ACTIVATION OF NEUTROPHILS, PLATELETS AND THE COAGULATION SYSTEM IN INTERMITTENT CLAUDICATION

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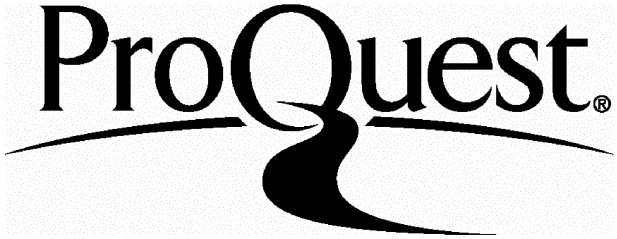

ABSTRACT

Intermittent claudication is a common condition, affecting 2-5% of the population. The prognosis for the affected limb is relatively benign, but morbidity and mortality from associated cardiovascular events is high, with up to 50% of patients suffering a myocardial infarction or stroke in the five years following diagnosis. Multivariate analysis has suggested that claudication itself may be a risk factor for these events.

The pathophysiologies of claudication and cardiovascular events are reviewed, with particular emphasis on the role of neutrophils, platelets and the coagulation system. From this we developed a hypothesis that claudication may activate, or increase the potential to be activated of, neutrophils, platelets and the coagulation system; and that this may contribute to the increased cardiovascular morbidity and mortality seen in claudicants. The aim of this work was to investigate activation, and, in particular, the activation potential of these elements in claudicants, before and after exercise.

We assessed cellular and humoral activation in claudicants before and after exercise; and compared this with controls. We used sensitive flow cytometric techniques to investigate neutrophil and platelet activation, with a whole blood method to reduce preparation artefact. These investigations were run alongside more established techniques. Newer, more sensitive, assays were used to assess activation of the coagulation system.

We found evidence of increased potential for neutrophil activation, as shown by increased superoxide production and hydrogen peroxide generation, following exercise in claudicants. Claudicants showed increased thromboxane levels following exercise in both the neutrophil and platelet studies. However, there was no further clear evidence for activation or priming of platelets from the studies of surface glycoproteins or platelet release factors. Differences in levels of markers of activation of the coagulation system were found between claudicants and controls, but no effect of exercise was seen. From this we concluded that claudication may increase the risk of cardiovascular events by priming neutrophils. There was no equivalent evidence to suggest priming of platelets or the coagulation system in response to claudication, although neutrophil activation may in turn activate platelets and the coagulation system.

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III.3 **Results**

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c)Platelet size
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LIST OF ABREVEATIONS

5-HT	5-Hydroxy-tryptamine
ABPI	Ankle Brachial Pressure Index
ADP	Adenosine Di-Phosphate
ANOVA	Analysis Of Variance
APTT	Activated Partial Thromboplastin Time
ATP	Adenosine Tri-Phosphate
BSA	Bovine Serum Albumin
CI	Confidence Intervals
βTG	Beta-Thromboglobulin
DCFH-DA	Dichlorofluoroscin Diacetate
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme-linked Immunosorbent assay
EDGF	Endothelial cell Derived Growth Factor
FDPs	Fibrin Degradation Products
FpA	Fibrinopeptide A
FITC	Fluorescein Isothiocyanate
Fb	Fibrinogen
GPRP	Glycyl-propyl-L-arginyl-L-proline
H_2O_2	Hydrogen Peroxide
IHD	Ischaemic Heart Disease
IGF-1	Insulin-like Growth Factor-1
IgG	Immunoglobulin G
LDL	Low Density Lipoprotein
Lp(a)	Lipoprotein (a)
LTB_4	Leucotriene B4
MI	Myocardial Infarction
MDGF	Macrophage Derived Growth Factor
MFI	Maximum Fluorescence Intensity
MoAb	Monoclonal Antibody
OPD	o - Phenylenediamine
PAF	Platelet Activation Factor
PDGF	Platelet Derived Growth Factor
PAI-1	Plasminogen Activation Inhibitor-1
PF4	Platelet Factor 4
PGI_2	Prostacyclin
PRT	Pressure Recovery Time
PRP	Platelet Rich Plasma
PPP	Platelet Poor Plasma
PBS	Phosphate Buffered Saline
PMA	Phorbol Myristate Acetate
PVD	Peripheral Vascular Disease
TAT	Thrombin-Anti-Thrombin
TIA	Transient Ischaemic Attack
SO	Superoxide
SPAA	Stagnation Point flow Adhesion Aggregometry

t-PA	Tissue Plasminogen Activator
TxA_2	Thromboxane A2
TxB_2	Thromboxane B2
u-PA	Urinary Plasminogen Activator
vWF	von Willebrand Factor
WBC	White Blood Cell
WCC	White Cell Count

CHAPTER ONE: INTRODUCTION

SECTION ONE: INTERMITTENT CLAUDICATION

I. 1.1 INTERMITTENT CLAUDICATION

a) Definition Intermittent claudication describes a clinical syndrome of cramp-like muscular pain, usually affecting the lower limbs, induced by exercise. This increases steadily causing a limp (Latin, *claudicare* = to limp) and eventually the patient is forced to stop. The pain is relieved by a few minutes rest, but if exercise is resumed the pain recurs after a similar distance. Commonly the pain occurs in the calf muscles but is also described in the thigh and buttocks. The essential features of claudication are: the pain is always experienced in the functional muscles group; is reproducibly precipitated by a consistent amount of exercise and is relieved by rest[1].

b) Historical Perspectives This condition was first reported in 1831 by Bouley, a Parisian veterinary surgeon, who described a case of intermittent limping in a horse. Subsequent dissection of the animal revealed bilateral femoral artery occlusions. In 1858 Charcot applied the term intermittent claudication to symptoms in man, and supported Bouley's observation that pain was due to circulatory insufficiency revealed by exercise, and so began our understanding of the pathology of claudication[2].

c) Aetiology Claudication develops whenever blood flow to the exercising muscle mass is unable to meet the increased metabolic demands. It is caused by a fixed arterial occlusion, or significant stenosis, proximal to the affected muscle bed. At rest the blood supply is adequate to meet the metabolic demands of the tissue. However, upon exercise this demand rises markedly, the normal exercise-induced increase in muscle blood flow is impaired and cannot meet the increased need. As a result the muscle becomes hypoxic, the interstitial pH falls and waste products build up resulting in pain. The most common cause of this impaired blood flow is atherosclerosis, which will be discussed later. Less common causes include thromboangiitis obliterans, or Buerger's disease, vasculitis, fibrosis (retroperitoneal or due to radiation), cystic advential disease, popliteal entrapment syndrome or late effects of arterial trauma[3].

d) Epidemiology Most patients with intermittent claudication are not referred to hospital and hence accurate estimates of the prevalence of claudication can only be obtained from population studies. Prevalence can be assessed in two ways, either by symptom enquiry or by physiological testing. While symptom enquiry is simple, quick and can therefore be used to study very large numbers, it measures symptomatic disease only and will underestimate the prevalence of peripheral arterial disease.[4] Furthermore, several studies have shown up to a third of patients with claudication based on questionnaire do not have true claudication when assessed by a doctor.[5,6,7,8,9,10] In addition, patients perception of the significance of their claudication varies widely. Studies report 50 - 90% of patients with intermittent claudication do not complain of this symptom to their doctor, considering it to be part of the ageing process.[7,11] In a further study, one third of patients with angiographically

proven lower extremity arterial occlusion denied claudication despite extensive questioning.[5] Hence, the symptom of claudication is relative: the patient must have a significant arterial stenosis or occlusion and exercise sufficiently to induce muscle ischaemia.[6] The most commonly used questionnaire to for intermittent claudication was devised in 1962 by Professor G Rose at the London School of Hygiene and Tropical Medicine[12]. Despite only moderate sensitivity it remains the most valid way of making international comparisons of the prevalence of intermittent claudication[4,13,14]. The reported prevalence of claudication varies widely. A review of international population studies revealed a range from 0.9% in N America to 6.9% in Moscow in men aged 40 - 60 years. This may represent some geographical difference, but problems with translation, age stratification and health worker effects make comparisons difficult[4]. Reported prevalences of 0.9% in sedentary office workers[11] compared with 2.1% in agricultural workers[9] suggests an occupational component. Prevalence also varies with age[9,15,16]. McDaniel and Cronenwett found claudication occurred in about 1.8% of people under 60 years, in 3.7% of those between 60 and 70 years, and in 5.2% of those over 70 years[17]. These figures have been confirmed in a further international review[18]. The prevalence of claudication is more common in men than in women[7,9,19,20,21] and this difference becomes more marked with increasing disease severity[18].

Only two studies have followed up a normal population to determine the incidence of intermittent claudication. The Framingham Study[22] reports an incidence of 0.2% in men aged 45-55 years, increasing to 0.5% in those aged 55-65 years. The Basle study[23] found a five year incidence for occlusive peripheral arterial disease, assessed by history and examination, of 4% in the younger group (35-44 years) and 18% in the

older group (>65 years). However, only a third of those with detectable disease reported symptoms of claudication.

I.1.2 NATURAL HISTORY OF INTERMITTENT CLAUDICATION

Interest in peripheral arterial disease and its natural history occurred with the development of vascular surgery. Consequently there is very little data on the natural history of patients with intermittent claudication without the influences of surgery[1].

a) Local disease Morbidity associated with the local effects of peripheral vascular disease is usually determined by assessing any deterioration of symptoms and by measuring the amputation rate. Early studies tend to suggest progression is unusual. Boyd prospectively followed up 1440 patients with intermittent claudication and reported an amputation rate of 12.2% after 10 years[24]. Further studies, although smaller and with shorter follow-up times, reported lower amputation rates around 7%, with approximately 20% of patients suffering a deterioration of their symptoms[25,26,27,28]. These earlier studies have been criticised because of lack of objective assessment of peripheral vascular disease. It has been suggested that more recent studies, with objective assessment of disease, show higher levels of disease progression[1]. However, although one study records 60% of patients with symptomatic deterioration, the follow-up period was only 2.5 years, and only 2.2% of patients proceeded to amputation[29]. Longer studies, also with objective assessment of arterial disease, have shown approximately 20 - 25% patients with worsening symptoms and an amputation rate around 5 - 7%[10,29,30,31,32,33,34,35]. In an attempt to partly control the

influence of surgical management, Dormandy et al reviewed ten studies, which were not primarily surgical. They concluded in approximately 75% of patients the disease will stabilise shortly after presentation and 25% will deteriorate[18]. However, stable symptoms do not necessarily indicate stable disease. The underlying atheromatous process almost certainly progresses, although the patient adapts psychologically and physiologically, and may develop further collateral circulation[4,18]. The incidence of amputation depends on the population studied. In hospital based studies patients tend to have more severe disease, their symptoms having been sufficient to warrant referral. This bias is important because numerous studies have shown that the severity of arterial disease, assessed objectively at presentation, is the most important factor predicting future outcome[25,29,31,35]. Thus, hospital based work records higher amputation rates, around 5-7%, compared to population studies, approximately 1-2%[4,18]. Furthermore, outcome is influenced by risk factor management and surgical intervention. Both of which are more likely to occur in hospital based work.

Unfortunately, despite a relatively optimistic outlook for the local disease, most claudicants will die prematurely from events secondary to generalised atherosclerosis.

b) Systemic disease Atherosclerosis is a generalised disease, a fact which is emphasised by the incidence and severity of associated coronary and cerebral vascular disease found in claudicants.

i) *Concurrent disease* Early studies looking at the incidence of concurrent coronary disease have been limited by a lack of suitable screening tools. The prevalence of coronary artery disease diagnosed from history and electrocardiogram

(ECG) ranges from 19%[26] to 47%[36]. Use of more sophisticated tests such as Treadmill stress testing[37] and Dipyridamole-stress thallium imaging[38] gave levels of 62% and 63% respectively. Only one study has used the 'gold standard' of coronary angiography and reported some degree of coronary artery atherosclerosis in 90% of patients undergoing surgery for claudication[39]. Importantly, 14% of patients with no history or ECG findings of coronary artery disease had severe surgically correctable coronary lesions. However, the patients in this study were all being assessed pre-operatively and hence represent a group with more severe peripheral vascular disease. A more realistic figure suggests approximately 50% of patients presenting with claudication have coronary artery disease sufficient to be detected by simple clinical techniques[18]. The incidence of concurrent cerbrovascular disease also varies with the screening technique used. Duplex ultrasound, used by many as the only pre-operative assessment of carotid disease prior to carotid endarterectomy, detected significant cerebrovascular disease in 52% of claudicants pre-operatively. Interestingly, 25% of these had >60% stenosis[40].

ii) *Non-fatal coronary and cerebrovascular events* In view of the above it is not suprising that claudicants have a high incidence of cardiovascular events. However, the data on non-fatal events is sparce[18]. Only two studies have looked at an unselected population of claudicants and distinguished between angina and myocardial infarction (MI)[26,41]. These studies reported 14 and 28.7% incidence of non-fatal cardiac events, and a 5 and 6.1% incidence of non-fatal cerebrovascular events respectively. The Basle study[42] found 16% non-fatal cardiac and 12% non-fatal cerebrovascular events, although many of these patients did not have symptoms of claudication. The PACK study prospectively followed up 1969 patients receiving

placebo for at least 1 year. They recorded 36 (1.8%) non-fatal MI, 12 (0.6%) fatal MI, 27 (1.4%) non-fatal major strokes and 8 (0.4%) fatal strokes[43].

iii) *Fatal coronary and cerebrovascular events* Follow-up studies of claudicants report a loss of 10 years in life expectancy[44,45,46]. In male claudicants followed-up in population studies the five year cumulative mortality rates ranged from 4.8%[47] to 17%[48]. Higher rates still are seen in hospital based studies. In a review by Dormandy et al overall mortality at 5, 10 and 15 years is given as approximately 30%, 50% and 70% respectively[18]. In studies where patient mortality was compared to a parallel, age and sex matched control population, the overall risk of dying within 5 years was 2 - 3 times greater in claudicants; the difference being greater in surgical series[9,10,26,28,30,48,49,50]. Mortality rates tend to be higher in men than women[9,10,30], but the relative risk of death was similar[10]. Mortality also increases with age, although the relative risk fell with age[7,10,28,30]. Similar findings have been noted in subjects followed up for 10 years[10,23,26,28,48] and 21 years[51]. Ischaemic heart disease is the commonest cause of mortality, accounting for between 35 and 60% of deaths. Cerebrovascular disease causes between 7 and 17%, and other vascular events (such as ruptured aneurysms and visceral infarction) around 8% of deaths[10,20,23,28,30,50].

c) Possible reasons for increased morbidity and mortality At first sight the cause of increased mortality rates found in claudicants appears obvious; an atherosclerosis related event. Some data suggests an increase in the incidence of and death from neoplasia in claudicants, but these rates are normalised when controlled for smoking. Possible mechanisms behind the increased morbidity and mortality in claudicants include:

i) *Associated vascular disease* Intermittent claudication is a manifestation of a generalised condition, and hence, it is not suprising that claudicants have an increased mortality from MI and stroke; claudication acts as a marker of coronary and cerebrovascular disease. However, the answer may not be as straightforward as this. Reports of ischaemic heart disease (IHD) show that mortality rates are three times higher in-patients with coronary heart disease and claudication when compared to patients with coronary heart disease alone[52]. Although this suggests an independent effect on mortality due to claudication, associated risk factors must be taken into account.

ii) *Associated risk factors* The development of peripheral vascular disease is associated with certain risk factors, in particular smoking, hypertension and diabetes[4,7,8,9,16,19,22,53-64]. Although there is some discrepancy between the risk factors linked with IHD, cerebrovascular disease and claudication, the above three are strongly associated with each group. Any group of claudicants is therefore likely to have increased levels of risk factors associated with cardiovascular death. Thus, claudication maybe considered as a marker for risk factors for raised cardiovascular mortality rather than a causal agent.

iii) *More aggressive disease* Alternatively, the presence of claudication may herald a more aggressive form of atherosclerotic process. However, there is sparse evidence for this view. Unpublished data from Luscombe et al[53] reports 25% of claudicants with carotid disease detected by Duplex as having a >60% stenosis. Hertzer[39] studied a group of patients prior to vascular surgery using angiography and reported 29% with advanced and 28% with severe coronary artery disease. However,

both these studies were uncontrolled and hospital based. The close association of claudication and other presentations of atherosclerosis make it very difficult, if not impossible, to answer this question. Interestingly, Brevetti et al found the more severe the peripheral vascular disease, as measured by Ankle Brachial Pressure Index (ABPI), the higher the cardiovascular morbidity[65].

d) Intermittent claudication as an independent risk factor An alternative possibility is that claudication may act, at least in part, as an independent risk factor for cardiovascular events or death. Although an earlier study[9] found no independent role for claudication, subsequent work has identified claudication as an independent risk factor for death[10,22,52,66]. The Whitehall Study, which followed 18 388 subjects for 17 years, showed increased cardiovascular mortality in claudicants after adjusting for cardiac ischaemia, blood pressure, serum cholesterol, smoking and glucose tolerance[46]. Furthermore, the Framingham Study found claudication imposed a penalty after MI in men, and was a significant predictor of survival following first MI in women[67]. These findings were confirmed by the SPRINT Study Group which followed 3 695 patients after their first MI. After controlling for risk factors and cardiac status, intermittent claudication was independently linked to re-infarction[68]. The Lausanne Stroke Registry reported that claudication predicts stroke following MI[69]. Similarly, studies of patients with Transient Ischaemic Attacks or non-disabling stroke have found (TIAs) claudication to independently predict further stroke, MI and vascular death[70].

From this it can be concluded that intermittent claudication is associated with an increased mortality, and probably morbidity, from cardiovascular disease. Whilst part

of this increased mortality relates to associated disease and risk factors, there is evidence for intermittent claudication acting as independent risk factor for mortality. Although it would be logical to assume a similar relationship for non-fatal cardiovascular events such data is not available. Intermittent claudication is a common condition and a better understanding of the mechanism of any independent effect may help target future therapeutic strategies. Investigation of the mechanism of such an independent effect is likely to be difficult; the effect may well be small and the effect of confounding variables, such as risk factors and concurrent disease, comparatively large.

I.1.3 PATHOPHYSIOLOGY OF EXCESS CARDIOVASCULAR MORBIDITY AND MORTALITY IN CLAUDICANTS

To consider possible ways in which claudication may act independently to increase mortality and morbidity, the pathophysiology of atherosclerosis, cardiovascular events and claudication itself will be discussed.

a) Atherosclerosis "Atherosclerosis is a degenerative disease, characterized by the accumulation of cells, matrix fibres, lipids, and tissue debris in the intima, which may result in the narrowing of the lumen and obstruction of blood flow or ulceration, embolisation, and thrombosis"[71].

Large scale epidemiological studies have identified certain risk factors for the development of atherosclerosis[72], the most important being hypertension,

hypercholesterolaemia and smoking. Lesser factors including diabetes, obesity, hypertriglyceridaemia, sedentary lifestyle, stress and family history have a more variable correlation with atherosclerotic disease. Although commonly found in-patients with atherosclerosis they cannot be separated as independent predictors of disease[71]. Interestingly, one study has found fibrinogen to be a better predictor for the development of ischaemic heart disease than cholesterol[73].

i) *Aetiology* The pathogenesis of the atherosclerotic plaque remains obscure. Whilst many theories abound, each presented as discrete entities, more evidence is accumulating to suggest an inter-relation between many of the current proposed mechanisms. The exact initiating factor for the development of an atherosclerotic plaque is unclear, and may well comprise a number of different mechanisms, but two features appear to be common to most theories. They are, an increase in the movement of lipid and lipoprotein into the sub-intimal space and monocyte adhesion to the endothelium followed by migration through into the sub-intimal space[74,75]. Although considered a generalised condition, atherosclerosis does show a predilection for certain 'lesion-prone sites'[75]. Interestingly, these sites show increased endothelial permeability to albumin[76,77], fibrinogen[78] and Low Density Lipoprotein (LDL) cholesterol[79,80], have a thinner endothelial glycocalyx coat and increased monocyte recruitment and endothelial cell turnover [75,81-83]. These sites also correspond to the areas of lipid accumulation seen in young children and hyperlipidaemic states[75]. Hence, it could be argued these sites of endothelial dysfunction are 'natural', probably secondary to local haemodynamic factors[75].

Endothelial dysfunction Endothelial dysfunction can also arise due to exogenous factors, including toxins, chemical irritation (e.g., factors from cigarette smoke), immunological trauma, viruses and hypertension. Such dysfunction may then manifest itself as increased passage of lipid into the subintimal space and expression of adhesion proteins for monocytes on the endothelium[74]. Thus, by one or more of the above mechanisms there is an ingress of lipid, particularly LDL, and monocytes into the sub-endothelial space.

Monocyte Activation In the sub-endothelial space the monocytes undergo a complex process of activation/differentiation to become macrophages. The trigger for this conversion is not clear, but may involve LDL and its oxidised form[84]. Within the arterial wall LDL is oxidised by free radicals from smooth muscle cells, macrophages or endothelial cells[84]. The resulting molecule stimulates endothelial cells and smooth muscle cells to produce monocyte chemotactic protein-1, increasing monocyte adherence and migration[85], transforms monocytes to macrophages and has a further effect to inhibit the migration of macrophages out of the vessel wall[75]. Macrophages have a scavenger receptor with a high affinity for oxidised LDL and no down-regulation mechanism. Consequently, there is continued uptake of modified lipoprotein with the formation of 'foam-cells'; an essential feature of atherosclerosis[75]. Furthermore, oxidised LDL is cytotoxic and maybe responsible for further endothelial cell damage and foam cell necrosis leading to the formation of the extracellular lipid core. The formation of an extracellular lipid core is an important stage, representing the transition from the reversible 'fatty streak' to a less easily reversible, more mature lesion[74,75].

Smooth Muscle Cell Proliferation Further maturation involves proliferation of smooth muscle cells and the increased production of collagen and proteoglycans to form the 'fibrous' part of the mature fibro-fatty atherosclerotic lesion. Smooth muscle cells migrate into the sub-endothelial space from the media, in response to chemotactic agents[86,87], including Platelet Derived Growth Factor (PDGF) and Insulin-like Growth Factor-1 (IGF-1)[74]. Here, they undergo proliferation and probably transformation from contractile to secretory phenotype[74]. Smooth muscle proliferation is induced by a variety of proposed mitogens[74], of which PDGF maybe the most important[88]. This cationic protein, found in the α-granules of platelets, was isolated from experiments studying platelet deposition on areas of denuded endothelium. It was found to be mitogenic and chemotactic for smooth muscle cells[89]. Subsequent work has shown PDGF, or PDGF-like molecules, can be produced by macrophages, endothelial cells and smooth muscle cells[88]. Interestingly, hypercholesterolaemic serum may trigger production of PDGF by normal endothelium, possibly explaining the smooth muscle cell proliferation which maybe found at sites of fatty streak formation[90].

Into the above synopsis of the two main atherogenesis theories has to be integrated the work suggesting a role for platelets and the coagulation system, which are discussed in more detail in Section Two. Whilst, it is possible to conceive links between most of the current views on atherogenesis, the fine details and exact mechanisms are far from clear.

ii) *Natural history* In pathological terms atherosclerotic lesions begin with endothelial dysfunction, leading to formation of the early 'fatty streak' lesion. Plaque

progression is characterised by foam cell necrosis, formation of an extracellular lipid core, smooth muscle cell proliferation and the deposition of connective tissue. Further progression occurs with plaque fissuring and thrombosis which can either further narrow or completely occluded the lumen. Despite what appears to be an inexorable sequence of events in pathological terms, the natural history of the disease can follow a different course. It has been shown that the arterial lumen expands in response to narrowing by atherosclerotic plaques[91,92]. This is a natural adaptive response of the artery to reduced blood flow; mediated through reduced shear stress[71,93]. When the plaque occupies 40% of the total artery lumen a critical point is reached, the mechanism fails and narrowing begins[94]. Furthermore, despite appearances at operation or post-mortem, intra-luminal pressure often results in a smooth luminal contour[95]. Hence, quite marked disease in pathological terms may produce very little in the way of clinical effects. However, some cases show both clinical and pathological deterioration, even with modification of associated risk factors. Whilst some of this maybe due to gradual disease progression, much of the clinical events relating to atherosclerosis are thought to arise from 'lesion complications', namely thrombosis; with sudden narrowing or occlusion of vessels or formation of emboli. Why some cases of atherosclerosis lead to clinical disease and others do not is not clear.

b) Mechanisms leading to cardiovascular events Most of the mortality and morbidity from atherosclerosis arises because of myocardial or cerebral infarction. Infarction occurs when the blood supply to an area of tissue is inadequate for vital functions. This can occur from occlusion of the supplying artery with insufficient collateral circulation. Possible mechanisms for such occlusion are: i) disease

progression, ii) acute thrombosis, or iii) thromboembolism. The majority of work in this area has been done looking at the coronary circulation, however, excepting that thromboembolism maybe more significant in stroke, the principles are probably similar in all thrombotic disease states.

i) *Disease progression* Arterial occlusion may arise through continued disease progression either by continued action of the initial insult, mural thrombosis on small plaque fissures or thrombosis in the absence of plaque disruption. It may be that all three of these processes act simultaneously, but there is good evidence that mural thrombosis at the site of plaque disruption is important in the progression of atherosclerosis. Autopsy studies of the coronary arteries in patients with IHD, who died from their disease or unrelated causes, reveal fresh mural thrombi, layered thrombi over plaque fissures as well as previously healed fissures with various stages of thrombosis and thrombus organisation[96,97]. Use of antibodies has demonstrated products related to platelets and fibrin in advanced and fibrous plaques[98,99], and experimental work has shown the incorporation of mural thrombi into atherosclerotic plaques[100]. Thrombosis may also occur in the absence of plaque disruption. Evidence for this comes from the finding that severely stenotic plaques tend to be very fibrotic and stable[101]. It has been proposed that haemodynamic factors, such as shear stress may lead to platelet aggregation and result in thrombotic occlusion of such lesions[102-105].

Despite the 'ability' of disease progression to occlude vessels, it appears to have limited clinical significance. Gradual formation of stenoses is likely to be associated with the development of collateral circulation. By the time the stenosis becomes

critical and/or occlusive, the majority of blood flow is through collaterals and occlusion has little clinical effect[105-107]. In conditions of critical stenoses in both normal and collateral circulation occlusion may produce infarction, but the majority of events are thought to arise due to the formation of an occluding thrombus on a ruptured atherosclerotic plaque.

ii) *Occluding thrombosis on ruptured atherosclerotic plaques* Whilst occlusive thrombosis due to plaque disruption is probably the more severe end of the spectrum, which includes disease progression through mural thrombosis, there are certain interesting differences. It is well established that thrombosis secondary to plaque rupture plays a major part in myocardial infarction[97,108-112]. A prospective angiographic study has shown that severe stenoses are three times more likely to occlude, but that this rarely causes infarction. In contrast, 85% of infarct-related lesions were considered not haemodynamically significant[113]. Thus, it appears disruption of smaller plaques is more important in the pathogenesis of MI[111,114]. These smaller plaques tend to be soft with a high concentration of cholesterol at the base; thinning of the fibrous cap overlying the lipid core precedes rupture[109,115]. The pathophysiology of plaque rupture is unclear. Studies by Davies et al showed fissures frequently occur at the junction of the fibrous cap and the normal vessel wall. It is postulated that a lack of underlying collagen support makes the cap more prone to rupture[116]. In addition, stress from disordered blood flow and sudden changes in intraluminal pressure or tone may contribute to rupture[100].

The amount and duration of thrombus deposition is dependent on various local and systemic factors. In cases of superficial plaque disruption a small, labile thrombus is

formed, and probably results in mural thrombus or very transient occlusive thrombus. Conversely, deep rupture exposes collagen and tissue factor, and produces a larger, relatively persistent occlusive thrombus[104]. Studies have shown increased platelet deposition, from shear-induced aggregation, with increased stenosis. This suggests that acute platelet response to plaque disruption depends in part on the degree of stenosis following disruption[100]. Another local factor influencing thrombosis is the presence of residual, partly lysed, thrombus. This not only narrows the lumen, increasing shear rates, but is strongly thrombogenic, causing increased platelet deposition and activation of the coagulation system[100]. In addition, to the physical effect of arterial occlusion factors released due to plaque disruption, platelet aggregation and thrombosis[100] can produce arterial vasoconstriction thus further impairing myocardial oxygen supply and demand.

c) Pathology of Cardiovascular events The major causes of morbidity and mortality in patients with intermittent claudication are myocardial infarction and stroke (cerebral infarction). Infarction is a process of tissue death, which occurs when the blood supply to that tissue is inadequate to supply the vital needs of the tissue. The degree of ischaemia and subsequent infarction depends on various factors. Firstly, the metabolic needs of the tissue. Unfortunately, both heart and brain tissue are very sensitive to ischaemia, with three to four minutes being enough to cause irreversible damage to nerve cells. Furthermore, neither cardiac muscle nor neuronal tissue is capable of regeneration. Secondly, the speed and degree of arterial occlusion are important. A complete occlusion tends to result in more tissue extensive damage than a severe stenosis. Rapid occlusion of an artery, such as occurs with plaque rupture and thrombosis, allows little time for the opening up of collateral vessels, compounded in the heart and brain by a paucity of such

collaterals. At a histological level, the dead parenchymal cells swell and then autolyse; any diapedesed red cells haemolysing. There is then a brisk inflammatory response with infiltration of neutrophils and macrophages, in response to chemotactic agents released from the dying cells. Subsequently, dead cells are removed by phagocytes and replaced with granulation tissue and eventually mature scar tissue[117].

The clinical effects of infarction obviously depend on site and size. The site of an infarct is determined by the anatomy of the vasculature and the site of the occluding lesion. The size of an infarct is also determined by these factors, but in addition the general level of perfusion, the presence of collateral vessels and tissue pressure play a part. Furthermore, the size of an infarct can evolve. The pathophysiology behind this is thought to relate to the presence of a peri-infarct area, or penumbra. Cells in this area may show morphological and/or functional abnormalities. However, they differ from cells central to the infarct area in that they are salvageable; the damage being reversible. Such peri-infarct tissue is vulnerable to further insult, as the oxygen supply is just enough to maintain viability. Any further impairment in blood supply or oxygen delivery can lead to increased cell death and an enlargement of the infarct size. Interestingly, the presence of neutrophils within and around the infarction can have a detrimental effect on the survival of the peri-infarct tissue, and this is discussed further in Section Two.

To consider why claudication might promote the above processes and increase the risk of stroke or MI, it is necessary to look at the pathophysiology of Intermittent Claudication.

I.1.4 PATHOPHYSIOLOGY OF INTERMITTENT CLAUDICATION

a) Physiology of clinical symptom Intermittent claudication is the commonest presenting symptom of peripheral vascular disease. This symptom complex arises because of impaired blood supply to the effected muscles. At rest the blood supply is adequate to provide the vital needs of the muscle. However, on exercise the metabolic requirements of the tissue increase. Normally, the muscular vessels dilate to increase flow. In the presence of atherosclerosis, with narrowing of the supplying vessels, this increase in flow cannot occur. As a consequence the muscle becomes deprived of oxygen, anaerobic metabolism predominates and there is a build up of waste products and lactic acid. Under these conditions the muscle becomes dysfunctional, leading to a limp and a "dead" feeling in leg, and sensory nerves are stimulated, possibly by the acidic conditions, to produce pain. On resting the metabolic requirements are reduced, the oxygen debt is slowly paid back and conditions normalise with return of function and relief of pain. McCully et al showed, that on walking, muscle desaturation occurs quicker and takes up to four times as long to recover in claudicants compared to normal young and old people[118]. We have also noted (unpublished) it can take up to 15 minutes for femoral venous oxygen levels to return to resting state following exercise in claudicants. Thus, during a "claudication cycle" the effected muscles become ischaemic with exercise followed by reperfusion or reoxygenation on rest. This ischaemia-reperfusion cycle is a well-recognised pathological phenomenon and the average claudicant experiences repeated episodes each day. This may have important implications in the natural history of patients with intermittent claudication.

b) Ischaemia-reperfusion Ischaemia is a common clinical event with potentially serious consequences. Interruption of the blood supply to a tissue deprives the cells of oxygen, the fuel essential for the generation of the high-energy phosphate bonds required for normal cell function. Lack of oxygen leads to anaerobic metabolism with a build-up of lactic acid and a reduction in the generation of Adenosine Tri-phosphate (ATP) molecules. A depletion of cellular energy results in a failure of homeostatic mechanisms and eventual cell death. Re-establishing blood supply, which restores the energy supply and removes toxic metabolites, is necessary for recovery from ischaemic injury. However, there is good evidence showing that reperfusion of ischaemic tissue causes more tissue damage than ischaemia alone. Parkes and Granger demonstrated that the histological changes of injury were worse after 3 hours ischaemia and 1 hour reperfusion than after 4 hours ischaemia[119]. This phenomenon is called the ischaemia-reperfusion injury.

i) *Pathophysiology* Ischaemia-reperfusion injury is thought to be initiated by the generation of oxygen free radicals. Ischaemia leads to an accumulation of hypoxanthine, a metabolite of ATP, and xanthine oxidase within the tissues. Xanthine oxidase requires oxygen as a substrate to convert hypoxanthine to xanthine, and generates superoxide anion. Upon reperfusion this reaction causes a large burst of superoxide production, which in turn leads to the production of other free radicals within the cells. The main effect of these free radicals is thought to be the generation of chemotactic activity directing activated neutrophils into the reperfused tissue[120-122]. The importance of neutrophils as a mediator of the ischaemia-reperfusion injury is demonstrated by the protective effect of prior neutrophil depletion or blocking their microvascular adherence[122-125]. Exactly how superoxide induces neutrophil activation

and migration is unclear. Oxygen derived free radicals cause lipid peroxidation and damage cell membranes. This may induce release of arachidonic acid metabolites[126-128] and cytokines[129], activation of complement[130], and altered expression cellular adhesion molecules[131]. Many of these inflammatory mediators are chemotactic for, and cause activation of, neutrophils[127,128]. These activated neutrophils may adhere and act locally causing damage by the release of free radicals, peroxidase and proteolytic enzymes[120,132] including elastase, collagenase and cathepsin G. Local injury is characterised by oedema, increased microvascular permeability and reduced, or occluded, micro-circulation. Alternatively, neutrophils may circulate, impact in distant microcirculations and cause remote tissue injury[133-136]. One manifestation of this injury is non-cardiogenic pulmonary oedema, due to abnormal pulmonary capillary permeability to protein[133,137]. Experimental evidence suggests this is due to the sequestration of activated neutrophils with the pulmonary vasculature. Although the precise mechanisms behind this pulmonary sequestration are unclear, it does demonstrate that ischaemia reperfusion generates circulating activated neutrophils.

ii) *Ischaemia-reperfusion in intermittent claudication* Much of the work studying ischaemia-reperfusion has looked at the effect of prolonged, severe ischaemia in relatively large muscle masses. However, moderate ischaemia can produce a similar response. Erlansson et al showed incomplete ischaemia induces similar microvascular changes to those seen with complete ischaemia[138]. Although, claudication involves a comparatively small muscle mass and the ischaemia is short-lived, there is evidence to suggest ischaemia-reperfusion injury does occur in intermittent claudication. Firstly, evidence of free radical formation has been demonstrated by the finding of increased plasma levels of lipid peroxides in exercising claudicants

compared to controls[139,140]. Neutrophil activation has been suggested by the reduced filterabilty of neutrophils and the raised levels of serum lysozyme[140,141], released upon neutrophil degranulation, following exercise in claudicants. Experimental work has shown increased neutrophil adhesion to endothelial cells, increased microvascular permeability and endothelial cell swelling in model of claudication[142,143]. Interestingly, there was evidence of increased permeabilty and endothelial cell swelling in systemic sites. Clinical studies of claudicants have shown increased urinary excretion of albumin compared to controls[139]. This is in keeping with the idea of increased microvascular permeability, possibly mediated by oxygen derived free radicals which are known to increase vascular permeability[120,133]. Thus, there is evidence to suggest not only a local ischaemia-reperfusion injury but also a remote effect. Whilst the size of the response is small compared to cases of prolonged limb ischaemia, the process is repetitive occurring many times each day.

Thus, at a cellular level, the activity and interaction of platelets, the coagulation system and neutrophils plays an important role in the development of cardiovascular events. Activation or priming of these 'systems' may amplify the response to plaque rupture and increase the resulting cardiovascular event. Claudication may activate or prime these systems, via ischaemia and/or reperfusion, or some other mechanism, and this may explain, in part, the independent effect of intermittent claudication on cardiovascular morbidity and mortality. Before outlining the further investigation of this idea, Section Two will review the current information on the role of neutrophils, platelets and the coagulation system in intermittent claudication and

SECTION TWO: NEUTROPHILS, PLATELETS AND THE COAGULATION SYSTEM IN ATHEROSCLEROSIS AND INTERMITTENT CLAUDICATION.

I.2.1 NEUTROPHILS AND INTERMITTENT CLAUDICATION

White blood cells act, with the complement and immunoglobulin systems, to protect the body against infection. Broadly speaking they can be divided into two groups: phagocytes and immunocytes. Neutrophils make up the majority of the phagocyte group. A large circulating cell, neutrophils have a characteristic dense lobulated nucleus, with pale cytoplasm containing many granules. Their function is to migrate to areas of infection or inflammation, and through a process of phagocytosis and digestion destroy bacteria and cellular debris. In atherosclerotic events neutrophils appear to be producing an inflammatory response to ischaemia and infarction, and "inadvertently" aggravating the situation.

