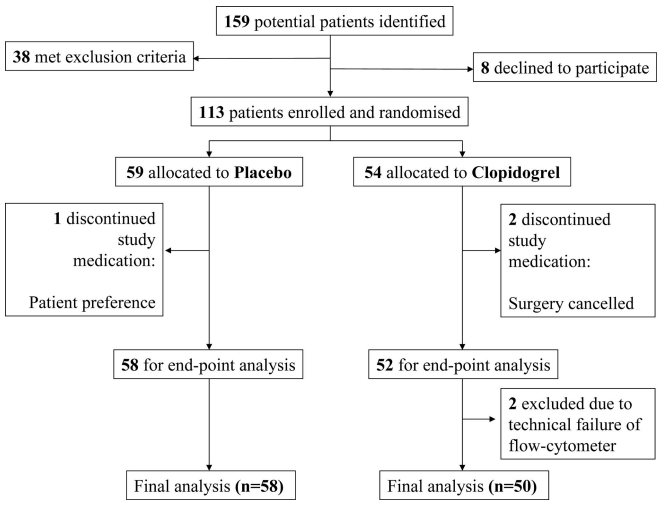

FIGURE 1. Trial profile.

TABLE 1. Baseline Characteristics of Patients According to Interventional Group

Variable	Clopidogrel n = 50	Placebo n = 58	*P*
Age (yr)	68 ± 2	68 ± 2	0.83
Male sex	39 (78)	45 (78)	0.96
Critical limb ischemia			
Ankle-brachial pressure index <0.2	34 (68)	31 (53)	0.12
Skin changes (ulcer/gangrene)	27 (54)	34 (59)	0.63
Rest pain	42 (84)	49 (84)	1.00
Operation			
Bypass	32	41	0.71
Amputation	14	14	0.71
Combined bypass and angioplasty	4	3	0.71
Lees revised cardiac risk index ≥3[25]	40	43	0.47
Cardiac risk factors			
Diabetes mellitus	19 (38)	19 (33)	0.57
Hypertension	41 (82)	48 (83)	0.91
Hypercholesterolemia	31 (62)	37 (64)	0.85
Family history of ischemic heart disease	5 (11)	8 (14)	0.55
Current smoker	20 (40)	31 (53)	0.36
Serum creatinine (μmol/L)	98 ± 4	106 ± 6	0.27
Ischemic heart disease	20 (40)	31 (56)	0.16
Cerebrovascular disease	11 (22)	15 (26)	0.64
Drugs			
Aspirin	50 (100)	58 (100)	1.00
Statin	35 (70)	45 (78)	0.37
Beta-blockade	13 (26)	12 (21)	0.43
Angiotensin-converting enzyme inhibition	19 (38)	24 (41)	0.72

Variables reported as mean ± SD or n (%) and analyzed with unpaired *t* test or χ^2 analysis as appropriate.

groups, platelet activation declined within 24 hours of surgery ($P \leq 0.005$), but clopidogrel treatment was associated with greater reductions throughout the immediate postoperative period ($P = 0.0019$; Fig. 2). To assess pharmacological efficacy of the trial intervention,

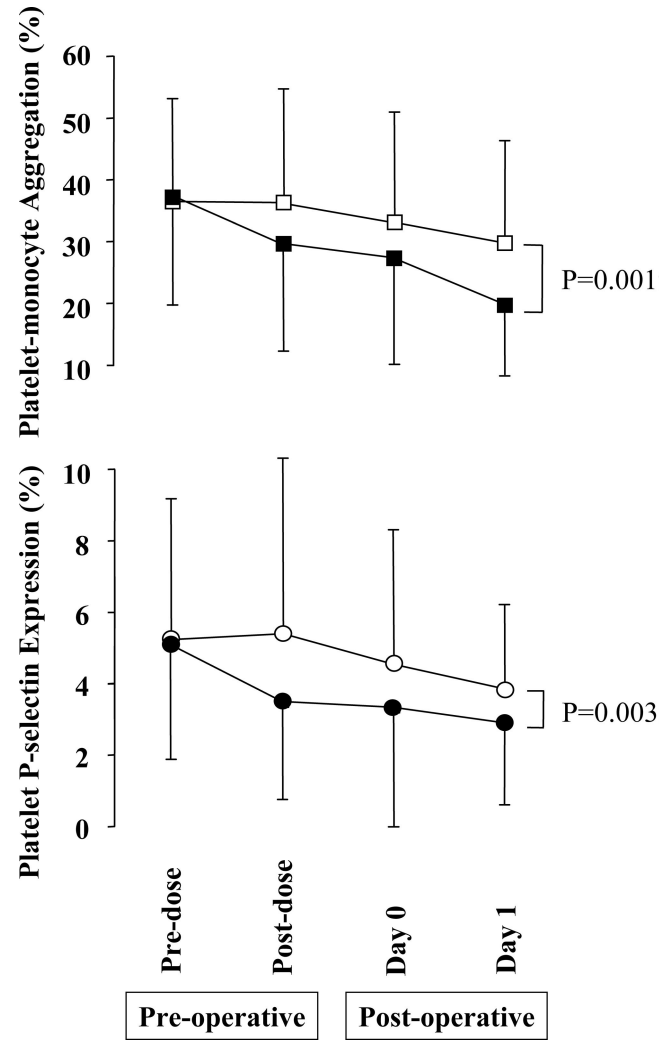

FIGURE 2. Platelet-monocyte aggregates (squares; $P < 0.0001$ for both groups) and platelet P-selectin expression (circles; $P \leq 0.005$ for both groups) over the perioperative period in patients receiving placebo or clopidogrel. Clopidogrel (closed symbols) caused a greater reduction in both platelet-monocyte aggregates ($P = 0.0019$) and platelet P-selectin expression ($P = 0.0035$) compared with placebo (open symbols). Symbols represent mean ± SD; analysis by 2-way ANOVA.

ex vivo platelet aggregation to 5 μM adenosine diphosphate was performed in a subgroup of trial participants (n = 10 per group). This confirmed that clopidogrel inhibited adenosine diphosphate-induced aggregation (59% ± 20%–33% ± 18%, $P < 0.0001$) throughout the peri-operative period ($P = 0.0015$; data not shown).

Myocardial Injury

Of the 108 trial subjects, 18 (16.7%) suffered an elevated plasma troponin concentration (>0.032 ng/mL): 8 (16.0%) received clopidogrel and 10 (17.2%) placebo (relative risk [RR] 0.93, 95% confidence intervals [CI] 0.40–2.17; $P = 0.86$). Nine (8.3%) patients (4 clopidogrel and 5 placebo) had an elevation of plasma troponin concentration before surgery, and 9 patients (4 clopidogrel and 5 placebo) suffered a postoperative rise in troponin. Of those 9

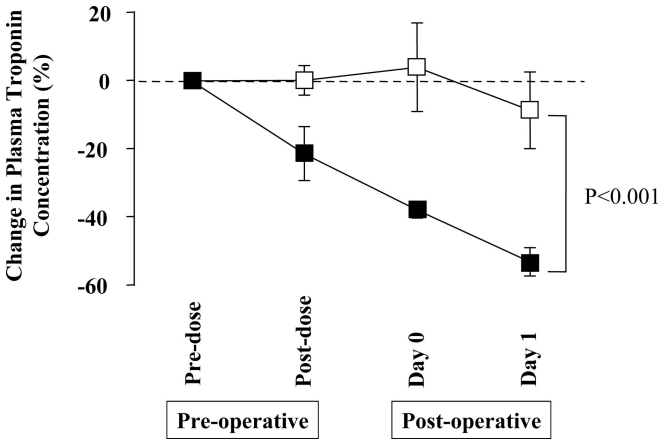

FIGURE 3. Change in plasma troponin concentrations in patients with preoperative troponin elevations on placebo (open squares) or clopidogrel (closed squares). Symbols represent mean ± SD, $P < 0.001$; 2-way ANOVA, clopidogrel (n = 4) versus placebo (n = 5).

patients who sustained a preoperative troponin rise, plasma troponin concentrations fell with clopidogrel therapy but remained unchanged or increased in those on placebo (Fig. 3). Patients with postoperative elevation in plasma troponin concentrations had greater platelet-monocyte aggregates (40% ± 4% vs. 30% ± 2%; $P = 0.033$).

Clinical Outcomes

Of the 108 trial participants, 7 patients (6.5%) sustained an acute myocardial infarction: 3 (6.0%) in the clopidogrel group and 4 (6.9%) in the placebo group (RR: 0.87, 95% CI: 0.20–3.7, $P = 0.85$). There were no in-patient deaths, intracranial hemorrhages or incidences of inotrope use. There was no difference in life-threatening major bleeding between treatment groups (7 [14%] clopidogrel and 6 [10%] placebo; RR: 1.35, 95% CI: 0.49–3.76; $P = 0.56$), although those who received clopidogrel had an increased risk of major nonlife-threatening bleeding (11 [22%] clopidogrel and 4 [7%] placebo, RR: 3.19, 95% CI: 1.1–9.4; $P = 0.024$; Fig. 4). Twenty (40%) patients receiving clopidogrel underwent red-cell transfusion compared with only 8 (14%) on placebo ($P = 0.0019$). Restricting analyses to transfusions administered in accordance with the SIGN guidelines, there remained an increased transfusion rate in the clopidogrel group (14 [28%] clopidogrel and 7 [12%] placebo; RR: 2.32, 95% CI: 1.02–5.29; $P = 0.037$). There was no difference in minor bleeding between the 2 groups (17 [34%] clopidogrel and 12 [21%] placebo; RR: 1.64, 95% CI: 0.87–3.10; $P = 0.12$). Five patients suffered gastrointestinal bleeding (hematemesis or malaena); 4 of whom were receiving placebo. Two patients received intraoperative protamine—1 in each intervention arm.