I.2.1.1 Role Of Neutrophils In Atherosclerotic Disease

a. Atherogenesis Monocyte-derived macrophages, and to lesser degree T-lymphocytes appear to be the main leucocytes involved in atherogenesis. Monocytes are believed to respond, in part to the endothelial injury/dysfunction and migrate into the intima, becoming macrophages. Here they release and respond to various humoral factors, forming foam cells and stimulating smooth muscle cell proliferation[74,75,88]. The

role of the T-lymphocytes is unclear. In the absence of evidence for a specific antigen-led immune response, they are thought to act as part of the inflammatory process. Animal models of atherogenesis have shown neutrophil adhesion and migration in response to endothelial injury[144], and neutrophil activation has been linked to increased vascular permeability, thought to be an early event in atherosclerosis[142]. However, there is little evidence for a role for neutrophils in the pathogenesis of atherosclerosis in humans.

b. Progression and Complications of Atherosclerosis

There is increasing evidence that white cells, in particular neutrophils, play an important role in vascular disease and are integral to the mechanism of tissue injury in ischaemia and infarction[145,146].

i) *Epidemiological observations* The Caerphilly study, which looked at prognostic factors in nearly 800 men, found a positive correlation between white blood cell (WBC) count and the prevalence of coronary heart disease[147]. A similar finding was reported by the Multiple Risk Factor Intervention Trial (MRFIT)[148]. Incident epidemiological studies have shown powerful and consistent positive correlations between WBC count and the risk of coronary heart disease[149,150], myocardial infarction[147,151-153] and stroke[154]. Examination of the differential counts showed a particularly strong association with the neutrophil count[149,154]. The Caerphilly and Speedwell Collaborative Study showing WBC count as a strong, independent predictor of coronary heart disease and infarction in both patients with and without evidence of pre-existing ischaemic heart disease[153]. Post myocardial infarction studies have found a higher WBC count in those patients who subsequently re-infarcted[155,156], died suddenly[155]

or died within 1 year[157]. In these studies it could be argued that the raised WBC count reflects the size of the first infarct; the bigger the infarct, the bigger the inflammatory response and hence WBC count. In turn, the larger the infarct the greater the morbidity and mortality, thus linking the WBC count and clinical picture[158]. However, results from the incident studies above suggest this cannot be the entire story. There is a strong correlation between smoking and WBC count[159], but Friedman et al[151] concluded that only two-thirds of the predictive value of WBC count could be explained by the effect of smoking. Further studies controlled for associated risk factors, including smoking, have confirmed WBC count as a strong independent risk factor for coronary heart disease[160], myocardial infarction[153], reinfarction[155,156], sudden cardiac death[147-149,152,160] and all-cause mortality[157].

Interestingly, a decline in WBC count has been reported to reduce the risk of coronary heart disease[160]. This protective nature of reduced white cell counts is supported by epidemiological studies of the Yemenite Jews. This group commonly has benign constitutional neutropenia and a low level of vascular events[161].

ii) Clinical studies Epidemiological studies have been supported by evidence from hospital reports. Cole et al reported patients with a WBC count of greater than 15 x 10^9/l had a risk of death four times greater than those with normal counts[162]. Haines et al found patients who died within 1 year of infarction had a significantly higher WBC than survivors[163]. Similar finding have been reported for patients with stroke[164,165]. Kostis et al found an independent, positive correlation between WBC count and the severity of coronary heart disease determined by angiography[166]. Whilst Maisel et al found WBC count on admission in patients with infarction predicted early ventricular fibrillation[167].

Furthermore, WBC count is also reported to correlate with the risk of re-thrombosis following peripheral vascular bypass grafting[168] and the presence of peripheral vascular disease[169].

Thus, the correlation between WBC count and vascular disease is likely to be meaningful, an elevation of WBC count being associated with an increased risk of vascular disease and thrombotic events.

iii) *Pathophysiology of white blood cell action* The mechanism behind this increased risk unclear. One possibility is that the WBC count is a marker for one of the disease processes (recognised or unrecognised) which cause vascular injury. However, there is increasing evidence that WBC, especially neutrophils, play a pathogenic role in the process of ischaemia and infarction.

a) *Ischaemia and Infarction* Interruption of the blood supply to a tissue leads to cellular dysfunction, oedema and eventual cellular death, with loss of membrane integrity and leakage of proteins, enzymes and free radicals[170-172]. These products of cell death precipitate an inflammatory response, with accumulation and infiltration of neutrophils and macrophages[158,173,174]. The blood supply of adjacent or peri-infarct tissue is often critically reduced[120], the cells being viable but vulnerable to further insult. As collateral blood supply opens up these ischaemic regions are reperfused inducing further neutrophil migration[174] via the ischaemia-reperfusion mechanism. The percentage salvage of cells within this peri-infarct region determines the eventual size of infarct, and hence in part it's clinical impact. There is a large amount of evidence accumulating to suggest that neutrophils play an important part in determining cell survival in peri-infarct tissue; with

or without interventional reperfusion. The majority of this evidence comes from animal models of myocardial ischaemia and reperfusion, which have shown animals made neutropenic developed smaller infarcts[123] and fewer arrythmias[175]. Depletion of circulating neutrophils using antiserum[176] or white cell filters[134,177], blockade of neutrophil adhesion using antibodies to CD 11b[178], CD 18[179] or ICAM-1[180], and inhibition of activating and chemotactic factors such as leucotriene B_4 (LTB_4)[125] and thromboxane A_2 (TxA_2)[181], all reduce neutrophil accumulation and subsequent infarct size. A monoclonal antibody to CD 18 has also been shown to reduce the cerebral infarct size in an animal stroke model[182].

b) *Mechanisms of white cell action* Plugging of microvessels by leucocytes contributes to the no-reflow phenomenon, seen following ischaemia and attempted or biological reperfusion[183,184], which contributes to the progression of tissue damage during myocardial infarction[185,186], and stroke and cerebral ischaemia[187,188]. Neutrophils are strongly linked to vascular disease because of their size, dramatic response to activation and prevalence; they are the most numerous leucocyte population[189]. They may interfere with cell survival in the peri-infarct zone in a number of ways, including physical obstruction, release of chemical agents and interaction with other blood components. It seems likely that these mechanisms probably act in concert.

Physical aspects With a diameter of around 8μm, neutrophils have to deform considerably to pass through the average capillary with a diameter of about 3 - 6μm. They are considerably stiffer than red cells due to their actin cytoskeleton and high cytoplasmic viscosity[145]. Consequently, white cells exert a rheological effect, which is

disproportionate to their number[146]. Studies of capilllary transit by white cells shows slowing and transient stopping of flow, even under normal conditions[190,191], and this doesn't appear to have any adverse effects. In low perfusion states, such as the peri-infarct region, leucocytes become trapped and cause prolonged disturbance of microvascular flow[146,192]. Although, even a relatively stiff neutrophil will eventually pass through the capillary because it's cytoplasm is viiscoelastic. Neutrophil activation increases these rheological effects[193] further impairing microvascular perfusion[194]. Furthermore, activated neutrophils can adhere to the endothelium[195,196], resulting in capillary plugging[145,197,198] as the low perfusion pressure fails to overcome the combined effects of adhesion and neutrophil stiffness[145]. They can also produce narrowing of pre-capillary arterioles or post-capillary venules[199,200] and formation of aggregates[201,202], which can impact in slightly larger vessels.

Evidence for the importance of rheological mechanisms in vascular events comes mainly from animal experiments, with some indirect observations in patients. Infarction triggers neutrophil accumulation which, based on the biorheological properties of white cells, would increase vascular resistance. This has been confirmed in an experimental model, which also demonstrated a fall in resistance with neutrophil depletion[175]. Furthermore, ibuprofen, which blocks neutrophil accumulation, reduces infarct size[203]. Neutrophil adhesion is considered essential to capillary plugging[189], evidenced by the reduction of infarct size in animals treated with anti CD18, which blocks adhesion[179,182]. Patient studies have reported abnormal neutrophil deformability and filterability post myocardial infarction and stroke[204-206], and increased neutrophil aggregation in patients with myocardial infarction and angina[207,208]. Although indirect measures, they suggest impaired neutrophil rheology in infarction.

Biochemical aspects Neutrophils are attracted to the site of infarction, and thence activated, by the products of cellular death. Further neutrophil activation occurs in the peri-infarct tissue in response to free radical release, generated by ischaemia reperfusion. In turn, these activated neutrophils release enzymes and free radicals directly onto the endothelial cell, resulting in membrane and cellular dysfunction, and even death[146,158,209,210]. Lysozymal enzymes, like elastase, degrade matrix molecules and, along with factors such as LTB_4, produce increased endothelial permeability[208,211]. This results in endothelial cell swelling, and tissue and interstitial oedema, which can further impair microcirculation[145]. The products of neutrophil activation can disrupt the microcirculation still further. Leucotriene B_4 initiates aggregation, the release of free radicals and lysosomal enzymes[211]. The action of TxA_2 is usual offset by prostacyclin (PGI_2) produced by endothelial cells. However, PGI_2 generation is reduced by lipid peroxides[212] tilting the balance towards vasoconstriction and platelet aggregation. This maybe further exacerbated by the 5-lipoxygenase metabolites of arachidonic acid, leucotriene D_4, C_4 and E_4[213]. Factors including LTB_4, TxA_2, complement and platelet activating factor are strongly chemotactic for neutrophils and induce further accumulation and adhesion[158,173]. Experimental work, using an animal infarction model, has shown that inhibition of activating and chemotactic factors such as LTB_4[125] and TxA_2[181], reduces neutrophil accumulation and infarct size. Post-infarction studies in patients have reported increased elastase[208] and LTB_4 levels[214].

Interaction with other blood components Through interaction with the other components of blood, neutrophils may magnify the initial thrombotic event. Activated white cells can in turn activate platelets via free radical release and the action of TxA_2 [215]. The relevance of this to the *in vivo* situation is supported by experimental

work showing that neutrophil depletion protects ischaemic myocardium against platelet deposition[216]. Leucocytes also interact with erythrocytes and components of the coagulation-fibrinolysis system to promote intravascular thrombosis[217]. In turn, platelets involved in the initial thrombotic event can activate neutrophils via release of 5-Hydroxytryptophan (5HT), adrenaline and Platelet Activation Factor (PAF)[158].

Hence, although neutrophil accumulation at the site of an infarct is part of the natural inflammatory/healing mechanism, their presence can be highly detrimental. Impairment of the microcirculation, especially in the vulnerable peri-infarct area, can exacerbated hypoxia, lead to cell death and increase the infarct size. This in turn may lead to increased morbidity and mortality.

I.2.1.2 Neutrophils In Intermittent Claudication

Studies of the role of neutrophils in claudication can be divided into two types. Those considering measurements in patients at rest, i.e., static or population studies, and those measuring factors before and after an episode of claudication, i.e., dynamic studies.

a) Population/ Static Studies

There are fewer studies of white cell counts in peripheral vascular disease and claudication, compared with reports in coronary heart disease. Dormandy et al reported claudicants have higher WBC counts compared to controls[43], while Edwards et al found significantly higher neutrophil counts in resting claudicants[218]. Other studies have supported these findings[168,219,220].

The Edinburgh Artery Study reported increased plasma leucocyte elastase levels in claudicants compared to controls, and a positive correlation with the severity of peripheral arterial disease, but made no mention of WBC counts[221]. This has been suggested to represent a chronic, low level white cell, in particular neutrophil, activation[222]. Studies of smaller numbers have reported reduced filterability or deformability[223-225] in claudicants endorsing the idea of chronic activation. Indirect evidence for this activation, via release of free radicals, has come from reports of increased lipid peroxide levels[226,227] and reduced antioxidant capacity in claudicants[226].

b) Dynamic Studies

White blood cell counts are known to increase in exercise in normal subjects due to a shift of cells from the marginated pool into the freely circulating pool[193]. This shift occurs partly because of altered flow patterns but also due to adrenaline and cortisol mediated effects[228-230]. Reports of exercise induced changes in white cell counts have been conflicting. Edwards et al reported a significant increase in neutrophil count in claudicants following exercise compared to controls[218]. Cuiffetti et al also showed a significant increase, but with a shift towards mononuclear cells and no significant change in granulocyte levels[231-233]. Exercise will induce an increase in leucocytes, whilst the ischaemia- reperfusion occurring in the muscle will sequester leucocytes, due to activation with or without adhesion, and tend to reduce leucocyte counts. Hence, differences in the intensity of exercise and severity of ischaemia may partly explain these conflicting results.

Evidence of increased neutrophil activation following claudication has come from numerous studies[234]. Ciuffetti et al showed reduced white cell filterability in claudicants after exercise, which was negatively correlated to the degree of ischaemia measured using trancutaneous oximetry[225]. Neumann et al found increases in neutrophil count, proportion activated and neutrophil rigidity in the femoral vein of the claudicating leg. These changes were subsequently seen in systemic blood samples[236]. Other reports of decreased filterability[139,141,237,238], plus studies showing increased elastase[239] and serum lysozyme activity[141] support the idea that exercise activates neutrophils in claudicants. Experimental work, using an animal model of claudication, has shown increased neutrophil-endothelial cell adhesion and endothelial cell swelling, both locally and systemically[142,143].

Activation is thought to occur due to free radicals generated in response to the ischaemia and subsequent reperfusion[120] of the skeletal muscle of the legs during claudication[240]. Evidence to support the generation of free radicals during claudication has come from reports of increased lipid peroxides[139], malondialdehyde[140] and plasma oxidant activity[237] following claudication. Ciuffetti et al noted malondialdehyde levels, a marker of free radical production, correlated with decreases in leucocyte filterability, a marker of white cell activation[140]. In addition, Capecchi et al showed improved filterability of whole blood following exercise in claudicants treated with a xanthine oxidase inhibitor[241]; reducing free radical generation from ischaemia-reperfusion[120].

Much of the work on white blood cell activation in claudication has discussed the potential impact of activation on the mortality and morbidity in this group from

myocardial infarction or stroke. A limiting factor in many of the studies is the short-term nature of the data, i.e., assessment of activation is performed within 0-5 minutes post-exercise. There is little data on the time course of the activation, which has important implication for a proposed risk of increased mortality and morbidity. In addition, studies measuring released factors may simply be measuring activation within the limb, and as such have no implication on systemic effects.

I.2.2 PLATELETS IN ATHEROSCLEROSIS AND INTERMITTENT CLAUDICATION

The importance of platelets in thrombosis and haemostasis has been long recognised[242]. However, their complexity and reactivity has meant that it is only recently that an understanding of their physiology has begun to emerge. In the non-activated state platelets are small, anuclueated discoid cells around 9.5fl diameter. They are derived from megakaryocytes in the bone marrow, have a circulation time of 9-10 days and a normal count ranges from 150 - 400 x $10^9/l$[243]. Platelets form a vital part of our haemostatic system. They react vigorously on exposure of sub-endothelial tissue by adhering to the damaged tissue, whereupon they alter shape and release the contents of their granules. These act to increase vascular tone, reducing blood loss, and attract more platelets, which aggregate to form a haemostatic plug[244]. Activated platelets provide a procoagulant surface for the coagulation cascade and contribute to the generation of thrombin[245-248] and thence, fibrin to stabilise the plug. Platelet activation comprises four phenomena: adhesion, shape change, secretion and aggregation. These events do not always happen together or in sequence. Adhesion requires specific sub-endothelial

components, plasma proteins such as fibrinogen and von Willebrand factor and the activation or expression of certain platelet membrane glycoproteins. Shape change is associated with elongation and the formation of projections; this initially reversible change helps cover the damaged area. Upon degranulation this change becomes permanent. Secretion or degranulation occurs in response to numerous stimuli including collagen, thrombin, adenosine diphosphate (ADP), adrenaline and TxA_2. Factors released include platelet factor 4 (PF4), beta-thromboglobulin (βTG) and PDGF from alpha granules, and various adenine nucleotides from the dense granules. These factors and others promote aggregation and coagulation[239]. An integral part of adhesion and aggregation is the expression of various glycoproteins on the surface membrane.

I.2.2.1 Role Of Platelets In Atherosclerosis

a) Atherogenesis

The response to injury hypothesis of atherogenesis was initially proposed by Virchow in 1856[250] and has found increasing popularity in recent years[74,75,84,88,102,251]. In its current form it manages to include the majority of experimental and clinical evidence, and in particular, incorporate the hyperlipidaemic theory. Early experimental work generated fatty-fibrous plaques in response to endothelial denudation caused by homocysteinaemia[252], chronic indwelling catheters[253] and balloon angioplasty[254]. Platelets were known to adhere to the exposed sub-endothelium[255] and undergo the release reaction[256,257]. Smooth muscle proliferation, an essential component of atherogenesis, is maximal in the 48 hours post-injury. This is consistent with the presence of adherent platelets, and as the platelets begin to disappear, smooth muscle cell mitotic activity drops. Subsequent work isolated a mitogenic factor released from platelets, labelled

PDGF, which was shown to stimulate smooth muscle cell proliferation[258]. Further evidence to support a role for platelets in atherogenesis came from findings that platelet consumption at injury sites correlated with plaque formation[252] and that inhibition of platelet function[259,260], or thrombocytopaenia[254,255], resulted in smaller plaques. In addition, pigs with von Willebrand's disease (a condition of impaired platelet adhesion) showed less atherosclerosis[261].

However, subsequent work has suggested endothelial denudation is unlikely to occur in human atherogenesis, and that the "injury" is probably more functional, such as increased permeability, than structural[88]. The role of platelets in such non-denuding injury is less certain. Platelet adhesion to apparently normal endothelium has been reported[262], but most studies have been unable to demonstrate this[102]. Furthermore, PDGF is produced by several other cells including monocytes and smooth muscle cells, and these sources are thought to be more important in the mitogenic effect on smooth muscle cells[88]. Faggiotto et al have reported platelets in the fatty streaks generated in hypercholesterolaemic primates[263]. However, the general lack of evidence for a role for platelets in non-denuding injury, particularly in hyperlipidaemic models, has meant platelets have fallen out of favour in modern theories of atherogenesis.

b) Progression and Complications of Atherosclerosis

Platelets are thought to play a more significant role in the progression of established disease and in the complications, i.e., thrombotic events, of atherosclerosis[102,264].

i) Platelets and Disease Progression Jφrgensen et al have shown experimental mural thrombi become organised into lesions ranging from those rich in smooth muscle cells, to advanced lipid containing plaques[265] Post-mortem studies in humans have also found evidence that atherosclerotic lesions can grow through mural thrombi[96,102,266,267], and histological studies of atherosclerotic vessels has shown large amounts of fibrin and fibrin degradation products[98]. Angiographic[268,269], angioscopic[112] and pathological[96,108,109,270] studies have all linked plaque rupture with mural thrombosis. The precise mechanism of rupture remains unclear although lipid content[264,271] and position[115], macrophage action[271-273] and haemodynamics[115,271] have all been postulated as causative agents. Upon plaque rupture platelets adhere to the exposed connective tissue and are integral in precipitating thrombosis. In cases of non-occlusive thrombosis the thrombus becomes incorporated and undergoes fibrotic organisation, thus contributing to the plaque[96,97,103,115,264,274]. Platelets have also been reported to aggregate in response to high shear rates[275], such as those found across pre-existing stenoses or those newly formed by mural thrombosis. This mechanism may further contribute to mural thrombosis and plaque progression.

ii) Platelets and Complications of Atherosclerosis The clinical complications of atherosclerosis usually arise due to thrombotic occlusion of a vessel, but also occur in response to spasm or thrombo-embolism[264]. Occlusive thrombi are nearly always found in arteries supplying infarcted tissue, and the majority arise on fissured plaques[96,97,115,276-279]. Retrospective[280-282] and prospective[283] angiographic studies, and, more recently, post thrombolysis[284-287] work has suggested the majority of clinically significant thrombotic occlusions occur due to rupture of plaques in moderate, not severe, stenoses. Severe stenoses are usual associated with collateral formation and hence occlusion has a

minimal effect[264]. It is unclear exactly what determines whether fissuring of a plaque leads to complete occlusion or to partial occlusion with plaque progression. However, the depth of fissuring[104,287], the area of disruption, blood flow[287] and platelet activity[286] may have an effect. Thrombo-embolism may arise from a shallow fissure in conditions of high flow, where a newly formed mural thrombus breaks off and embolises distally producing acute ischaemia[96]. Vasoconstriction is also thought to play an important role, particularly in acute coronary syndromes[264,288], and has been shown to contribute to coronary arterial occlusion post-thrombolysis[289]. Platelets activated by plaque rupture release vasoactive amines, which cause arterial spasm[284,290-292], particularly in the presence of an existing stenosis[293]. This not only reduces the luminal diameter, but also may contribute to platelet aggregation by generating high shear rates[116,275,294]. Haemodynamic, scintigraphic and arteriographic studies in patients with angina at rest or acute myocardial infarction have shown that some episodes of ischaemia or infarction are caused by primary reduction in coronary flow due to either increased arterial tone[288,295,296] or to phasic platelet aggregation at the site of stenosis[297-299]. Inhibition of platelet release agents TxA_2 and serotonin has been shown to reduce vasospasm and platelet aggregation in a model of coronary thrombosis[280-283]. In clinical studies, patients with unstable angina demonstrated increased transcardiac levels of TxA_2[285] suggesting a vasospastic component to the condition[264]. Furthermore, Maseri et al used electrocardiographic, haemodynamic and angiographic monitoring to suggest the importance of vasospasm in ischaemic heart disease[288].

Thus, platelets are thought to play an important role in acute thrombotic events, either by aggregation and occlusion of the vessel, or by release of vasoconstrictor substances, or both[284]. Enhanced platelet activity has been associated with smoking[300-302], stress[303,304],

catecholamine activity[286,304,305], hypercholesterolaemia[306,307] and inherited coronary artery disease[308], all of which are linked to cardiovascular thrombotic events. Hyper-reactive or stimulated platelets may result in an enhanced response to plaque rupture. In turn, this may cause a more aggressive thrombotic response and increase the likelihood of complete vessel occlusion[116,286]. The importance of platelets is further supported by the beneficial effects of aspirin in acute, and chronic, coronary syndromes[309], and by the on-going research into blocking alternative routes of platelet activation[116].

c) Platelet Activation In Cardiovascular Disease

The majority of work investigating the role of platelets in cardiovascular disease has been in coronary artery disease. Studies of platelet adhesiveness, circulating platelet aggregate ratios and platelet survival time in coronary heart disease have yielded conflicting results, partly due to methodology and population differences. Furthermore, marked inter-laboratory variation and poor reducibility of results has meant their use in assessing platelet function has largely been abandoned[244].

Platelet aggregation has also been measured in numerous studies, again with conflicting results. Increased aggregation, in particular to ADP, has been reported in both acute and chronic coronary artery disease[310-320], although others have found no increase[321], or decreased responses[322]. Studies using epinephrine or collagen, in particular, have produced variable results; the majority of collagen studies showing normal aggregation[244]. Mehta et al studied patients with angina and found increased platelet aggregation and prostaglandin formation compared to controls[323]. Mikhailidis et al reported increased aggregation, and thromboxane release, in patients with confirmed

myocardial infarction compared to controls. They also found increased responses in patients with no infarction but a history of vascular disease[324].

Markers of platelet release, βTG and PF4, have also been studied in ischaemic heart disease. Although the majority of work has shown increased βTG or PF4 levels following myocardial infarction[325-338], patient values show considerable overlap with controls[328-335]. Patients with coronary artery disease, but no recent symptoms, tend to have normal levels of βTG[244,326,337,339,340], although there are some reports of elevated levels[321]. Angina is associated with an increase in βTG and/or PF4 initially, but a rapid return to normal[321,328,339,341-343]. There is also some trend towards higher levels in more severe disease. Mehta and Mehta found higher increases in patients with coronary heart disease following a positive stress test compared to those with a negative test[321]. However, Nichols et al found no correlation of βTG and PF4 levels with the angiographic degree of coronary artery disease[337].

Thromboxane A_2 is a potent vasoconstrictor and platelet proaggregant. Elevated levels of its metabolite have been reported in patients with unstable angina[285,344-349], variant angina[350,351] and recent myocardial infarction[346]. Conversely, Sobel et al found no increase in Thromboxane B_2 (TxB_2) during coronary spasm[339] and Chierchia et al reported failure of a TxA_2 blocker to relieve coronary spasm[352]. Hirsch et al found elevated levels of TxB_2 in patients following recent angina[285], and others have reported increased levels following pacing induced ischemia[345,347,350] and exercise induced angina[353], but there is little evidence for raised plasma levels in angina patients at rest. Montalescot et al found no increase in urinary metabolites of thromboxane associated

with atrial pacing, but basal levels were significantly higher in patients with coronary artery disease compared to controls. They also reported increased levels of TxB_2 in coronary sinus blood, but no increase in βTG or PF4, suggesting generation of thromboxane locally but no α-granule release[354].

Data on platelet function in cerebrovascular disease is limited compared to that on coronary artery disease. Adhesion[355-357], aggregation[318,358-365], and platelet aggregate ratio[364,366-368] studies have produced conflicting results. Elevated levels of βTG have been found following stroke or TIA[369-373], returning towards normal levels several months after the event[371,374,375]. Platelet survival time tends to be reduced following acute stroke or TIA, returning to normal during recovery. All these result may represent the effect of a thrombotic event, and, as yet, there is no data suggesting hyperresponsive platelets as a predictor for cerebral thrombotic events.

In summary, there is good pathological, experimental and clinical evidence to support a role for platelets in the pathophysiology of acute thrombotic events. However, their role in stable, chronic disease, symptomatic or not, is less clear. Differences in techniques and methodology, patient investigation and study populations make comparison of studies difficult. There appears to be reasonable evidence to support platelet activation in or after symptomatic disease, but platelet behaviour in resting subjects remains unclear.

d) Problems Of Cause And Effect

Although the majority of results link platelet activation with thrombotic events and symptoms, they are retrospective and do not distinguish between cause and effect. Hyper-

responsive platelets as a causal agent in cardiovascular disease are biologically feasible[376]. However, any differences in platelet function in the resting disease state are likely to be very small. Traditional methods of assessing platelet responsiveness may be too insensitive, or prone to preparation errors, to detect such a difference. Newer, more sensitive techniques, requiring minimal preparation may allow more detailed assessment of platelet function in cardiovascular disease. Furthermore, prospective studies of subjects "pre-disease" or with risk conditions for cardiovascular disease are required to support a causal role.

e) Evidence For Causality

Evidence of platelet stimulation has been reported in diabetes[377], hypertension[378] and hyperlipoproteinaemia[379,380]; all risk factors for cardiovascular disease. In the only prospective study of platelets and cardiovascular disease, Thaulow et al measured platelet count and aggregation, and then followed the subjects for a mean of 13.5 years. They found significantly higher cardiovascular mortality in subjects with higher platelet counts and increased aggregation responses independent of other risk factors[381]. Further support for a predictive role of platelet function in cardiovascular disease comes from Tofler et al who reported an increase in platelet aggregability in the morning[286], which is the time of maximum cardiovascular events[382]. Three other studies have suggested platelet hyperaggregability[383,384] and volume[385] can predict cardiovascular events. The effect of aspirin, which inhibits platelet function, to reduce cardiovascular events[386], fulfils the therapeutic manipulation criterion[376] to support platelets as causative agent. The potential importance of enhanced platelet responsiveness to plaque rupture has been suggested indirectly by the work of Tshoeppe et al. They found flow cytometric markers of platelet

activation correlated with the risk of acute ischaemic events following coronary angioplasty[387].

Leaving aside the question of platelet activation and the cause of cardiovascular ischaemia and thrombosis, there is good evidence to suggest that coronary ischaemia, i.e., angina, is associated with platelet activation itself. We reasoned skeletal muscle ischaemia might also stimulate platelets, perhaps priming them to be hyper-responsive to subsequent stimuli, such as coronary artery plaque rupture.

I.2.2.2 Platelet Activation In Intermittent Claudication

Awareness of the role of platelets in the development of atherosclerosis and its thrombotic complications has lead to numerous studies looking at platelet activation in ischaemic heart, cerebrovascular and peripheral vascular disease. Such studies can be divided into:

a) Population Studies

Enhanced platelet activation and reactivity in patients with peripheral vascular disease (PVD) compared to controls has been demonstrated using measures of TxB_2, βTG, PF4, circulating platelet aggregates and aggregation methods including a whole blood technique. Cella[388] et al and others[389-391] have reported reduced platelet survival times in claudicants compared to controls, particularly in the presence of peripheral vascular thrombosis[391]. Increased circulating platelet aggregates have been reported in acute[392] and chronic[366,393] peripheral vascular disease, although Amodeo et al found no increase in

chronic disease[367]. Ambrus et al[393] found increased circulating aggregates but no difference in aggregation studies with a range of agonists. They postulated that the sensitised or activated platelets tended to form aggregates, which were then rapidly removed from the circulation. The remaining platelets then tended to have reactivity similar to normal individuals. This possibility exists in all ex-vivo studies comparing platelet activation in patient and controls. Increased platelet aggregation with ADP, in patients with peripheral vascular disease, has been reported by Zahavi et al[389] and others[314,394-396], but Cella et al[388] and others[397,398] found conflicting results. While increased heparin induced platelet aggregation[399] and aggregation using a whole blood technique[400] has been noted in patients with severe peripheral vascular disease. Elevated levels of thromboxane have also been noted in peripheral vascular disease[389,401].

Bevan et al[402] and others[388,389,396,403-409] found increased levels of βTG, and PF4[388,405,406,409-411], in patients with peripheral vascular disease. However, others found no difference[398]; Johnston et al[244] showed no increase in βTG, and Kaplan et al[403] failed to find increased levels of PF4. Interestingly, Zahavi[389] and Blättler et al[396] were able to demonstrate a correlation between βTG level and the severity of disease, but Catalano et al[412] and Baele et al[413] were unable to repeat this finding. Using flow cytometric techniques, Mookerjee et al[414] found increased GP53 expression in platelets from PVD patients, which enhanced further with stimulation. Devine et al[415] noted increased platelet-associated factor XIII in PVD patients, whilst Ejim et al[416] showed increased fibrinogen binding to platelets of PVD patients in response to thrombin stimulation. However, Galt et al[397] found no increased P-selectin expression, and also no difference in βTG levels or aggregation studies between PVD patients and controls.

b) Dynamic Studies

i) Normals Platelet count increases in exercise due to release of stored platelets in the spleen, lung and bone. Studies on the effects of exercise on platelet aggregation and markers of platelet activation have produced conflicting results. Differences in methodology, exercise parameters, both duration and intensity, and subject fitness make comparisons between studies difficult[417]. Of interest is the finding that platelet activation may relate to anaerobic metabolism during exercise, and that activation is higher above the anaerobic threshold[418,419]. Furthermore, adrenaline released in response to exercise and pain has been shown to sensitise platelets to activation by physiological agonists[420]. Claudicants function under anaerobic conditions every time they develop pain on exercise and this may explain any finding of activation in patients. However, the majority of studies of platelet function in normal subjects use strenuous, exhaustive or extended exercise making direct comparison with patient studies very difficult.

ii) PVD Patients There are very few studies, which compare platelet activation and reactivity in PVD patients both before and after exercise. Wennmalm et al[421] found no increase in TxM, the urinary dinormetabolite of TxB_2, immediately following exercise but a significant increase 20 minutes after exercise. Using plasma measurements of βTG and PF4, Baele et al[413] reported increased levels in claudicants compared to controls, and a significant increase in βTG 5 minutes after exercise. Lack of increase in PF4 was attributed to its rapid clearance, and used to support the validity of the βTG results. Others[410,422] have also reported increases in βTG following claudication,

and Di Perri[422] also noted an increase in TxB_2. Importantly, these changes were less pronounced, at the same distance, after a 3 week training programme[422]. However, in a similar study Minar et al[423] found no increase in βTG or PF4 on claudication. Edwards et al reported increased levels of TxB_2 between 15 and 30 minutes post-exercise, compared to controls[218].

I.2.3. THE COAGULATION SYSTEM IN ATHEROSCLEROSIS AND INTERMITTENT CLAUDICATION

a) Haemostatic System

The normal haemostatic response is a protective mechanism, essential to life, which occurs upon vascular injury. It involves a complex, closely linked interaction between blood vessel wall, circulating platelets and blood coagulation factors.

Briefly, the injured vessel, and the surrounding vessels, vasoconstrict slowing blood flow to the area and reducing blood loss. This is a reflex action, enhanced by vasoactive amines, TxA_2 released from platelets and possibly by products of fibrin formation. The reduced flow rate improves contact between the injured area and platelets and coagulation factors. Platelets adhere to the exposed connective tissue releasing the contents of the granules, which enhance platelet adherence and aggregation. The result is the formation of a platelet plug covering the injury site.

Extension of the process is limited by normal endothelium, probably through the action of prostacyclin.

This rapid response produces a temporary control of bleeding or coverage of the injury site. The unstable platelet plug is now re-inforced by cross-linked fibrin strands formed by the activation of the coagulation system.

b) Coagulation System

The function of the coagulation system is to form a cross-linked fibrin polymer mesh, which stabilises the platelet plug. the system is comprised of two parts: a coagulation system to form fibrin, and a control system to limit the extent of its formation.

i) *Formation of Fibrin* Blood coagulation involves a biological amplification system in which a few initiator substances sequentially activate a cascade of circulatory precursor proteins to produce fibrin. Physiologically, coagulation starts with activation of factor VII by tissue factor found on perivascular tissue exposed by injury. This initiates sequential activation, with amplifying feedback pathways, of a cascade of enzymes and co-factors culminating in the formation of thrombin from prothrombin. An active serine protease, thrombin hydrolyses fibrinogen to form fibrin monomers. These monomers initially form polymers through weak hydrogen bonds, but the action of activated factor XIII results in cross-linkage and the formation of a strong fibrin mesh. This acts to stabilise the platelet mass and form the definitive haemostatic plug. The localisation of factor VII to perivascular tissue, the role of phospholipid membranes exposed by platelet

aggregation and the binding of thrombin to platelets, act to maximise coagulation at the site of injury. The amplification system is so powerful that one mol of activated factor VII can generate up to 2 x 10^8 mol of fibrin. If uncontrolled the process could produce complete vessel occlusion and life-threatening thrombosis.

ii) *Control of the coagulation system* This is achieved by a number of mechanisms:

Blood Flow Flowing blood removes any circulating factors, thus maintaining low concentrations of factors outside of the thrombus.

Inhibitors Activated factors, in particular thrombin, are inactivated by circulating inhibitors. The most powerful of these is anti-thrombin III, which binds thrombin to form an inactive stable complex. Other inhibitors include, α_2-macroglobulins, α_2-antiplasmin and α_2-antitrypsin. Thrombin itself inhibits its own generation by binding to thrombomodulin on endothelial cells and activating protein C. This acts with protein S to destroy co-factors V and VIII. In addition, products of fibrinolysis (see following) acts as competitive inhibitors of thrombin and fibrin polymerisation.

Fibrinolysis The fibrinolytic system acts to breakdown fibrin and hence limit the extent of the evolving thrombus. Mainly through the action of tissue-plasminogen activator (t-PA) which is released by endothelial cells, but also through the action of factors XII and XI, plasminogen is converted to plasmin. Tissue-plasminogen activator binds to fibrin, and this coupled with the binding of plasminogen to thrombus, strongly localises the generation of plasmin to the fibrin clot. Plasmin is a serine protease capable of digesting fibrinogen, fibrin and factors V

and VIII, limiting thrombus extension by destruction of fibrin and by blocking its formation. In turn, t-PA is inactivated by Plasminogen Activator Inhibitor-1 (PAI-1), whilst plasmin is inactivated by α_2-macroglobulins and α_2-antiplasmin, thus limiting widespread destruction of fibrinogen and other coagulation factors.

I.2.3.1 The Role Of The Coagulation System In Atherosclerosis

Thrombosis, and activation of the coagulation system, plays an integral part in atherosclerosis and it's complications. However, although invoked in the aetiology and pathogenesis of atherosclerosis this role remains controversial[424,425].

a) Atherogenesis

Von Rokitansky, in 1852, first suggested a role for the coagulation system in atherosclerosis when he postulated that atheroma was caused by mural deposits of fibrin[426]. His theory was taken up and modified by Duguid[427], and others[428-432] who suggested atheroma was the results of microthrombosis on vessel walls. In the 1950's Astrup[433] and Copley[434] proposed impaired fibrinolysis may result in persistent fibrin deposits at the site of injury which promote atherosclerosis, which is in keeping with the modern "intimal injury hypothesis" of Ross[251,258].

Evidence for a role of the coagulation and fibrinolytic systems comes from a variety of sources including, microscopic studies of plaque morphology, in vitro cell studies, and epidemiological studies of risk factors. Numerous morphological and

immunohistochemical studies have demonstrated the presence of fibrin or fibrinogen within early and late atherosclerotic plaques[98,435-442]. Interesting work by Tanaka et al revealed deposition of fibrin(ogen) associated with LDL in the walls of cerebral arteries. Deposition was most marked at the bifurcation of the middle cerebral artery, within or sub-endothelial, and was more frequent with age and intimal thickness[425,443]. More recent work by Bini et al using monoclonal antibodies found little fibrinogen/ fibrin I or fibrin II, and no fibrin degradation products (FDPs) in normal aortic wall. Early lesions and fibrous plaques contained fibrinogen/ fibrin I and fibrin II around vessel wall cells and macrophages, but no FDPs, whilst in advanced plaques all elements were found. They suggested increased fibrin formation and degradation maybe associated with atherosclerotic progression[98,442]. Work by Smith et al[437] and Shekhonin et al[439] has suggested that within the intima there is continuous formation and breakdown of cross-linked fibrin producing fragments which may have atherogenic potential. This is supported by the finding of raised FDPs in "healthy" subjects given intravenous fibrin specific plasminogen activator[444], and by release of FDPs from normal intima treated with plasmin[445]. Furthermore, measurements of activation peptides and enzyme inhibitor complexes, indicate a small proportion of the coagulation enzymes are continually activated[446].