There were 2 reoperations for bleeding in the placebo group and 1 in the clopidogrel group. Although there was an increase in wound leak in those patients who received clopidogrel (13 [26%] vs. 3 [5%]; RR: 5.03, 95% CI: 1.52–16.6; $P = 0.0024$), there was no difference in incidence of wound infection at 3 months ($P = 0.80$). Clopidogrel therapy did not increase the length of operation ($P = 0.60$) or hospital stay ($P = 0.72$). Subgroup analysis of patients undergoing revascularization compared with amputation revealed no significant differences in clopidogrel versus placebo in terms of perioperative adverse cardiovascular events or bleeding outcomes (major life threatening, major nonlife threatening or minor). There were no incidences of early graft failure in either group.

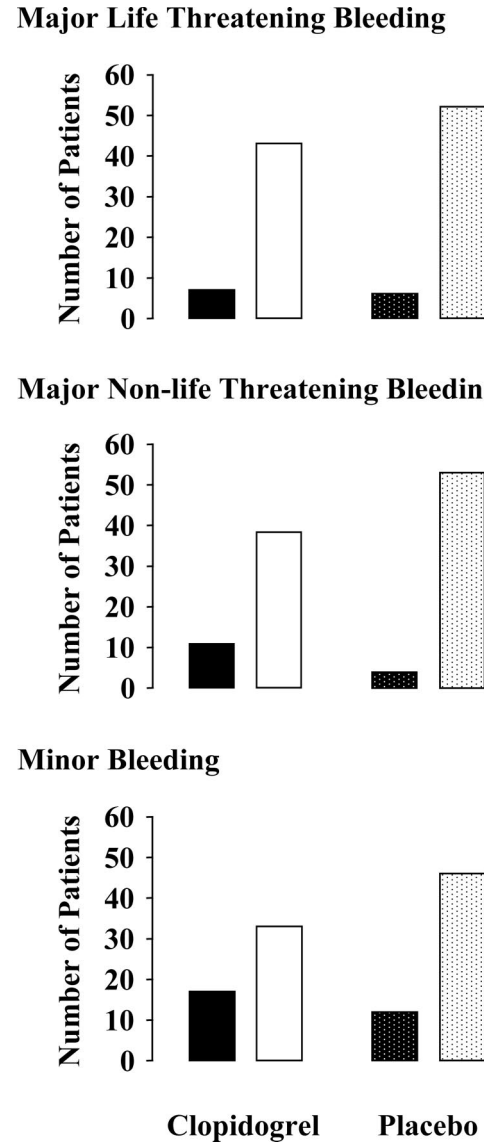

FIGURE 4. Bleeding Outcomes. Major life-threatening (upper panel; RR: 1.35, 95% CI: 0.49–3.76, $P = 0.56$), major nonlife-threatening (middle panel; RR: 3.19, 95% CI: 1.08–9.4, $P = 0.024$), and minor (RR: 1.64, 95% CI: 0.87–3.1, $P = 0.12$) bleeding (closed bars) in patients on clopidogrel (left-hand panels, nonstippled) or placebo (right-hand panels, stippled); χ^2 analysis.

DISCUSSION

We have conducted the first double-blind randomized controlled trial of perioperative dual antiplatelet therapy in patients undergoing surgery for critical limb ischemia. We demonstrate improvements in biomarkers of platelet activation and myocardial injury without causing unacceptable bleeding complications. These data form the first objective assessment of the risks and benefits of perioperative dual antiplatelet therapy in patients undergoing high-risk vascular procedures.

Peripheral arterial disease affects nearly 30 million people in Western Europe and North America. In up to 3-quarters of cases, patients have coexistent coronary artery disease and a 3-fold increased risk of cardiovascular events and death.[26] In a recent large

observational study (n = 5460), patients with peripheral arterial disease scheduled for open vascular surgery had a worse prognosis (2.4-fold increase in cardiovascular morbidity) than matched patients with severe myocardial ischemia referred for percutaneous coronary intervention.[27] Interestingly, the occurrence of perioperative cardiac complications following vascular surgery was associated with long-term cardiac death. There is therefore a clear unmet need to reduce cardiovascular events in patients with peripheral arterial disease, especially in the perioperative period.

In agreement with previous studies,[2–5] we report a high incidence of perioperative myocardial infarction (6.5%) and troponin elevation (16.7%) in patients undergoing surgery for critical limb ischemia. We also found that postoperative elevations in troponin were associated with increased levels of postoperative platelet activation. However, we were surprised to find that markers of platelet activation fell postoperatively (both in clopidogrel and placebo groups) and that half of the troponin-positive events occurred before surgery. This occurred in the absence of renal dysfunction. This suggests that rather than surgery being an additional thrombotic stimulus, removal of ischemic tissue may actually reduce platelet activation in the majority of these patients. These findings highlight the prevalence of "silent" preoperative myocardial injury, and the important systemic prothrombotic consequences of critical limb ischemia, as well as the need for preoperative intervention.

This study aimed to explore a proof of concept. Preclinical studies of platelet activation in coronary patients have informed the design of large scale trials which have subsequently shaped clinical practice. These trials required several thousands of patients to demonstrate a small but significant clinical benefit from antiplatelet regimes. Despite suffering significant cardiovascular risk, there has been little study of platelet activation in high risk vascular surgical patients. The potential for adverse bleeding would seem greater for patients undergoing open surgery under antiplatelet therapy. Consequently, any large scale trial powered to examine clinical end points should be justified by "pilot" data suggesting that some cardiovascular benefit could be achieved without excessive bleeding.

Through the use of surrogate biomarkers, we have demonstrated the potential benefits of dual antiplatelet therapy in the peri-operative period. Clopidogrel reduced sensitive markers of platelet activation (Fig. 2) that are associated with increased clinical risk as well as the progression of atherothrombosis.[11–17,28,29] Although clopidogrel did not have an overall effect on the number of peri-operative troponin-positive events (8 clopidogrel vs. 10 placebo; $P = 0.86$), half of the troponin elevations occurred preoperatively and prior to the administration of the study medication. Subsequent institution of clopidogrel therapy was associated with a marked reduction in troponin concentrations (Fig. 3). However, we readily acknowledge that our study was not primarily powered to assess cardiac troponin and clinical outcomes and whether these improvements in surrogate biomarkers translate into clinical benefit remains to be established.

All therapies have potential benefits and risks, and it is inevitable that antiplatelet therapies will be associated with increased bleeding complications. Although it may be regarded as potentially reversible, the importance of major bleeding must not be underestimated as it remains an independent predictor of adverse clinical outcome.[30–32] Although vascular patients are at high risk of cardiovascular events, bleeding concerns are a major disincentive for the investigation of perioperative intensive antiplatelet regimes, and perhaps underlie the paucity of such data. Most published reports are observational,[33,34] although randomized-controlled trials of dual antiplatelet therapy have been performed in patients undergoing carotid endarterectomy[35] and peripheral angioplasty,[36] and report no major increase in bleeding complications. However, there have been no randomized-controlled trials of dual antiplatelet therapy in surgery for critical limb ischemia, where the potential for both perioperative bleeding and cardiac complications is greater. We have successfully delivered such a trial and confirmed that while bleeding is increased, there was no excess of life-threatening bleeds, reoperations, or wound infections. Our results are consistent those reported by the CURE trial,[10] where those patients who continued clopidogrel therapy within 5 days of coronary artery bypass had a 2-fold increased relative risk of major bleeding. Arguably our pilot data suggests that the bleeding risks of dual antiplatelet therapy in the peri-operative period are modest, and perhaps often over estimated.

Increasingly patients are undergoing surgery for critical limb ischemia with a recent history of a cerebrovascular event, acute coronary syndrome, or percutaneous coronary intervention, and will be receiving intensive antiplatelet regimes. Their perioperative management must protect them from both coexisting pathology as well as the associated risks of surgery. Our study has shown that it is feasible to perform vascular surgery for critical limb ischemia, under dual antiplatelet therapy with an acceptable bleeding profile. Although the absolute clinical benefits of such a regimen need to be validated in a large-scale clinical trial, we believe that our study provides evidence for the beneficial role of perioperative antiplatelet agents in protecting these patients against cardiovascular complications.

Patients receiving epidural or spinal anesthesia were not recruited into the study due to the theoretical risk of epidural hematoma. We are aware that many vascular units aim for epidural or spinal anesthesia, thus precluding many patients from perioperative antiplatelet therapy. However, there is currently no level-one evidence for superior cardiovascular outcome with neuraxial blockade compared with general anesthesia. The main benefit lies in reducing respiratory complications associated with abdominal surgery.[37] Although many of the patients who were recruited smoked, none had significant chronic obstructive airway disease (based on lung function testing) and none were undergoing abdominal or emergency surgery. Use of both trial medication and general anesthesia was therefore deemed appropriate.

Persistent platelet reactivity despite antiplatelet therapy has been proposed as a risk factor for the recurrence of ischemic events following PCI. Recent mechanistic and clinical data suggest that higher loading and maintenance doses of clopidogrel may achieve a more rapid and greater degree of platelet inhibition that translates into improved clinical outcomes, but this is yet to be formally evaluated in an adequately powered randomized trial.[38] We administered a relatively high preoperative loading dose of clopidogrel (600 mg) to ensure efficacy of the intervention during surgery. It was hypothesized that if additional perioperative antiplatelet therapy was to be of any therapeutic advantage then this should be demonstrated with the largest degree of platelet inhibition. Previous studies have reported increased platelet activation[29,39–42] and cardiovascular events occurring within the first 5 days following surgery. We therefore rationalized that clopidogrel therapy would be of most benefit when given preoperatively and continued in the immediate postoperative period. It is possible that we could still achieve atherothrombotic protection with reduced bleeding complications by administering lower doses of clopidogrel and this requires further clarification. However, given the relatively high incidence of troponin-positive events before surgery, we would recommend initiation of therapy prior to surgery.

In conclusion, we have demonstrated that perioperative dual antiplatelet therapy has beneficial effects on reducing biomarkers of atherothrombosis without increasing life-threatening bleeds in patients with critical limb ischemia. We propose that large-scale randomized controlled trials are needed to establish whether dual

antiplatelet therapy can improve clinical outcomes in high-risk patients undergoing vascular surgery.

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Perioperative platelet and monocyte activation in patients with critical limb ischemia

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Background: **Patients with critical limb ischemia (CLI) have a high rate of adverse cardiovascular events, particularly when undergoing surgery. We sought to determine the effect of surgery and vascular disease on platelet and monocyte activation in vivo in patients with CLI.**

Methods: **An observational, cross-sectional study was performed at a tertiary referral hospital in the southeast of Scotland. Platelet and monocyte activation were measured in whole blood in patients with CLI scheduled for infrainguinal bypass and compared with matched healthy controls, patients with chronic intermittent claudication, patients with acute myocardial infarction, and those undergoing arthroplasty (n = 30 per group). Platelet and monocyte activation were quantified using flow cytometric assessment of platelet-monocyte aggregation, platelet P-selectin expression, platelet-derived microparticles, and monocyte CD40 and CD11b expression.**

Results: **Compared with those with intermittent claudication, subjects with CLI had increased platelet-monocyte aggregates (41.7% ± 12.2% vs 32.6% ± 8.5%, respectively), platelet microparticles (178.7 ± 106.9 vs 116.9 ± 53.4), and monocyte CD40 expression (70.0% ± 12.2% vs 52.4% ± 15.2%; $P < .001$ for all). Indeed, these levels were equivalent (P-selectin, 4.4% ± 2.0% vs 4.9% ± 2.2%; $P > .05$) or higher (platelet-monocyte aggregation, 41.7% ± 12.2% vs 33.6% ± 7.0%; $P < .05$; platelet microparticles, 178.7 ± 106.9 vs 114.4 ± 55.0/μL; $P < .05$) than in patients with acute myocardial infarction. All platelet and monocyte activation markers remained elevated throughout the perioperative period in patients with CLI ($P < .01$) but not those undergoing arthroplasty.**

Conclusions: **Patients undergoing surgery for CLI have the highest level of in vivo platelet and monocyte activation, and these persist throughout the perioperative period. Additional antiplatelet therapy may be of benefit in protecting vascular patients with more severe disease during this period of increased risk. (J Vasc Surg 2010;52:697-703.)**

Clinical Relevance: **Peripheral arterial disease is increasingly common and is associated with a significant risk of cardiovascular complications, especially at the time of surgery. Despite this, patients are poorly provided with evidence-based therapies such as antiplatelet and lipid-lowering medications. Platelets play a key role in the pathogenesis of atherothrombosis, with elevated levels of in vivo platelet activation prognostic of adverse clinical events. This study demonstrates, for the first time to our knowledge, significantly greater levels of platelet activation in patients with severe peripheral arterial disease compared with patients with acute myocardial infarction or patients undergoing other moderate- to high-risk surgical procedures. This further emphasizes the need for improved risk stratification and cardioprotection of this vulnerable group.**

Patients with peripheral arterial disease (PAD) are at increased risk of adverse cardiovascular events.[1] This risk increases with the severity of disease, with progressive reductions in the ankle brachial pressure index (ABPI) being an independent predictor of cardiovascular outcome.[2,3] Cardiovascular risk factor management is therefore the first-line treatment of patients with PAD. These patients are particularly at risk during the perioperative period. Myocardial infarction (MI) is the commonest life-threatening complication of major vascular surgery, with a reported perioperative incidence of 8% to 40%, depending on the diagnostic criteria.[4-6] This is comparable to the 30-day death and reinfarction rate of 8% to 20% seen in patients with acute coronary syndromes.[7] Despite this, patients with PAD often fail to receive evidence-based medical therapies. There is clearly an unmet need for improved risk stratification of these patients with appropriately tailored intensive medical regimens.

Atherogenesis and its complications are complex processes involving inflammatory and thrombotic mechanisms.[8-10] Monocyte adhesion to a damaged endothelium is a central step in the initiation and progression of atherosclerosis and appears to play a key role in plaque destabilization. In contrast, platelets can adhere to atherosclerotic lesions in the presence or absence of an overlying endothelium[8] and contribute to thrombus formation that is responsible for acute cardiovascular events. Hence, platelet and monocyte activation both predispose to plaque growth, instability, and rupture.

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Table I. Inclusion and exclusion criteria

	Healthy volunteers	*Claudicants*	*Critical limb ischemia*	*Non-ST elevation MI*	*Arthroplasty*
Inclusion criteria	Age >50 y	Age >50 y Intermittent claudication	Age >50 y Rest pain and/or skin ulceration or necrosis	Age >50 y Cardiac chest pain plus ECG changes plus plasma troponin I > 0.2 μg/L	Age >50 y
		ABPI <1 and >0.2 Rutherford- Baker II, III	ABPI ≤ 0.2 Rutherford-Baker IV-VI		
Exclusion criteria	PAD	Dual antiplatelet therapy	Dual antiplatelet therapy	PAD	PAD
	IHD, CVD Smoking Hypertension Diabetes Lipid-lowering agents Antiplatelet therapy Antihypertensive agents Warfarin	Warfarin	Warfarin		IHD, CVD Smoking Hypertension Diabetes Lipid-lowering agents Antiplatelet therapy Antihypertensive agents Warfarin

ABPI, Ankle-brachial pressure index; *CVD,* cerebrovascular disease; *ECG,* electrocardiogram; *IHD,* ischemic heart disease; *MI,* myocardial infarction.

Platelets and monocytes also directly interact to form platelet-monocyte aggregates that promote expression of vascular cell adhesion molecules and increase leucocyte adhesion to the inflamed endothelium.[11] In addition, the CD40/CD40 ligand dyad, which is expressed on the surface of activated monocytes and platelets, is a major inflammatory trigger that promotes release of inflammatory cytokines, adhesion molecules, and procoagulant activity.[12] Disruption of CD40/CD40L or platelet-monocyte aggregation leads to the retardation of atherosclerotic lesions in animal studies.[13,14]

Raised levels of platelet-monocyte aggregates and monocyte CD40 have been detected in smokers, patients with diabetes mellitus, acute coronary syndromes, and in those at risk of rethrombosis after percutaneous coronary interventions.[15-18] They are surrogate markers of clinical risk and are predictive of adverse cardiac events. Several studies have assessed platelet activation in peripheral atherosclerosis and demonstrated a progressive increase in activation with increasing severity of disease.[19-21] However, there have been relatively few reports of platelet activation and cellular inflammation at the time of operation,[22-24] and none, to our knowledge, have compared PAD patients with other high-risk populations.

We wished to investigate platelet and monocyte activation in patients with PAD specifically at the time of surgery and compare this to levels of activation in other high-risk populations. The high incidence of perioperative adverse cardiovascular events in patients with severe PAD could be mediated by increased systemic inflammation and platelet activation. These patients may potentially benefit from risk stratification and appropriately tailored medical regimens, as occurs in patients with coronary atherosclerotic disease. This exploratory study may support the conduct of interventional projects aimed at reducing surrogate markers of clinical risk, before clinical trials of intensive antiplatelet and anti-inflammatory strategies.

METHODS

This observational, cross-sectional study was performed with the approval of the local ethics committee and in accordance with the Declaration of Helsinki. All participants provided written informed consent.

Participants. Participants were recruited from five groups (n = 30 per group): (1) patients with nonthromboembolic CLI, (2) patients with chronic intermittent claudication, (3) patients with a non-ST segment elevation MI, (4) otherwise healthy patients undergoing hip or knee arthroplasty, and (5) healthy volunteers. The inclusion and exclusion criteria are summarized in Table I.

Patients with CLI, defined by the presence of rest pain or skin ulceration, or both, and an ABPI ≤0.2, who were scheduled to undergo infrainguinal bypass or amputation were recruited from the surgical vascular unit. Patients who had symptoms of intermittent claudication with an ABPI of <1 and >0.2, were recruited from the outpatient claudication clinic.

The 60 PAD patients received maintenance aspirin (75 mg daily) and statin therapy for at least 6 weeks before inclusion. We wished to examine platelet activation under the standard medical regimen; therefore, patients receiving clopidogrel or warfarin were excluded. At present, the only

evidence-based indication for dual antiplatelet therapy in PAD is coexisting history of recent (<6 months) coronary artery stenting or stroke.

Patients presenting to the coronary care unit and diagnosed with a non-ST elevation MI, defined as chest pain with electrocardiographic (ECG) changes and elevated plasma troponin I concentration (>0.2 μg/L), were recruited.

We wished to examine the contribution of surgical stress (without underlying PAD) to in vivo platelet activation. After discussion with a panel of consultant vascular surgeons and anesthetists, we concluded knee or hip arthroplasty represented surgery of a similar magnitude to peripheral bypass or amputation and was likely to be performed in patients of a comparable age. Patients aged >50 years undergoing elective arthroplasty were approached. To limit the effect of atherosclerosis on platelet activation, we excluded patients with a history of diabetes, hypertension, ischemic heart disease or stroke, smoking, or antiplatelet, antihypertensive, or statin use. Patients undergoing arthroplasty did not receive perioperative heparin according to the unit policy.

Sequential healthy individuals aged >50 were recruited.

Blood sampling. Single baseline samples were taken from all 150 participants. In patients with non-ST segment elevation MI, samples were taken ≤24 hours of hospitalization and after the initiation of dual antiplatelet medication with aspirin and clopidogrel. To compare perioperative platelet and monocyte activation, three blood samples were taken from patients undergoing vascular or orthopedic surgery preoperatively, immediately postoperatively, and on the day after the operation.