Proliferation of smooth muscle cells is considered an integral part of the development and progression of atherosclerotic lesions[447]. Although several named growth factors for smooth muscle cells have been identified, including PDGF, macrophage derived growth factor (MDGF) and endothelial cell derived growth factor (EDGF), there is evidence fibrin may also act to promote proliferation and lipid accumulation[445,448]. Using a chick chorioallontoic membrane model, plasmin digests of cross-linked

fibrin[445,449] and extracts of gelatinous plaques, purified to retain fibrinogen and fibrin-related activity[450], have been shown to stimulate DNA synthesis. Ishida and Tanaka demonstrated a dose dependent proliferation of smooth muscle cells in culture in response to fibrin, in the absence of plasminogen. In the presence of plasminogen growth was observed for 24 hours, but by 48 hours cells had begun to degenerate and detach. This event coincided with the detection of FDPs in the culture, and subsequent studies demonstrated the inhibitory effect of FDPs on cell growth[451]. In addition, thrombin has also been shown to have a stimulatory effect on smooth muscle cell growth[452,453].

Fibrin deposition has been shown to injure endothelial cells in culture[454], either through direct action[455] or via FDPs[454,456], and there is evidence FDPs may enhance microvascular permeability[457]. However, Stebhens[424] argues against extrapolation of these findings to the in vivo situation, claiming there is little or no evidence for deposition of fibrin on normal endothelium. There are angioscopic reports of mural thrombi over intact intima in the coronary arteries of living patients with unstable angina[112], and post-mortem studies showing thrombi on apparently normal intima[458]. However, these reports must be viewed with great care as the intima in both cases cannot be truly called normal and is not comparable to the intima at the start of the atherosclerotic process. The adhesion of macrophages to the endothelial surface and their migration into the subendothelium is considered an initial event in some models of atherogenesis[459]. Within the subendothelium macrophages may produce growth factors mediating smooth muscle cell proliferation[460], as well as coagulation and fibrinolysis factors[461,462]. Fibrinogen is thought to play an integral role in macrophage adhesion[463] and there is evidence that LDL and fibrin, along with various FDPs act as

chemotactic agents for monocytes and macrophages[425], and may enhance macrophage lipid accumulation[445]. Defibrinogenation of a rat model of atherogenesis, significantly reduced the size of myointimal lesions following injury[464]. However, this finding was not supported using a rabbit model[465].

Although there is evidence that the products of coagulation and fibrinolysis can be found in atherosclerotic plaques and influence the behaviour of cells integral to the atherosclerosis, our lack of understanding of the pathogenesis of this process mean this evidence is circumstantial at best.

b) Progression and Complications of Atherosclerosis

Evidence for a role of the coagulation and fibrinolytic systems in the progression and complications of atherosclerosis is stronger.

i) Coagulation and Disease Progression Rupture or fissuring of atherosclerotic plaques, results in exposure of collagen, lipid, smooth muscle cells and tissue factor. This leads to activation of platelets and the coagulation cascade, resulting in thrombosis. In smaller vessels this may lead to occlusion thrombus, but where the lumen is larger, the blood flow faster or the plaque disruption minimal, the resultant thrombus becomes organised and incorporated into the atherosclerotic process[97,266,267]. Microscopic studies have demonstrated such organising thrombus, and this has been cited as the reason for finding fibrin(ogen) and FDPs within atherosclerotic plaques[98,437]. Whilst this explains the later lesions it does not account for findings in early lesions. Repetitive mural thrombosis due to plaque fissuring may result in layering of thrombi and gradual occlusion of the lumen[96].

ii) Coagulation and Complications of Atherosclerosis The majority of complications arising from atherosclerosis do so because of thrombotic occlusion of vessels supplying important structures, i.e., myocardial infarction and stroke. It is now generally accepted that myocardial infarction (the most common acute atherosclerotic event studied) results from thrombotic occlusion of one or more part of the coronary arterial system. Evidence for this has come from post-mortem studies[96,97,266,267], angiographic[111,277,279] and angioscopic[112] work and has been supported by findings post-therapeutic lysis[466,467]. Thus, plaque disruption followed by occlusive thrombosis results in tissue infarction. Although there is evidence of natural thrombolysis, it is usually too late to salvage all injured tissue[468].

Whilst it could be argued that this represents a "normal" physiological response of the coagulation system to vessel wall "disruption", there is evidence from studies using biological markers of coagulation and fibrinolysis that the systems may be dysfunctional in these patients, resulting in an enhanced thrombotic response. Several prospective epidemiological studies have demonstrated a correlation between plasma biological markers of haemostasis and the development of ischaemic cardiovascular events. The Northwick Park Heart Study found an association between plasma concentration of fibrinogen and the level of factor VII coagulant activity, and both fatal and non-fatal myocardial infarction. This remained significant after multivariate analysis and exclusion of subjects with known atherosclerotic disease at entry to the study. Furthermore, a positive correlation was reported between plasma concentration and cardiovascular events[469]. Fibrinogen was associated with subsequent myocardial infarction and stroke in the Gothenberg Study, but after multivariate analysis only the

association with stroke remained significant[470]. The Framingham Study reported on 554 men and 761 women followed for ten years. They reported a significant association of fibrinogen with the risk of coronary artery disease for men and women, but for stroke the association was only significant for men[471]. These findings have been supported by other studies[472,473], including the GRIPS study in which fibrinogen remained significantly associated with cardiovascular disease after multivariate analysis which considered LDL-cholesterol; one of the strongest predictors for coronary heart disease[474].

It is worth noting that the Northwick Park Heart Study also found a tendency towards reduced fibrinolysis and cardiovascular disease, but this was not significant, and an association with factor VIII in the early follow-up. They also found no association with Factor V or antithrombin III levels[469]. The Gothenberg study also looked at Factor VIII, plasminogen, fibrinolysis and Factor II-VII-X linkage, but found no association with cardiovascular disease[470].

In some cases fibrinogen has a strong hereditary determination[475,476], which would argue against fibrinogen levels as an effect of atherosclerotic disease. This is supported by a recent report looking at a possible hereditary link between raised fibrinogen and cardiovascular risk. Raised fibrinogen levels were found in male university students whose fathers had suffered a myocardial infarction before the age of 55 years, when compared with controls[477]. Along similar lines, Hoffman et al reported elevated factor VII activity in first degree relatives of patients with premature ischaemic heart disease[478].

Cross-sectional studies comparing normal controls and patients with atherosclerosis have shown increased levels of fibrinogen in patients with coronary heart disease[479-486] and peripheral vascular disease[487-491]. Although in some of these studies fibrinogen could be argued to be a marker for other risk factors, Handa et al found increased fibrinogen levels in coronary disease patients after adjustment for other risk factors[492]. Patients with cerebrovascular disease, as evidenced by transient ischaemic attacks, have raised plasma viscosity[493] and raised fibrinogen[494]. Some studies have noted a positive correlation between fibrinogen concentration and the degree of ischaemic heart disease[338,482,492,495], although others have not[496]. Following stroke[497-499] and myocardial infarction[500], fibrinogen levels are raised, and this rise has been shown to correlate with infarct size in myocardial infarction[501]. These increases probably represent an acute phase response rather than a causal finding, and it has been argued that increased fibrinogen levels in coronary artery disease relate to previous detected or silent infarction. However, Broadhurst et al found increased levels of fibrinogen and factor VII clotting activity in coronary artery disease patients, both with and without evidence of previous infarction[495]. This work is particularly interesting because factor VII activity does not increase in acute or chronic illness[502] and usually falls following myocardial infarction[503], suggesting it's elevation maybe causal. Other studies have found increased factor VII associated with coronary heart disease[504,505] with increased levels in acute MI and unstable angina when compared to stable disease, indicating a correlation with severity. Miller et al looked at 3000 men at high risk for fatal coronary heart disease and found a positive and significant association with factor VII coagulant activity, factor VII antigen, activated factor VII, factor IX activation peptide, prothrombin fragment 1 + 2 and fibrinopeptide A[506]. In a study of 1,077 hospital patients, Peabody et al reported increased fibrinogen levels and

reduced fibrinolysis, as measured by euglobulin lysis time and fibrin plate lysis assays, in those patients with clinical features of atherosclerosis[507].

Reduced fibrinolytic activity has been reported in the wall of atherosclerotic vessels compared to "normal" collateral vessels[508]. The concept of reduced fibrinolysis in atherosclerotic disease has been supported by studies reporting increased PAI-1 in the blood of patients with coronary artery disease[509-515]. Furthermore, levels of PAI-1 have a circadian rhythm, which corresponds to that of myocardial infarction[382,516]. Follow up studies of patients IHD have shown that high plasma concentrations of PAI-1 are independently related to reinfarction[517,518]. Whilst the DART study showed raised fibrinogen levels are associated increased all-cause mortality in patients post-infarct; the precise cause of death was not detailed[157].

Studies of patient groups with conditions known to be risk factors for atherosclerosis have shown dysfunctional coagulation or fibrinolysis. Type I and type II diabetics have been reported to have raised levels of fibrinogen[519-522] and impaired fibrinolysis[523,524], although Auwerx et al found increased PAI-1 in non-insulin dependent diabetics only[525]. Elevated fibrinogen levels have been reported in smoking[526,527], hypertension[528,529], hypercholesterolaemia[530,531], stress[532], hypertriglyceridaemia[533] and obesity[534], all of which are risk factors for atherosclerosis. In addition, impaired fibrinolysis has been associated with obesity[535] and hypertriglyceridaemia[536], possibly due to increased PAI-1 levels[537,538]. Increased levels of lipoprotein (a) (Lp(a)) are a known risk factor for coronary artery disease[539,540] and stroke[541]. Interestingly, Lp(a) is structurally similar to plasminogen[542], and it has been suggested Lp(a) exerts its effect by interfering with

the binding of plasminogen or plasmin to cells[543,544] or fibrin binding sites[545], hence producing a hypofibrinolytic state. Decreased fibrin binding of t-PA and reduced plasminogen activation by Lp(a) has been reported[545,546].

Some of these changes are undoubtedly secondary to the atherosclerotic process itself and hence an aetiological role is difficult to argue, but whether the abnormalities are primary or secondary they argue strongly for a role of coagulation in progression and complications of atherosclerosis.

I.2.3.2 Intermittent Claudication And The Coagulation System

The majority of work investigating the role of the coagulation and fibrinolytic systems in atherosclerosis has been done in patients with coronary heart disease. However, some studies have considered peripheral vascular disease, with the aim of identifying evidence of dysfunction. Such studies can be divided into:

a) Population Or Static Studies

i) Fibrinogen and PVD Case-control studies have shown patients with PVD have significantly higher plasma levels of fibrinogen than controls[223,487-491,547-555]. Epidemiological studies have also shown raised levels of fibrinogen in patients with PVD, although the findings have not been as statistically powerful[221,555,556]. The Scottish Heart Health Study found increased levels of fibrinogen in male claudicants, but the difference failed to reach statistical significance, possibly due to the small number of claudicants[555]. However, a more detailed analysis of data from the Scottish Heart Health

Study demonstrated a relation between tertiles of fibrinogen and the presence of intermittent claudication, and that the odds ratio remained significant even after adjustment for other cardiovascular risk factors[557]. The Edinburgh Artery Study found a significant relationship between fibrinogen and peripheral arterial disease and claudication, but only the association with peripheral arterial narrowing, as detected by ABPI, remained significant after multivariate analysis[221]. This study also showed a significant trend of increasing fibrinogen with increasing degree of disease[558], which has been supported by other reports[169,488,553,559]. As with coronary heart disease the relationship between fibrinogen and PVD is confounded by other risk factors for atherosclerosis. However, in the Edinburgh Artery Study, elevated fibrinogen remained negatively related to the ABPI after adjusting for smoking, age, sex, diabetes and serum lipids[221]. Longitudinal studies of claudicants have demonstrated that increased fibrinogen is associated with a worse prognosis in untreated[552] and surgical[560] patients, and with an increased cardiovascular mortality[561,562]. Fibrinogen has been described as a powerful screening test for asymptomatic stages of peripheral vascular disease[563], and suggested as a guide to those claudicants at risk of deterioration and death[561]. Whilst this data shows a relationship between fibrinogen and PVD, it doesn't distinguish between cause and effect. However, data from the Framingham Study has shown raised plasma fibrinogen in subjects who subsequently developed PVD[471,556] and this has been supported by data from the Speedwell study[554].

Further evidence for the importance of fibrinogen and the coagulation system in claudication comes from therapeutic work, which showed intravenous Ancrod or fibrinolytic agents, which break down fibrinogen, maybe clinically effective in claudication[564].

ii) Other coagulation factors and PVD There is limited work on the levels of other coagulation factors in PVD. Woodburn et al found patients with symptomatic PVD had a significantly higher level of von Willebrand factor and a significantly lower level of Factor VII compared to controls. They also found a positive and negative correlation, respectively, with the severity of disease on angiography[169]. Reduced levels of Antithrombin III have been reported in PVD[565] although Christie et al reported increased levels[548]. Bannerjee et al measured factors V, VII and VIII, antithrombin III and fibrinolytic activity in their longitudinal study of claudicants. There was no association between these factors and increased cardiovascular mortality[561].

iii) In-vitro clotting studies Using the recalcification time of blood after incubation with endotoxin to detect changes in coagulability, Spillert et al reported reduced times, indicative of increased coagulability, in patients with PVD compared to healthy controls[566].

iv) Products of coagulation and PVD Further evidence for activation of the coagulation system in PVD has come from studies measuring products or by-products of the enzyme cascade. Fibrinopeptide A (FpA), a marker of thrombin mediated conversion of fibrinogen to fibrin, levels have been reported to be higher in claudicants compared to controls[404,410,559,567]. Conversely, the Edinburgh Artery Study, and others, found no association between FpA and symptomatic PVD[221,551]. Thrombin-Anti-Thrombin (TAT) complexes are formed when thrombin is generated by activation of the coagulation system. Levels have been reported to be increased in claudicants[559]. Johansson et al found no significant difference in levels of TAT between patients and controls, although there

was a trend to higher levels in the patient group[551]. De Buyzere et al found significantly higher levels of F1 + 2 fragments, formed when prothrombin is cleaved to produce thrombin, and TAT complexes in claudicants compared to controls[568].

v) Fibrinolytic system and PVD Fibrin degradation products (FDPs), such as D-dimer, are formed after breakdown of fibrin, and hence represent a marker of activation of the fibrinolytic system. Studies have reported increased levels in patients with PVD[569]. Woodburn et al found D-dimer levels were significantly higher in symptomatic claudicants compared to controls, and that there was a positive correlation between the level and the severity of PVD on angiography[169]. Other studies suggest that in stable claudicants, the amount of thrombin generation and fibrin degradation is related to the severity of disease[559,570,571] and predicts progression and future coronary risk[562,572].

Fibrinolytic activity has been shown to be reduced in atherosclerotic vessels from the lower limb when compared to macroscopic "normal" vessels[507]. Studies have found reduced fibrinolytic activity and potential in patients with PVD[508,573,574]. Earnshaw et al found reduced fibrinolytic activity in patients with recent peripheral thrombosis and ischaemic rest pain, but no difference between claudicants and controls[575]. Woodburn et al found elevated PAI-1 levels in claudicants compared to controls[169], and Johansson et al reported an independent association after multiple regression analysis[551]. Conversely, Blann et al found no association[576], but they measured PAI-1 antigen, not activity. This data suggests a link between activation of coagulation and fibrinolysis with peripheral vascular disease. However, the mechanism for this link is unclear.

b) Dynamic Studies

i) Normals The effect of short term exercise in normal subjects is usually associated with a shortening of the Activated Partial Thromboplastin Time (APTT) and an increase in Factor VIII. There is also an increase in blood fibrinolysis as shown by increased t-PA and decreased PAI-1 release from the endothelium[417,577]. The overall impression is that, in normal subjects, the balance between the coagulation and fibrinolytic systems is maintained[577].

ii) PVD patients There is very limited data on the effect of exercise on the coagulation and fibrinolytic systems in claudicants. Gallino et al reported claudicants had increased levels of FpA at rest compared to controls, and a significant increase, compared to resting values, following exercise[410]. However, De Buyzere et al failed to detect any increase in F1 + 2 fragments, TAT complexes or D-dimer levels following exercise in claudicants, although all these parameters were elevated pre-exercise[568]. Similarly, Woodburn et al found no increases in von Willebrand factor, t-PA or PAI-1 levels in claudicants after treadmill exercise[578].

SECTION THREE: SUMMARY, HYPOTHESIS AND AIMS

I.3.1 SUMMARY

Intermittent claudication is a common and due to impaired blood supply to the lower limb, usually caused by atherosclerosis. The outlook for the affected limb is relatively good, but intermittent claudication is associated with an increased risk of myocardial infarction and stroke. The fact that atherosclerosis is a generalised condition affecting the coronary and cerebral circulations is usually quoted as the explanation for the increased morbidity and mortality in claudicants. However, evidence from multivariate analysis suggests an effect independent of co-existing diseases, cardiovascular risk factors or the extent of coronary or cerebral atherosclerosis. Claudication itself may be an independent risk factor for these cardiovascular events.

Activation of neutrophils, platelets and the coagulation system are intimately involved in the pathophysiology of thrombosis and infarction. Platelets and the coagulation system cause thrombosis and neutrophils are intimately involved in the response to and outcome of infarction. Intermittent claudication is a mild, repetitive form of ischaemia-reperfusion. In this situation oxygenation of previously ischaemic tissue results in the generation of oxygen free radicals, with subsequent cellular and humoral activation, and increased tissue damage. There is evidence that neutrophil activation is fundamental to this phenomenon, and that platelet activation and thrombosis may contribute to the injury.

There is ample evidence supporting a role for neutrophils, platelets and the coagulation system in both atherogenesis and the progression and complications of atherosclerotic disease. Most of this comes from experimental and clinical work on the coronary circulation and myocardial infarction, with some work suggesting similar mechanisms at other sites. Differences in neutrophils, platelets and the coagulation system have also been reported in peripheral vascular disease, and claudication has been shown to activate neutrophils, generate arachidonic acid metabolites, activate coagulation factors and increase microvascular permeability.

I.3.2 HYPOTHESIS

Activation, or increased potential to be activated, i.e., priming, of neutrophils, platelets or the coagulation system could result in an amplified response to pathological events. In these circumstances the response to plaque rupture could be more pronounced. A fissured plaque which would otherwise have formed a thrombotic cap and gradually remodelled, may evolve into an occlusive thrombus in the presence of a "primed" neutrophils, platelets and/or coagulation system. Enhanced coagulation may stabilise a developing thrombus, preventing early lysis. Similarly, a "primed" system may impair recovery of the peri-infarct tissue due to micro-thrombosis and/or increased numbers of activated neutrophils. Such priming could turn a subclinical event into a symptomatic stroke or myocardial infarction.

We hypothesised that if claudication were to activate and/or "prime" neutrophils, platelets and/or the coagulation system this may explain some of the increased cardiovascular risk found in claudicants.

I.3.3 AIM OF THESIS

To investigate whether intermittent claudication causes activation and/or priming of blood cells, and activation of the coagulation system. In detail the work will:

1. To determine whether exercise precipitates activation and/or priming of neutrophils in claudicants, and compare this with healthy, matched controls.

We planned to measure neutrophil counts, and other leucocyte populations, before and after exercise, as this may influence other measurements. Markers of neutrophil activation will be measured, mainly to compare baseline differences and to consider local activation. To measure neutrophil activation in response to stimulation we aim to use an established microplate technique and a more sensitive flow cytometric technique. We also want to study the duration of any activation, and will measure changes over two hours after exercise.

2. To determine platelet activation and priming in systemic blood from claudicants and controls, at rest, after a single episode of exercise and after repeated exercise.

These studies are limited by constraints of techniques used to assess activation. Differences between the two groups, and changes following exercise, are likely to be small and subtle, particularly in stable claudicants, and sampling artefact and the basic nature of some of the measures may mask such changes. We aim to use a flow cytometric technique, as this involves minimal preparation, and is more likely to detect subtle differences. Experiments will be run in parallel with more established techniques to measure platelet activation and assess activation in response to stimuli. Flow cytometry, as a method of studying platelet reactivity, is new to our laboratory. Hence, before outlining the experimental design we will discuss flow cytometry and the necessary set-up and standardisation procedures.

3. To determine whether repeated exercise causes activation and/or priming of the coagulation system in systemic blood from claudicants, and compare this with controls.

A measure of the response of the coagulation system to stimulation would be ideal, but currently available techniques are very crude and would be unlikely to detect any small differences we might expect in claudication. Consequently, we plan to measure activation using new, highly sensitive assays of activation as an indirect measure.

4. Does aspirin influence the activation and/or priming of platelets and/or the coagulation system in claudicants at rest, after a single episode of exercise and after repeated exercise.

Many patients with atherosclerotic disease are taking aspirin, which may have a profound effect on platelet activation, and may affect the coagulation response. We aim to look at platelet and coagulation responses in patients taking and not taking aspirin.

CHAPTER TWO: NEUTROPHIL ACTIVATION IN INTERMITTENT CLAUDICATION

II.1. RESEARCH QUESTION

Our literature review reveals clear evidence of white cell activation, particularly neutrophils, in claudication. These studies demonstrate neutrophil activation has occurred, presumably in response to the ischaemia-reperfusion events within the leg, but they have not demonstrated an increased potential for neutrophils to be activated at remote sites, such as the coronary circulation. We undertook this work to look for evidence of neutrophil priming, in response to claudication. The presence of such primed neutrophils may, in part, explain the increased incidence of cardiovascular events in claudicants.

II.2. METHOD

We used two methods to measure neutrophil priming. The more traditional microplate measure of superoxide has several preparation steps, increasing the risk of spurious results and/or missing a small difference. Consequently, we also used a flow cytometric technique to measure neutrophil hydrogen peroxide production. This uses whole blood, minimising preparation artefact, and is generally thought to be a more accurate measure of neutrophil activation. In addition, we measured elastase release and thromboxane production as markers of neutrophil activation within the leg.

PATIENTS AND CONTROLS

Sixteen claudicants and twelve healthy controls were studied (See Appendix 2). The patients, comprising 15 men and 1 woman, of mean age 64 (range 57-72) years, were recruited from our Vascular outpatient population. All patients had at least a six-month history of stable claudication due to femoropopliteal disease. This was assessed clinically in all but two patients, who had had angiograms, and was based on history, signs and palpation of pulses. Whilst it was acknowledged that some degree of proximal or distal disease may also be present, the main disease was femoropopliteal. All patients had an Ankle Brachial Pressure Index (ABPI) of less than 0.8. Patients with clinical evidence of ischaemic heart disease, diabetes mellitus, chronic renal failure or uncontrolled hypertension were excluded from the study. All patients were assessed to confirm they were limited by claudication alone, as opposed to chest pain or dyspnoea, on a treadmill exercise test.

Control subjects, 11 men, 1 woman, of mean age 67 (range 58-77) years, were recruited from old general surgical patients. All had had a previous operation more than six months earlier. Nine had undergone inguinal hernia repair, two excision of lipoma and one excision of a sebeceous cyst. Controls had no evidence or history of cardiovascular disease, all had an ABPI of greater than 0.95 and were not limited on treadmill exercise. They were all assessed based on history, examination, blood and urine tests to exclude any active inflammatory or infective process, diabetes, hypertension or chronic renal failure.

Two patients were smoking at the time of the study and 13 were ex-smokers claiming to have given up at least two months prior to the study. Of the controls, one was currently smoking, 3 were ex-smokers and the remainder had no significant smoking history.

All subjects were seen at a preliminary visit to assess their walking capacity and general health. All subjects were asked to avoid aspirin, non-steroidal anti-inflammatory drugs or free radical scavengers for 14 days prior to the study. Written, informed consent was obtained from all subjects.

STUDY DESIGN

This was an unmatched case-control study comparing neutrophil activation pre- and post-exercise in claudicants and controls. All subjects were brought to our Vascular Studies Unit by taxi, at approximately 0900 hours, and had been specifically asked to avoid exercise that morning. On arrival all subjects rested in the supine position for 20 minutes. An ante-cubital vein was cannulated with an 18-G cannula and a resting/ pre-exercise blood sample was taken. The ABPI was measured and subjects then walked on a treadmill set at a 10% slope at a speed of 2.5 km/hr. Claudicants walked until stopped by pain. Controls walked for 5 minutes, which was the average walking time of the patient group during preliminary studies. Following exercise subjects again lay supine, and a post-exercise ABPI was recorded immediately. Further blood samples were taken immediately post-exercise and at 5,10,15,30,60, and 120 minutes post-exercise.

The venflon was flushed before and after each sample with Normal saline. The blood sample for analysis was taken after an initial 2ml sample had been removed and discarded. All samples were placed in cooled tubes, containing the appropriate anticoagulant (see below) and then placed on ice until separation.

LABORATORY METHODS

Blood was taken for the measurement of plasma elastase, white cell and differential counts and neutrophil free radical production. Measurement of free radical production was performed by assessing superoxide release and hydrogen peroxide generation by neutrophils in response to stimulation.

White cell counts and differentials These were performed using a portion of the EDTA sample run through a Coulter® MAXM counter.

Plasma elastase A 4ml sample was taken into EDTA and gently mixed. The sample was stored on ice until it could be separated, between 15-20 minutes post-sampling. This was achieved by centrifugation at 1400g for 10mins. Plasma was removed, aliquoted into 250µl measures and frozen at -80°C. Elastase was measured using an 'in-house' ELISA assay, based on the method of Browser and Harpel[579]. Plates were coated with 100µl sheep anti-human elastase (IgG, Binding Site, Birmingham, UK) diluted 1 in 1000 in carbonate/bicarbonate buffer and incubated for 24 hours at room temperature. On the following day, plates were washed four times with PBS/Tween and blocked with 100µl 1% BSA in PBS for one hour. The plates were washed again, and

100µl standards (Merck elastase calibrators, diluted 1 in 100 in PBS/Tween) and plasma samples (diluted 1 in 20 in PBS/Tween) added, in triplicate. The range of standards was 0 to 320ng/ml. The plates were incubated for two hours, washed, then 100µl peroxidase conjugated anti-human α-1 antitrypsin (Binding Site, Birmingham, UK), diluted 1 in 1000 in PBS/Tween, added. After one hours' incubation, the plates were given a final wash prior to addition of the substrate solution. A 100µl aliquot of 0.5mg/ml OPD, in phosphate citrate buffer containing sodium perborate (Sigma Chemical Co, Poole, UK) was then added to each well, the plates covered in foil and incubated for 15 minutes. The reaction was stopped by the addition of 50µl 4M sulphuric acid and the plates read at 492nm.

Superoxide release This was determined using a microplate technique based on a superoxide dismutase inhibitable reduction of ferricytochrome c[580,581]. A 4.5ml blood sample was gently mixed with 0.5ml of Heparin and placed on ice. The sample was then mixed with 0.5ml of 6% Dextran and left for 45 minutes at room temperature to sediment the red blood cells. The white blood cells form a "buffy-layer" between the red cells and the supernatant. This layer is removed and spun at 100g for 12 minutes to pellet the leucocytes. The supernatant was discarded and the cells resuspended in distilled water for 30 seconds. This effects hypotonic lysis of the residual red cells. Iso-osmolarity is restored with 0.6% KCl. The suspension was then spun at 160g for 4 minutes and the process of hypotonic lysis repeated. Finally, cells were resuspended in 2ml Hanks buffered Saline. Cell viability was assessed using Trypan blue exclusion technique, and revealed>90% viability for this technique. A 20µl aliquot cell suspension is added to 380µl of acetic acid and methyl violet. A neutrophil count was then determined for each

sample using a Neubauer counting chamber; number of cells in the outer four squares x 0.05 = cells x 10^6/ml. counting technique. Samples were then diluted with Hanks buffered Saline to give a fixed count of 2 x 10^6 cells /ml. Neutrophil superoxide release is then determined using a microplate technique based on a superoxide dismutase inhibitable reduction of ferricytochrome c. Two reaction solutions are mixed:

Solution 1 (-SOD): 64µl Distilled water
320µl cytochrome c (Sigma Chemical Co, Poole, UK)
4.75ml Phorbol myristate acetate (Sigma Chemical Co)

Solution 2 (+SOD): 64µl Superoxide dismutase (Sigma Chemical Co)
320µl cytochrome c (Sigma Chemical Co)
4.75ml Phorbol myristate acetate (Sigma Chemical Co)

A microplate is set up with 85µl of solution 1 in each of the wells in the top four rows, and 85µl of solution 2 in the lower four. A 74µl aliquot of cell suspension is added to each well. The plate is covered and incubated at 37°C with constant rotation. Phorbol myristate acetate (PMA) causes release of the superoxide radical, which acts to reduce ferricytochrome c from an orange to a pink colour. Superoxide dismutase breakdown superoxide and prevents the colour change. The colour is measured at 550nm using a microplate reader. The difference between stimulated and unstimulated samples is used as a measure of superoxide production.

Hydrogen peroxide generation This was measured using a flow cytometric technique to measure intracellular hydrogen peroxide formation[582]. A 2.7ml blood sample was gently mixed with 0.3 ml of Heparin and placed on ice. The sample was then mixed with 0.3ml of 6% Dextran and left to sediment for 45 minutes at room temperature. The sediment was removed and cell viability assessed. This separation technique gave >95% viability by Trypan blue exclusion test. Neutrophil counts were determined and samples diluted to give a cell count of 2 x 10^6/ml. Two 0.5ml samples of each cell suspension are incubated for 15 minutes with 0.5ml Dichlorofluoroscin diacetate (DCFH-DA, Kodak, Liverpool, UK) to give a final concentration of 9.6μg/ml. PMA, final concentration 60ng/ml, is added to one of the duplicates, an equivalent volume of Hanks to the other, and the suspensions incubated for a further 15 minutes. DCFH-DA is deacetylated by the neutrophil membrane to produce dichlorofluoroscin, a non-fluorescent compound. In the presence of hydrogen peroxide, this compound is oxidised to the fluorescent dichlorofluoroscein. PMA is a powerful neutrophil stimulant inducing formation of hydrogen peroxide. The fluorescence of the neutrophil population in each sample is then measured using a Coulter EPICS® XL Flow Cytometer. Neutrophils are gated for based on forward and side scatter, and the Mean Fluorescence Intensity (MFI) is determined for this region. The difference in MFI between the two samples is used as a measure of hydrogen peroxide formation.

Thromboxane B_2 Samples were collected as above into tubes containing citrate and aspirin, to prevent *in-vitro* formation of thromboxane A_2. As above, the tubes were stored on crushed ice both pre- and post-sampling. At intervals, between 5 and 20 minutes following collection, samples were centrifuged at 2 500g at -4°C. The central

part of the supernatant was separated into aliquots of 0.5mls and stored at -80°C. Thromboxane B_2 is stable metabolite of Thromboxane A_2, a product of arachidonic acid, and was measured using a commercially available ELISA kit (ACE™, Cayman Chemical Co, Ann Arbor, MI, USA). All specimens were run in duplicate and the mean value determined.

STATISTICAL ANALYSIS

This was performed with the help and advice of the Department of Medical Statistics, University Hospital of South Manchester. Primary analysis was by Repeated Measures analysis of Variance to look at changes over time. Secondary analysis was using Multiple Comparison Testing to look for differences between individual time points. $P < 0.05$ was taken as statistically significant. The flow cytometry data required a square-root transformation to approximate to a normal distribution.

II.3. RESULTS

Patient Details

Patients with diabetes, ischaemic heart disease, uncontrolled hypertension or renal failure were excluded to avoid the confounding effect of these conditions on neutrophil function.

	CONTROLS	PATIENTS
n	12	16
M:F	11:1	15:1
Mean Age (Range)	67 (58-77)	64 (57-72)
Smoking: Current	1	2
Ex	3	13
Non	8	1
Aspirin	2	6
Hypertension (Controlled)	0	5

In all subsequent graphs the *x*-axis represents the sample timing, from pre-exercise to 0, 5, 10, 15, 30, 60 and 120 minutes post-exercise. The exercise period is taken as 5 minutes (time of control subjects exercise), hence the plotted time points are pre-exercise (or 0) and 5,10, 15, 20, 35, 65, 125 minutes post-exercise. The *y*-axis shows the parameter being measured.

White cell counts and differentials

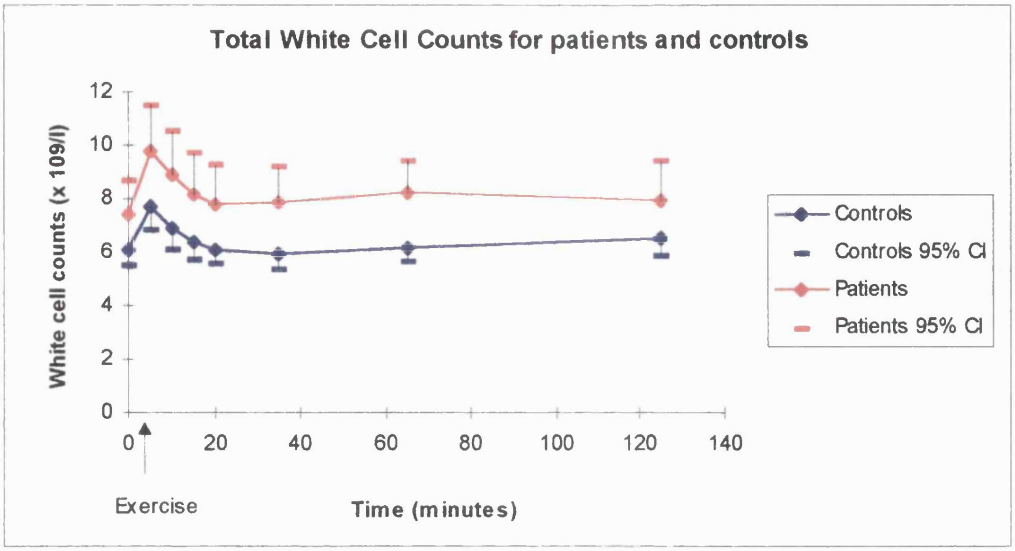


Fig 2.1 White Cell Counts in controls and patients over time, with 95% Confidence Intervals (CI)

The white cell count (Fig 2.1) and neutrophil counts (Fig 2.2) showed that claudicants tended to have higher counts than controls, but this failed to reach significance ($p > 0.05$). Both controls and patients showed a significant trend over time ($p < 0.05$), with a marked increase in counts immediately post-exercise. The counts then gradually returned to resting levels around 20 minutes post-exercise.

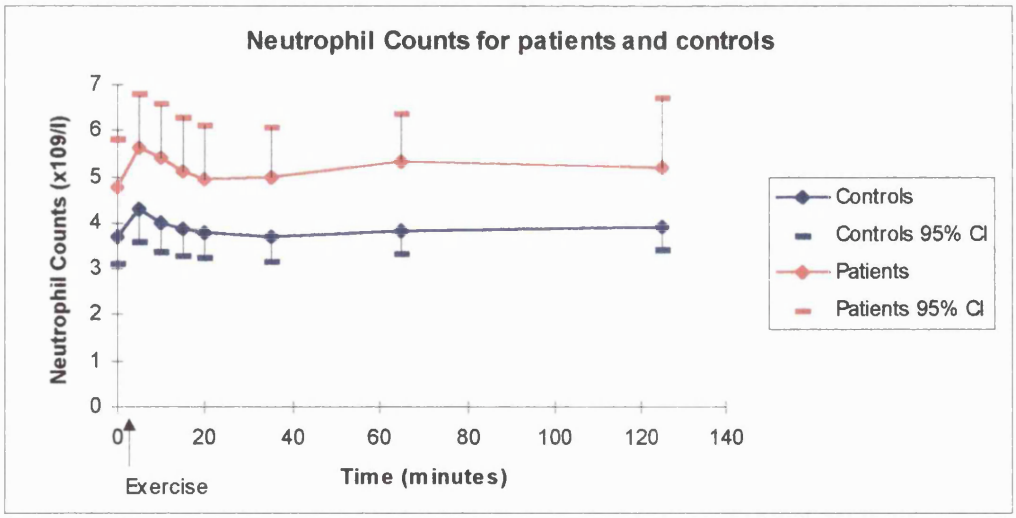


Fig 2.2 Neutrophil counts in controls and patients over time, with 95% CI

However, if neutrophils are plotted as a fraction of total white cell count it can be seen that both controls and patients showed a fall in the ratio of neutrophils to white cell immediately post-exercise which returned to normal by 25 minutes (Fig 2.3).