Blood was drawn by venipuncture of a large antecubital vein using a 19-gauge needle. Care was taken to ensure a smooth blood draw without venous stasis. Samples were processed immediately. Blood for assessment of platelet-monocyte aggregates, platelet expression of P-selectin, and monocyte CD40 and CD11b, was collected into tubes containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK, Cambridge Biosciences, Cambridge, UK). Blood for the assessment of platelet microparticles was collected into sodium citrate. Platelet-poor plasma was prepared by centrifugation at 2000*g* at 4°C for 10 minutes and confirmed by a platelet count of $<10^7$/L.

Assessment of in vivo platelet activation. Flow cytometric measurements of platelet-monocyte aggregates and platelet surface expression of P-selectin were performed as described previously.[15,16] Immunolabeling was performed in whole blood ≤5 minutes of collection. Directly conjugated monoclonal antibodies were obtained from DakoCytomation (Cambridge, UK) and Serotec (Oxford, UK). To assess platelet-monocyte aggregates, 60 μL of blood were incubated for 15 minutes with a fluorescein isothiocyanate (FITC)-conjugated anti-CD42a monoclonal antibody (platelet marker) and a phycoerythrin (PE)-conjugated anti-CD14 monoclonal antibody (monocyte marker) before fixation and erythrocyte lysis with 500 μL of FACSLyse solution (Becton Dickinson, Oxfordshire, United Kingdom).[17]

Cells were measured by flow cytometry (EPICS XL2; Beckman-Coulter). Samples were analysed with EXPO 32 software (Cytometry Systems). Platelet-monocyte aggregates were detected by gating for cells that were positive for both CD14 PE and CD42a FITC. Platelet surface expression of P-selectin was assessed by gating for cells that were positive for both FITC-conjugated anti-CD42a monoclonal antibody, (platelet marker) and PE-conjugated anti-CD62P monoclonal antibody (thrombin receptor-activating peptide [TRAP 1], immunoglobulin G1). Isotype controls were used to reduce error from nonspecific binding.

Platelet microparticles were identified by both size and expression of platelet markers CD41 (glycoprotein [GP] IIb) and CD31 (GPIIIa; platelet endothelial cell adhesion molecule-1). Aliquots (25 μL) of platelet-poor plasma were incubated for 30 minutes with a PE-conjugated anti-CD31 monoclonal antibody and a FITC-conjugated anti-CD41 monoclonal antibody (Serotec, Oxford, UK), before dilution with phosphate-buffered saline to form a volume of 1 mL.

Platelet microparticles were gated according to their size (events <1.0 μm) by assessment of their forward light scatter. TruCOUNT beads of 1.0 μm (Becton Dickenson) of a known concentration were used to calculate the volume of sample analyzed over 120 seconds at medium flow rate. This allowed the absolute number of platelet microparticles to be measured. Isotype controls were used to reduce error from nonspecific binding. Platelet microparticles were detected by gating for events that were sized <1 μm (based on forward scatter) and positive for both CD31 and CD41.

Assessment of in vivo monocyte activation. Monocyte activation was assessed by flow cytometric measurement of percentage of monocyte CD40 expression and mean fluorescent intensity (MFI) of monocyte CD11b expression, as described previously.[15,16] Immunolabeling was performed in whole blood within 5 minutes of collection. To evaluate CD40 and CD11b on monocytes, blood was diluted 1:2 with phosphate-buffered saline and incubated with the following monoclonal antibodies: anti-CD14:FITC (Serotec), anti-CD40:PE (Serotec), anti-CD11b:PE (Serotec), and appropriate isotype-matched controls for 20 minutes before fixation and erythrocyte lysis with 500 μL of FACSLyse solution. Monocytes were identified by gating for CD14-positive cells.

Statistical analysis. Data are shown as scatter plots or mean ± standard deviation. Data were analyzed by analysis of variance, χ^2 and Bonferroni post hoc tests, where appropriate, using Prism 4 software (GraphPad, La Jolla, Calif). Statistical significance was taken as a two-sided value of $P < .05$.

Table II. Participant demographics

Variable[a]	*Healthy volunteers (n = 30) Mean ± SD*	*Intermittent claudication (n = 30) Mean ± SD*	*Critical limb ischemia (n = 30) Mean ± SD*	*Acute coronary syndromes (n = 30) Mean ± SD*	*Arthroplasty (n = 30) Mean ± SD*
Age, y [a]	59 ± 3	60 ± 4	68 ± 2	58 ± 2	57 ± 4
	No. (%)	*No. (%)*	*No. (%)*	*No. (%)*	*No. (%)*
Male sex [b]	16 (53)	22 (73)	23 (77)	20 (67)	17 (57)
CV risk factors					
Hypertension	0	18 (60)	24 (80)	20 (67)	0
Diabetes	0	7 (23)	10 (33)	8 (27)	0
CAD	0	10 (33)	16 (53)	13 (43)	0
Current smoker	0	17 (57)	16 (53)	16 (53)	0
Medications					
Aspirin	0	30 (100)	30 (100)	30 (100)	0
Clopidogrel	0	0	0	30 (100)	0
Statin therapy	0	30 (100)	30 (100)	24 (80)	0
ACE inhibitor	0	3 (10)	12 (40)	21 (70)	0
β-blocker	0	2 (6.7)	6 (20)	16 (53)	0

ACE, Angiotensin converting enzyme; *CAD,* coronary artery disease; *CV,* cardiovascular.
[a] $P = .38$, one-way analysis of variance.
[b] $P = .24$, χ^2 analysis.

RESULTS

Participants were predominantly middle-aged men, with groups having similar distribution of ages and no significant difference in mean age (Table II). In keeping with their clinical presentation, patients with peripheral arterial and coronary heart disease had a range of cardiovascular risk factors and medications that were not present in the healthy volunteers or patients undergoing orthopedic surgery (Table II). Of the 30 patients with CLI, 18 underwent femoral-popliteal bypass and 12 underwent below knee amputations. None underwent endovascular procedures. Dry gangrene was present in 17, but none had wet gangrene.

Baseline platelet activation. Platelet activation markers were lowest in healthy volunteers and patients scheduled for arthroplasty (Fig 1). Baseline platelet-monocyte aggregation (41.7% ± 12.2%) and platelet microparticles (178.7 ± 106.9) were highest in patients with CLI compared with all other groups (Fig 1). Although patients with CLI had higher values of platelet P-selectin than healthy volunteers or those undergoing arthroplasty ($P < .001$), there was no demonstrable difference between these patients and those with claudication or non-ST elevation MI (4.4% ± 2.0% vs 4.2% ± 2.0% and 4.9% ± 2.2% respectively; $P > .05$; Fig 1).

Baseline monocyte activation. Monocyte activation markers were lowest in healthy volunteers and patients scheduled for arthroplasty (Fig 2). Baseline monocyte expression of CD40 (70% ± 12.2%) was highest in patients with CLI compared with all other groups (Fig 2). Baseline monocyte CD11b was greatest in patients with CLI compared with all groups except those with claudication, where it was equivalent (56.6 ± 18.3 vs 50.5 ± 13.9, respectively; $P > .05$; Fig 2).

Perioperative platelet and monocyte activation. Throughout the perioperative period, levels of all platelet and monocyte markers remained greater in patients with CLI than in those undergoing arthroplasty (Fig 3 and 4; $P < .0001$). Platelet and monocyte activation rose immediately postoperatively in patients undergoing joint arthroplasty ($P < .05$) before falling on the first postoperative day (Fig 3 and 4). In contrast, platelet activation fell immediately after surgery in patients undergoing infrainguinal revascularization or amputation ($P < .05$; Figure 3), whereas monocyte activation remained unchanged (monocyte CD40, $P > .05$) or rose on day 1 (monocyte CD11b; $P < .05$; Fig 4).

There was no statistically significant difference in markers according to type of surgery performed for CLI (bypass or amputation) and no difference in postoperative trend (subanalysis not shown).

DISCUSSION

Consistent with previous studies,[19-21] we have demonstrated that platelet and monocyte activation is increased in patients with PAD. We have shown for the first time, to our knowledge, that patients undergoing surgery for CLI have even greater levels of platelet and monocyte activation than patients being treated for acute MI. In addition, perioperative platelet and monocyte activation is markedly increased in these patients and exceeds the increase in platelet activation and inflammation attributable to surgery itself. This study supports the need for an increased appreciation of the cardiovascular risks associated with these patients and an improvement in cardioprotective management, especially in the perioperative period.