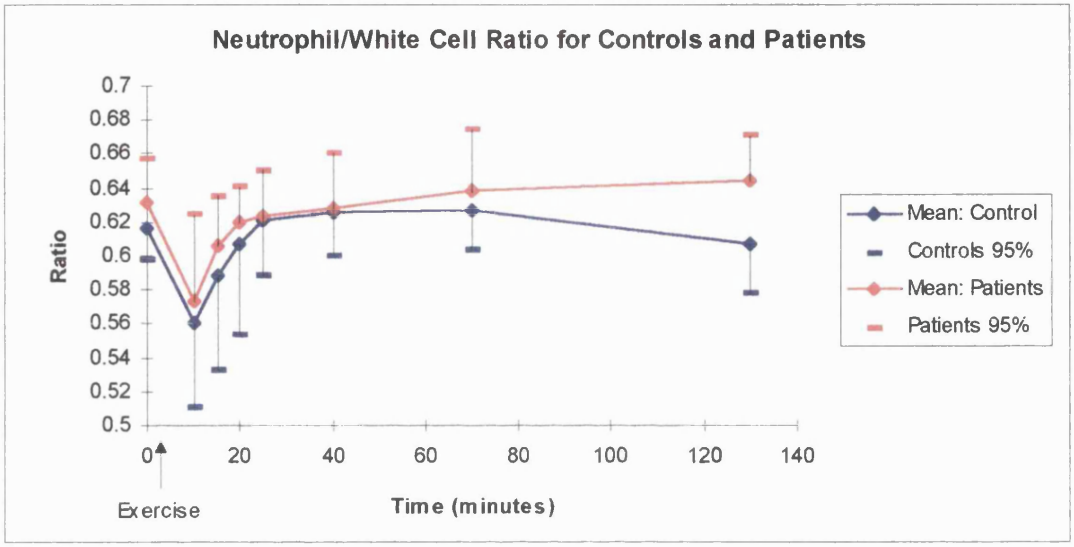


Fig 2.3 Neutrophil counts as a fraction of total white cell count in controls and patients over time.

This would suggest that other leucocyte types might show greater increases following exercise. For both lymphocytes (Fig 2.4) and monocytes (fig 2.5) claudicants tended to have higher counts than controls, but this failed to reach significance ($p > 0.05$). Both controls and patients showed an increase in levels post-exercise, again returning to resting levels approximately 20 minutes after exercise. Statistically, there was a significant trend over time ($p < 0.05$), but no difference between the groups, although increases were slightly more pronounced in the patient group, particularly for monocytes.

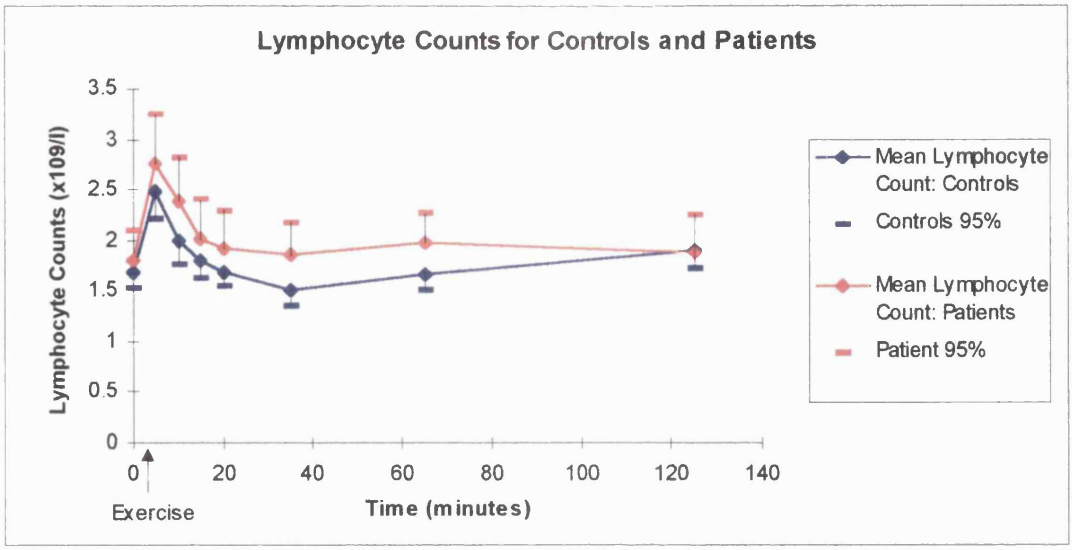


Fig 2.4 Lymphocyte counts in controls and patients over time, with 95% CI.

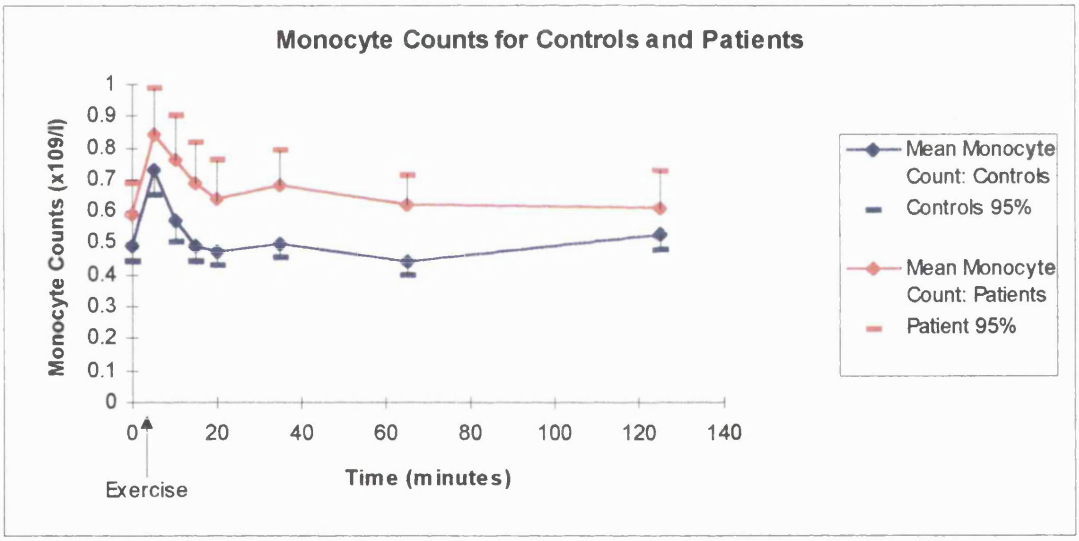


Fig 2.5 Monocyte counts in controls and patients over time, with 95% CI.

When lymphocyte and monocyte counts are plotted as a fraction of the total white cell count, a relative increase in these cell types is seen. Controls showed a higher fraction of lymphocytes at all points compared to controls, but both groups showed similar patterns of increase with exercise (Fig 2.6). The ratio of monocytes to white cells showed a marked increase in patients post-exercise, compared to controls, beginning 5 minutes post-exercise and lasting to 15 minutes post-exercise (Fig 2.7).

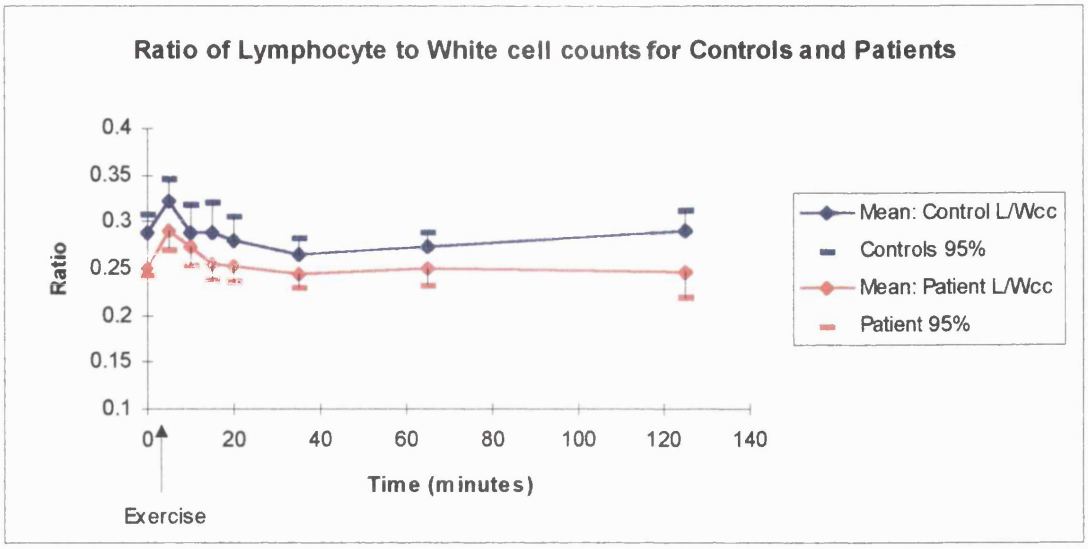


Fig 2.6 Lymphocyte counts as a fraction of total wcc in controls and patients over time, with 95% CI.

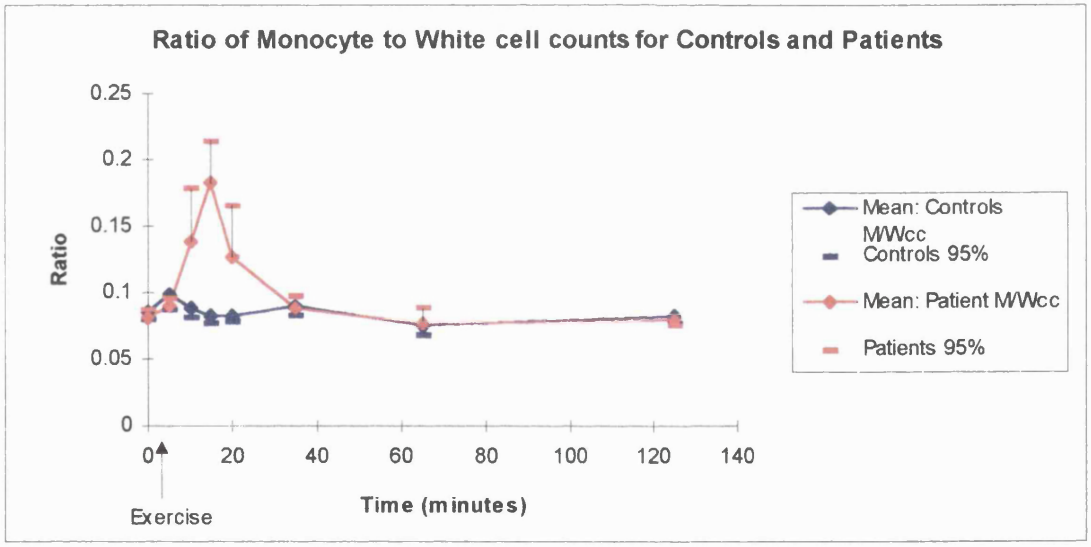


Fig 2.7 Monocyte counts as a fraction of total wcc in controls and patients over time, with 95% CI.

Plasma elastase

Both patients and controls showed a significant pattern over time ($p < 0.05$), with a marked increase in elastase immediately post-exercise. This rapidly returns to baseline and there are no further changes over time (Fig 2.8). There was no significant difference

between the patient and control group ($p > 0.05$). Elastase production was standardised for both white cell and neutrophil count (Fig 2.9 & 2.10). Both these plots showed a similar pattern. Claudicants showed little or no increase in elastase levels immediately post exercise, but a clear fall in levels at 5 minutes post-exercise. Thereafter, values showed very little change from resting levels. Controls showed a clearer increase in levels immediately post-exercise, with a return to resting levels at 5 minutes post-exercise, and then a secondary peak at 35 minutes.

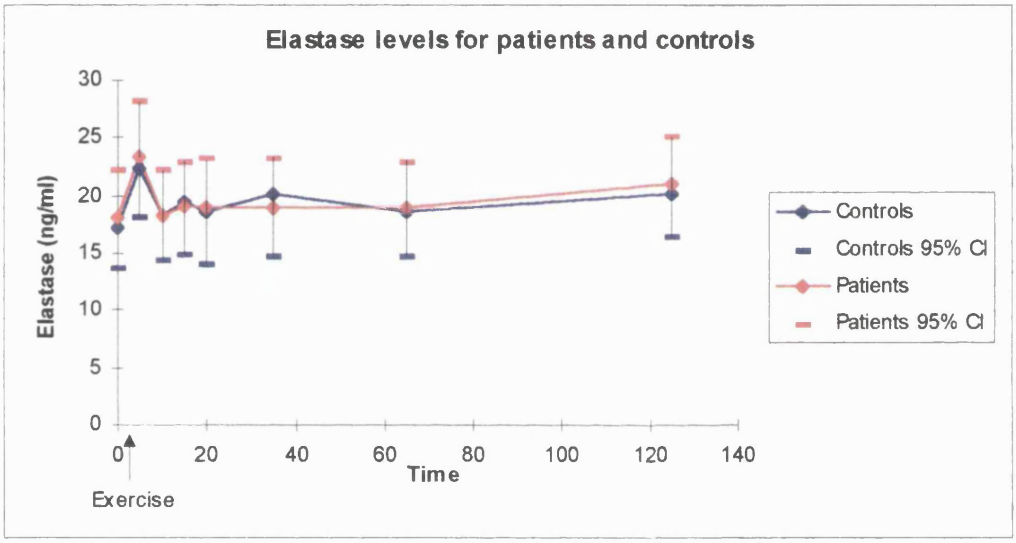


Fig 2.8 Plasma elastase levels in control and patient groups over time, with 95% CI.

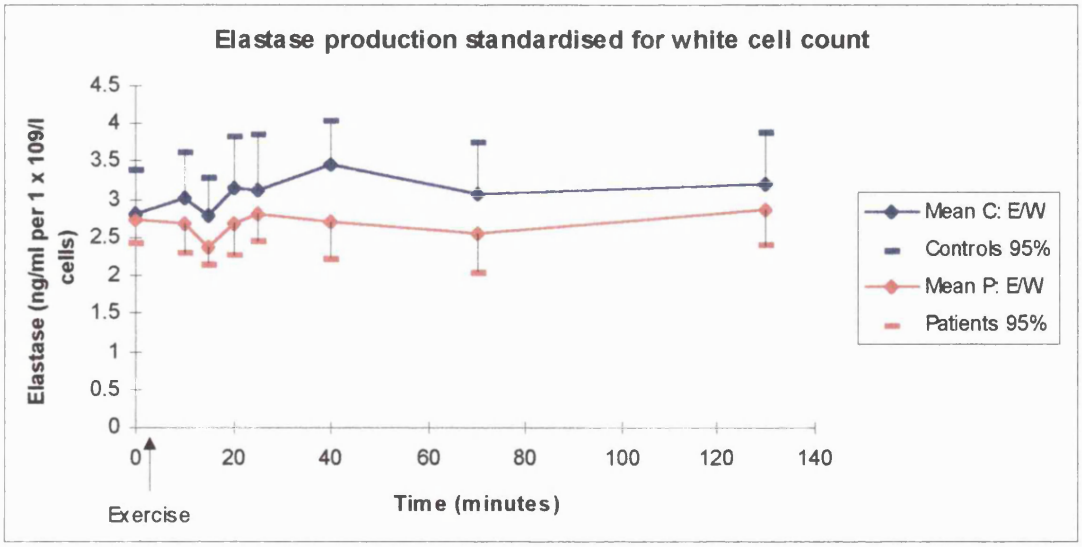


Fig 2.9 Elastase production standardised for white cell count in controls and patients over time, with 95% CI.

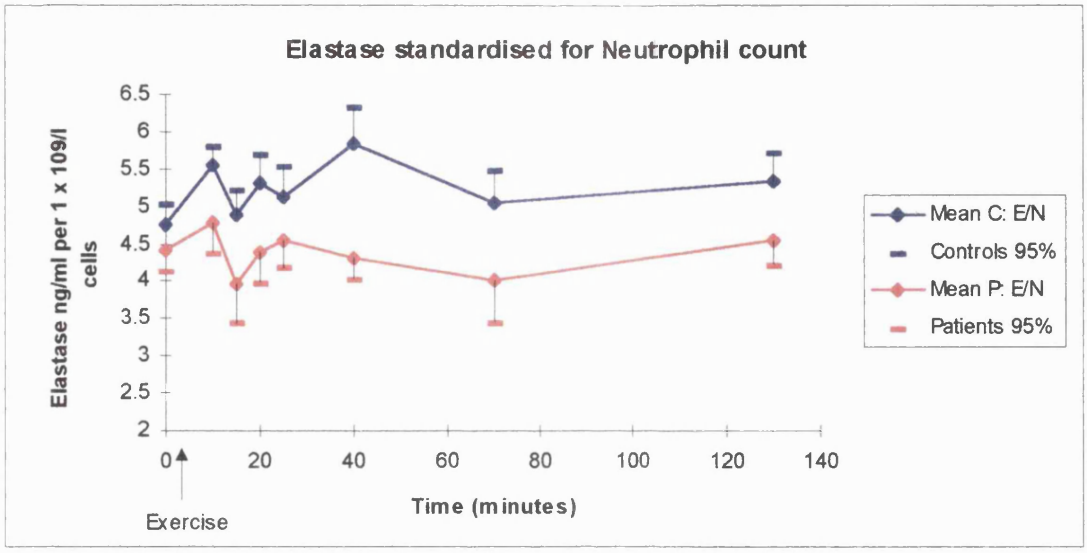


Fig 2.10 Elastase production standardised for neutrophil count in controls and patients over time, with 95% CI.

Superoxide production

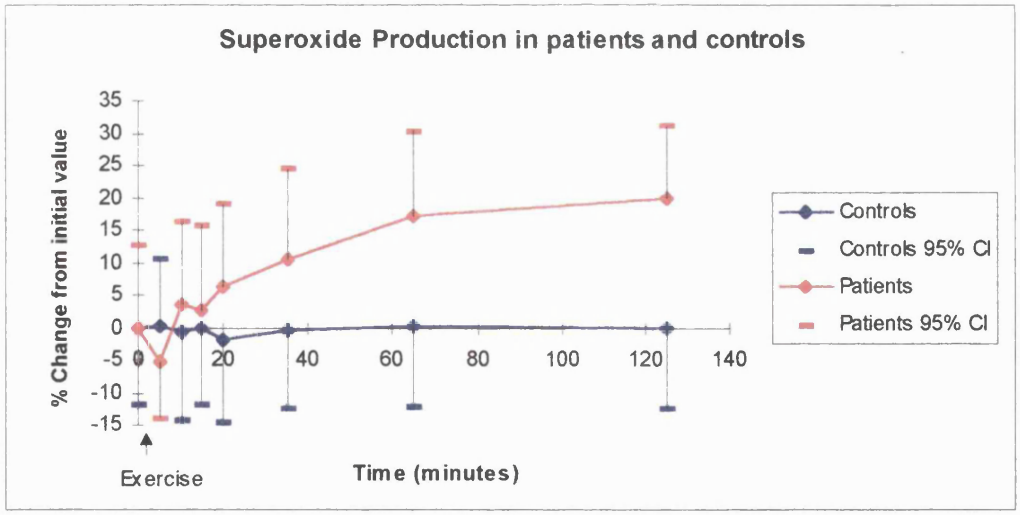


Fig 2.11 Neutrophil superoxide production, as a percentage of initial value over time, with 95% CI

Differences between individual subjects were greater than the changes over time, hence, results were plotted as a percentage change from initial value. Patients showed a significant increase in superoxide production over time; values 30 to 120 minutes post-

exercise significantly higher compared to pre-exercise ($p < 0.05$). The control group showed no change over time, or between time points ($p > 0.05$). However, there were no differences between patients and controls ($p > 0.05$).

Hydrogen Peroxide Generation

The flow cytometry data was plotted as the change in MFI, and showed no significant changes in the control group over time or between time points ($p > 0.05$). However, the patients showed significant changes over time with values from 10 to 60 minutes post-exercise significantly higher compared to pre-exercise ($p < 0.05$). Furthermore, the patient values were higher than the control group at all time points, and this was statistically significant except immediately post-exercise ($p < 0.05$).

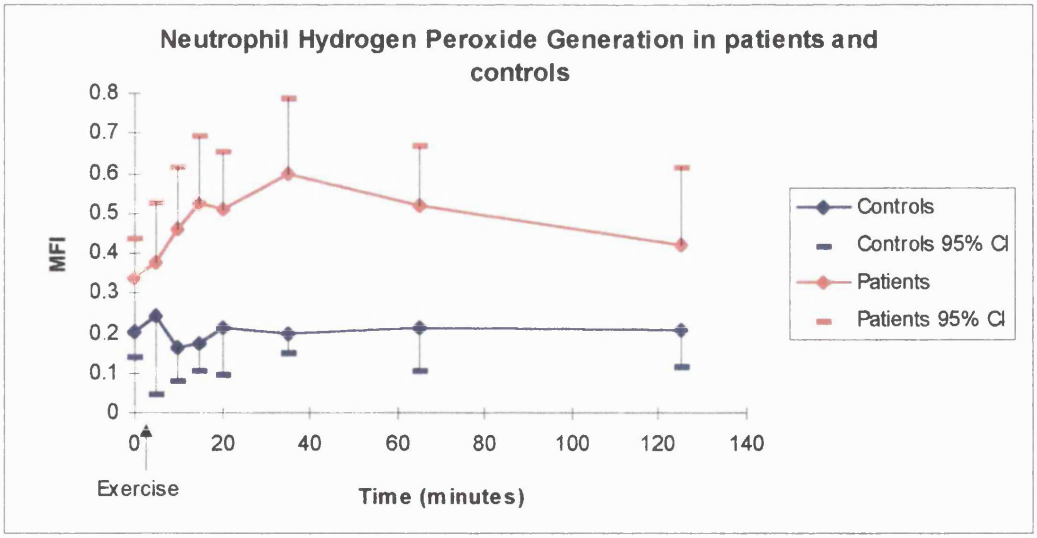


Fig 2.12 Hydrogen peroxide generation by neutrophils from controls and patients over time, with 95% CI.

Thromboxane A_2 release

Both patients and controls showed an increase in plasma thromboxane B_2 immediately following exercise, but this failed to reach significance. However, between 15 -35 minutes post-exercise patient thromboxane levels had reached significantly higher levels compared to resting values ($p < 0.05$).

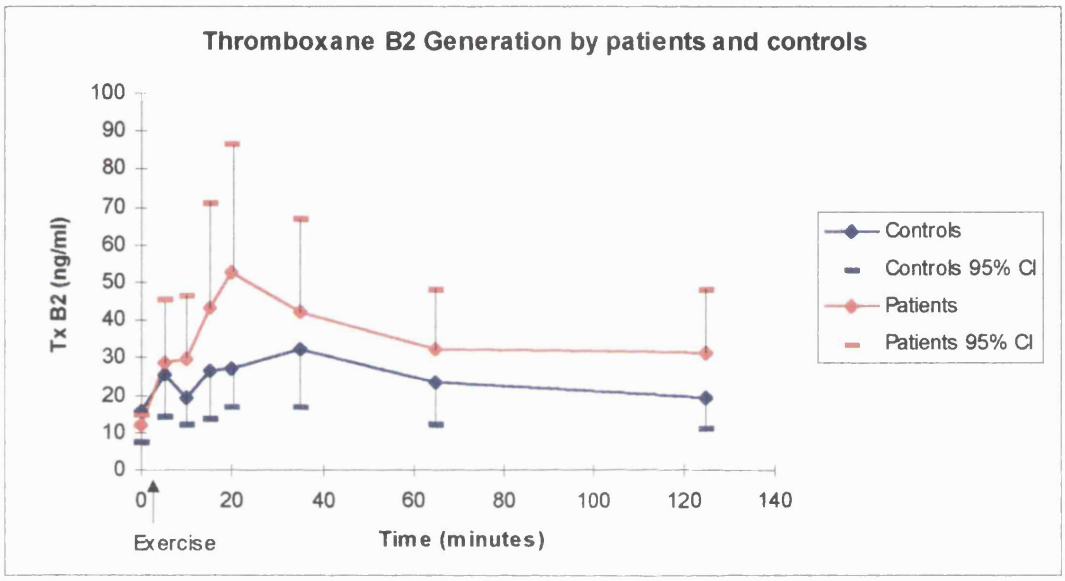


Fig 2.13 Thromboxane A_2 release in controls and patients over time, with 95% CI.

All patient thromboxane levels post-exercise were higher than controls, and this difference was significant at 10 and 15 minutes post-exercise ($p > 0.05$). When the use of aspirin was taken into account, claudicants showed a greater increase in thromboxane levels, but the small numbers made analysis impossible.

II.4. DISCUSSION

In summary, we found claudicants had a higher WBC count than controls, with both groups showing an increase immediately post-exercise. All cell types showed an increase but a proportionally greater increase in monocytes was seen in the claudicant group. Both patients and controls showed an increase in elastase levels immediately post-exercise, rapidly returning to normal. However, these changes became minor when corrected for cell count. Claudicants showed increased neutrophil activation, with both the microplate and the flow cytometric techniques, and produced more pronounced and prolonged increases in thromboxane B_2 levels following exercise, when compared to controls.

1. Implications of white cell counts and differential results.

Claudicants have higher WBC and neutrophil counts at rest when compared to controls, but this difference was not significant. This is in keeping with reports by Dormandy et al[43] and others[168,218-220]. Similarly, patient levels of lymphocytes and monocytes were slightly higher at rest. There were two smokers in the claudicant group and one in the control group, but their exclusion had little effect on the picture. Exercise produced a short lived, but significant, increase in WBC count in both groups and this represents the normal physiological response to exercise[193,228-230]. There is no evidence from our data that the response is any greater in claudicants. A similar rise was seen in neutrophil, lymphocyte and monocyte counts. These findings were unaltered when changes in blood volume, assessed using the haematocrit, were taken into account. The counts returned to resting levels quickly, and hence the rheological effect of the increased white cell count is short-lived. Patients experience claudication repeatedly, and this may, in part, maintain

chronically elevated white cell counts. However, our patients had a full nights rest prior to the study, but still showed higher levels of leucocytes than controls, and controls showed a proportional rise in counts with a similar short time course. Although, a delayed neutrophilia extending more than 4 hours following anaerobic exercise has been reported, this was found in normal subjects[583]. These factors argue against claudication as the cause of the elevated leucocyte count. Higher resting levels may be explained by the presence of an "inflammatory condition" like atherosclerosis, or any of its associated risk factors. Interestingly, repeated exercise is associated with better prognosis in atherosclerotic conditions. Part of this effect is due to improved rheology and conditioning effects, and it may be that increases in white cell count with exercise may be proportionally reduced by regular exercise. However, Ndon et al reported no change in the leukocyte response to exercise with training[584].

Neutrophils are known to become adherent and trapped within, even temporarily, ischaemic tissue[240]. Thus, claudication would be expected to produce a fall in neutrophil counts due to trapping within the muscle, and the increases in circulating neutrophil count are probably an underestimation of the true "mobilisation" of neutrophils. Interestingly, although we found an increase in absolute neutrophil counts immediately post exercise, comparison with the total white blood cell count revealed a relative neutropenia. Although this could be interpreted as due to neutrophil trapping, a similar finding in the control group, and in studies of healthy athletes post-exercise[583,585], argues against this. This relative neutropenia may represent increases in the other leucocyte populations. Both controls and patients showed an increase in absolute lymphocyte and monocyte counts post-exercise. When related to total white cell count, lymphocytes showed a proportional increase in both controls and patients, in keeping with other studies which

have reported a proportionally higher increase in lymphocyte counts and a relative neutropaenia in healthy subjects following exercise[583,585]. However, patients showed a marked increase in the proportion of monocytes following exercise when compared to controls. Other studies in claudication have also shown a significant increase with a shift towards mononuclear cells[231-233], and a neutropenia immediately post exercise. Although the relative increase is very pronounced the importance of this is difficult to gauge as the absolute numbers of monocytes remain small, but subsequent periods of exercise maybe associated with a greater increase in white cells[586], and a more significant role for monocytes.

2. Implications of elastase results.

Activated neutrophils express adhesion molecules, which enable them to stick to endothelial cells[587] and degranulate[195,226,240,588], releasing free radicals[226], peroxidases and proteolytic enzymes[589]. Using these products, such as lysozyme and elastase, to study leucocyte activation is limited because they give an indication of past, local events but no information about the activity of leucocytes in the systemic circulation. Interestingly, we found no real difference between controls and claudicants. Both show increase immediately post-exercise following which plasma elastase rapidly returns to baseline. However, when elastase levels are normalised for white cell and neutrophil count, controls show higher levels compared to patients. Furthermore, controls appear to have a biphasic response, with increases immediately post-exercise and at about 35 minutes, whereas, patients show a brief fall at 5 minutes post-exercise, but otherwise little variation. It is possible that the healthy controls produce higher increases in blood flow with exercise and destroy more effete neutrophils. The fall in patient elastase may arise

because of a greater absolute increase in neutrophil count, thus "diluting" the increase in elastase levels. This would suggest ischaemia within claudicating muscle is insufficient to cause degranulation and elastase release. These findings appear at odds with those of the Edinburgh study[221] and others[690,691] which found increased elastase levels in claudicants and the reports of Hickey et al who found increased lysozyme levels in claudicants[141]. The numbers in our study may have been too small to detect a difference or increased levels reported in the above two studies may have been due to exercise prior to the study period.

3. Neutrophil activation results.

Studies of systemic neutrophil activity have demonstrated decreased filterability[141,238] following exercise in claudicants compared to controls. Experimental work, using an animal model of claudication, has shown increased neutrophil-endothelial cell adhesion and endothelial cell swelling, both locally and systemically[142,143]. However, in all these studies the 'markers' of activation have been investigated immediately or shortly after exercise. The duration of these effects is unclear and it is possible that activated neutrophils may be 'filtered out' after a few passes through the microcirculation. Hence, their capacity to cause increased damage in say MI or stroke may be limited to the immediate post-exercise period.

Our study differs in that it considers both activation and the activation potential in neutrophils before and up to two hours post-exercise. We found no increase in unstimulated neutrophil activity, by either the superoxide (SO) or hydrogen peroxide (H_2O_2) techniques, following exercise. This differs from the finding of increased

filterability and adhesiveness post-exercise in other studies[141,142,238]. However, direct comparison of the studies is difficult. Firstly, our patients had all rested that morning, the study being their first episode of claudication. This information is not given in other reports, hence patients may have developed several episodes of ischaemia and reperfusion prior to their investigation. Secondly, increased filterability of neutrophils occurs with activation, but activation also causes free radical release, as demonstrated by increases in lipid peroxides[139,140]. Hence, such neutrophils maybe depleted of free radicals and not appear activated without further stimulation.

Stimulation of the neutrophils, albeit with a non-physiological stimulant, showed increased release of SO and H_2O_2 in claudicants compared to controls, the effect lasting up to 2 hours. The assumption is made that a similar increase would occur in response to physiological stimulation, and under physiological, i.e., *in vivo* conditions. These findings suggest that following claudication neutrophils are primed, and if further stimulated may produce a more "aggressive" release of free radicals. Cuiffetti et al[237] also looked at neutrophil SO production following claudication, but only in the immediate post-exercise period. They found a significant fall in neutrophil SO production, and a rise in plasma oxidant capacity indicating free radical release. The implication is that the free radicals are derived from neutrophils, which then become refractory to further stimulation. We also found an initial fall in neutrophil SO production but this rapidly returned to normal. Subjects in their study performed two consecutive exercise tests and this may explain the larger fall they observed. To our knowledge neutrophil H_2O_2 production, measured using flow cytometric techniques, has not been reported in claudicants. This technique is considered a more accurate and sensitive measure of neutrophil activation[582]. This is partly because cell preparation is less traumatic and because flow cytometry allows

assessment of individual cells. Using this technique, and the superoxide assay, we have shown an increase in neutrophil activation potential following claudication. This could be partly explained by the increase in numbers of circulating neutrophils, particularly if these extra cells had a greater activation potential. However, both groups showed an increase in neutrophils, and no increase in activation potential was found in the control group. Furthermore, the increase in neutrophil counts is over within 20 minutes, whereas H_2O_2 peaks at 40 minutes. Claudicants may have a pool of neutrophils with increased activation potential, but such cells would tend to marginate because of increased adhesive properties. Increased adhesiveness has been reported, and such cells bind firmly to the endothelium[199] and are not released with increased flow[158]. Furthermore, such 'sticky' cells would be expected to return to the marginated pool preferentially to normal cells. Alternatively, the trapping of activated leucocytes, particularly in the reperfusion stage, and the destruction of effete cells, would lead to an increased proportion of the normally marginated leucocytes. If these marginated white cells were comparatively juvenile, due to increased turnover, this may explain the finding of long-term increased activation potential. However, without further data on leucocyte turnover in peripheral vascular disease and the dynamics of the various leucocyte "pools" during exercise, in claudicants and controls, it is difficult to attribute our findings to shifts in leucocyte populations.

4. Implications of thromboxane results.

Elevated levels of thromboxane have been noted in peripheral vascular disease[389,401], but we found no difference between patients and controls at rest. Following exercise patients showed an increase 15-35 minutes post exercise, a similar finding to that reported by Wennmalm et al[421] and others[218]. Release of TxA_2 following claudication probably

represents local events. Activated neutrophils release TxA_2 which is chemotactic for further neutrophils, activates more neutrophils and stimulates adhesion to endothelial cells[128,592]. However, neutrophils are not the only source of TxA_2 in-vivo, and it is possible platelet release may have contributed to the elevated levels. The increased levels of TxA_2 may act systemically to stimulate circulating neutrophils, as suggest by Khaira et al[593], and/or may stimulate platelets, which are present in far greater numbers. Our results showed increased levels of thromboxane B_2 compared to controls despite three patients having been taking aspirin, based on their levels. The increase in TxB_2 levels is short lived in comparison to neutrophil activation, which could argue against a systemic effect. However, it could produce a "knock on effect", activating neutrophils which in turn activate further cells through an alternative mechanism.

5. Conclusion

Following exercise patients with claudication showed increased superoxide and hydrogen peroxide generation, in response to stimulation, when compared to controls. This enhanced activation potential was noted within 10 minutes of exercise using the flow cytometric technique, and from 30 minutes post-exercise with the cruder superoxide assay. The effect extended to the end of the time period with both techniques. Interestingly, the other markers of white cell activation showed a different pattern. Thromboxane levels increased following exercise in both controls and patients, a larger increase being seen in the patient group. However, levels in both groups had returned to almost normal about by 60 minutes post-exercise. Similarly, the changes in white cell population, both absolute and relative, had returned to normal by 60 minutes at the most. This different temporal relationship would be in keeping with the idea of local and

systemic events. The early, short-lived increases in thromboxane, and to a lesser degree elastase, are markers of events occurring in the leg. The similarity in the pattern of responses, although not the magnitude, may in part represent a normal response to exercise. The more long-term increase in activation potential of the neutrophils tends to suggest a systemic effect, or the presence of circulating "primed" neutrophils. Although both patients and controls showed increases in absolute elastase levels post-exercise, this pattern was lost when levels were normalised for white cell count. This may indicate that the exercise challenge was insufficient, even in claudicants, to produce full neutrophil de-granulation. Exercise was associated with a temporary increase in all white cell counts, but a relative fall in neutrophil counts in both groups. This relative neutropenia would appear to be part of the normal physiological response to exercise, and hence shifts in cell populations are unlikely to explain the increased cellular activation. Although, claudicants had a higher relative increase in monocyte counts, the absolute numbers, the shorter term response, and the specificity of the techniques for neutrophils, make it unlikely that monocytes contribute significantly to the cellular priming.

6. Implications of the finding of increased neutrophil activation after claudication.

Infarction is associated with neutrophil infiltration, as part of the inflammatory response to cellular death[594]. However, it is becoming apparent that they also influence the extent of damage through effects on the microcirculation[189]. Neutrophils exert some of their effect through their rheological properties[595]. Upon activation not only are these properties more pronounced, but neutrophils also show increased adhesion, release of free radicals and proteolytic enzymes causing endothelial cell swelling and damage. The overall effect is to impair the microcirculation, which in the vulnerable "penumbra" may

result in extension of the infarct[175,186-188]. This mechanism has most clearly been demonstrated in animal models of infarction where neutrophil depletion results in reduced infarct size[134,176].

Claudicants are known to have an increased risk of MI and stroke. The finding of neutrophil priming could explain some of this effect. In the presence of an equivalent degree of vessel occlusion, claudicants may have a more "aggressive" neutrophil response and potentially end up with more cellular damage. This could translate into a bigger infarction and greater clinical effect.

CHAPTER THREE: PLATELET AND COAGULATION SYSTEM ACTIVATION IN INTERMITTENT CLAUDICATION

III.1 RESEARCH QUESTION

There is good evidence for a role for platelets in atherosclerotic disease, particularly in the pathophysiology of cardiovascular events. However, evidence of platelet activation in claudicants, at rest or following exercise, is rather more equivocal; with evidence for increased activation potential, or priming, in platelets even more unclear. In line with our postulate we were looking for evidence of platelet activation and, in particular, potentiation of platelet activation by exercise in patients with claudication, at rest and/or following exercise. A fundamental problem in all work on platelet activation is that of preparation artefact, whereby platelets can be activated or even aggregate during sampling or preparation. Using a highly sensitive, but "minimal preparation" whole blood flowcytometric technique we hoped to reduce preparation activation, and detect any changes associated with claudication. Traditional tests of platelet activation were run for comparison.

Assessing changes in the activation potential of the coagulation system is difficult. Most currently available methods are insensitive, and unlikely to detect any small change in coagulation function. In view of this we attempted to assess the coagulation system using indirect methods, looking for evidence of activation using new, highly sensitive assays for markers of activation.

Flow cytometry, as a technique for studying platelet reactivity, is new to our laboratory. Hence, before outlining the experimental design we will discuss flow cytometry and the necessary set-up and standardisation procedures.

III.2 METHODS

III.2.1. FLOW CYTOMETRY

a) VALIDITY OF FLOW CYTOMETRY TO ASSESS PLATELET ACTIVATION

Activation of platelets produces a series of changes that leads to adhesion and aggregation. Stimulation with weak agonists such as ADP or adrenaline causes conformational changes in the platelet GPIIb-IIIa complex resulting in fibrinogen binding. In the presence of platelet-to-platelet contact aggregation will occur. Stronger agonists, such as thrombin or vessel wall collagen, cause degranulation with translocation of granule membrane glycoproteins onto the platelet surface[596]. We wanted to be able to measure both "degrees" of activation.

Platelets are very small, extremely labile and subject to artefactual activation *ex vivo* by separation or preparation techniques. Such artefacts can be minimised by methods to inhibit platelets, fix them and/or by reducing handling to a minimum. The first two methods can be used to assess 'major' activation since once granule contents have been released or expressed on the platelet surface inhibition or fixation does not alter them. However, such techniques cause changes in the levels of binding of fibrinogen to GPIIb-

IIIa[597]. To assess lesser degrees of platelet activation it is necessary to measure fibrinogen binding to platelets and this requires a minimal handling technique. However, it then becomes difficult to distinguish platelets amongst the other blood cells or from circulating proteins.

Flow cytometry provides a technique of assessing individual platelets, and their surface markers, within whole blood and a way of identifying them from the other components of blood. Whole blood techniques not only minimise handling but the absence of inhibitors and/or fixatives allows investigation of platelet responsiveness or activation in vitro[598,599]. The value of this technique in assessing platelet activation, and in particular fibrinogen binding, is demonstrated by its use to characterise the dynamics of binding of fibrinogen to GPIIb-IIIa, including the conformational changes in both the fibrinogen molecule and the receptor complex[596]. Furthermore, whole blood techniques have been used to assess platelet activation in patients having haemodialysis. Results show platelet activation in keeping with previous studies, and demonstrated hyporesponsiveness to stimulation with ADP[600]. Work on PVD patients showed no activation but increased responsiveness to stimulation with ADP[414]. Flow cytometry has also been used to study platelet activation in cardio-pulmonary bypass[597], pre-eclampsia, myeloproliferative disorders and in stored platelets used for replacement therapy[596].

b) **PRINCIPLES OF THE TECHNIQUE**

Flow cytometry is a system for sensing cells as they move in a liquid stream, through a laser, past a series of sensors. Measurement of light scatter and colour-discriminated fluorescence enables differentiation of cell types and the detection of fluorescent

molecules in the form of antibodies or dyes. Modern machines can analyse up to 20, 000 cells per second making flow cytometry a powerful technique for the study of cellular activity.

In simple terms a flow cytometer is made up of three components. One, a laser and a sensing system, the latter comprising a flow chamber and an optical assembly to detect light scatter. Two, a hydraulic system which controls the passage of cells through the sensing system. Three, a computer system that collects data from the sensing array and analyses the electrical signals. Cells suspended in an isotonic fluid are drawn into the flow chamber using a lamina/sheath flow technique. The fluid within the sheath creates a hydrodynamic focusing system, which draws the sample fluid into a thin stream, which passes through the laser beam and the focus point of the optical assembly. Adjustment of the sheath and sample pressures creates a stream where cells pass through the detection point one by one. Light from the laser strikes the individual cells and is scattered. The optical assembly collects, filters and directs various components of this scattered light on to photomultiplier tubes or sensors. These generate an electrical signal, in response to the light, which is then interpreted by the computer system[601].

Cells passing through the laser scatter light in all directions. Light scattered in a forward direction is proportional to size of the cell[602]. Some light enters the cell and is reflected and refracted by intracellular components. Such scatter, detected by a sensor at 90° to the laser beam, is considered proportional to the granularity of the cell. Using these light scatter properties cells can be discriminated, allowing the study of particular cell populations. Cells can also be labelled with fluorochrome-linked antibodies to surface receptors or antigens, DNA, enzymes, etc. These fluoresce when struck by the

monochrome light of the laser and generate a specific wavelength of emitted light. This is detected via filters and focusing systems and facilitates differentiation of cell types and detection of surface marker expression. Hence, using antibodies to markers expressed during cell stimulation, it is possible to study cellular activation. One of the particular advantages of a flow cytometer is that it is multiparameter. This means it is able to record several measurements on each cell and hence identify a specific group of cells within a heterogeneous population. In terms of platelet studies this obviates the need to separate platelets from whole blood samples, thus reducing the likelihood of activation due to separation procedures.

Platelet Analysis Platelets are small and their light scatter characteristic maybe mimicked by dust particles. This problem can be solved by filtering all buffers used to prepare the samples and the sheath fluid through a 0.22μm filter. As mentioned above the lability of platelets is a major difficulty and confounding factor in assessing platelet activation. Techniques to minimise the artefactual activation by preparation methods involve fixing the platelets, inhibiting them or minimal handling. The former two options can interfere with activation markers, particularly changes in the GPIIb-IIIa complex, which binds fibrinogen. For analysis of activation markers use of whole blood assays, a minimum-handling technique, is advised[603]. This relies on the ability of the flow cytometer to distinguish individual cell groups within a heterogeneous population. It avoids the need to separate out the platelets from other blood cells, thus reducing the risk of artefactual activation. The platelet population is determined by the light scatter characteristics of the cells, and their identity can be confirmed with a fluorescent antibody to GPIb; found on all platelets.

c) **WHOLE BLOOD ASSAY**

This technique was first described by Jennings et al[604] and developed by Shattil et al[597,598]. Several modifications of this method have been described[599,600,605].

i. *Sample collection* Blood is collected with minimum stasis into citrate. A 21-gauge or larger needle is used, the first 2mls of specimen are discarded and blood is only removed once the tourniquet has been released.

ii. *Preparation* Within 2 and 10 minutes of taking the sample a 5μl aliquot of whole blood is added to a tube containing appropriate amounts of HEPES-buffered saline, antibody, agonist, etc. (volumes depend on assay being performed and the concentration of the reagents required). Samples are incubated in the dark at 22-28°C for 20 minutes, and then diluted and fixed with 0.5ml of 0.2% formyl saline (filtered through a 0.22μm filter). All specimens are analysed in the flow cytometer within 2 hours of fixation.

iii. *Analysis* Prepared whole blood samples are then passed through the flow cytometer. Platelets are identified by their scatter and presented on a logarithmic scale scatter plot. The platelet population, circled, is distinct from area 1, which represents noise and dust, and from area 3 which represents red blood cells. An electronic bit map is created around area 2 (the platelet population) and analysed for differing light wavelengths indicative of fluorescent antibodies. High levels of fluorescence associated with a monoclonal antibody (MoAb) to GPIbα, confirms the cells as platelets.

Parameters of this assay have been set by work on detection of platelet-bound fibrinogen[596]. Blood left more than 10 minutes before dilution shows an increase in fibrinogen binding[600], whilst samples diluted in under 2 minutes seems to have slightly lower levels. Dilution of the whole blood sample reduces cell to cell contact and avoids aggregation of activated platelets. Assays performed outside the temperature range are associated with a significant increase in fibrinogen binding. Fixation with 0.2% formyl saline does not alter fibrinogen binding to unstimulated platelets, however, fixation of stimulated samples can be associated with a small increase in binding. In both circumstances the level of binding then remains stable for 2 hours at 22°C (or 24 hours at 4°C).

Platelet activation in vitro can be determined using the whole blood method by addition of 5μl of varying concentration of agonist prior to the incubation stage. The reactivity of distinct platelet samples can be assessed by comparing dose-response curves to certain agonists for each sample.

d) **STANDARDISATION OF METHOD**

Before embarking on flow cytometric studies of platelet activation and reactivity it was necessary to determine the concentration of antibodies and agonist required. In the case of antibodies this means optimising concentration such that there is adequate antibody levels to meet conditions of maximal stimulation but avoids using excess antibody, which may form immune complexes with soluble antigen[599,600], and can be an expensive commodity. Platelet glycoprotein expression, as detected by fluorescence labelled

antibodies, can be plotted as a dose response curve against concentration of agonist. One way of assessing platelet reactivity is to study the shift in the dose response curve to a specific agonist. Our aim was to choose three agonist doses, one on the upper part of the curve, one on the lower and one in the middle. This would allow us to plot approximate dose response curves and improve the likelihood of detecting up or down regulation. The set-up procedures allowed us to determine optimum antibody concentrations and three appropriate doses of our two agonists.

i. ***Determination of Antibody Concentrations*** Monoclonal antibodies were used to measure the expression of certain platelet surface glycoproteins. GPIbα is a surface glycoprotein specific to platelets and is used to confirm the initial flow cytometer settings, i.e., that we are scanning the platelet population. It also acts as a check at each time point for each patient to ensure we continued to study the same population of cells. Platelet activation was measured by the expression of fibrinogen, which binds to a surface glycoprotein GPIIb-IIIa in response to platelet activation, and P-selectin, which is released as a soluble factor as well as expressed on the surface of activated platelets.

The aim of this standardisation experiment was to determine the concentration of each MoAb necessary to saturate the receptors under conditions of maximal stimulation. This would ensure that measurement of activation is not limited by insufficient MoAb.

Method: Whole blood samples of 4.5mls were taken from 6 normal subjects. All subjects were healthy and had taken no medication within the last two weeks. Samples were taken without a tourniquet, using an 18 gauge needle, directly into syringes anticoagulated with 0.5mls of 0.0105mol/l tri-sodium citrate. All samples were processed within 10 minutes

of collection. Aliquots of 5μl of blood were added to 1.5ml epindorph tubes containing varying volumes of HEPES-buffered saline (HBS). HBS contains 0.145mol/l NaCl, 5mmol/l KCl, 1mmol/l $MgSO_4$, 10mmol/l HEPES at pH 7.4, and was filtered through a 0.22 μm filter.

GPIbα The tubes contained increasing doses of a fluorescein isothiocyanate (FITC)-conjugated IgG mouse monoclonal antibody (MoAb) (Immunotech, The Binding Site, Birmingham, UK) to the GPIbα glycoprotein. The experiment was performed with no agonist, and then repeated with a maximum stimulatory dose of adenosine diphosphate (ADP) (Final concentration 3 x 10^{-3}M), and α-thrombin (Final concentration 0.8units/ml) (Sigma Chemical Co Ltd, Poole, UK).

Fibrinogen and P-selectin This experiment had two parts, and each part had three arms. In the first part the tubes contained increasing doses of an FITC-conjugated polyclonal rabbit antibody to human fibrinogen (DAKO Ltd, High Wycombe, UK). In the first of the three arms the tubes had no agonist present, in the second they contained ADP (Final concentration 3 x 10^{-3}M), and in the third they contained α-thrombin (Final concentration 0.8units/ml). In the second part of the experiment increasing doses of a FITC-conjugated IgG1 mouse MoAb to P-selectin (Immunotech, The Binding Site, Birmingham, UK) were run in the three 'agonist' arms as described above.

To prevent clot formation when using α-thrombin, samples also contained 0.125mmol/l of a synthetic peptide, glycyl-propyl-L-arginyl-L-proline (GPRP) (Sigma Chemical Co Ltd, Poole, UK). This acts to inhibit fibrin polymerisation and consequent clot formation.

All samples were run in duplicate. After gentle mixing the samples were incubated, in the dark, at room temperature (22-26°C) for 20 minutes. The reaction was stopped by the addition of 500µl of 0.2% formyl saline. The reaction was then further diluted with 1.5ml of 0.2% formyl saline.

Samples were analysed within 2 hours of collection using a Coulter EPICS Profile II flow cytometer (Coulter Electronic Ltd, Luton, UK). The machine was calibrated each day using "Immuno Check" and "Standard Brite" beads (Coulter Electronic Ltd, Luton, UK) to check light scatter and fluorescence respectively. Platelets were detected based on light scatter properties. An electronic bit-map was placed around the platelet population and five thousand platelets were analysed from each sample. The mean fluorescence intensity (MFI) was measured and plotted against the concentration of antibody. The optimum concentration of GPIbα was determined first, and thereafter, an electronic bit-map was placed around the platelet population and adjusted so that greater than 98% of particles analysed were positive for GPIb. Five thousand platelets were analysed from each sample. Using 2% negative cut off levels for fluorescence, set with appropriate isotype FITC-conjugated control antibodies, the percentage of cells positive for each antibody was measured. This was then plotted against antibody concentration, or dilution factor. Each specimen is run in duplicate and the average value for each sample is calculated. The overall mean percentage positive cell value is then determined for each concentration of MoAb, both with and without agonist. Using this data a dose-response curve is plotted to determine the minimum concentration of MoAb necessary to label the majority of receptors

Results The titration of GPIbα against binding, as measured by Maximum Fluorescence Intensity (MFI) is shown in Fig 3.1. Maximum binding was seen with antibody concentrations greater than 1 in 7 dilution. No difference was seen with or without agonist, or between the two agonists. Hence, a dilution of 1 in 7, or 10μl was used in all subsequent experiments.

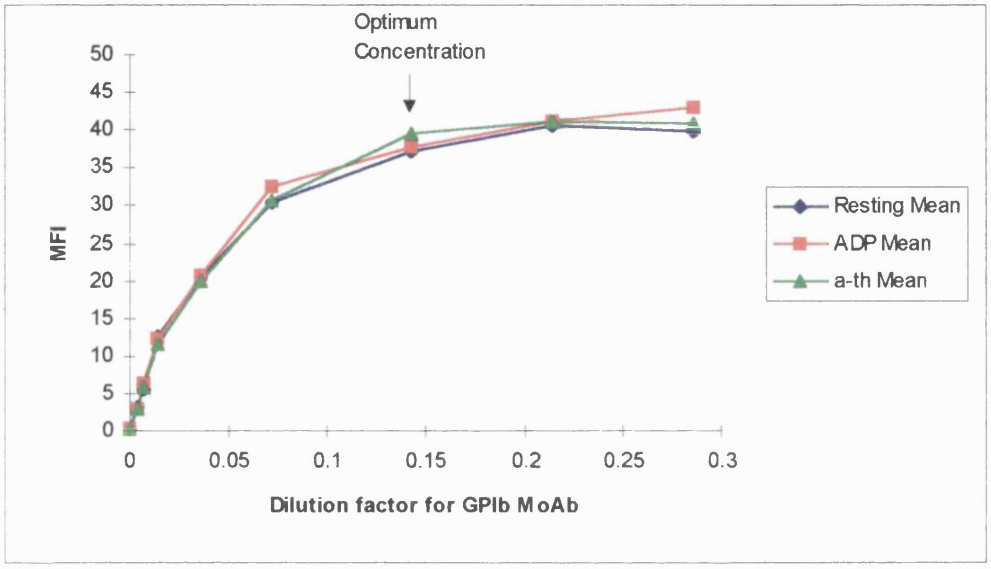


Fig 3.1 Mean Fluorescence Intensity for each group plotted against dilution factor of GPIb MoAb.

The overall mean percentage of positive cells is then determined for each concentration of MoAb, both with and without agonists. Using this data dose-response curves are plotted to determine the minimum concentration of fibrinogen MoAb necessary to label the majority of bound fibrinogen (Fig 3.2), and the minimum concentration of P-selectin MoAb necessary to label the majority of bound P-selectin (Fig 3.3).

Fibrinogen binding to resting platelets was low (< 20%) at concentrations of antibody below 1 in 70 dilution, but rose sharply to 70% binding at 1 in 28 dilution (Fig 3.2). This represents the optimum concentration of antibody, where most or all of the bound fibrinogen is labelled and there is little waste of antibody. Hence, 5 μl of a 1 in 2 dilution

in 70µl of Fb antibody was used during the subsequent flow cytometric studies. Similarly, from Fig 3.3, 10µl in 70µl, or a 1 in 7 dilution, of P-selectin MoAb was used for subsequent flow cytometric studies.

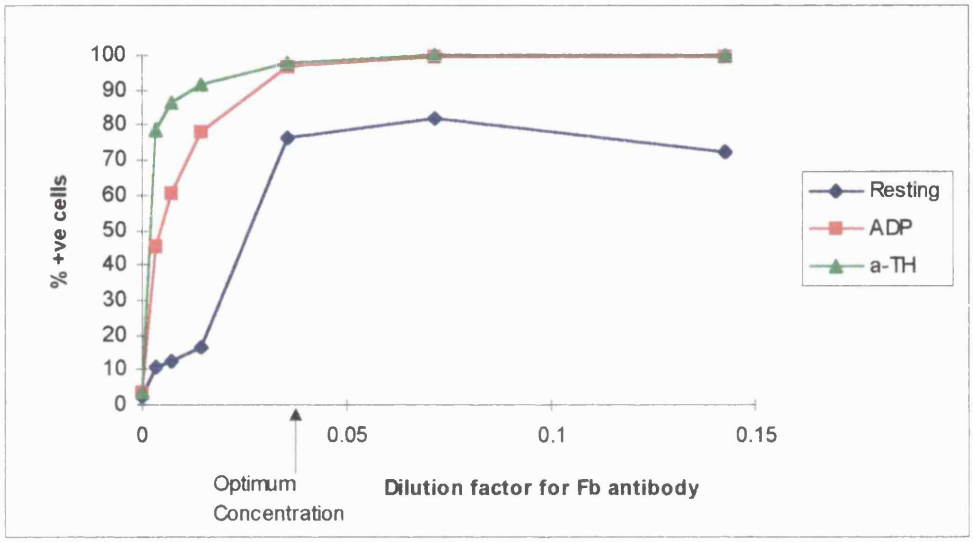


Fig 3.2 Percentage of positive cells for each group plotted against dilution factor of Fibrinogen MoAb.

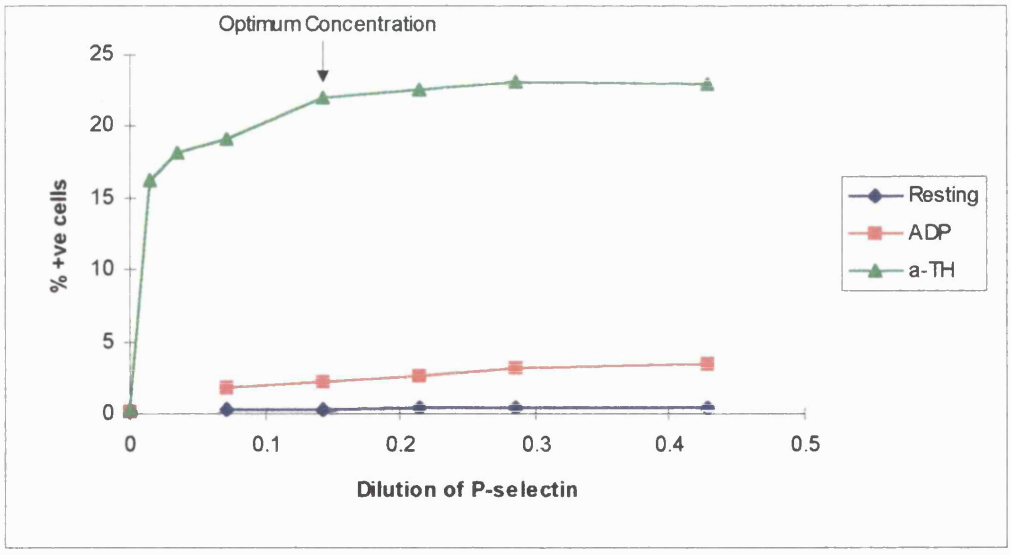


Fig 3.3 Percentage of positive cells for each group plotted against dilution factor of P-selectin MoAb

ii. *Determination of Agonists Concentrations* There are numerous possible agonists to cause platelet activation. Adenosine diphosphate is fairly standard and a considerable amount of work has been done using it, and α-thrombin is a likely physiological agonist in the pathophysiology of atherosclerosis and its progression. The aim of this standardisation experiment was to determine the working range of agonist concentrations, ADP and α-thrombin, for the expression of each marker of platelet activation, namely bound fibrinogen and P-selectin.

Methods Blood samples were taken from ten normal subjects under the same conditions as discussed above. Aliquots of 5µl of blood were added to a mix of buffer and varying concentrations of either ADP, 1 x 10-8 to 3 x 10-5M, or α-thrombin, 0.025 to 0.8 units/ml. Two experimental arms were set-up, one using 5 µl of half-strength MoAb to bound fibrinogen, the other using 10 µl of neat MoAb to P-selectin. Platelet activation was assessed, by the expression of bound fibrinogen or P-selectin, in response to a range of concentrations of each agonist. Samples were handled as discussed above.

Results Dose response curves for each agonist against the expression of each platelet marker are shown in Fig 3.4 & 3.5.

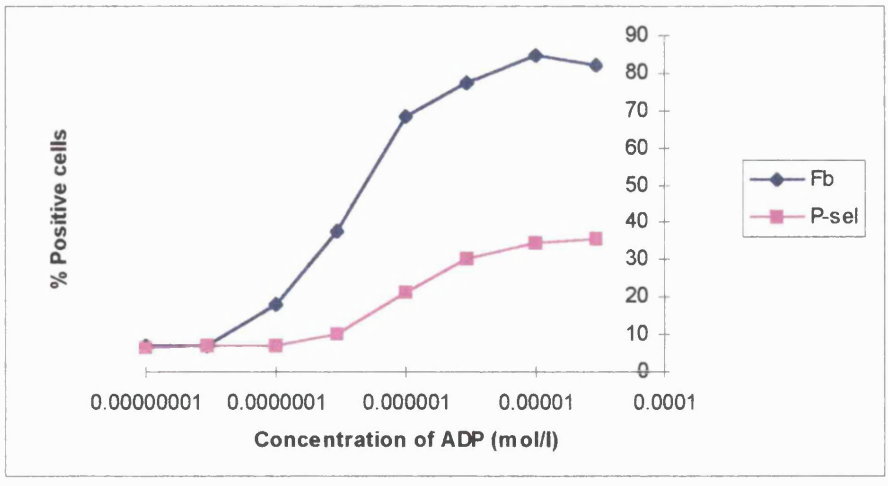


Fig 3.4 Dose-response curve for Fibrinogen binding and P-selectin expression in response to ADP.

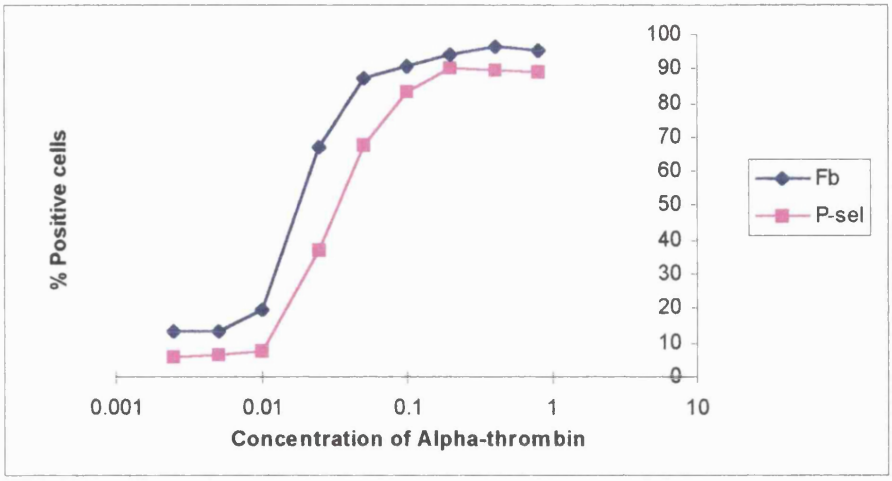


Fig 3.5 Dose-response curve for Fibrinogen binding and P-selectin expression in response to α-thrombin.

The eventual aim of this work was to study platelet activation at rest and in response to agonist stimulation, under different clinical situations. It was possible that the expression of the platelet markers of activation could be up or down- regulated in these different situations. With this in mind three concentrations of agonist were chosen, at the lower, mid and upper portions of the dose-response curve. The three concentrations of ADP, for both fibrinogen and P-selectin binding are 1 x 10^{-6}M, 3 x 10^{-7}M and 1 x 10^{-7}M.. For fibrinogen binding the concentrations of α-thrombin were 0.01 units/ml, 0.02 units/ml and 0.03 units/ml, whereas for P-selectin binding they were 0.01 units/ml, 0.025units/ml and 0.05 units/ml.

III.2.2 GENERAL

a) **SUBJECTS**

i. *Patients* Twelve men with claudication were recruited from our Vascular outpatient population (See Appendix 2). Ages ranged from 52 to 79 years (Mean = 66.4). A detailed history and examination was performed. They all had at least a six-month history of stable claudication predominantly due to femoropopliteal disease, based on clinical assessment, and were limited on exercise by claudication alone. Care was taken to exclude those with recent history of percutaneous intervention, acute deterioration, ischaemic ulceration, tissue loss or gangrene. Patients with recent myocardial infarction, cerebrovascular events or a clinical picture suggesting reno-vascular disease were also excluded. Each patient had an ECG performed to exclude silent infarction. In addition, blood was taken to assess renal and hepatic function, and basic haematological parameters. A urine specimen was taken for urinalysis, and if appropriate, microscopy and culture. Necessary treatment was instigated if infection was detected. Patients with laboratory or clinical evidence of significant disease, including diabetes, hypertension, chronic renal failure and infection or inflammatory conditions, were excluded. Patients fitting the above criteria were then asked to perform an exercise test on the treadmill. This confirmed their exercise was limited by claudication. The patients' pre and post-exercise ankle-brachial-pressure-indices (ABPI) were recorded, as well as the pressure recovery time, duration and speed of exercise. Any non-steroidal anti-inflammatory drugs, other than aspirin, were stopped two weeks prior to the study, and patients taking β-blockers were excluded.

The aim was to select a group of patients whose primary manifestation of atherosclerosis was intermittent claudication. Attempts were made to exclude any factors, which may

influence cellular activation, such as recent arterial event, acute illness or chronic organ dysfunction. It was likely that all of these patients had some degree of atherosclerosis elsewhere, but provided the above investigations were normal it was considered sub-clinical.

ii. *Controls* Twelve controls were recruited from our General surgical outpatients (See Appendix 2). Again there were 12 men, aged from 58 to 77 years (Mean = 67.3). We asked patients who had undergone a non-vascular procedure at least six month ago. Eleven had undergone inguinal hernia repair and one excision of papillomatous lesion. A detailed history and examination was performed. A similar screening process was used to exclude any patients with significant laboratory or clinical disease. Furthermore, all controls were assessed to exclude any latent arterial disease. ABPIs were measured at rest and after a 5-minute exercise test. All resting ABPIs were > 0.95, and remained stable or increased on exercise. All controls were able to walk on the treadmill for more than 5 minutes without limitation. Any non-steroidal anti-inflammatory drugs were stopped two weeks prior to the study, and controls taking β-blockers were excluded.

All patients and controls were seen and assessed at least two weeks prior to enrolment in the study. All subjects gave fully informed written consent to the experiment. In addition, all subjects were assessed for the ease of venepuncture from both arms. This was important because many markers of platelet activation can be elevated by poor or difficult venesection.

b) **STUDY DESIGN**

This was an unmatched case-control study comparing platelet and coagulation system activation pre- and post-exercise in claudicants and controls. Five of the patients were taking Aspirin, 300mg, at the start of the study. Aspirin is known to have inhibitory effects on some but not all aspects of platelet activation. Consequently, a decision was made to make the patient arm of the study a crossover design with respect to aspirin usage. Patients taking aspirin at the start of the study completed the following test and then stopped for 4 weeks and the study was repeated. Those not receiving aspirin for the first test were commenced on Aspirin 300mg od for 4 weeks prior to the second test. Thus, controls were compared with the patients taking aspirin and the same patients not taking aspirin.

	TEST/ STUDY 1	TEST/ STUDY 2
CONTROLS 1 – 12	✓	**O**
PATIENTS 1 – 5	✓ (On Aspirin)	✓ (Not on Aspirin)
PATIENTS 6 - 12	✓ (Not on Aspirin)	✓ (On Aspirin)

Fig 3.6 Outline of crossover

Each test involved the following (See Fig 3.7):

i. Subjects (patients or controls) were collected from home on the day of the study by taxi. They were asked to avoid exercise prior to their appointment and to abstain from tea, coffee or cola drinks, to avoid any effect of caffeine on the measures. Upon arrival in the department subjects were asked to pass a urine sample. They were then seated in the waiting area and given 1/2 pint of water to drink. After 30 minutes they were transferred to the laboratory.

ii. In the laboratory subjects rested on a couch. After 10 minutes an ABPI measure was determined for the affected limb. A blood sample was taken from an ante-cubital vein using an 18G needle and no tourniquet. The first 2 mls of this, and all samples, was

discarded. This was labelled time-point one. Samples were taken without stasis into various tubes.

iii. Subjects then walked on a treadmill. Claudicants walked until limited by pain whilst controls walked for 5 minutes. This was the mean exercise time of the claudicant group determined during the pre-study visits. The time, speed and distance walked were recorded. All subjects immediately returned to the couch at the end of the exercise period where a post-exercise ABPI was measured. The pressure cuff was left in place and measurements repeated every 30 seconds until the ABPI returned to pre-exercise levels. This time, for the ankle pressure to return to pre-exercise levels was recorded and called the Pressure Recovery Time (PRT). Where necessary measurements were performed whilst blood samples were being taken.

iv. Further blood samples were taken at 5 and 15 minutes post-exercise. All samples were taken with a 17G needle in an attempt to reduce sampling activation. Sequential samples were taken from alternate arms and each new sample was taken from a more distal point than the last one. This was an attempt to avoid any local activation due to previous venepunctures.

v. At 30 minutes post-exercise another blood samples was taken and the subjects underwent a further exercise test. Pressures were recorded pre- and post exercise as before and further samples taken at 5 and 15 minutes following the second exercise period.

vi. At the end of this time subjects were asked to pass a further urine specimen.

The following table gives an overview of the test that was undertaken on each control and each patient, both taking and not taking aspirin.

PLATELET MEASUREMENTS	Pre-Ex 1	Exercise 1	5 mins Post-Ex 1	15 mins Post-Ex 1	30 mins Post-Ex 1	Exercise 2	5 mins Post-Ex 2	15 mins Post-Ex 2
Platelet Count	✓		✓	✓	✓		✓	✓
Platelet Volume	✓		✓	✓	✓		✓	✓
Platelet Markers: Thromboxane B2	✓		✓	✓	✓		✓	✓
PF4	✓		✓	✓	✓		✓	✓
βTG	✓		✓	✓	✓		✓	✓
Platelet Glycoprotein								
- Fibrinogen: Unstimulated	✓		✓	**O**	✓		✓	**O**
ADP (1 x 10-6M)	✓		✓	**O**	✓		✓	**O**
ADP (3 x 10-6M)	✓		✓	**O**	✓		✓	**O**
ADP (1 x 10-7M)	✓		✓	**O**	✓		✓	**O**
α-thrombin (0.03u/ml)	✓		✓	**O**	✓		✓	**O**
α-thrombin (0.02u/ml)	✓		✓	**O**	✓		✓	**O**
α-thrombin (0.01u/ml)	✓		✓	**O**	✓		✓	**O**
- P-selectin Unstimulated	✓		✓	**O**	✓		✓	**O**
ADP (1 x 10-6M)	✓		✓	**O**	✓		✓	**O**
ADP (3 x 10-6M)	✓		✓	**O**	✓		✓	**O**
ADP (1 x 10-7M)	✓		✓	**O**	✓		✓	**O**
α-thrombin (0.05u/ml)	✓		✓	**O**	✓		✓	**O**
α-thrombin (0.025u/ml)	✓		✓	**O**	✓		✓	**O**
α-thrombin (0.01u/ml)	✓		✓	**O**	✓		✓	**O**
Platelet Aggregation (Max & 3 mins)								
ADP (1.0μmol/l)	✓		✓	**O**	✓		✓	**O**
ADP (2.5μmol/l)	✓		✓	**O**	✓		✓	**O**
Adrn (0.005mmol/l)	✓		✓	**O**	✓		✓	**O**
Adrn (0.0025mmol/l)	✓		✓	**O**	✓		✓	**O**
α-thrombin (5.0u/ml)	✓		✓	**O**	✓		✓	**O**
α-thrombin (5.5u/ml)	✓		✓	**O**	✓		✓	**O**
α-thrombin (6.0u/ml)	✓		✓	**O**	✓		✓	**O**
α-thrombin (6.5u/ml)	✓		✓	**O**	✓		✓	**O**

(✓ - Test performed, **O** – No test performed, βTG – Beta-thromboglobulin, PF4 - Platelet factor 4, u/ml – units per millilitre, Agg^{n} – Aggregation, Adrn – Adrenaline)

COAGULATION MEASUREMENTS	Pre-Ex 1	Exercise 1	5 mins Post-Ex 1	15 mins Post-Ex 1	30 mins Post-Ex 1	Exercise 2	5 mins Post-Ex 2	15 mins Post-Ex 2
APTT	✓		✓	✓	✓		✓	✓
TAT	✓		✓	✓	✓		✓	✓
F1 & 2 Fragments	✓		✓	✓	✓		✓	✓
d-dimer	✓		✓	✓	✓		✓	✓

(✓ - Test performed)

Fig 3.7 Tabular summary of platelet and coagulation study

The study was designed in this way to include an assessment of the affect of repeated exercise; a more natural course of events in claudicants. The sampling time points were chosen arbitrarily, influenced by the practicalities of patient measurements, sampling and the time available for laboratory work. A time period of 30 minutes between exercise periods was used because we had noted cellular excitation up 60 minutes following exercise in earlier experiments on neutrophils.

c) MEASUREMENTS AND LABORATORY TECHNIQUES

i. Physical Measurements

Ankle Brachial Pressure Index (ABPI) - This is a standard non-invasive measure of the severity of peripheral vascular disease[606]. Measurements are made after a period of rest supine on a couch. A sphygmomanometry cuff is placed around the upper arm. A standard 8 MHz Doppler probe, from a hand-held Doppler (Hutchinson Ltd, USA), is placed so as to insonate the best arterial signal from the brachial artery. The cuff is then inflated until the signal disappears. The pressure is then gradually reduced until the arterial signal returns. This pressure is the "brachial pressure". This procedure is then repeated at the ankle. The dorsalis pedis and posterior tibial arteries are insonated separately and the one with the highest pressure is used for all subsequent measurements. The ankle pressure reading is then divided by the brachial reading to give the ABPI.

Pressure Recovery Time (PRT) - The pressure recovery time is considered by some to be a more accurate measure of the severity of peripheral vascular disease[141,607]. Following exercise the ankle pressure measurement falls because of vasodilatation, secondary to

metabolite build-up, in the presence of a stenosis. Once the patient ceases exercise, the muscle ischaemia slowly resolves and the pressure returns to pre-exercise levels. The time taken to recover depends on the severity of the ischaemia, and hence provides an indication of disease severity.

Exercise parameters - in each case the speed and duration of walking were recorded along with the distance walked. Measurements were made from the digital read-out on the treadmill, the end-point readings were taken from when the treadmill stopped. All subjects walked at an incline of 10%. Although we aimed for a similar walking speed for all subjects, some patients required slightly slower speeds, and the speed control on the treadmill made small adjustments very difficult. In the end, patient walking speeds ranged from 2.4 - 3 km/h compared to 2.6 - 2.9km/h for controls.

ii. Microalbuminuria

Shearman et al reported low-level albuminuria, undetectable by "dip-stix" testing, in response to intermittent claudication[139]. It is hypothesised that the generation of inflammatory mediators and activation of neutrophils in response to muscle ischaemia causes endothelial damage and increased vascular permeability. This in turn is thought to increase glomerular permeability to albumin, and result in microalbuminuria[608]. Thus, microalbuminuria maybe a marker of a systemic response to claudication and may represent a measure of the severity of claudication. Urinary albumin was measured using a radioimmunoassay (Diagnostic Products Corporation, Wallingford, UK). Results were expressed as a urinary- creatinine ratio as described by Hickey et al. This compensates for changes in urinary flow during the study[141].

iii. Platelet size A 4.5ml sample was taken into Na EDTA (standard full blood count Vacutainer) and passed through a Coulter® MAXM machine in the haematology laboratory, within an hour of sampling. Platelet count and mean volume was measured as part of the routine full blood screen. These values were measured at each time-point.

iv. Platelet markers

Surface glycoproteins - Due to the practicalities of the flow cytometric technique it was not possible to process blood from all six sampling time-points. Consequently, flow cytometric analysis of platelet activation was performed at time-points 1, 2, 4 and 5, i.e., pre-exercise period one and five minutes post-exercise, and then at 30 minutes post-exercise, or pre-exercise period two, and 5 minutes post-exercise period two.

Four point five millilitres of blood was taken, without a tourniquet and using an 18 gauge needle, directly into syringes anticoagulated with 0.5mls of 0.0105mol/l tri-sodium citrate. The syringe was gently tipped to mix the anticoagulant, and transferred to the laboratory. Here, aliquots of 5μl of blood were added to 1.5ml epindorph tubes containing varying volumes of HEPES-buffered saline (HBS), antibody and agonist. All samples were processed within 10 minutes. All samples are run in duplicate. After gentle mixing the samples were incubated, in the dark, at room temperature (22-26°C) for 20 minutes. The reaction was fixed by the addition of 500μl of 0.2% formyl saline. The reaction was then further diluted with 1.5ml of 0.2% formyl saline.

At each time point blood was run against three concentrations of ADP, 1 x 10^{-6} M, 3 x 10^{-7} M and 1x 10^{-7} M, and either fibrinogen antibody or P-selectin MoAb at the standard concentration (Fig 2.2). Blood was also run against three concentrations of alpha-thrombin, 0.03 units/ml, 0.02 units/ml and 0.01 units/ml with the standard concentration of fibrinogen antibody. Differences in the dose-response curve for thrombin and P-selectin resulted in three different concentrations of α-thrombin, 0.05 units/ml, 0.025 units/ml and 0.01 units/ml, being used with the standard amount of P-selectin MoAb (Fig 2.3). To prevent clot formation when using α-thrombin, samples also contained 0.125mmol/l of a synthetic peptide, glycyl-propyl-L-arginyl-L-proline (GPRP) (Sigma Chemical Co Ltd, Poole, UK). In addition, three further duplicates were run. One containing buffer and blood alone, the second, buffer, blood and fibrinogen antibody and the third, buffer, blood and P-selectin MoAb.