PAD affects nearly 30 million people in Western Europe and North America, and up to three-quarters of these patients have coexistent coronary artery disease and a threefold increased risk of cardiovascular events and death.[25] Despite attempts to raise awareness of PAD as an important marker of cardiovascular risk, patients are often poorly

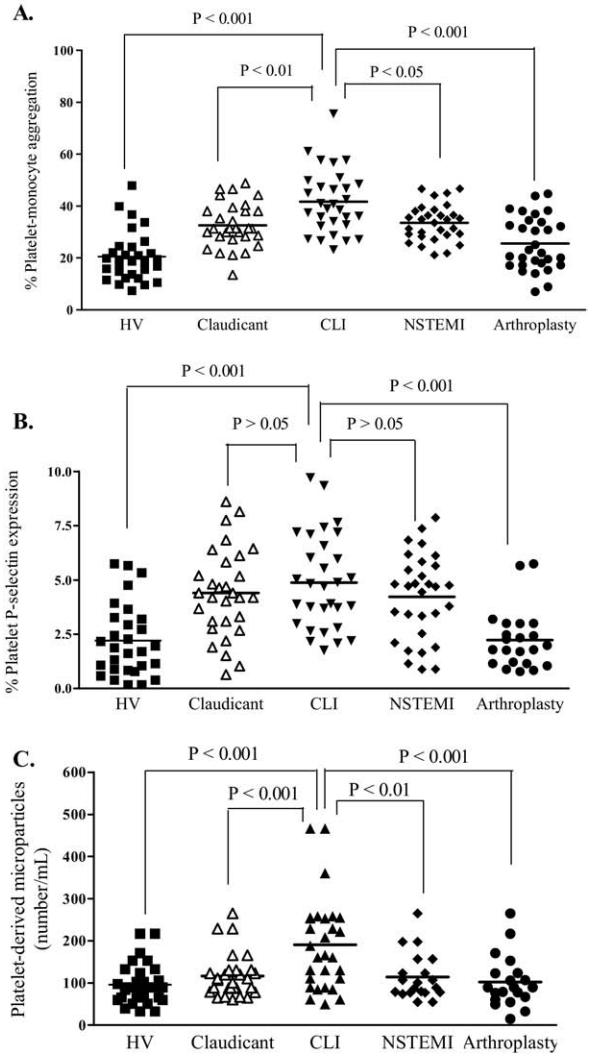

Fig 1. Scatter dot plots are shown for resting in vivo platelet activation in healthy volunteers (*HV*, ■), claudicants (Δ), patients with critical limb ischemia (*CLI*, ▼), patients with non-ST elevation myocardial infarction (*NSTEMI,* ♦), and patients undergoing arthroplasty (●). **A,** Percentage of platelet-monocyte aggregation. **B,** Percentage of platelet P-selectin expression. **C,** Platelet-derived microparticles (/mL). The *horizontal lines* represent population means (n = 30 per group). Analysis by one-way analysis of variance with Bonferroni post-tests.

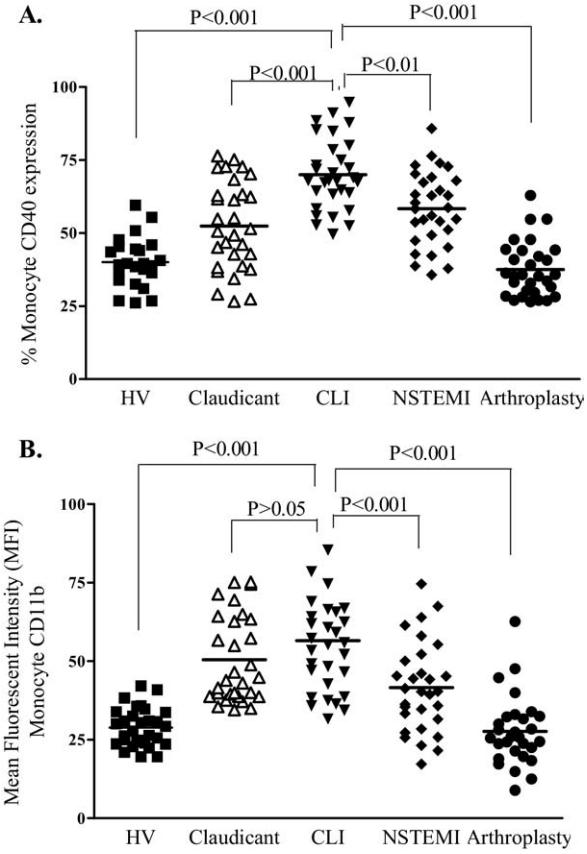

Fig 2. Resting in vivo monocyte activation in healthy volunteers *(HV,* ■*)*, claudicants (Δ), patients with critical limb ischemia *(CLI,* ▼*)*, patients with non-ST elevation myocardial infarction *(NSTEMI,* ♦*)*, and patients undergoing arthroplasty (●). **A,** Percentage of monocyte CD40 expression. **B,** Monocyte CD11b (mean fluorescent intensity, MFI). Horizontal lines represent population means (n = 30 per group). Analysis by one-way analysis of variance with Bonferroni post-tests.

provided with evidence-based therapies such as antiplatelet and lipid-lowering medications.[26-29] The reasons for this are unclear, but appear to be related to a lack of awareness amongst health professionals of the severity of the disease.

Our study demonstrated that patients with CLI have a greater elevation in systemic markers of platelet and monocyte activation than comparator groups. We acknowledge that the healthy volunteers and patients undergoing arthroplasty did not receive antiplatelet agents or lipid-lowering drugs, which can affect platelet activation. However, patients with PAD have a high incidence of adverse cardiovascular events despite existing medical therapy. Indeed, the elevations in platelet activation were seen despite the standard medical regimen of aspirin and statin therapy and were even greater than those seen in patients with acute MI. It may be that there is scope for further management of platelet activation in these patients.

Patients undergoing peripheral vascular surgery have a particularly high incidence of perioperative cardiovascular events. This has been attributed to increased platelet and monocyte activity caused by the surgical process itself. Patients without cardiovascular risk factors undergoing arthroplasty (and therefore no reason for elevated baseline platelet activation) were recruited to assess the effect of surgery alone on platelet activation. Our study showed that throughout the perioperative period, platelet and monocyte activation markers were higher in patients undergoing vascular surgery than in those undergoing arthroplasty. However, although platelet markers rose postoperatively in patients undergoing arthroplasty, we were surprised to see

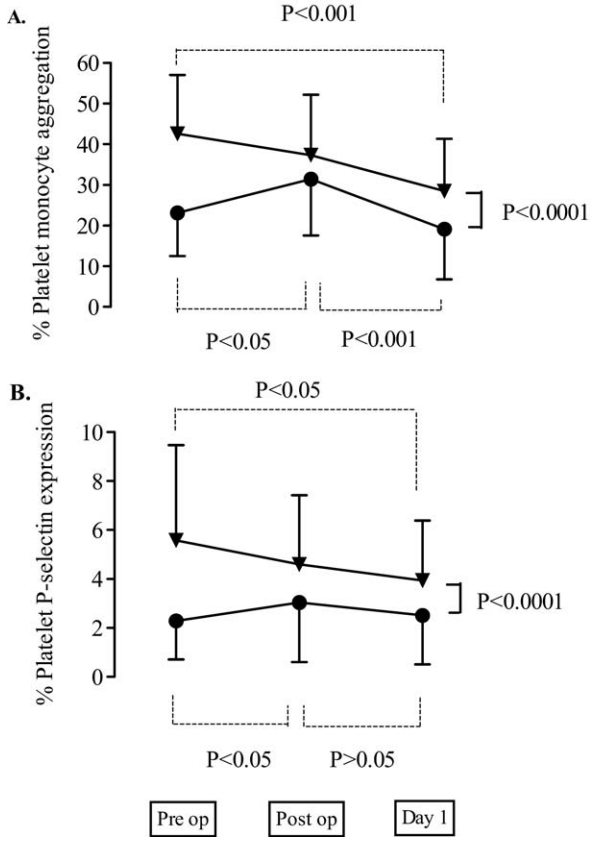

Fig 3. Perioperative in vivo platelet activation is shown in patients undergoing surgery for critical limb ischemia (▼) and arthroplasty (●). Platelet activation in patients undergoing infrainguinal bypass or amputation was greater than in patients undergoing arthroplasty throughout the perioperative period ($P < .0001$). In vivo platelet activation measured by **(A)** percentage of platelet-monocyte aggregation ($P < .05$) and **(B)** percentage of P-selectin expression ($P < .05$), rose immediately postoperatively in patients undergoing arthroplasty but fell in those with critical limb ischemia ($P < .001$). Mean ± standard deviation; analysis between patient groups by two-way analysis of variance with repeated measures; analysis between time points for the same patient group by one-way analysis of variance with Bonferroni post-tests.

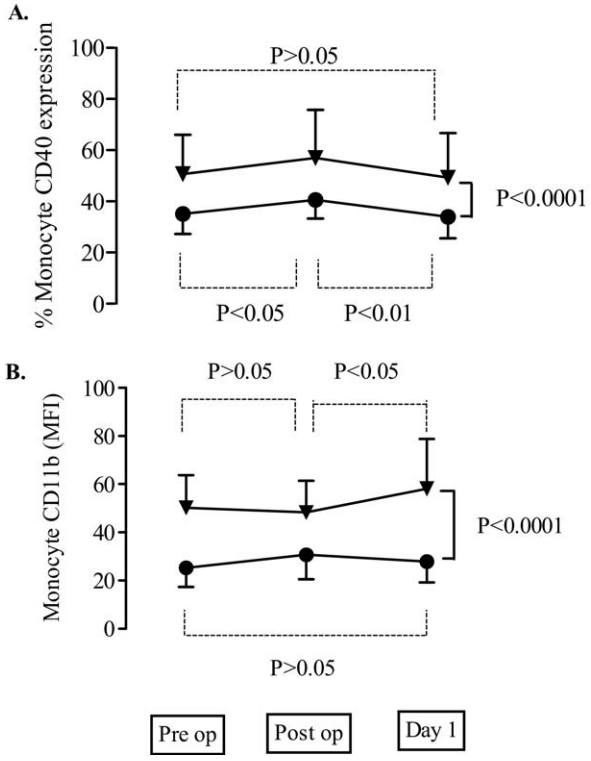

Fig 4. Perioperative monocyte activation in patients undergoing surgery for critical limb ischemia (▼) and arthroplasty (●). Monocyte activation in patients undergoing infrainguinal bypass or amputation was significantly greater than in patients undergoing arthroplasty throughout the whole perioperative period ($P < .0001$). **A,** Percentage monocyte CD40 expression tended to rise immediately postoperatively in orthopedic patients before returning to baseline ($P < .05$) **B,** There was no significant postoperative change in monocyte CD11b (mean fluorescent intensity) in arthroplasty patients ($P > .05$), but levels appeared to rise on day 1 after surgery in those with critical limb ischemia ($P < .05$).

that platelet activation fell immediately after surgery in those with CLI. Patients with PAD, especially CLI, have high baseline levels of activated platelets and monocytes. This is due to the underlying endothelial dysfunction and tissue ischemia. We therefore propose that the lack of response after surgery in these particular patients could be due to the removal of thrombotic stimulus by amputation or revascularization. These patients may benefit from increased platelet inhibition before surgery.