Samples were analysed within 2 hours of collection using a Coulter EPICS Profile II flow cytometer (Coulter Electronic Ltd, Luton, UK). The machine was calibrated each day using "Immuno Check" and "Standard Brite" beads (Coulter Electronic Ltd, Luton, UK) to check light scatter and fluorescence respectively. Platelets were detected by their light scatter properties. An electronic bit-map was placed around the platelet population and adjusted so that greater than 98% of particles analysed were positive for GPIb. Five thousand platelets were analysed from each sample. Using 2% negative cut off levels for fluorescence, set with appropriate isotype FITC-conjugated control antibodies, the percentage of cells positive for each antibody was measured. An FITC-conjugated IgG antibody (Coulter Ltd, UK) was used as a negative for the Fibrinogen antibody, whilst an IgG antibody (Immunotech, Birmingham, UK) was used with the P-selectin MoAb.

Plasma markers - Three plasma markers of platelet activation were measured at all six time-points. The factors measured were thromboxane B_2 (TxB_2), beta-thromboglobulin (βTG) and platelet factor 4 (PF4).

i. *βTG and PF4* Samples were collected, without tourniquet, into tubes containing 0.109M sodium citrate and citrate, supplemented with theophylline, adenosine and dipyridamole (Diatube®H, Diagnostica Stago, Asnieres-sur-Seine, France). The tubes are supplied vacuumed, but to reduce platelet activation on sampling the vacuum was released. The tubes were stored on crushed ice both pre- and post-sampling. Although reported stable in these conditions for one hour, all samples were centrifuged between 5 and 20 minutes following collection. Samples were centrifuged at 15 000g at -4°C for 30 minutes. The central part of the supernatant was separated into aliquots of 0.5mls and stored at -80°C. Beta-thromboglobulin and PF4 were measured using commercially available ELISA kits (Asserachrom® βTG, Diagnostica Stago, Asnieres-sur-Seine, France). All specimens were run in duplicate and the mean value determined.

ii. *TxB_2* Samples were collected as above into tubes containing citrate and aspirin, to prevent *in-vitro* formation of thromboxane A_2. As above, the tubes were stored on crushed ice both pre- and post-sampling. At intervals, between 5 and 20 minutes following collection, samples were centrifuged at 15 000g at -4°C. The central part of the supernatant was separated into aliquots of 0.5mls and stored at -80°C. Thromboxane B_2 was measured using a commercially available ELISA kit (ACE™, Cayman Chemical

Company, Ann Arbor, MI, USA). All specimens were run in duplicate and the mean value determined.

v. *Platelet Aggregation* Due to the volumes of blood required and the logistics of platelet aggregation studies samples were taken at time-points 1, 2, 4 and 5. Platelet aggregation was assessed in response to addition of ADP (Final concentrations 1.0μmol/l and 2.5μmol/l), Adrenaline (Final concentrations 0.005mmol/l and 0.0025mmol/l) and alpha-thrombin (Final concentration 5.5units/ml) Limitation on the total volume of blood used in this study resulted in the determination of only two measures of platelet aggregation; the maximum aggregation and the aggregation after 3 minutes.

The aggregometer was switched on 20-25 minutes prior to use to allow warming to 37°C. The optical reference levels for each chamber was set using water blanks. A 20 ml blood sample was taken at each time point into tubes containing 0.5ml of 0.0105mol/l tri-sodium citrate. Samples 1 and 2 were processed together, and then samples 4 and 5. The samples were centrifuged, within 15minutes of collection, at 250g for 10 minutes. The resulting supernatant (platelet rich plasma (PRP)) was removed, using a plastic pipette, and kept at room temperature for 30 minutes. The remaining sample was centrifuged at 1400g for 10 minutes to produce a supernatant of platelet poor plasma (PPP). This was also removed with a plastic pipette. A platelet count for the PRP was obtained using a Coulter MAXM machine. All PRP samples were then diluted with PPP to achieve a platelet count of 250 x 10^9/l. Four cuvettes of 0.05 ml of PPP were placed in incubating wells. At each time-point studied, the aggregation of PRP in response to various concentration of different agonist was studied. Cuvettes of 0.45ml of PRP were aliquoted

for each test point and placed in the incubator. After at least 5 minutes in the incubator a magnetic stirring bar was placed in each cuvette, the stirring rate was set at 1000 rpm.. Prior to each sample of PRP run, a cuvette of PPP was placed in the optical chamber and the "PPP" switch pressed, to set the 0% level. Then the PRP cuvette was inserted and the appropriate concentration of agonist added. Aggregation was measured as the maximum percentage fall in optical density and at 3 minutes in each case.

vi. ***Markers of Coagulation Activation*** A 4.5ml blood sample was taken into 0.5mls of 0.0105mol/l tri-sodium citrate (standard clotting studies Vacutainer). Activated partial thromboplastin time was determined in the haematology laboratory using standard techniques. The remaining sample was then centrifuged at 2 500g for 10 minutes at -4°C. The central part of the supernatant was separated into aliquots of 0.5mls and stored at -80°C. When adequate numbers had been collected to "batch" for analysis, or within a month, D-dimer, F1 & 2 fragments and TAT were measured using commercially available ELISA kits (Asserachrom, Stago). All samples were run in duplicate and mean values determined for each subject at each time point.

The results were plotted against time and comparisons made between patients and controls, over time, between exercise periods and between patients receiving and not receiving aspirin.

d. STATISTICAL ANALYSIS

Statistical analysis was performed with the help and advice of Dr B Uddin, Department of Mathematics and Statistics, University of New South Wales, Sydney, Australia.

III.5 RESULTS

i. Physical Measurements

Exercise parameters

Patients walked similar distances for the two exercise periods, and on and off aspirin. This data also demonstrates that the exercise time chosen for controls was similar to that achieved by the claudicant groups.

	First Exercise Period			Second Exercise Period		
	Speed (km/hr)	Time (mins)	Distance (m)	Speed (km/hr)	Time (mins)	Distance (m)
Controls	2.75	5.025	232	2.75	5.025	232
Patient (No Aspirin)	2.73	4.82	227	2.76	5.25	254.6
Patient (Aspirin)	2.74	5.03	237	2.74	4.76	231.7

Fig 3.8 Exercise parameters (speed, distance walked and time of exercise) for controls and patients.

Ankle Brachial Pressure Index (ABPI)

These results were analysed using repeated measures t-test of the differences, significance level is taken as $p < 0.05$, and results are given with confidence intervals.

At rest the controls had a "normal" Ankle brachial Pressure Index (ABPI) of 1.071 (1.007 - 1.135) compared to a value of 0.558 (0.382 - 0.734) in those claudicants not on aspirin and 0.579 (0.409 - 0.751) in claudicants on aspirin. There was, not suprisingly, a significant difference between controls and claudicants, whether they took aspirin or not

($p < 0.001$). However, aspirin did not influence ABPI measurements in claudicants ($p < 0.05$). Following exercise the control group ABPIs increased significantly to 1.199 (1.013 - 1.359) ($p = 0.008$). Conversely, the ABPIs fell in the claudicants; to 0.237 (0.05 - 0.424) in the group not on aspirin, and to 0.231 (0.029 - 0.436) in those taking aspirin. These were both significant falls ($p < 0.001$) but again aspirin made no difference. The second exercise period produced similar results as the first, and although the absolute figures were slightly different, this difference was not significant ($p = 0.978$).

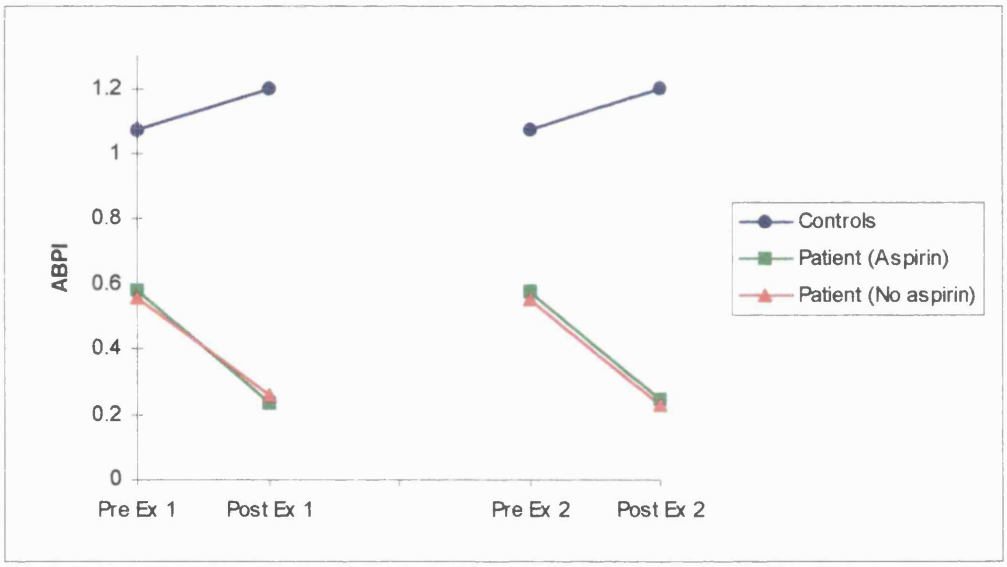


Fig 3.9 Changes in Ankle Brachial Pressure Indices (ABPI) following two periods of exercise in controls and in patients, with and without aspirin.

Pressure Recovery Time (PRT)

Following the first exercise period, patients on aspirin took 8.6mins (3.5 - 14.5) for their ABPIs to return to normal, compared to 8mins (3.0 - 14.0) after the second period. Those patients not on aspirin took 8.6mins (3.76 - 21.3) and 9mins (3.5 - 20) to recover after the two exercise periods respectively.

	PRT	PRT
	Post Ex 1	Post ex 2
Controls	0	0
Patient (Aspirin)	8.6	8
Patient (No aspirin)	8.6	9

Fig 3.10 Pressure Recovery Times (PRT) in minutes for controls and patients.

ii. Microalbuminuria

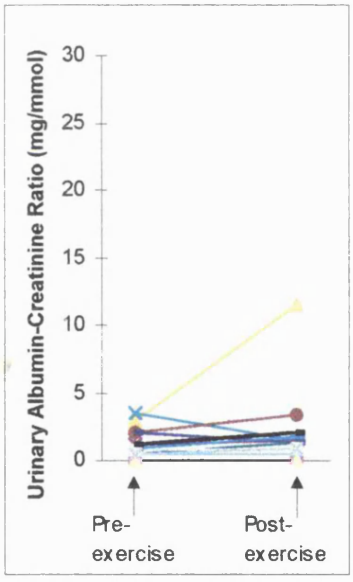


Fig 3.11 Microalbuminuria in controls pre- and post-exercise

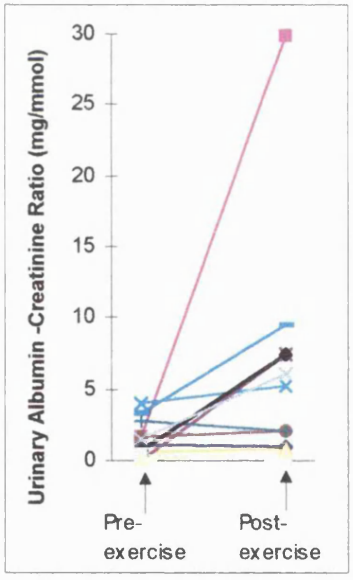


Fig 3.12 Microalbuminuria in patients taking aspirin pre- and post-exercise

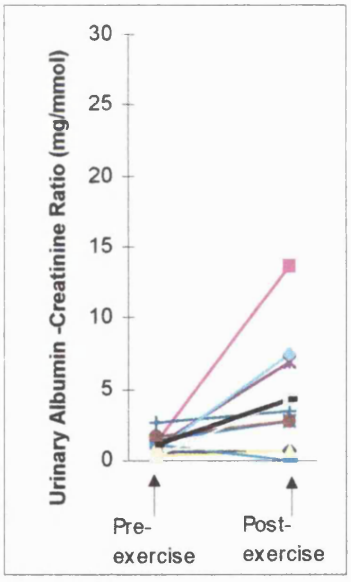


Fig 3.13 Microalbuminuria in patients not taking aspirin pre- and post-exercise

At rest, or prior to exercise, there was little difference in microalbuminuria between controls, 1.225 (0.496 - 1.95) and patients, those on aspirin 1.455 (0.542 - 2.367) and those not 1.064 (0.618 - 1.51). Following the two periods of exercise microalbuminuria

increased to 2.017 (0.03 - 4) in controls. Claudicants on aspirin increased their excretion of albumin to 5.99 (0.22 - 11.77), whilst patients not on aspirin increased to 4.25 (1.2 - 6.65). Differences where analysed using paired t-test for the differences. The no aspirin group showed a significant difference ($p = 0.04$), but the patient group on aspirin and the controls showed no significant differences ($p = 0.11$ & $p = 0.32$ respectively).

iii. Platelet count and size

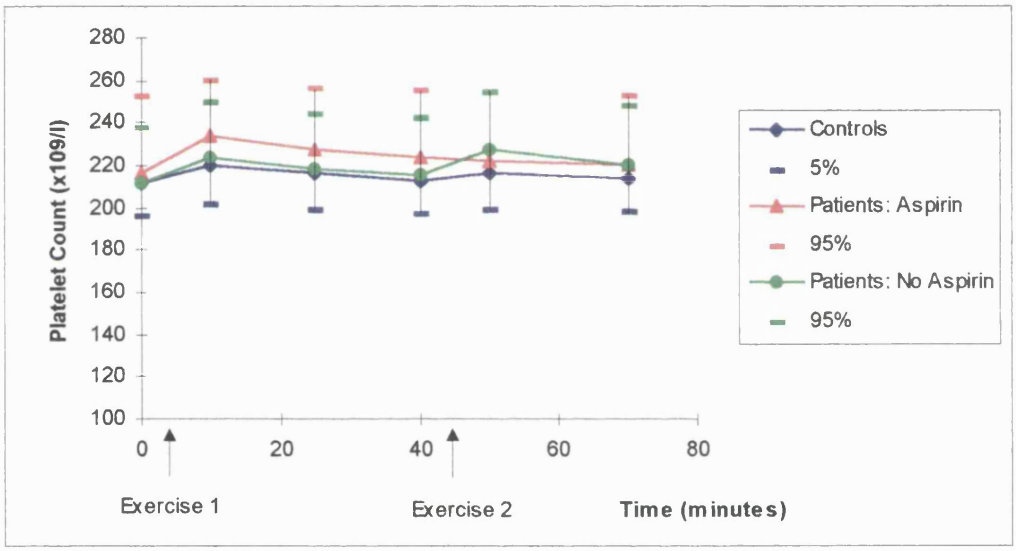


Fig 3.14 Platelet counts in controls and patients, with and without aspirin, over the course of the study, with 95% Confidence Intervals (CI).

Following the first exercise period all groups showed a small increase in platelet count. However, one-way repeated measures analysis of variance revealed no significant difference over time in any of the groups (controls: $p = 0.99$, patients on aspirin: $p = 0.99$, and patients not on aspirin: $p = 0.097$), and using two-way repeated measures analysis of variance (balanced design) showed no difference between the groups, with respect to group ($p = 0.57$), time ($p = 0.92$), or group by time ($p = 1$).

Mean platelet volumes in the control group were initially 8.84 fl(8.36 - 9.24), remained static following the first exercise period and then progressively fell to 8.28 fl(7.84 - 8.72) by the end of the study ($p = 0.51$, one-way repeated measures analysis of variance). Both claudicant groups showed a slightly different pattern. Two way repeated measures ANOVA revealed a difference with respect to group ($p < 0.001$), but not time ($p = 0.55$) or group by time ($p = 1$). Initially the patient groups had higher platelet volumes than controls; 9.66 fl(9.00 - 10.30) for patients on aspirin and 9.32 fl(8.58 - 10.26) for those not on aspirin. These values, and the pattern, are significantly different to control group ($p = 0.003$, multiple comparison testing) but not significantly different compared to each other. Following the first exercise period both claudicant groups showed a slight rise in platelet volume to 9.71 fl(9.05 - 10.35) for patients on aspirin and 9.55 fl(8.76 - 10.50) for those not on aspirin at 15 minutes post-exercise. Thereafter, values fell to 9.22 fl(8.55 - 9.85) for patients on aspirin and 9.05 fl(8.28 - 10.02) for those not on aspirin by the end of the study. One way repeated measures analysis of variance applied to both groups shows no significant differences over time ($p = 0.91$ and 0.97 respectively).

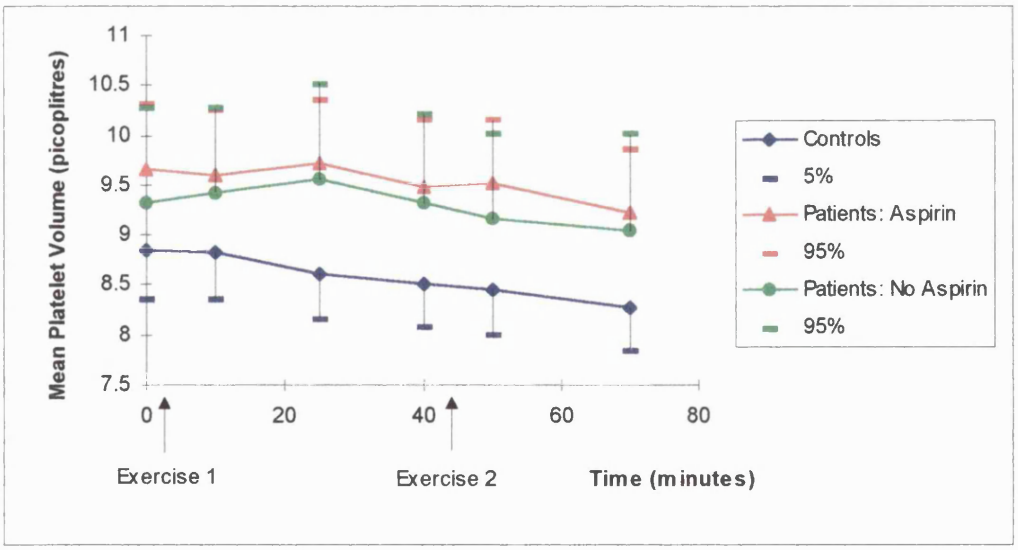


Fig 3.15 Mean platelet volumes in controls and patients, with and without aspirin, over the course of the study, with 95% (CI) .

iv. Platelet markers

Surface glycoproteins

Analysis of this data was difficult, partly due to the repetitive measurement data, but also because of the large variation in results between subjects in each of the groups.

Fibrinogen binding in unstimulated platelets

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	2.39 (2.18-2.61)	2.2 (1.89-2.52)	2.54 (1.77-3.32)	1.81 (1.33-2.29)
Patients: Aspirin	2.37 (1.61-3.13)	2.76 (1.73-3.79)	3.72 (1.78-5.67)	4.23 (0.74-7.73)
Patients: No aspirin	2.56 (1.91-3.2)	3.72 (1.78-5.67)	4.23 (0.74-7.7)	2.56 (1.91-3.2)

Fig 3.16 Fibrinogen binding, expressed as % positive cells, in unstimulated platelets, in patients and controls over time, with 95% CI.

There is very little effect of the first period of exercise on the binding of fibrinogen to unstimulated platelets in any of the groups (Fig 3.16). Controls show very little change with the second exercise, although patient groups show a slight tendency to increased binding both pre and post the second exercise. However, one way repeated measures analysis found no significant difference over time in controls ($p = 0.13$), patients on aspirin ($p = 0.49$) and patients not on aspirin ($p = 0.52$). Two-way repeated measures analysis of variance found no differences between the groups with respect to time ($p = 0.09$), group ($p = 0.2$) or group by time ($p = 0.09$).

P-selectin binding in unstimulated platelets

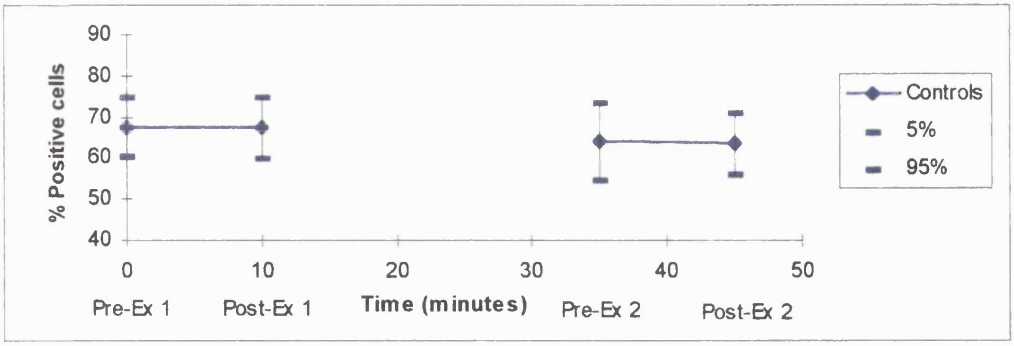
	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	2.49 (2.12-2.87)	2.09 (1.72-2.46)	2.29 (1.64-2.94)	2.27 (1.47-3.07)
Patients: Aspirin	2.2 (1.89-2.5)	1.74 (1.44-2.04)	1.72 (1.32-2.12)	1.6 (1.17-2.04)
Patients: No aspirin	2.49 (2.25-2.75)	2.15 (1.83-2.46)	2.43 (1.6-3.25)	2.06 (1.49-2.62)

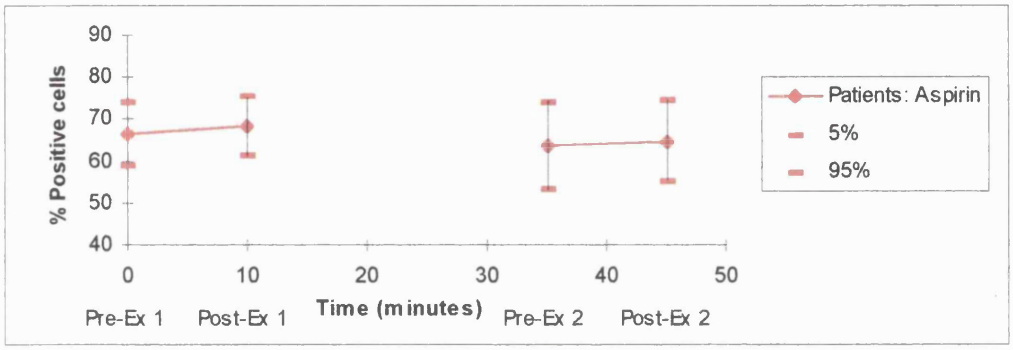
Fig 3.17 P-selectin binding, expressed as % positive cells, in unstimulated platelets, in patients and controls over time, with 95% CI.

Very little change is seen in the binding of P-selectin in unstimulated platelets following exercise, with time or between groups (Fig 3.17). One way repeated measures analysis performed to assess changes within the groups, found no significant difference over time in controls ($p = 0.76$), patients on aspirin ($p = 0.07$) and patients not on aspirin ($p = 0.52$). Two-way repeated measures analysis of variance found no differences between the groups with respect to time ($p = 0.26$), group ($p = 0.20$) or group by time ($p = 0.65$).

Fibrinogen binding with ADP

Fibrinogen binding with ADP was studied at three different concentrations of ADP and pre- and post-exercise for the two exercise periods. Binding of fibrinogen with 1×10^{-6} mol/l ADP (Fig 3.18) showed an increase in each of the groups after the first exercise period. However, immediately prior to the second exercise test all groups had nearly returned to their starting values. The second exercise test produced a fall in fibrinogen binding in controls and patients not on aspirin, but an increase in the aspirin group.





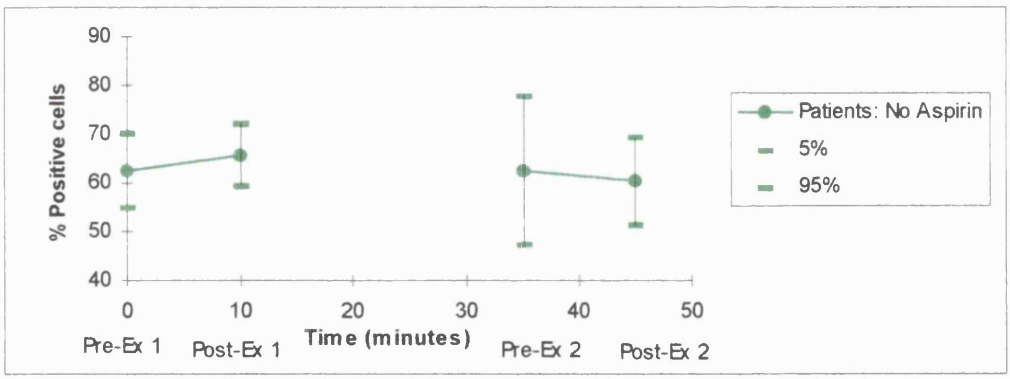


Fig 3.18 Platelet binding of Fibrinogen in response to 1 x 10^{-6}mol/l ADP, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

One way repeated measures analysis of variance found no significant differences over time in the controls ($p = 0.79$), patients taking aspirin ($p = 0.83$) or patients not receiving aspirin ($p = 0.82$). Two way repeated measures ANOVA to compare the groups found no difference with respect to group ($p = 0.73$), time ($p = 0.74$) or group by time ($p = 0.77$).

A similar response was seen with 3 x 10^{-7}mol/l ADP (Fig 3.19), although the effects of exercise were less marked. Again the aspirin group was the only one to show an increase

in fibrinogen binding with the second exercise test. One way repeated measures analysis of variance found no significant differences over time in the controls (p = 0.8), patients taking aspirin (p = 0.81) or patients not receiving aspirin (p = 0.99). Two way repeated measures ANOVA to compare the groups found no difference with respect to group (p = 0.68), time (p = 0.15) or group by time (p = 0.65).

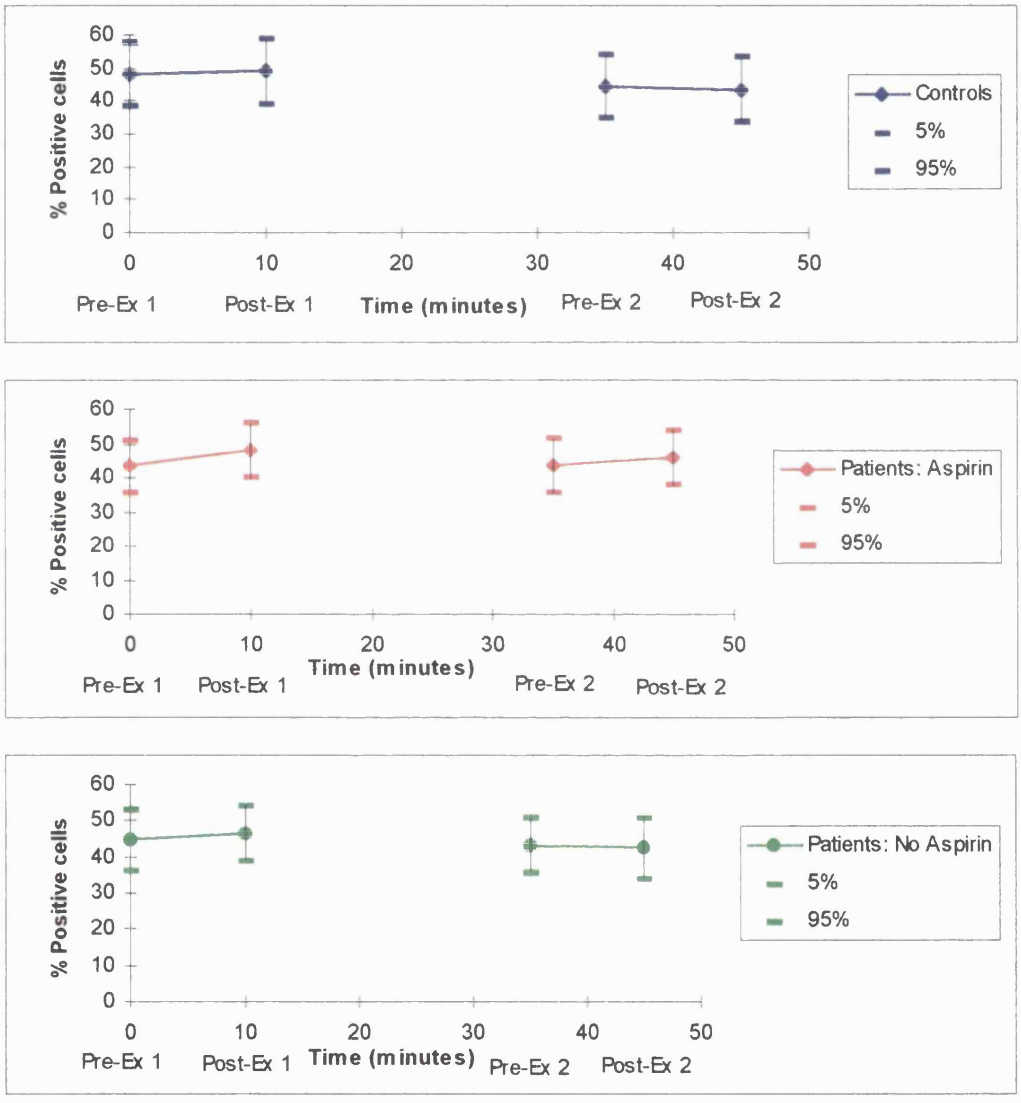


Fig 3.19 Platelet binding of Fibrinogen in response to 3 x 10^{-7}mol/l ADP, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

Fibrinogen binding in response to the lowest concentration of ADP, 1 x 10^{-7}mol/l (Fig 3.20), showed essentially no change, although the aspirin group tended to show a fall in binding at this concentration. Statistical analysis, using one-way repeated measures

analysis of variance, of the individual groups over time found no significant differences in controls ($p = 0.67$), patients on aspirin ($p = 0.91$), or patients not taking aspirin ($p = 0.87$). Two way repeated measures analysis of variance was used to look for differences between the groups. No differences were found with respect to group ($p = 0.86$) or group by time ($p = 0.62$), but a difference was found with respect to time ($p = 0.02$).

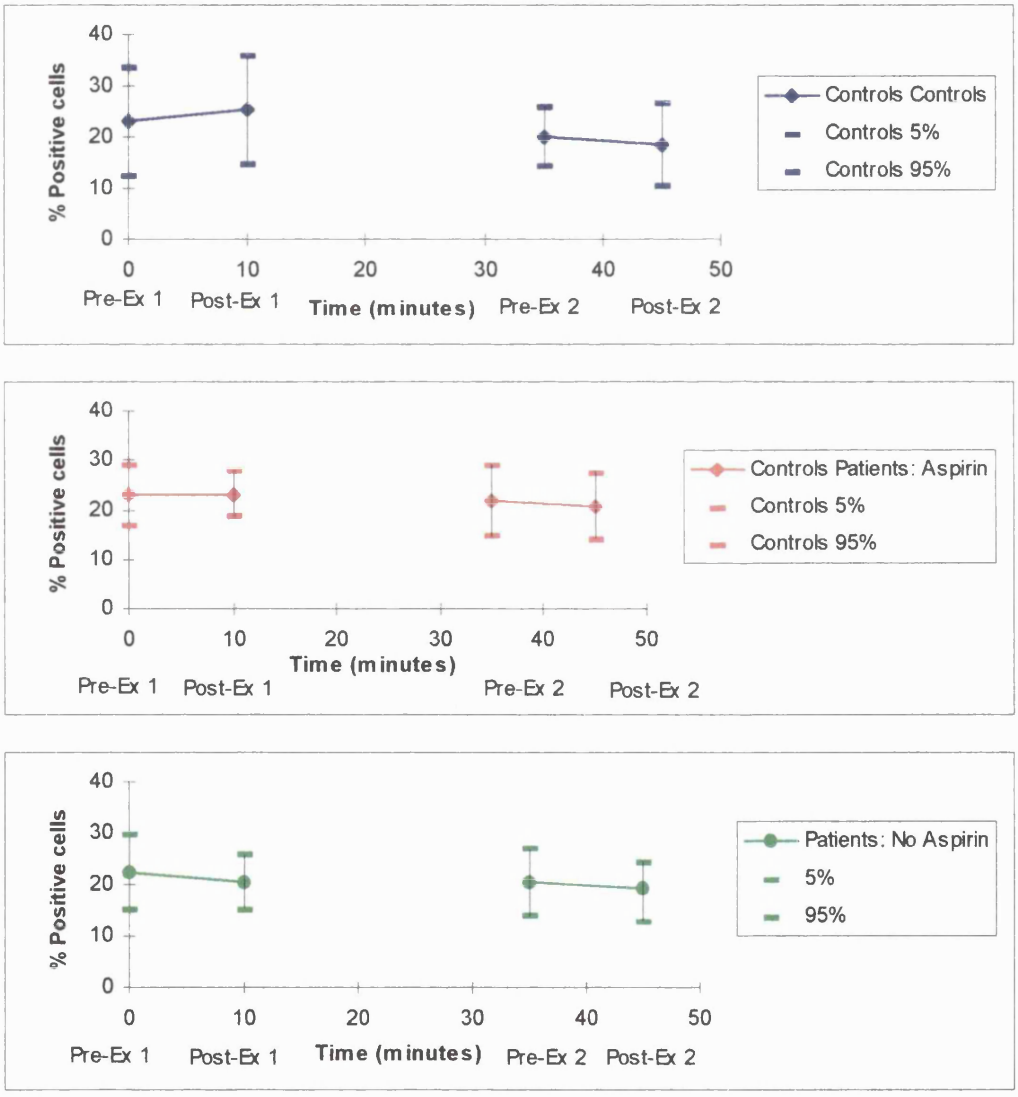
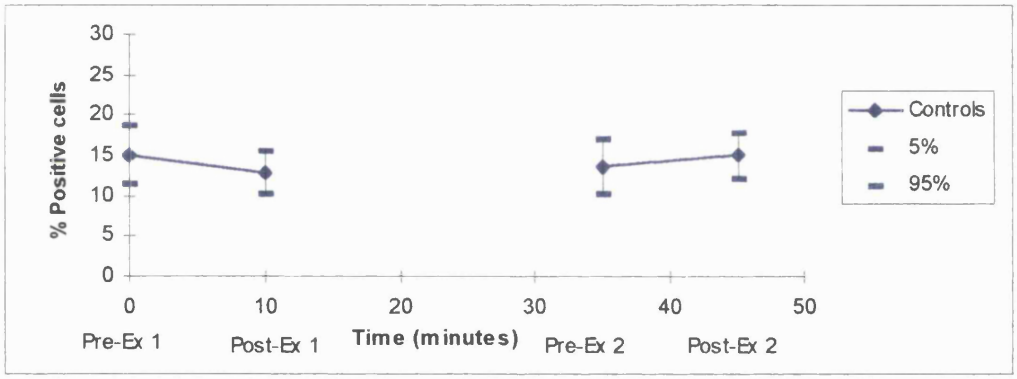
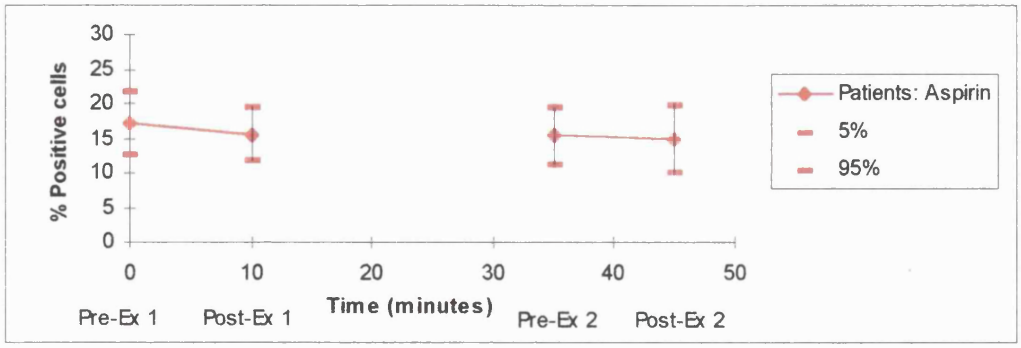


Fig 3.20 Platelet binding of Fibrinogen in response to 1 x 10^{-7}mol/l ADP, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

P-selectin binding with ADP

The binding of P-selectin in response to ADP was markedly lower than fibrinogen binding, with the same agonist concentrations (Fig 3.21, 3.22 and 3.23). Actual values showed small, non-significant changes in response to exercise, and there was no detectable difference between exercise periods. With P-selectin binding it appears all groups show a reduced response after the first exercise period, whilst the control and non-aspirin group demonstrate a tendency to an increased response after the second exercise period. However, none of these descriptive changes was statistically significant.





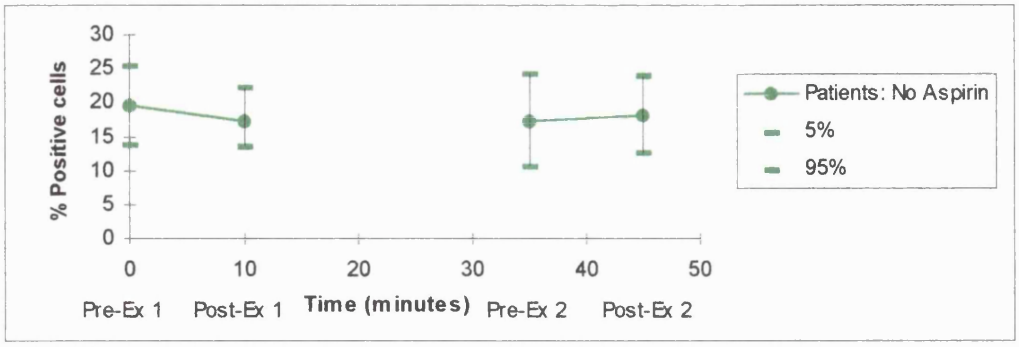
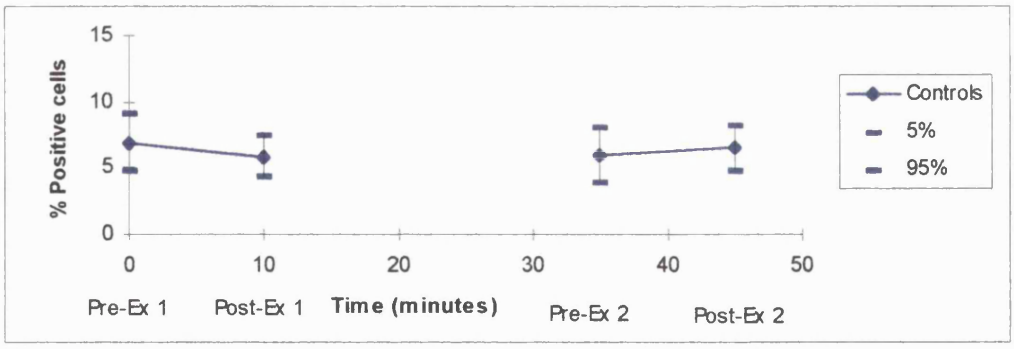
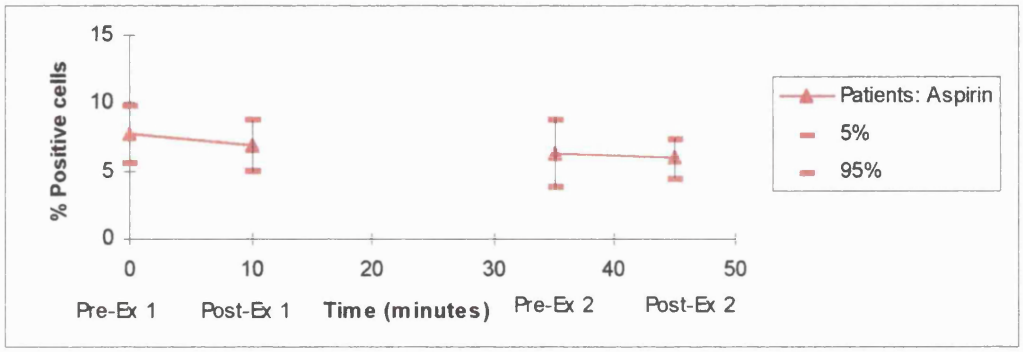


Fig 3.21 Platelet binding of P-selectin in response to 1 x 10^{-6}mol/l ADP, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

With the maximum concentration of ADP, 1 x 10^{-6}mol/l, one-way repeated measures ANOVA found no significant differences over time in either controls, patients on aspirin and patients not on aspirin ($p = 0.66$, $p = 0.85$ & $p = 0.92$ respectively). Two way repeated measures analysis of variance found no significance between the groups with respect to group or group by time ($p = 0.18$ & 0.86 respectively), but a significant difference with respect to time ($p = 0.05$).





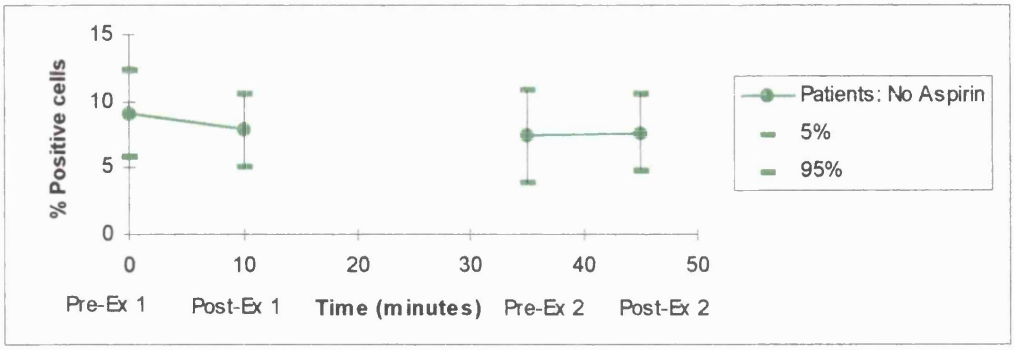
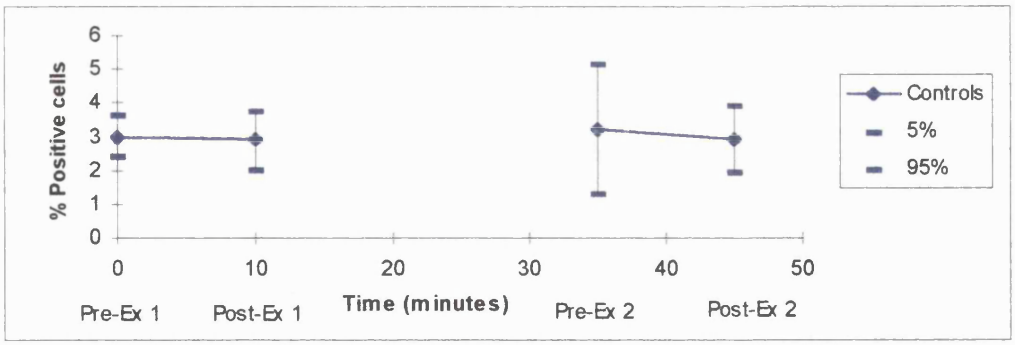
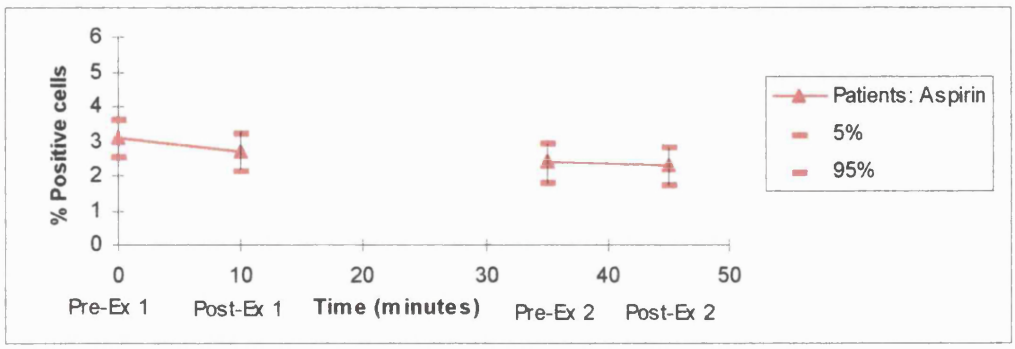


Fig 3.22 Platelet binding of P-selectin in response to 3 x 10^{-7}mol/l ADP, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

For the middle concentration of ADP, 3 x 10^{-7}mol/l, one-way repeated measures ANOVA found no significant differences over time in either controls, patients on aspirin or patients not on aspirin ($p = 0.79$, $p = 0.51$ & $p = 0.83$ respectively). Two way repeated measures analysis of variance found no significance between the groups with respect to group or group by time ($p = 0.74$ & 0.43 respectively), but a significant difference with respect to time ($p < 0.01$).





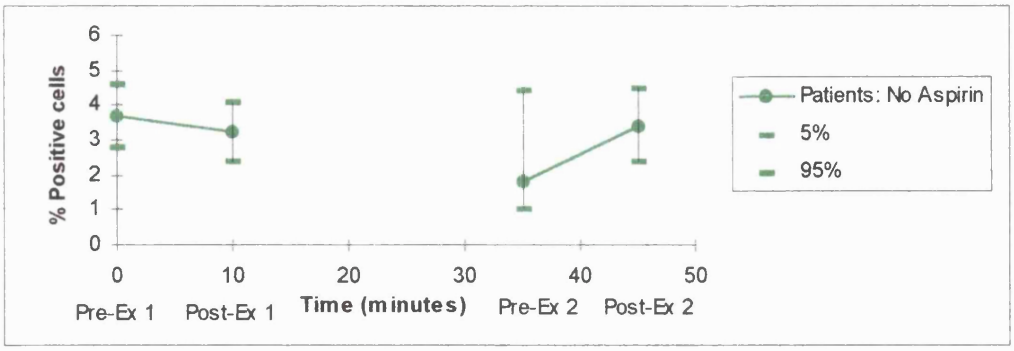


Fig 3.23 Platelet binding of P-selectin in response to 1 x 10^{-7}mol/l ADP, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

One-way repeated measures ANOVA found no significant differences over time in either controls, patients on aspirin and patients not on aspirin ($p = 0.97$, $p = 0.79$ & $p = 0.87$ respectively). Two way repeated measures analysis of variance found no significance between the groups with respect to group, time or group by time ($p = 0.51$, $p = 0.34$ & $p = 0.56$ respectively).

Fibrinogen binding with α- thrombin

The pattern of binding of fibrinogen following exercise varied with the concentration of α- thrombin. The first exercise period was associated with a fall in fibrinogen binding in all groups and at all three concentrations of α- thrombin, except a slight increase in the non-aspirin group at the higher concentration. However, the second exercise period produced differing responses. At the highest concentration of α-thrombin all groups showed reduced binding pre-exercise, but an increase after exercise (Fig 3.24). This effect was also seen in the control group at the middle concentration (Fig 3.25), but the other groups and all groups at the lower concentration (Fig 3.26) showed a decline in binding after the second exercise. One and two way ANOVA were performed to analyse the data.

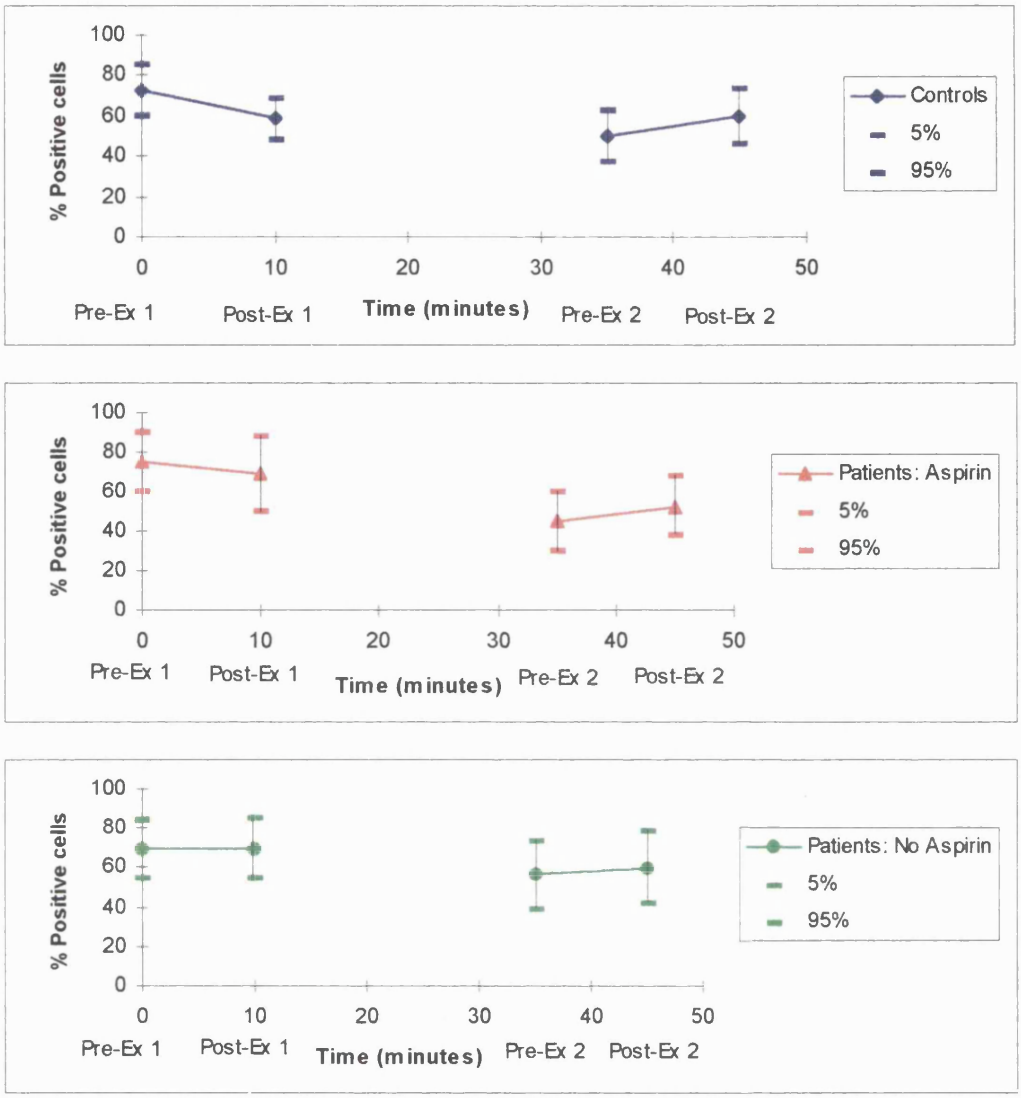


Fig 3.24 Platelet binding of Fibrinogen in response to 0.03units/ml α-thrombin, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

With 0.03 units/ml of α-thrombin, one way repeated measures analysis of variance showed a significant difference over time in patients receiving aspirin ($p = 0.03$), the difference occurring between time point 0 and prior to the second exercise period (Tukey's pairwise comparison, significance level $p = 0.05$). There were no difference in the other groups (controls: $p = 0.09$ and patients not on aspirin: $p = 0.57$). Two way repeated measures analysis of variance found no differences between the groups with respect to group or group by time ($p = 0.69$ & $p = 0.49$), but a difference with respect to time ($p < 0.01$).

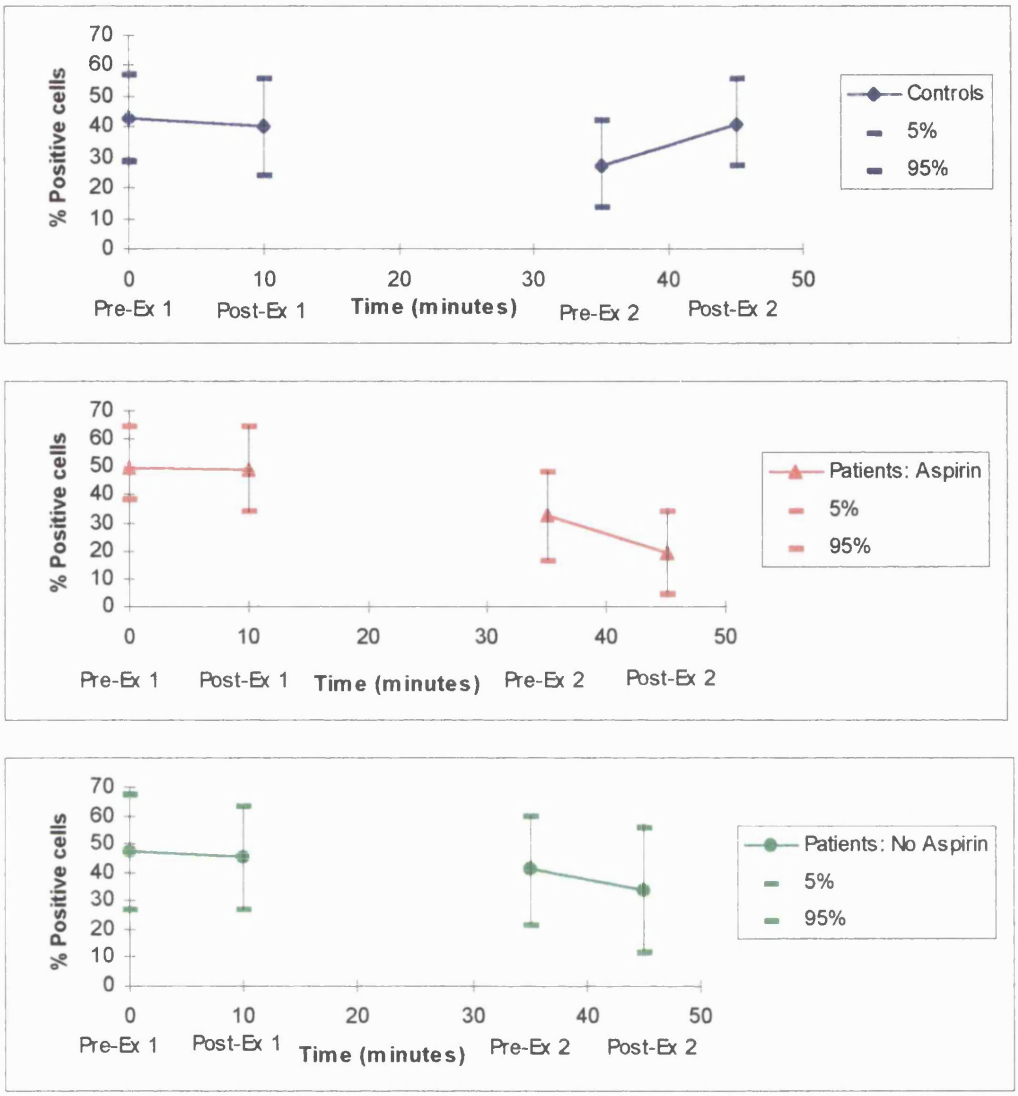


Fig 3.25 Platelet binding of Fibrinogen in response to 0.02units/ml α-thrombin, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

At the middle concentration of α-thrombin (Fig 3.25) patients taking aspirin again showed a significant difference over time when analysed with one-way repeated measures ANOVA ($p = 0.02$). The difference occurred between the values at 0 and 40 minutes and the value at 50 minutes (Tukey's pairwise comparison, significance level $p = 0.05$). There were no differences in the other groups (controls: $p = 0.42$ and patients not on aspirin: $p = 0.80$). A general linear model was used for two-way analysis due to missing values. There were no significant difference between the groups with respect to group, time or group by time ($p = 0.73$, 0.05 & 0.5 respectively).

One-way repeated measures analysis of variance found no differences within any of the groups at the lowest concentration of α-thrombin (Fig 3.26) (controls: p = 0.73, patients on aspirin: p = 0.48 & patients not on aspirin p = 0.66 respectively). Two way repeated measures analysis of variance found no differences between the groups with respect to group, time or group by time (p = 0.59, 0.54 & 0.83 respectively).

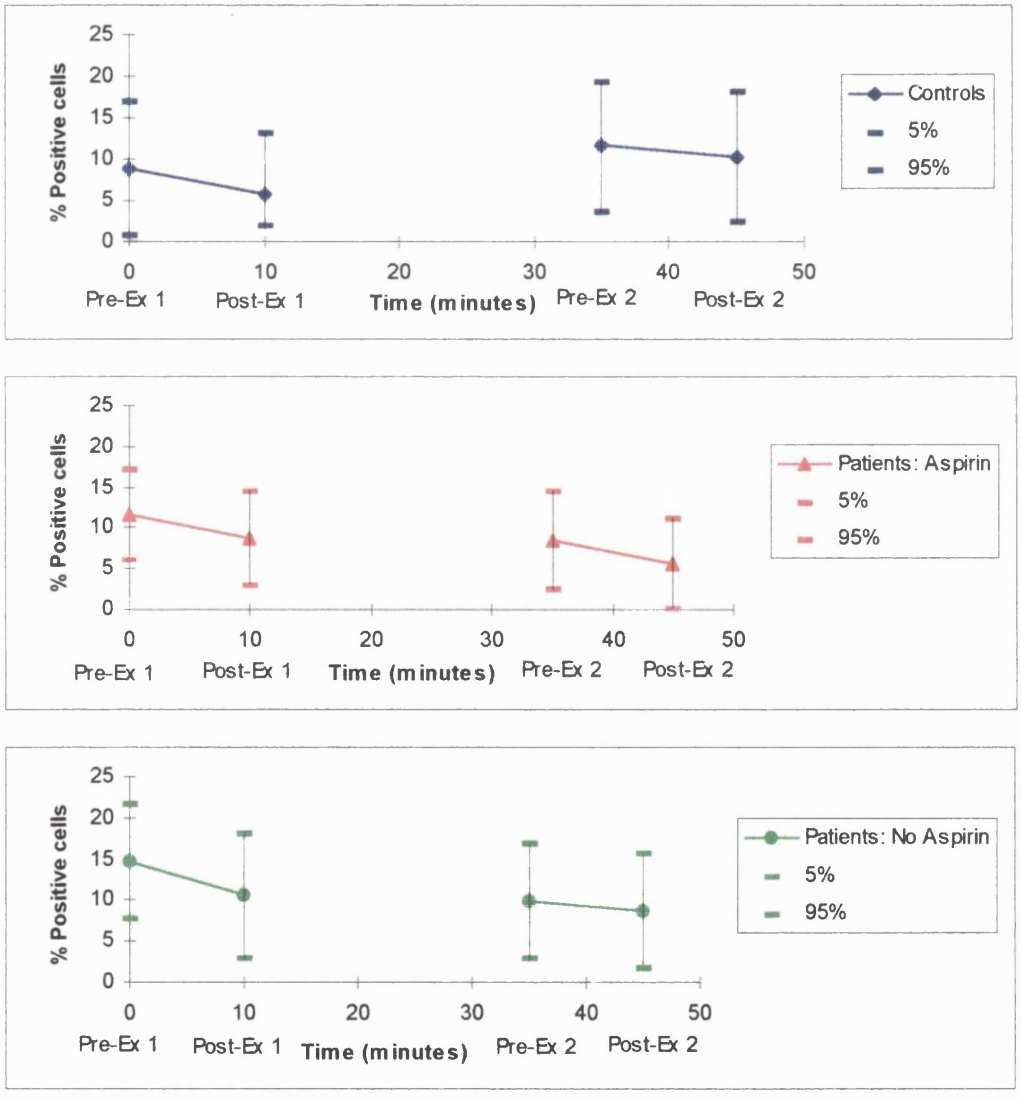


Fig 3.26 Platelet binding of Fibrinogen in response to 0.01units/ml α-thrombin, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

P-selectin binding with α-thrombin

Both the controls and the patient group on aspirin tended to show reduced binding of P-selectin after the first exercise period (Fig 3.27). This continued to 30 minutes post first exercise, or prior to the second exercise. There was then some increase in binding to approximately starting levels. The patient group not taking aspirin showed an increase in binding with both exercise periods, but a decrease between exercises.

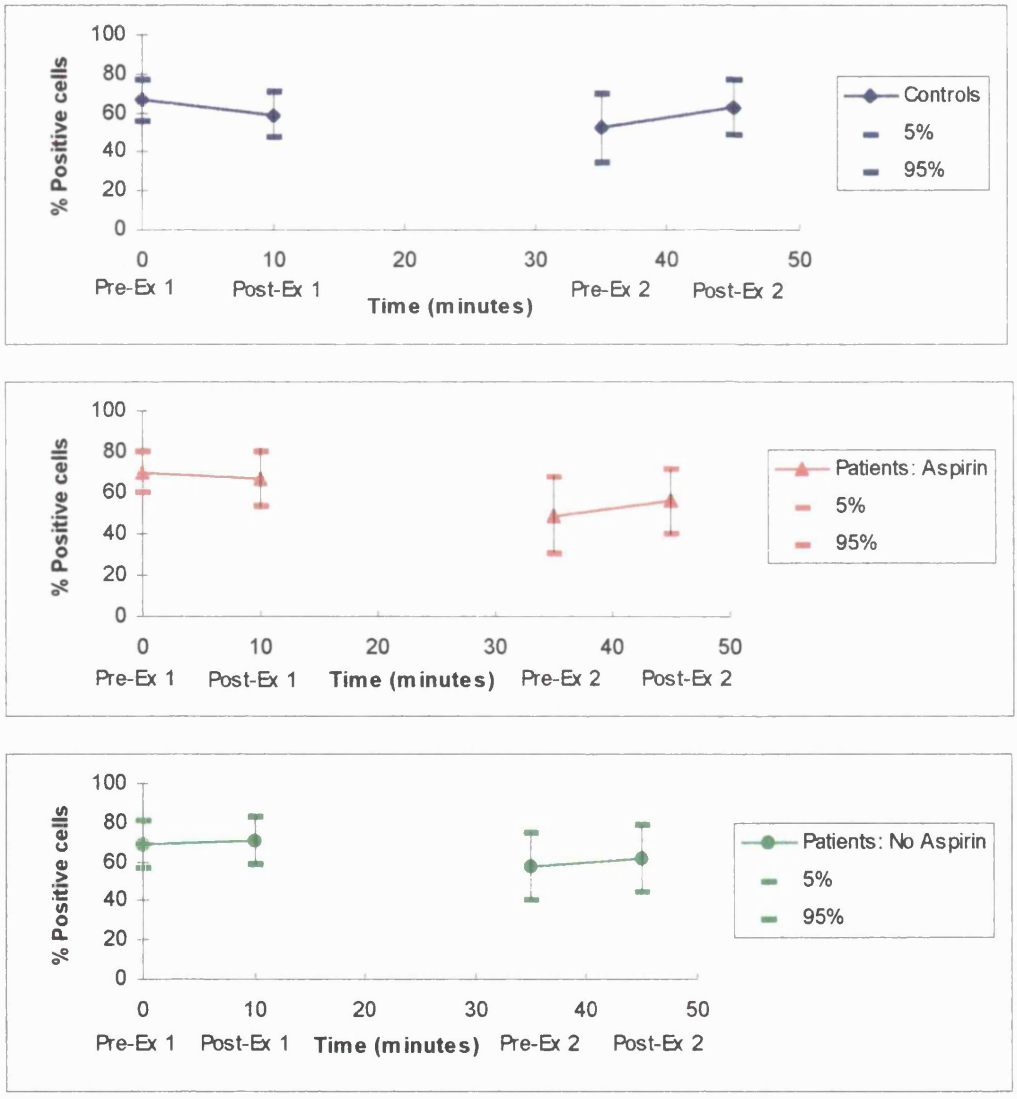


Fig 3.27 Platelet binding of Fibrinogen in response to 0.05units/ml α-thrombin, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

However, despite these apparent trends, one way repeated measures ANOVA found no significant differences within any group with respect of time (controls: $p = 0.42$, patients on aspirin: $p = 0.11$, patients not on aspirin: $p = 0.47$). Two way repeated measures ANOVA found no significant differences between the groups with respect to group ($p = 0.73$) or group by time ($p = 0.93$), but a difference with respect to time ($p = 0.05$).

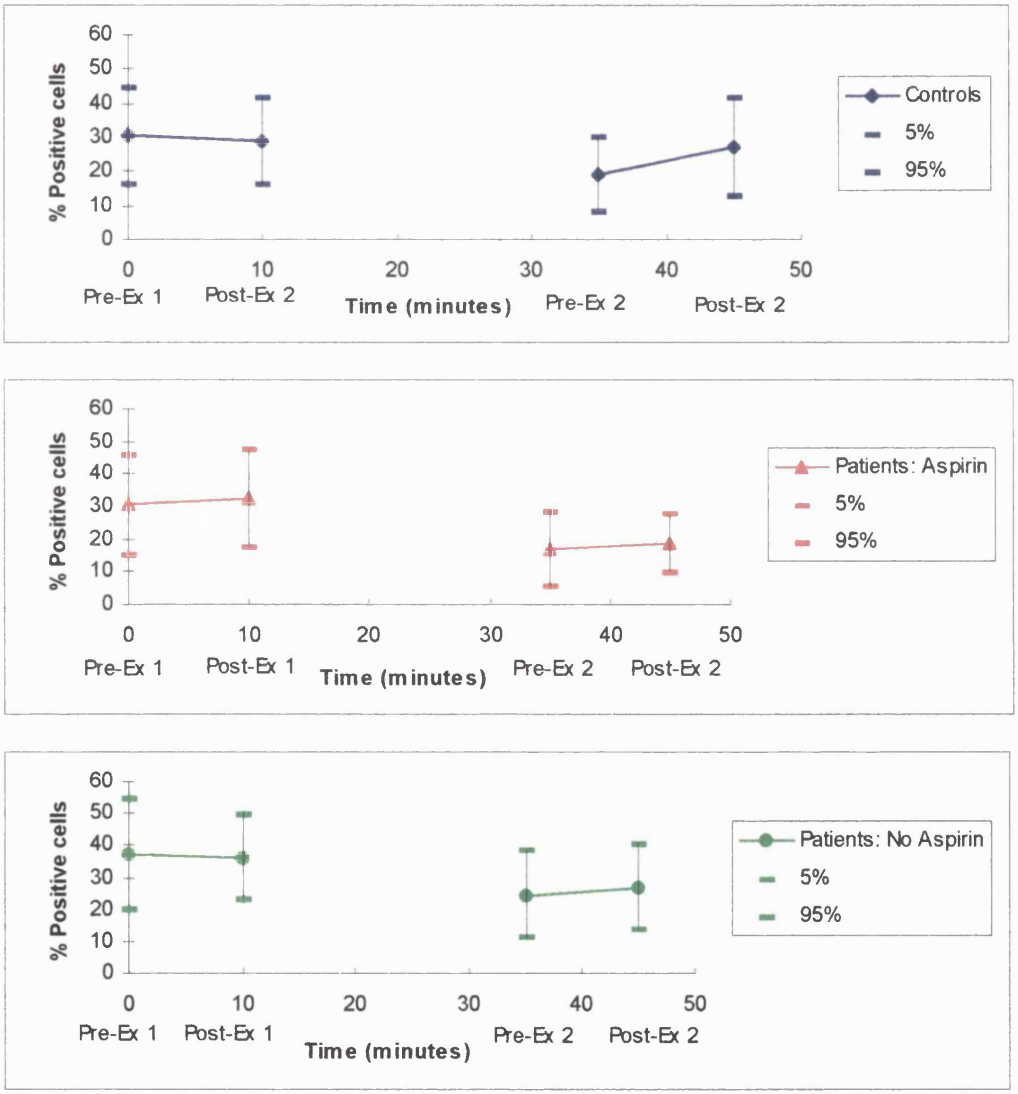
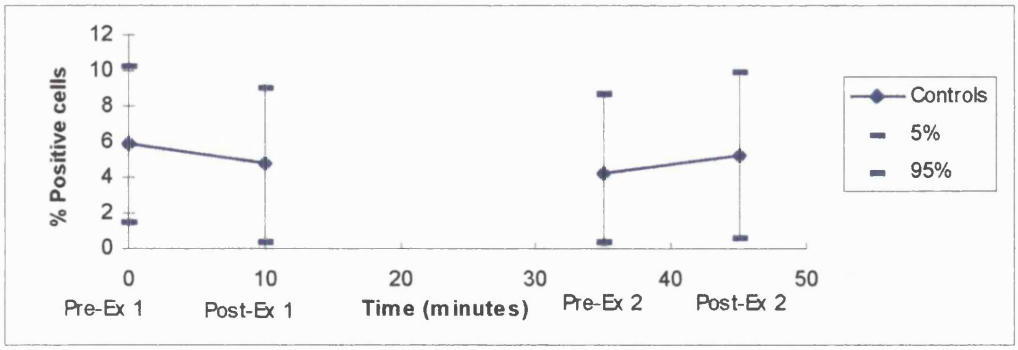


Fig 3.28 Platelet binding of Fibrinogen in response to 0.02units/ml α-thrombin, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

Binding with 0.02units α-thrombin (Fig 3.28) showed little changes following the first exercise period, but markedly reduced binding between the exercise times. The second exercise period was associated with a slight increase in binding.

Analysis of changes over time within the groups, using one-way repeated measures analysis of variance, found no differences in the controls ($p = 0.56$), patients on aspirin ($p = 0.15$) or patients not taking aspirin ($p = 0.43$). As might be expected two way repeated measures analysis found differences between the groups with respect to time ($p = 0.01$), but not with respect to group ($p = 0.47$) or group by time ($p = 0.92$).

At the lowest concentration of α-thrombin P-selectin (Fig 3.29) binding shows a fall following the first exercise period, with different starting levels of binding between the groups. There is little change in binding between the exercise periods, and little change following the second exercise period in the two patient groups. Controls show an increase in binding with the second exercise period. One way repeated measures ANOVA found no differences over time in the control group ($p = 0.95$), the patient group on aspirin ($p = 0.15$) or the patients not taking aspirin ($p = 0.15$).



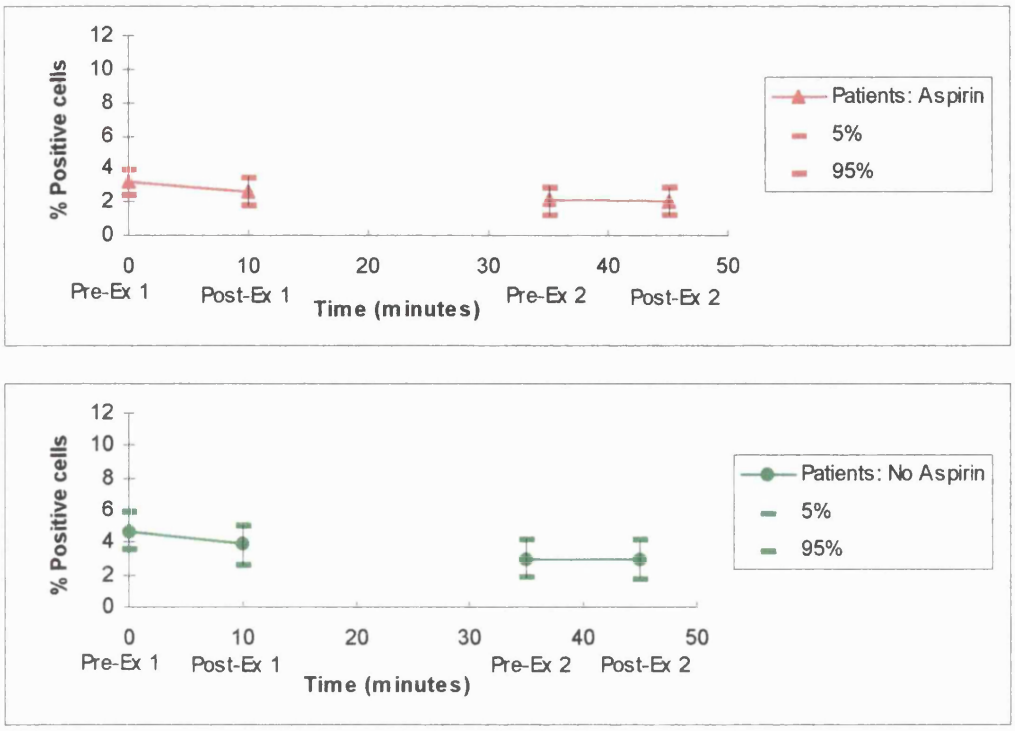


Fig 3.29 Platelet binding of Fibrinogen in response to 0.01units/ml α-thrombin, expressed as the percentage of positive cells, over time for controls and patients, with 95% CI.

Two way repeated measures analysis of variance found no significant differences between the groups with respect to group ($p = 0.42$) or group by time ($p = 0.7$, but a significant difference with respect to time ($p = 0.02$).

Plasma markers

Thromboxane B_2 (Fig 3.30)

Patients taking aspirin, which inhibits generation of thromboxane A_2, had little or no thromboxane A_2 generation initially or over time following exercise (the ELISA gives < 10 pg/ml as its lowest measure, hence values are not exactly zero). Both patients not receiving aspirin and controls had similar resting levels of thromboxane B_2, which were higher than in patients taking aspirin. These two groups showed little change over the course of the study until the final time point when levels increase markedly. On statistical

analysis using one-way repeated measures ANOVA no significant difference over time in any of the three groups was achieved (controls: $p = 0.08$, patients on aspirin: $p = 0.16$, patients not on aspirin: $p = 0.38$). Analysis of differences between the groups found a significant difference with respect to group ($p = 0.02$), but not with respect to time or group by time ($p = 0.07$ & 0.16 respectively).

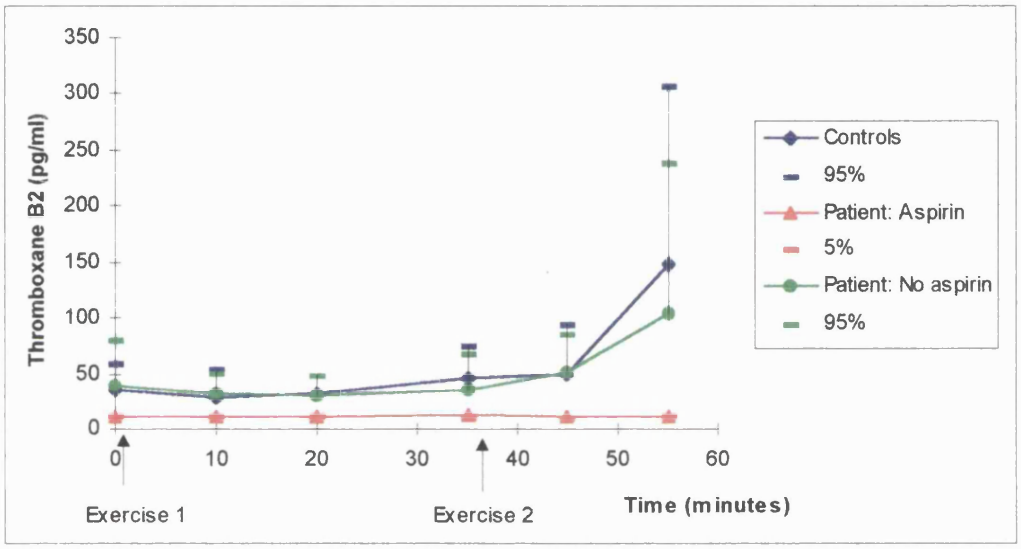


Fig 3.30 Concentration of thromboxane B_2 (picograms/ml) in controls and patients, with and without aspirin, over the course of the study, with 95% CI.