Cardiovascular disease is a critical public health issue. The prevalence of the disease and increased awareness of the cost-benefit associated with the management of cardiovascular risk have led to the concept of potential screening programs for vascular disease.[30] In addition, recommendations are required for the most appropriate use of interventions and therapies for patients with different manifestations of PAD. The use of imaging technologies or biomarkers could help risk-stratify patients and guide management. This study demonstrates that sensitive markers of in vivo platelet and monocyte activation—known to be predictors of clinical risk—are markedly elevated in patients with severe PAD at the time of surgery, despite current medical therapy. This is in line with reports of the increased cardiovascular risk associated with reducing ABPI.[2]

Further work is required, however, to demonstrate the link between platelet activation and adverse cardiovascular outcomes in this specific patient group. We are therefore conducting a trial of dual antiplatelet therapy (aspirin plus clopidogrel) vs aspirin alone, in patients undergoing surgery for CLI. The effect of intensive antiplatelet therapy on platelet markers, cardiac troponin, and high-selective C-reactive protein will be noted. This may inform the design of larger-scale clinical trials powered at examining clinical end points in this population.

We would like to thank all the clinical staff of the vascular unit, and in particular, Roderick T. A. Chalmers, Simon C. A. Fraser, and Zahid Raza; the Wellcome Trust Clinical Research Facility and the Clinical Biochemistry and Haematology Departments of the Royal Infirmary of Edinburgh.

AUTHOR CONTRIBUTIONS

Conception and design: AB, AN, SH, DN
Analysis and interpretation: AB, AD, DN
Data collection: AB, NC, AD
Writing the article: AB, AN, OJG, DN
Critical revision of the article: AB, AN, NC, SH, OJG, AD, DN
Final approval of the article: AB, AN, NC, SH, OJG, AD, DN
Statistical analysis: AB
Obtained funding: AB
Overall responsibility: DN

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Regular Article

Platelet activation in patients with peripheral vascular disease: *Reproducibility and comparability of platelet markers* ☆

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ABSTRACT

Background: Many markers of platelet activation have been described but their reproducibility and comparability in patient populations are poorly defined.
Objectives: We sought to compare markers of platelet and monocyte activation with platelet-monocyte aggregates, a proposed gold standard of *in vivo* platelet activation, and assess their reproducibility in patients with peripheral arterial disease: a population with substantial platelet activation, inflammation and risk of thrombotic events.
Patients/Methods: Thirty patients with peripheral vascular disease attended on two occasions to permit within-day and between-day comparisons. *In vivo* platelet and monocyte activation were determined by flow-cytometric quantification of platelet-monocyte aggregation, platelet surface expression of P-selectin and CD40L, platelet-derived microparticles, and monocyte surface expression of CD40 and CD11b. Plasma concentrations of platelet-derived microparticles, soluble P-selectin and CD40L were measured by enzyme-linked immunosorbant assays.
Results: Platelet-monocyte aggregation (36.7 ± 7.86%), and platelet surface expression of P-selectin (5.8 ± 1.65%) and CD40L (3.3 ± 1.45%) demonstrated comparable within-day (mean difference ± co-efficient of reproducibility; 0.9 ± 15.4%, 0.21 ± 1.65% and 0.2 ± 2.8% respectively) and between-day reproducibility (2.0 ± 12.4%, 0.10 ± 2.25% and 0.9 ± 6.4% respectively). Platelet-monocyte aggregates correlated well with other platelet ($r = 0.30$-0.50, $P < 0.02$) and monocyte ($r = 0.27$-0.47, $P < 0.03$) activation markers. Flow cytometric and assay quantified platelet-derived microparticles showed poorer reproducibility (co-efficient of reproducibility >40).
Conclusions: In patients with peripheral arterial disease, measurements of platelet-monocyte aggregates have good reproducibility and consistently reflect other markers of platelet and monocyte activation.

Introduction

Atherothrombosis is the leading cause of mortality in the western world. Platelets play a major role in the inflammatory and thrombotic progression of atherosclerosis [1,2]. Indeed, increased platelet activity is present in patients at increased risk of atherothrombotic events and predicts adverse cardiovascular events [3–8]. The measurement of platelet activity is therefore crucial to our understanding of the pathophysiology of atherothrombosis, the prediction of adverse cardiovascular events, and the development of novel therapeutic interventions.

Despite the development of several techniques, there is still no generally accepted ideal measure of platelet activation. A variety of methods exist, including platelet aggregometry, point of care devices, flow cytometric assessment of platelet surface antigens, and plasma markers of platelet activation: all have advantages and disadvantages [9]. Historically considered the gold standard, platelet aggregometry requires the preparation of platelet rich plasma and a high sample volume. Centrifugation and washing procedures may produce cell loss and artefactually activate platelets. Many of the point-of-care systems assess *ex vivo* platelet aggregation to various exogenous agonists. Although more labour intensive, flow cytometry is emerging as the new sensitive gold standard with measurement of surface expression of platelet antigens providing an assessment of *in vivo* platelet activation. It requires only a small sample volume, is performed on whole blood, and allows analysis of platelets in their physiological milieu. One of the most commonly studied markers of platelet activation is the α-granule membrane protein, P-selectin, that is present only on the surface of activated degranulated platelets. However,

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in vivo degranulated platelets rapidly lose their surface P-selectin, but continue to circulate and function [10]. In contrast, P-selectin-positive platelets very rapidly bind to leucocytes (mainly monocytes) via their constitutively expressed counter receptor, P-selectin glycoprotein ligand-1 (PSGL-1) [6]. Hence circulating platelet-monocyte aggregates provide a more sensitive measure of *in vivo* platelet activation [11]. In addition platelet-monocyte aggregation may alter leucocyte function, causing monocyte arrest on the endothelium and potentially enhance the growth of atherosclerotic plaques [12].

Many methods and protocols for the measurement of platelet activation have been described [13–17]. Importantly, aspects of the techniques themselves can lead to artefactual platelet activation, thus altering the end result. However, there are few reports of test reproducibility. Even when studying the same platelet marker, the methodology and end unit of measurement may differ, making it extremely difficult to interpret and compare results. An appreciation of these issues is crucial for the meaningful interpretation of interventional studies, and the development of anti-platelet therapies.

In the present study we sought to assess the reproducibility of an established protocol (**3,4,6**) in measuring platelet-monocyte aggregation in a patient population expected to have elevated resting platelet activation. In addition, we wished to compare the reproducibility and correlation of other markers of platelet activation to a proposed gold standard of platelet-monocyte aggregation.

Methods

The study was performed with the approval of the local ethics committee, in accordance with the Declaration of Helsinki and the written informed consent of all participants.

Subjects

Peripheral venous blood was obtained from 30 patients with peripheral arterial disease. Inclusion criteria were (i) symptoms of claudication, without rest pain or ulceration, (ii) reduced ankle brachial pressure ratio, and (iii) evidence of arterial stenosis on Doppler scanning.

Study design

In order to assess reproducibility, four samples were taken from each subject – two were taken on the same day one hour apart (within-day reproducibility), and two were taken the following day at the same time points (between-day reproducibility).

Platelet activation was assessed by measuring percent platelet-monocyte aggregation andplatelet expression of P-selectin and platelet-derived microparticles (no./μL) using flow cytometry. Platelet-derived microparticles, and plasma soluble P-selectin and CD40L concentrations were also measured by enzyme-linked immunosorbant assays (ELISAs). Monocyte activation was measured via flow cytometric measurement of percent monocyte CD40 expression and mean fluorescent intensity of monocyte CD11b expression.

Blood collection

Blood was drawn by venepuncture of a large anticubital vein using a 19-gauge needle. Care was taken to ensure a smooth blood draw without venous stasis. Samples were processed immediately. Blood samples for assessment of platelet-monocyte aggregates, platelet expression of P-selectin and monocyte CD40 and 11b expression, were collected into tubes containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK, Cambridge Biosciences). Flow cytometric measurement of platelet microparticles was performed on platelet poor plasma (PPP). Blood was collected into 10 mL sodium citrate tubes and PPP prepared by centrifugation at 2000 *g* at 4 ^{0} C for 10 min and confirmed by a platelet count of $<10^{9}$/L/(dilution with autologous plasma as required). Blood for the assay assessment of platelet microparticles and soluble P-selectin and CD40L was collected into tubes containing sodium citrate.

Immunolabelling and flow cytometry

Flow cytometric measurements of platelet-monocyte aggregation, platelet surface expression of P-selectin, and monocyte CD40 and 11b expression were performed as described previously [3,4,6]. Immunolabelling was performed in whole blood within 5 min of collection. Directly conjugated monoclonal antibodies were obtained from DakoCytomation (Cambridge, UK) and Serotec (Oxford, UK). In order to assess platelet-monocyte aggregates, 60 μL of blood were incubated for 15 min with a FITC-conjugated anti-CD42a monoclonal antibody (GRP-P, platelet marker) and a PE-conjugated anti-CD14 monoclonal antibody (Tuk-4, monocyte marker) before fixation and erythrocyte lysis with 500 μL of FACSLyse solution (**6**). Samples were processed using a BeckmanCoulter flow cytometer and at least 2,500 cell events were analysed by EXPO32 software. Platelet-monocyte aggregates were detected by gating for cells that were positive for both CD14 PE and CD42a FITC. Platelet surface expression of P-selectin was assessed by gating for cells that were positive for both FITC-conjugated anti-CD42a monoclonal antibody (platelet marker) and PE conjugated anti-CD62P monoclonal antibody (TRAP 1, IgG1). Isotype controls were used to reduce error from non-specific binding. To evaluate CD40 and CD11b on monocytes, blood was diluted 1:2 with PBS and incubated with the following monoclonal antibodies: anti-CD14:FITC (Serotec), anti-CD40:PE (Serotec), anti-CD11b:PE (Serotec) and appropriate isotype-matched controls for 20 min.

Platelet microparticles were identified by both size and expression of platelet markers CD41 (GPIIb) and CD31 (GPIIa; PECAM). Aliquots (25 μL) of PPP were incubated for 30 min with a PE-conjugated anti-CD31 monoclonal antibody and a FITC-conjugated anti-CD41 monoclonal antibody (Serotec, Oxford, UK), before dilution with phosphate buffered saline to form a volume of 1 mL. Platelet microparticles were gated according to their size (events <1.0 μm) by assessment of their forward light scatter. TruCOUNT beads of 1.0 μm (Becton Dickenson) of a known concentration were used to calculate the volume of sample analysed over 120 s at medium flow rate. This allowed the absolute number of platelet microparticles to be measured. Isotype controls were used to reduce error from non-specific binding. Platelet microparticles were detected by gating for events that were <1 μm in size (based on forward scatter) and positive for both CD31 and CD41.

ELISAs

Platelet-derived microparticles

Platelet-derived microparticles were assessed using a time-resolved immunofluorometric assay previously reported by Michelsen *et al.* [18]. This method quantifies the amount of platelet-microparticle-located CD41 (GPIIb) antigen in detergent-treated platelet-free plasma (μg/L). In brief, PPP is filtered to remove any platelet micro-particle larger than 0.1 μm (Ultra-free-MC Filter Units, Millipore, Billerica, MA, USA). The GPIIb/GPIIIa complex (CD41/CD61) is then released from the microparticle membrane and solubilized by mixing 1 volume PPP and 1 volume Delfia Assay buffer containing 1% of the non-ionic detergent Igepal CA-630. Two different monoclonal antibodies to GPIIb (CD41) are used, one labelled with Europium (Diatec, Oslo, Norway), and the other conjugated with biotin (clone DD4.1, Southern Biotechnology, Birmingham, AL, USA). Samples (50 μL) of the solubilized GPIIb/IIIa were then added to a Delfia streptavidin-coated plate (Perkin-Elmer Life Sciences, Boston, MA, USA) and 150 μL of antibody mixture added. Following incubation for 2 hours at room temperature, the wells are washed and Delfia Enhancement solution (200 μL/well) added prior to measurement of time-resolved fluorescence in a Victor^{2}1420 (Perkin-Elmer Life Sciences, Boston, MA,USA).

Soluble plasma markers

Soluble human P-selectin and CD40L were assessed using ELISAs from RnD systems and Bender Med Systems respectively.

Statistical analysis

The Bland Altman method was used to analyse the differences between paired measurements and to test the reproducibility of each measurement [19]. The co-efficient of reproducibility was calculated as 1.96 x the standard deviation of the differences between pairs of measurements in the same subjects. Statistical significance was assessed using a paired student's *t*-test. Correlation between variables was analysed using Pearson and Spearman's correlation coefficients as appropriate. The statistical package employed was GraphPad Prism, Version 4 (La Jolla, USA). Statistical significance was taken as a two-sided P value <0.05.

Results

All 30 patients were male, aged 58 ± 4 years and had a mean ankle-brachial pressure index of 0.38 ± 0.18. All patients were receiving maintenance aspirin (75–300 mg daily) and statin therapy, and were current smokers.

Reproducibility

Repeated measurements were similar for all markers used (Table 1) $P>0.05$. Flow cytometric measurements of per cent platelet-monocyte aggregation, and platelet surface expression of P-selectin and CD40L, showed good reproducibility (Table 1), with monocyte markers demonstrating a wider standard deviation (although statistically not significant). There was minimal difference when comparing within-day and between-day reproducibility although monocyte activation markers appeared to show better within-day reproducibility. Flow cytometric-derived measures of platelet-derived microparticles appeared to demonstrate better within-day and between-day reproducibility than immunoassay-derived measures, although both measurement techniques demonstrated poor co-efficients of reproducibility in comparison to the other markers.

Correlation between platelet markers

Platelet-monocyte aggregates correlated with all activation markers suggesting that it represented a reliable measure of global activation (Table 2, Fig. 1). Platelet surface expression of P-selectin showed the best correlation with platelet-monocyte aggregation ($r=0.50$) although platelet CD40L and platelet-derived microparticles also showed statistically significant correlations. Monocyte activation markers also correlated with platelet-monocyte aggregation (monocyte CD11b $P=0.0002$; monocyte CD40; $P=0.0026$) but not with other markers of platelet activation ($P>0.05$), suggesting that monocyte activation may also determine platelet-monocyte aggregate formation.

Immunoassay-derived measures of platelet activation, including plasma concentrations of soluble P-selectin and soluble CD40L, demonstrated poor or no correlations with flow cytometric-derived measures of platelet activation, except platelet-monocyte aggregates (Table 3). Indeed, when assessing the same variable, platelet-derived microparticles or P-selectin, there was only a moderate correlation between the two measures, or none at all (Table 3).

Discussion

We have assessed the reproducibility and consistency of platelet activation markers in a patient population with high baseline levels of platelet activation. We have shown that most measures of platelet activation in patients with peripheral arterial disease have good reproducibility both within and between days. Platelet-monocyte aggregates appear to consistently reflect other markers of platelet and monocyte activation. We suggest that platelet-monocyte aggregates are reproducible and provide the best measure of *in vivo* platelet activation for clinical study.

Several reports have demonstrated an association between increased platelet activation and adverse cardiovascular events [3–8]. Proof-of-concept studies hypothesise that reductions in platelet activation by anti-platelet regimes should correlate with improved clinical outcomes. It is therefore useful to quantify the degree by which antiplatelet medications reduce platelet activation markers. However, clinically meaningful interpretation of these studies must account for the normal variation in marker levels that occur within patient populations and that associated with measurement techniques. A variety of methods are currently employed for the assessment of platelet activation and the reproducibility of techniques is poorly reported.

Table 1
Within day and between day reproducibility of platelet activation markers.

Variable	Mean	Mean Difference	SD of difference	Co-efficient of Reproducibility	P value
A. Within Day Reproducibility					
PMA (%)	36.7	0.9	7.86	15.4	0.53
Platelet P-selectin (%)	5.8	0.21	1.65	3.2	0.56
Platelet CD40L (%)	3.3	0.2	1.45	2.8	0.55
PMP: Flow (no./μL)	157.2	8.1	42.4	83.1	0.81
PMP: Assay (GPIIb/μg/L)	25.9	5.7	24.2	47.4	0.28
Monocyte CD40 (%)	69.5	0.2	7.2	14.1	0.88
Monocyte 11b (MFI)	47.4	3.7	11.9	11.4	0.16
Soluble P-selectin (ng/mL)	42.3	1.2	16.8	32.9	0.74
Soluble CD40L (ng/mL)	0.6	0.1	0.35	0.6	0.46
B. Between Day Reproducibility					
PMA (%)	37.3	2.0	6.35	12.4	0.08
Platelet P-selectin (%)	5.9	0.1	1.15	2.25	0.81
Platelet CD40L (%)	4.2	0.9	3.25	6.37	0.28
PMP: Flow (no./μL)	161.9	7.2	39.9	78.2	0.43
PMP: Assay (GPIIb/μg/L)	58.4	0.9	27.65	54.2	0.84
Monocyte CD40 (%)	69.6	2.5	24.1	47.2	0.39
Monocyte 11b (MFI)	49.9	0.8	11.45	22.4	0.68
Soluble P-selectin (ng/mL)	41.7	5.0	19.3	37.8	0.08
Soluble CD40L (ng/mL)	0.6	0.02	0.25	0.49	0.61

Table 2
Correlation of flow cytomteric-derived measures of platelet and monocyte activation.

Variable	Platelet P-selectin (%)	Platelet CD40L (%)	PMP (no./μL)	Monocyte CD40 (%)	Monocyte 11b (MFI)
PMA (%)	0.50 **P<0.0001**	0.41 **P = 0.0007**	0.27 **P = 0.028**	0.27 **P = 0.026**	0.47 **P = 0.0002**
Platelet P-selectin (%)		0.52 **P<0.0001**	0.08 P = 0.11	0.04 P = 0.095	0.24 P = 0.07
Platelet CD40L (%)			0.03 P = 0.88	0.00 P = 0.96	0.42 **P = 0.0013**
PMP (no./μL)				0.00 P = 1.0	0.08 P = 0.98
Monocyte CD40 (%)					0.16 P = 0.22

Pearson and Spearman correlation coefficient as appropriate.
Significant correlations shown in bold.

Certain processing stages in the assessment of platelet activation markers can contribute to artefactual activation. Differences in technique make it difficult to compare results across studies, even when examining the same subject population. In addition, units of measurement often vary (e.g.% platelet expression, versus mean fluorescent intensity). Therefore, for a given patient population, the range of platelet activation levels often varies widely between studies. Indeed, our reported mean sCD40L level of 0.6 ng/mL differs from some of the reported studies by a factor of both 10^{-1} [20] and 10^{1} [21], although it is in agreement with other groups [22]. If platelet activation and inflammatory markers are to be utilised in guiding interventional studies, readers must have a clear appreciation of the limits of the methological techniques, and how they compare against an accepted 'gold standard'.

In the present study, we utilised a two-colour whole blood technique incorporating erythrocyte lysis and fixation to quantify platelet-monocyte aggregates [3,4,6]. We have previously reported a number of methodological considerations in the preparation and processing of samples for flow-cytometric quantification of platelet-monocyte aggregates, and this technique has shown good reproducibility in healthy

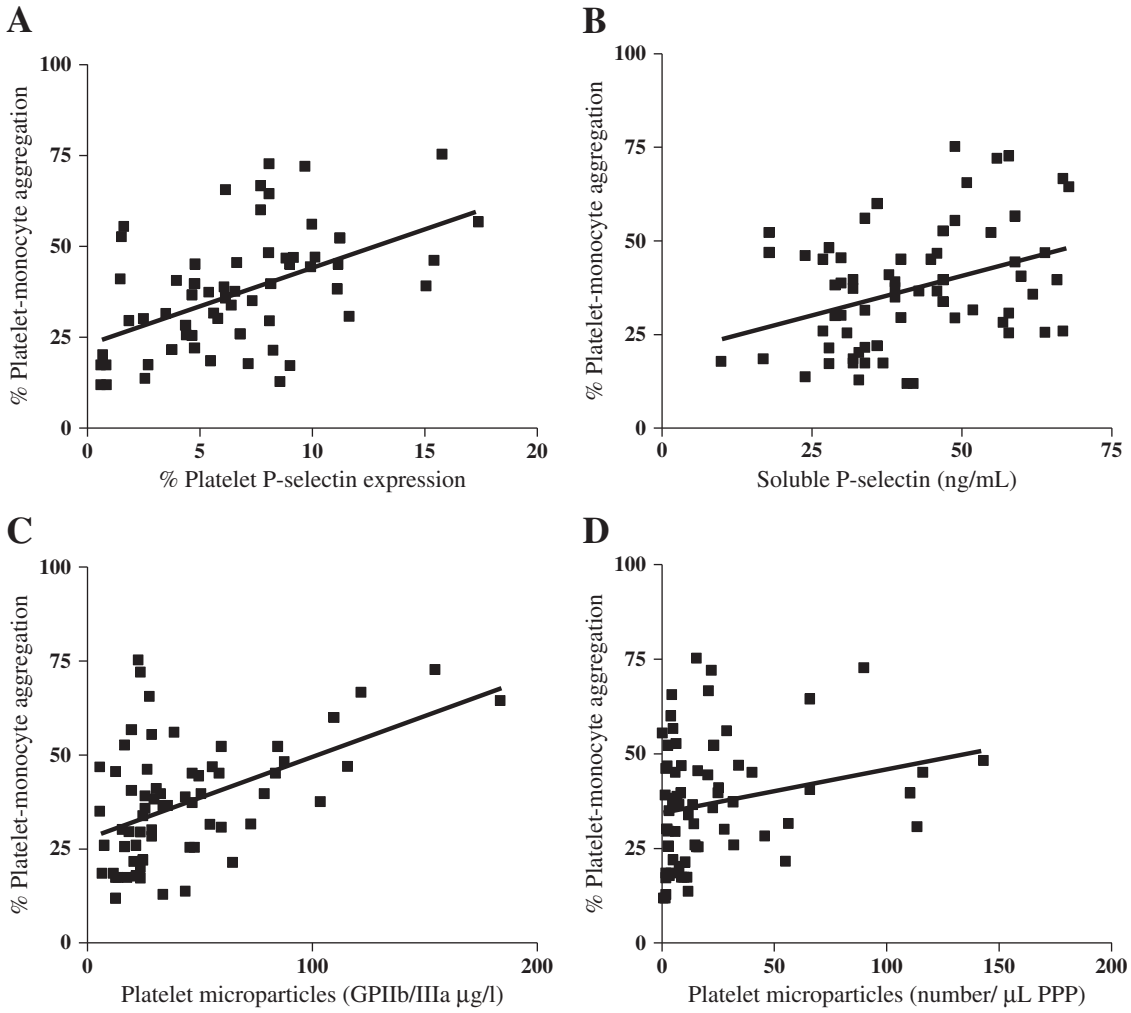

Fig. 1. Comparison of percent platelet-monocyte aggregation *versus* (A) per cent platelet P-selectin expression (r = 0.50, P<0.0001), (B) plasma soluble P-selectin concentrations (ng/mL; r = 0.37, P = 0.002) (C) immunoassay-derived measures of platelet microparticles (μg/L; r = 0.44, P = 0.0002), and (D) flow cytometric-derived measures of platelet microparticles (no./μL; r = 0.27, P = 0.028).

Table 3
Comparison of Immunosorbant Assay-Derived and Flow Cytometric-Derived Markers of Platelet Activation.

	Immunoassay			
Flow Cytometry		**PMP: Assay (GPIIb μg/L)**	**Soluble P-selectin**	**Soluble CD40L**
	PMA (%)	**r = 0.44**	**r = 0.37**	**r = 0.30**
		P = 0.0002	**P = 0.002**	**P = 0.018**
	Platelet P-selectin (%)	**r = 0.30**	r = 0.05	r = 0.04
		P = 0.015	P = 0.69	P = 0.73
	Platelet CD40L (%)	r = 0.14	r = 0.20	r = 0.14
		P = 0.27	P = 0.11	P = 0.27
	PMP: Flow (no./μL)	r = 0.39	r = 0.03	r = 0.08
		P = 0.015	P = 0.41	P = 0.19

Pearson or Spearman correlation coefficient as appropriate.
Significant correlations shown in bold.

volunteers (co-efficient of variation, 7.8%) [23]. We collected whole blood through direct venepuncture as opposed to via a cannula to avoid turbulent flow and increased activation. We chose to anticoagulate blood with a direct thrombin inhibitor as opposed to heparin or citrate as these compounds have been shown to increase and decrease aggregates respectively. We also chose not to stimulate samples with agonists *ex vivo* as we wanted to assess resting circulating *in vivo* levels.

We appreciate that our procedures differed when it came to the detection of platelet microparticles (when platelet poor plasma had to be prepared from whole blood) and when assays were used instead of flow cytometry. It is inevitable that processing may differ with the detection of different markers, however, as long as the methodology is fully reported (with clarity over the stages known to affect outcomes e.g. anti-coagulant agent) then the relationship between measurements can still be examined.

Recently there has been increasing evidence to suggest that the role of platelets in atherosclerosis may be mediated by the production of microparticles [24,25]. Platelet-derived microparticles (diameter <1 μm and bearing platelet-derived surface antigens) are involved in several stages of atherosclerosis from coagulation (tissue factor release; inhibition of fibrinolysis) [26,27] to direct effects on the blood vessel wall (platelet adhesion) and angiogenesis [28,29]. In addition, increased platelet-derived microparticles have been demonstrated in individuals with a smoking habit, diabetes mellitus, cerebrovascular disease and acute coronary syndromes [7,8]. A variety of methods have been proposed for the assessment of platelet-derived microparticles. However, platelet microparticles are heterogeneous with respect to both size and composition, and can carry a range of antigenic markers from their parent platelet. We decided to compare flow cytometric and immunoassay based methods of quantification: both techniques identifying the same platelet antigen (CD41, GPIIb). The preparation of platelet poor plasma and use of counting beads is well validated and the assay has been reported as demonstrating good sensitivity for *in vitro* generated platelet microparticles [18]. Although reproducibility was good for other markers, our measures of platelet microparticles were less consistent. This is likely to reflect several factors. First, significant additional processing was required by these assays and this will inevitably increase variability. Second, the accuracy of flow cytometry is reduced when assessing particles <1 μm in size. Finally, our flow protocol identified microparticles that were positive for two platelet markers, CD41 (GPIIb) *and* CD31 (GPIIa, PECAM) whereas the immunoassay only identified microparticles positive for CD41 (GPIIb).

Although platelet P-selectin and CD40L demonstrated good reproducibility (co-efficients <7), platelet monocyte aggregates showed the best global correlation with other markers. Platelet-monocyte aggregates primarily form through the binding of P-selectin on the platelet surface to the leucocyte counter-receptor, P-selectin glycoprotein ligand-1 (PSGL-1), on monocytes. It is therefore not surprising that platelet expression of P-selectin shows good correlation with levels of platelet-monocyte aggregates. Platelet microparticles modulate monocyte interactions with other cell types by increasing expression of adhesion molecules [30,31]. The correlatation between microparticles and platelet-monocyte aggregates could reflect this, although the relationship is less strong. It is interesting to note the lack of correlation between soluble P-selectin and platelet expression of the molecule ((r = 0.05; P = 0.69). This has been previously reported [34]. Dissociation between platelet expressed and soluble P-selectin suggests that the soluble form cannot serve as a surrogate marker of platelet activation.

Inflammation plays a central role in the pathogenesis of atherosclerosis. Platelet-monocyte aggregation is not only a sensitive measure of platelet activation but also has important pro-inflammatory consequences. Platelet-monocyte adhesion activates (NF)-κB; a transcriptional activator thought to be important in the regulation of CD40 gene expression [32]. The correlation between platelet-monocyte aggregates and CD40 expression on monocytes may reflect this process. Induction of nuclear factor (NF)-κB also stimulates expression of Mac-1 or CD11b/CD18 [33]. This could be reflected in the excellent correlation between monocyte CD11b expression and platelet-monocyte aggregation (Table 3). Subsequent activation of CD11b is associated with neutrophil recruitment to sites of inflammation.

In conclusion, we have demonstrated that, in patients with peripheral arterial disease, our measurement of platelet and monocyte activation markers demonstrated good reproducibility. Platelet-monocyte aggregates consistently reflected other markers of platelet and monocyte activation and we propose them as a sensitive 'gold standard' of in vivo platelet activation. In contrast, measures of platelet-derived microparticles appeared less reproducible. We plan to employ these techniques in a study of different anti-platelet regimes in this patient population. The co-efficients of reproducibility will allow us to interpret the true contribution of such drugs to reductions in platelet activation. With increasing interest in the manipulation of platelet and inflammatory mediators for therapeutic gain, an understanding of the reproducibility and comparability of techniques for *in vivo* assessment of platelet activation is imperative.

Conflict of interest

There are no conflicts of interest.

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