Beta-thromboglobulin

Beta-thromboglobulin and platelet factor 4 are released from platelet granules and not suprisingly have nearly identical patterns of release (Fig 3.31, 3.32). This, and a ratio of βTG:PF4 of around 3.5-5, suggests sampling methods have been adequate. Both plots show patients not on aspirin to have the highest resting levels, followed by controls and finally patients taking aspirin. The first exercise period was associated with an increase in levels in all groups, with a fall to starting levels or below at 15 minutes post-exercise. By

30 minutes post-exercise values have returned to starting levels. The second exercise period is associated with a fall in levels of both markers, less pronounced in patients on aspirin. The final measurement is increased in all groups, again less so in the aspirin group. Thus, the variations with the second exercise period are almost completely reversed from the first exercise test.

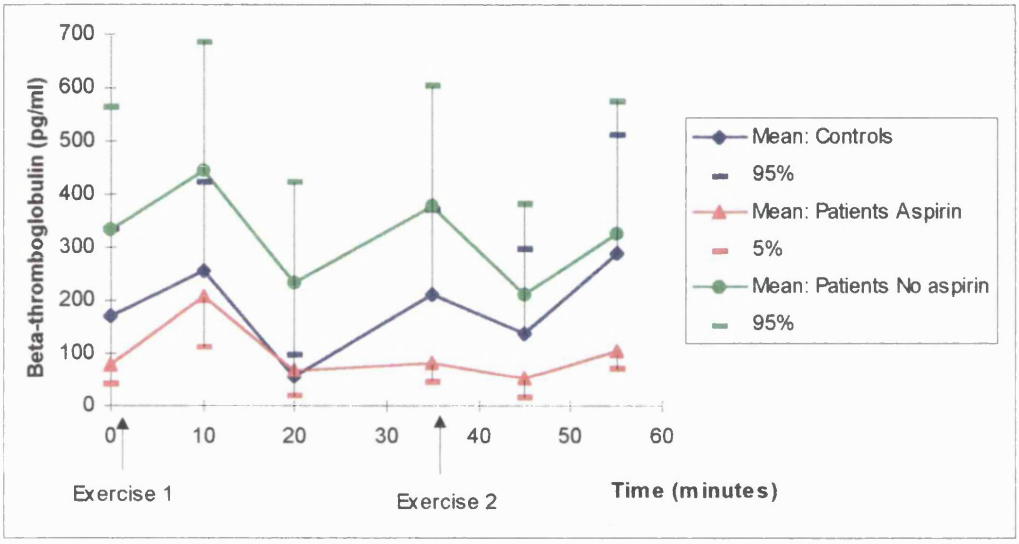


Fig 3.31 Concentration of beta-thromboglobulin (picograms/ml) in controls and patients, with and without aspirin, over the course of the study, with 95% CI.

Using one-way repeated measures analysis of variance the patient group taking aspirin showed significant changes over time ($p = 0.03$), with the value immediately following the first exercise period significantly different at a significance level of 0.05 (Tukey-HSD test). However, despite larger fluctuations of values neither the control or patient group not taking aspirin showed any significant differences over time ($p = 0.29$, and $p = 0.57$ respectively). This in part is due to the large intragroup variation of values. Comparison of the groups using two-way repeated measures analysis of variance found no difference with respect to group or group by time ($p = 0.14$ and $p = 0.76$ respectively), but a

difference with respect to time ($p < 0.01$), between patients receiving aspirin, and controls and patients not on aspirin (Tukey-HSD test, significance level $p < 0.05$).

Platelet Factor 4

The patterns of plasma PF4 were very similar to those of βTG. One way repeated measures ANOVA revealed no significant changes over time in controls ($p = 0.29$) or patients taking aspirin ($p = 0.32$). Patients taking aspirin showed changes over time ($p = 0.03$), with the 5 minute post first exercise period being significantly higher than resting or subsequent values (Tukey-HSD test, significance level $p < 0.05$). Comparison of the groups using two-way repeated measures analysis of variance found no difference with respect to time ($p = 0.06$), but a difference with respect to group ($p < 0.05$) and group by time ($p = 0.001$), between patients receiving aspirin and patients not on aspirin (Tukey-HSD test, significance level $p < 0.05$).

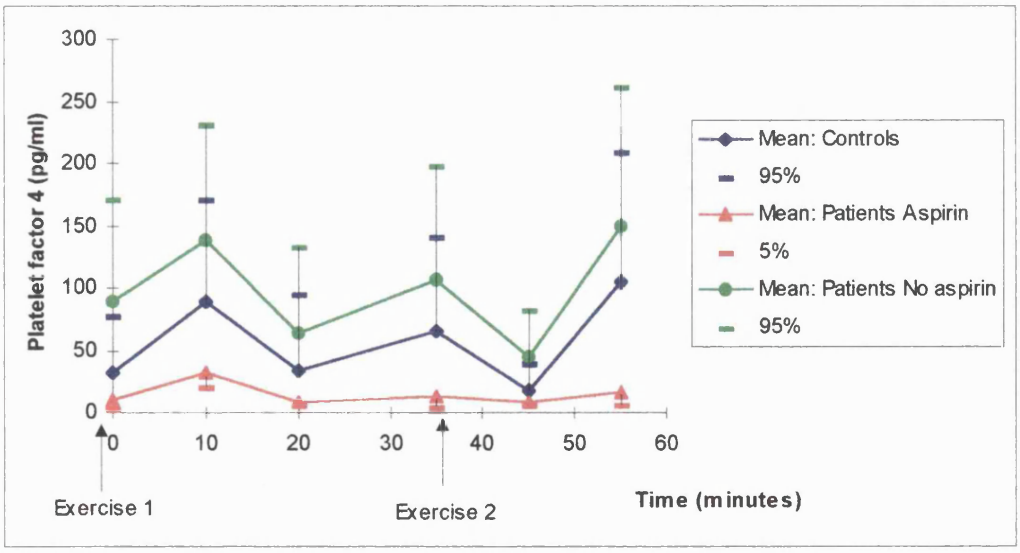


Fig 3.32 Concentration of platelet factor 4 (picograms/ml) in controls and patients, with and without aspirin, over the course of the study, with 95% CI.

v. ***Platelet Aggregation***

Aggregation with different agonists at varying concentrations is shown in tabular form, with means and confidence intervals. The results of the maximum aggregation studies are shown. Statistical analysis was performed using one-way repeated measures analysis of variance to assess differences within each group with respect to time. Where complete sets of paired data were available analysis was confirmed using paired t-test. Two-way repeated measures analysis of variance was used to compare differences between the groups. Any points of difference were then analysed using Tukey's multiple range test.

ADP 1.0μM

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	20.2 (6.67-33.67)	25.42 (9.68-41.15)	16.25 (8.38-24.12)	16.25 (8.38-24.12)
Patients: Aspirin	20.8 (11.76-29.88)	19.73 (9.83-29.63)	15.8 (6.23-25.4)	17.64 (7.53-27.74)
Patients: No aspirin	21.7 (3.91-39.51)	29.63 (7.99-51.26)	14.73 (5.69-23.77)	22.82 (0.91-44.72)

Fig 3.33 Maximum aggregation in response to 1.0μM ADP in patients and controls over time, with 95% CI.

Analysis within the groups over time found no significant difference for controls ($p = 0.58$), patients on aspirin ($p = 0.85$) or patients not receiving aspirin ($p = 0.66$). Two way repeated measures analysis of variance found no differences with respect to group or group by time ($p = 0.69$ & 0.43 respectively), but a difference with respect to time ($p = 0.01$).

ADP 2.5μM

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	61.17 (44.3-78.04)	64.88 (47.8-81.9)	62.88 (46.7-79.1)	57.2 (37.3-77.1)
Patients: Aspirin	50.4 (38.2-62.5)	53.05 (41.3-64.8)	46.1 (30.9-61.2)	42.5 (27.9-56.94)
Patients: No aspirin	55.9 (38.4-73.5)	56.4 (38.8-73.9)	55.3 (35.9-74.6)	57.9 (38.2-77.5)

Fig 3.34 Maximum aggregation in response to 2.5μM ADP in patients and controls over time, with 95% CI.

Analysis within the groups over time found no significant difference for controls ($p = 0.92$), patients on aspirin ($p = 0.61$) or patients not receiving aspirin ($p = 0.99$). Two way repeated measures analysis of variance found no differences with respect to group, time or group by time ($p = 0.27$, 0.06 & 0.14 respectively).

Adrenaline 0.0025mmol/l

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	55.1 (34.4-75.9)	49.1 (26.7-71.6)	54.2 (32.5-75.9)	49.5 (27.8-71.3)
Patients: Aspirin	22.2 (13.4-31.05)	23.3 (13.6-33)	24.5 (14.2-34.9)	24.5 (14.7-34.2)
Patients: No aspirin	50.6 (27.6-73.7)	42.5 (19.9-64.9)	37.5 (15.4-59.6)	47.7 (24.9-70.5)

Fig 3.35 Maximum aggregation in response to 0.0025mmol/l adrenaline in patients and controls over time, with 95% CI.

Analysis within the groups over time found no significant difference for controls ($p = 0.96$), patients on aspirin ($p = 0.98$) or patients not receiving aspirin ($p = 0.81$). Two way repeated measures analysis of variance found no differences with respect to time or group by time ($p = 0.17$ & 0.31 respectively), but a significant difference with respect to group ($p = 0.03$), the aspirin group having lower values than the other two.

Adrenaline 0.005mmol/l

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	59 (39.5-78.6)	65.5 (47.6-83.5)	60.8 (43.6-78)	55 (34.1-75.9)
Patients: Aspirin	27.5 (18.3-36.6)	27.9 (18.3-37.6)	27.7 (18.1-37.3)	27.9 (17.6-38.2)
Patients: No aspirin	57.4 (35.7-79.1)	50.2 (28.9-71.4)	49.5 (29.5-69.5)	54.4 (33.3-75.5)

Fig 3.36 Maximum aggregation in response to 0.005mmol/l adrenaline in patients and controls over time, with 95% CI.

Analysis within the groups over time found no significant difference for controls ($p = 0.86$), patients on aspirin ($p = 0.99$) or patients not receiving aspirin ($p = 0.93$). Two way repeated measures analysis of variance found no differences with respect to time or group by time ($p = 0.38$ & 0.38 respectively), but a significant difference with respect to group ($p = 0.005$), again due to the low values in the aspirin group.

Alpha-thrombin 5.0 units/ml

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	33.9 (7.3-60.5)	31.3 (6.3-56.3)	29.1 (5.9-52.3)	36.8 (6.9-66.7)
Patients: Aspirin	26.8 (2.03-51.6)	25.8 (2.9-48.6)	18 (-5.08-41.1)	29.9 (2.9-56.9)
Patients: No aspirin	28.4 (2.65-54.2)	25.8 (2.98-48.6)	18 (-5.08-41.1)	29.9 (2.95-56.87)

Fig 3.37 Maximum aggregation in response to 5.0units α-thrombin in patients and controls over time, with 95% CI.

Analysis within the groups over time found no significant difference for controls ($p = 0.97$), patients on aspirin ($p = 0.89$) or patients not receiving aspirin ($p = 0.89$). Two way repeated measures analysis of variance found no differences with respect to group, time or group by time ($p = 0.23$, 0.54 & 0.62 respectively).

Alpha-thrombin 5.5 units/ml

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	38.8 (11.1-66.5)	40 (14.3-65.8)	32.4 (8-56.8)	41.3 (11.4-71.2)
Patients: Aspirin	33.5 (7.4-59.6)	38.7 (10.5-66.9)	28 (10.6-45.4)	39.8 (12.1-67.4)
Patients: No aspirin	36.8 (10.1-63.6)	37.4 (10.1-64.6)	26.7 (1.24-52.2)	42.8 (13.6-72.1)

Fig 3.38 Maximum aggregation in response to 5.5units α-thrombin in patients and controls over time, with 95% CI.

Analysis within the groups over time found no significant difference for controls ($p = 0.96$), patients on aspirin ($p = 0.88$) or patients not receiving aspirin ($p = 0.84$). Two way repeated measures analysis of variance found no differences with respect to group, time or group by time ($p = 0.72$, 0.63 & 0.49 respectively).

Alpha-thrombin 6.0 units/ml

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	56.5 (32.1-80.9)	60.4 (36.4-84.5)	63.1 (44.3-81.8)	52.7 (26.9-78.5)
Patients: Aspirin	42.1 (17.9-66.3)	50.3 (25.6-74.9)	34.5 (11.4-57.6)	45.3 (19.5-71.1)
Patients: No aspirin	49.5 (23.9-75.2)	54.7 (26- 83.4)	47.7 (18-77.4)	52.8 (23.1-82.4)

Fig 3.39 Maximum aggregation in response to 6.0units α-thrombin in patients and controls over time, with 95% CI.

Analysis within the groups over time found no significant difference for controls ($p = 0.91$), patients on aspirin ($p = 0.77$) or patients not receiving aspirin ($p = 0.98$). Two way repeated measures analysis of variance found no differences with respect to group, time or group by time ($p = 0.85$, 0.09 & 0.49 respectively).

Alpha-thrombin 6.5 units/ml

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	78.5 (62.7-94.2)	81.9 (65.7-98)	81.3 (67.9-94.6)	62.6 (41.1-84.1)
Patients: Aspirin	61.2 (38.8-83.5)	63.5 (39.7-87.3)	56.8 (36.6-77.1)	51.9 (25.6-78.3)
Patients: No aspirin	62.3 (38.6-85.9)	62.8 (37.3-88.3)	58.5 (31.8-85.2)	65.9 (42.2-89.6)

Fig 3.40 Maximum aggregation in response to 6.5units α-thrombin in patients and controls over time, with 95% CI.

Analysis within the groups over time found no significant difference for controls ($p = 0.25$), patients on aspirin ($p = 0.86$) or patients not receiving aspirin ($p = 0.98$). Two way repeated measures analysis of variance found no differences with respect to time, group or group by time ($p = 0.43$, 0.67 & 0.38 respectively).

vi. Markers of Coagulation Activation

Analysis of repeated measures is complicated and can be difficult to interpret[C222]. We analysed the results using repeated measures analysis of variance techniques. A one-way analysis allowed comparison within each individual group over time, whilst a two-way analysis compared the patterns over time between the groups. If differences were found further analysis was performed to determine which group(s) were different.

Activated Partial Thromboplastin Time

Control subjects appear to have a higher APTT ratio at rest compared to the patient groups, and over the course of the study showed very little change ($p = 0.99$, one-way

analysis of variance) (Fig 3.41). Both patient groups showed a gradual decline in APTT ratio with time, however, the fall was not significant (patients on aspirin: $p = 0.88$, patients not on aspirin: $p = 0.85$; one-way analysis of variance). Despite the apparent difference between the control and patient groups there was no statistical difference between the patterns on two way repeated measures analysis of variance with respect to group, time or group by time ($p = 0.29$, $p = 0.44$ & $p = 0.35$ respectively). There was a slightly more pronounced fall in ratio immediately following exercise in patient groups however, this was not detected on analysis.

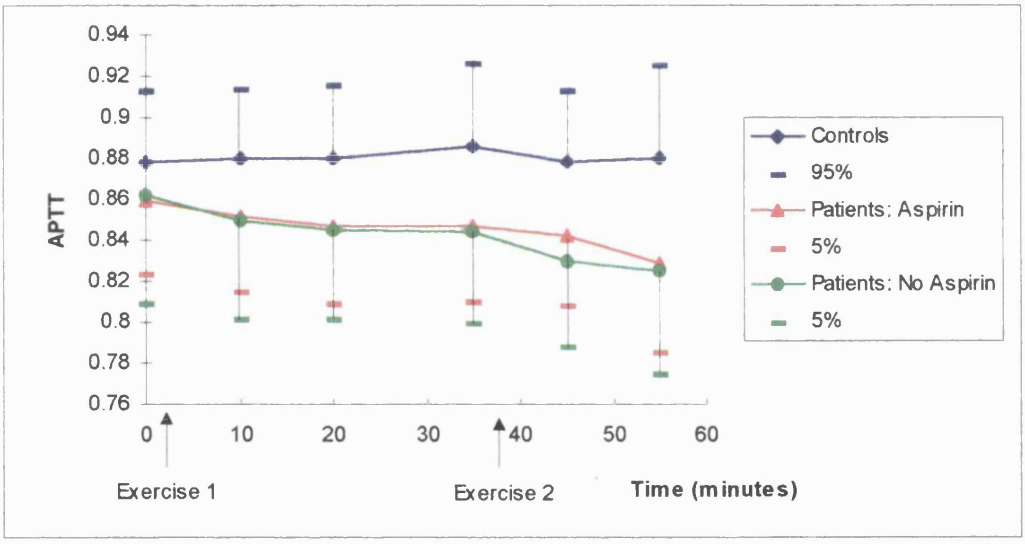


Fig 3.41 Activated partial thromboplastin time ratios in controls and patients, with and without aspirin, over the course of the study, with 95% CI.

D-dimer

Control D-dimer levels were lower than patient groups at rest and remained low and fairly constant during the study (Fig 3.42). One way repeated measures analysis of

variance (ANOVA) found no change over time in the control group ($p = 0.93$), or in either patient group (patients on aspirin: $p = 0.99$, patients not on aspirin: $p = 0.99$). There was a significant difference between the groups with respect of group on two way ANOVA, but no difference with respect of time or group by time. Tukey-HSD test, significance level 0.05, revealed a difference between patient groups and controls, but not between the patient groups.

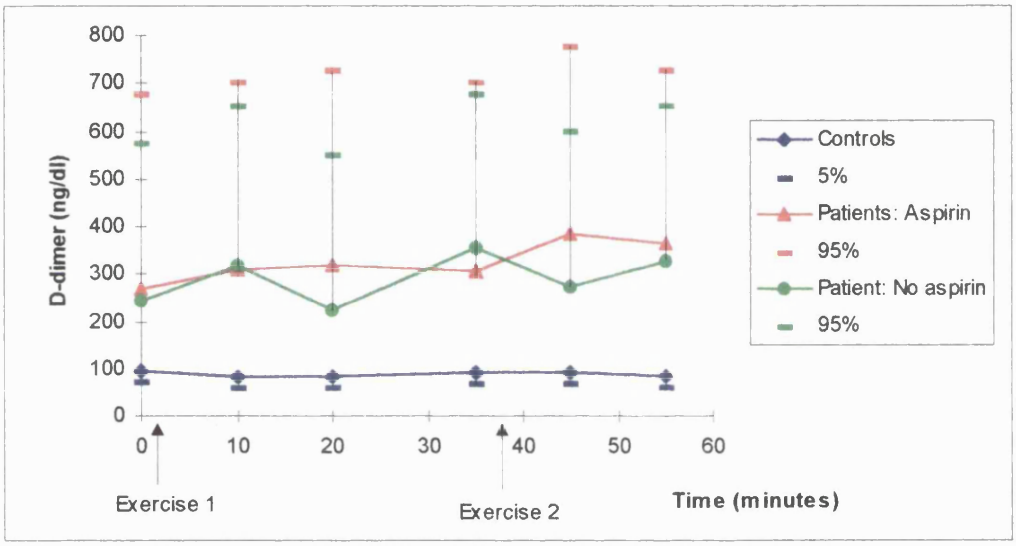


Fig 3.42 D-dimer levels (ng/ml) in controls and patients, with and without aspirin, over the course of the study, with 95% CI.

Although neither patient group showed significant changes over time, both showed an increase in levels after Exercise 1, but the no aspirin group falls after Exercise 2 while the aspirin group increases again. Both patient groups had higher D-dimer levels at the end of study compared to controls, but this was not statistically significant.

F1 & 2 Fragments

The results of the F1 & 2 fragment levels show no obvious pattern when plotted against time (Fig 3.43). Compared to controls and patients taking aspirin, the patients not taking aspirin have higher values. They show an increase in levels following each exercise period, with a return to "baseline" values in between these times. However, one-way analysis found no significant changes over time in this patient group ($p = 0.99$). The control group shows little change over time ($p = 0.94$). Following exercise the aspirin group shows a slightly later rise in F1 & 2 fragments, compared to patients not on aspirin. Levels then show a gradual decrease over time, with no apparent effect from the second exercise period. Despite any appearance of change over time, analysis revealed no statistically significant difference ($p = 0.85$). Two way analysis of variance was used to compare the groups, and no significant differences were found with respect to group, time or group by time ($p = 0.28$, $p = 0.6$ and $p = 0.63$ respectively).

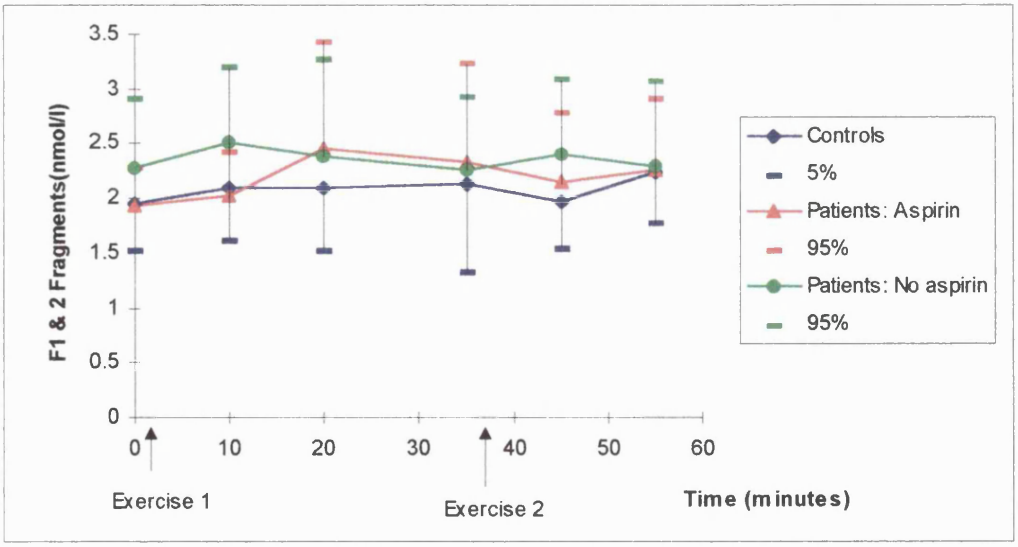


Fig 3.43 F1 & 2 fragments levels (nmol/l) in controls and patients, with and without aspirin, over the course of the study, with 95% CI.

Thrombin-Anti-Thrombin Complexes

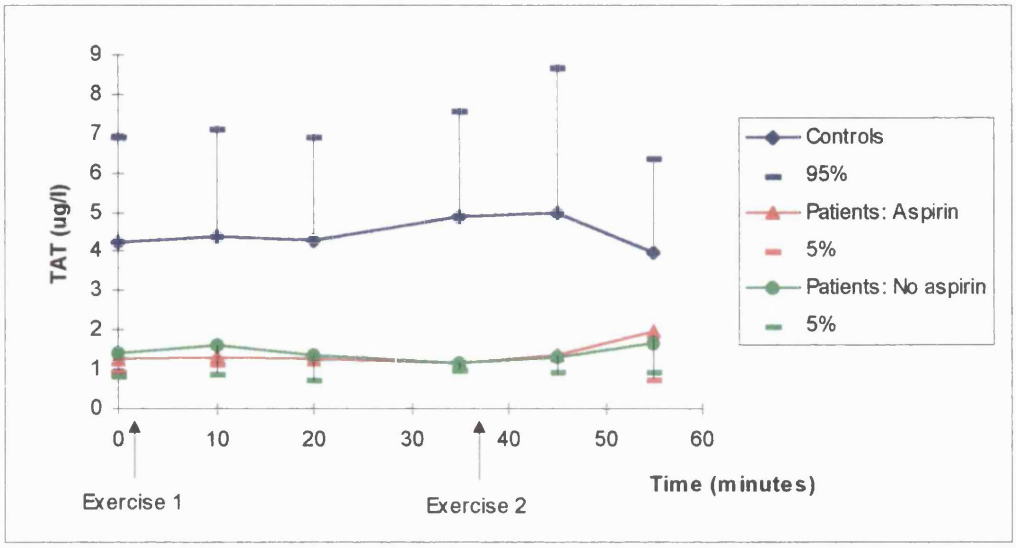


Fig 3.44 Thrombin-Antithrombin (TAT) complex levels (μg/l) in controls and patients, with and without aspirin, over the course of the study, with 95% CI.

The control group seems to have higher values of TAT complexes compared to either patient group, although none of the groups shows any obvious change over time, or in relation to the exercise periods (Fig 3.44). Statistical analysis found no changes over time in any of the groups (controls: $p = 0.99$, patients on aspirin: $p = 0.50$, patients not on aspirin: $p = 0.75$, one way analysis of variance). However, using two way repeated measures ANOVA there was a significant difference between the groups with respect of group, but not with respect of time or group by time. Tukey's multiple comparison test was applied, with a significance level of 0.05, and found controls were significantly different from the two patient groups ($p < 0.05$).

III.4 DISCUSSION

Summary

We found an increase in microalbuminuria in claudicants not taking aspirin compared to controls, but there was no significant difference when the patients were taking aspirin. There were no differences between the groups in platelet count, but a higher platelet volumes in patients compared to controls.

Flow cytometric studies showed no difference between patients and controls at rest, in the absence of agonists, for either fibrinogen or P-selectin binding. In the presence of the agonists there was no significant difference in fibrinogen or P-selectin binding between the groups, although there was a tendency to reduced fibrinogen binding and increased P-selectin binding in patients (particularly without aspirin) with ADP. The responses to α-thrombin were less clear, although P-selectin binding again appeared higher in patient groups, particularly at lower agonist concentrations. In response to exercise the two markers show different patterns. With ADP, fibrinogen binding tends to increase with the first exercise, whilst P-selectin binding tends to fall. With the second exercise period, fibrinogen binding tends to increase whilst P-selectin binding seems to fall (although not with low agonist concentrations) suggesting some "conditioning". Both markers tend to fall with α-thrombin after the first exercise period, with a further drop prior to, but little response following the second exercise period.

Thromboxane levels increased markedly at the end of the study period in both controls and patients not taking aspirin. Beta-thromboglobulin and PF4 showed identical patterns with increases after the first exercise, return to normal and an increase prior to the second exercise followed by a fall after the second exercise period. Platelet aggregation studies were difficult to interpret with no clear trends.

Coagulation studies revealed lower APTT and TAT levels in patients, compared to controls, over time, with higher d-dimer levels. There were no clear patterns to F1 + 2 fragment levels in either groups.

Study design

Patients were selected based on their disease stability. This excluded those with recent deterioration, tissue loss, angioplasty or surgery, all factors, which may alter the levels of markers. Controls were selected from patients undergoing minor surgery over 18 months previously to avoid the effect of their surgery. They were all assessed pre- and post-exercise to exclude peripheral arterial disease. In addition, all controls were carefully questioned to exclude occult ischaemic heart disease, the treadmill test and an ECG supported this. All subjects were screened to exclude hepatic or renal impairment, conditions known to alter the clotting system, and any acute disease processes, particularly infective or inflammatory processes, which may influence markers.

Ankle brachial pressure index and treadmill exercise testing is widely used and an accepted technique to assess claudication[609]. It is the standard exercise test in our

laboratory, and most closely duplicates the normal situation for the patients whilst allowing easy post-exercise measurements and sampling. We used the patients average walking time measured during pre-study assessment, to determine the exercise time for the controls.

The study was designed to ensure all subjects arrived completely rested at similar times in the morning. Subjects had been asked to avoid all exercise prior to arrival. This was an attempt to produce a degree of uniformity in the starting conditions. Other studies have reported fixed investigation times, but have failed to control for exercise prior to assessment. Assuming exercise does influence the markers measured, results could be confounded by this factor. Subjects were also asked to avoid caffeine containing drinks for 24 hours prior to the study, to avoid interaction with platelet, and possibly coagulation, activity.

The aim of the second exercise period was to evaluate the effect of repetitive exercise, a more representative situation. The second exercise period was chosen at 30 minutes following the end of the first period. This was an arbitrary time and a compromise. It allowed assessment of markers over an extended period post-exercise, consequent of previous work showing neutrophil activation up to 2 hours post-exercise, whilst trying to reproduce the clinical situation. Patients exercised to the point of pain preventing further walking. Again this was felt to be a more accurate clinical scenario. The ankle pressure drop following exercise and the pressure recovery time are both accepted measures of disease severity[141], and provides a repeatable, non-invasive assessment with negligible risk to the patient.

i. Physical Measurements

The ankle brachial pressure index results were to be expected. The controls were chosen for their lack of significant peripheral vascular disease and in their "normal" vasculature exercise results in increased muscle blood flow with an increase in pressure, and hence ABPI. The claudicants, however, respond to exercise with a fall in pressure and ABPIs. Although there are reports of aspirin producing clinical improvements in some individuals this has not been a general finding and was not the case in our study; although one of the patients did improve dramatically on aspirin to the point of becoming almost asymptomatic. There was no difference between the two exercise periods. Some patients report improved exercise tolerance following the "first walk of the day". However, others report a gradual deterioration in walking distance with each subsequent walk. Whilst it is possible that some form of conditioning (or pre-conditioning) occurs in some patients, differences in factors such as pain tolerance, walking style, terrain, rest periods and the environment make these reports difficult to interpret. The pressure recovery times were clearly different between controls and claudicants, but there was no difference between claudicants taking and not taking aspirin. This again demonstrates the lack of effect of aspirin in group studies, and is in keeping with the lack of difference in ABPI measurements.

ii. Microalbuminuria

Microalbuminuria is thought to reflect vascular permeability and has been used as a measure of the systemic effect of the ischaemia-reperfusion cycle seen in claudication[610]. In this study controls and both groups of patients had comparable levels of microalbuminuria at rest, but following exercise patients showed an increase compared to controls. This result confirms claudication causes an increase in microalbuminuria[610], but

demonstrates little or no effect from aspirin. This tends to negate a role for platelet activation in the pathophysiology of microalbuminuria in claudicants. Hickey et al argue this effect arises from humoral factors released from activated leucocytes, and or ischaemic muscle[610].

iii. Platelet count and size

Platelet counts changed little between groups, following exercise or over time, however, all groups showed a fall in platelet size. Platelets are normally stored in the lungs and spleen and maybe released from here or other sites in response to exercise[611]. However, if this is the case platelet counts would be expected to rise, not remain static. A possible reason maybe the destruction of effete platelets by the increased flow stresses associated with exercise. Platelets are known to adhere to areas of damaged or denuded endothelium, and although there is evidence of endothelial damage from claudication (markers such as vWF, thrombomodulin), there is no evidence (and it is difficult to conceive) that claudication damages the endothelium enough to cause platelet adhesion. Our work (Chapter 2) and that of others has shown evidence of neutrophil activation[139,141,234-236] and trapping in skeletal muscle during claudication[143,240,610]. Platelets are known to interact with neutrophils through surface ligands[612] and this may account for some of the fall in counts after exercise.

Patients tended to have larger platelets than controls although this failed to reach significance. Interestingly, the only other group reported to have larger platelets are patients following myocardial infarction[613]. Younger platelets may be larger[614] and hence this finding could be explained by increased platelet turnover in peripheral vascular disease[389-391]. The fall in platelet size seen in all groups over the course of the study is less

easy to explain. Exercise is associated with the release of stored platelets[611], which are reported to be enriched with large platelets[615,616]. This, or destruction of older, smaller platelets, would be expected to produce an increase, absolute or relative, in the platelet size with exercise. Schmidt and Rasmussen found large platelets were preferentially sequestered from the circulation following exercise, and this may explain the fall in size during the study[614].

iv. Platelet markers

The unclear platelet population dynamics during exercise and claudication may confound interpretation of studies of markers of platelet activation, e.g., are "mopped up platelets releasing their activation factors? and how reactive are "new" platelets? This maybe particularly important in looking at platelet responses to agonists, i.e., flow cytometry studies.

Surface glycoproteins

Platelets are notoriously difficult to study, particularly in vitro, because of their reactivity. We utilised the whole blood technique for the flow cytometric studies to minimise preparation and thus the risk of activation. We decided to study only two of the many platelet surface glycoproteins predominately for logistical reasons, in view of the multiple sample points and subjects. Fibrinogen binding and P-selectin were chosen in an attempt to give the broadest overview. Fibrinogen binding is an integral part of platelet involvement in thrombosis and occurs discretely from degranulation. P-selectin is involved in binding to endothelial cells and neutrophils and is expressed upon degranulation. We chose to use only two of the many agonists for platelet activation, again for logistical reasons. ADP is a well studied platelet agonist and is

formed in ischaemic tissue from the breakdown of ATP to release energy, in addition it is released by platelets themselves and acts as part of the amplification system in the platelet response to thrombosis. Thrombin is an essential part of the coagulation and thence clotting cascade.

The available data on platelet activation in claudication is varied in its conclusions. We were aware that "deactivation" or down- regulation of platelet responses to exercise and ischaemia were possible. For this reason we studied platelet surface markers at rest and after stimulation with agonist, furthermore, we used three concentrations of agonist. These values were taken from dose-response curves determined before the study. This was done to maximise the chances of detecting any change, small or large, in any direction, up or down-regulation.

From our results we have found no evidence of any significant changes in the resting expression of fibrinogen or P-selectin between patients and controls, or between patients on aspirin and those not on aspirin. This is in agreement with the findings of Galt et al who found no difference in the expression of several platelet markers between patients with PVD and controls[397]. We looked at the levels of glycoprotein expression in unstimulated platelets before and after exercise. Although there was a slight trend towards increased fibrinogen expression both before and after the second exercise period, there were no significant differences in any of the groups over time. The confidence intervals for the values relating to the second exercise period were much wider than the first exercise, possibly suggesting some patients were demonstrating a more pronounced response. However, the small numbers make sub-division of the study group impossible. Thus, we found no evidence that peripheral

vascular disease or claudication increases fibrinogen or P-selectin expression, or that aspirin has any effect on their expression.

The binding of fibrinogen in response to ADP showed no differences within or between the groups suggesting that neither peripheral vascular disease, with or without aspirin, nor claudication had no effect on the response of platelets to this stimulation. However, there was a trend towards reduced responses in the second exercise period. P-selectin binding in response to ADP also showed no effect of exercise/ claudication within any of the groups. However, at the upper two concentrations of ADP there were differences in the pattern of responses over time between the groups. This arises because the patient group on aspirin shows a smaller increase in expression with the second exercise period, thus producing a different pattern. However, the values between the groups and the pattern of group by time showed no differences. This is supported by the dose-response curves, which show very little change. Thus, we found no evidence that having peripheral vascular disease, on or off aspirin, has any effect on platelet expression of fibrinogen or P-selectin in response to ADP. Furthermore, we found no evidence to support the hypothesis that claudication causes stimulation, or priming, of platelets as assessed by responses to ADP.

Platelet glycoprotein expression in response to α-thrombin was more variable. Great care was require in preparation to avoid formation of fibrin. Despite GPRP several samples were not able to be assessed by flow cytometry, resulting in more missing data in the α-thrombin studies. Overall the thrombin studies were harder to interpret.

At the higher concentration of 0.03units/ml both patient groups showed a down-regulation of fibrinogen binding with the first exercise period, and a further reduction in expression prior to the second exercise period. However, the second exercise period produced an increase in expression of fibrinogen although not to initial levels. Despite similar patterns, only the patient group receiving aspirin showed significant differences. The control group showed a slight increase in expression for each period of exercise, with a small down-regulation between exercise periods. Although these changes were not significant, they resulted in a difference between the groups with respect to the patterns over time. With 0.02units of α-thrombin a similar pattern of reduced fibrinogen binding with exercise and over time was seen, however only the aspirin group showed significant differences. The control group showed little change with the first exercise period, but a marked drop between exercise periods. Again up-regulation was seen with the second exercise period. At the lowest concentration of α-thrombin all groups showed a similar pattern of reduced expression with exercise. However, the patients showed a progressive fall between exercise periods, whilst controls showed an increase. Hence, we found no evidence of increased platelet activation in response to α-thrombin between controls and patients, either taking or not taking aspirin, at rest. Following exercise the patients tended to show a fall in fibrinogen expression both over time and associated with exercise, although this was only significant in the group taking aspirin. This suggests claudication produces a down-regulation of platelet response to α-thrombin, arguing against our hypothesis of increased responses. A possible explanation could be that claudication activates "thrombin-responsive" platelets and reduces the number subsequently available to respond to exogenous agonist. However, our results would imply greater activation in

patients taking aspirin, whilst aspirin is associated with reduced clinical events. The expression of P-selectin in response to α-thrombin showed little differences between groups and little effect of exercise. Some differences between the groups were detected in their patterns over time, but again we found no evidence to support the hypothesis that claudication primes platelet responses to α-thrombin.

Hence, overall we have found no evidence to support the idea that claudication is associated with platelet activation, or increased activation in response to ADP or α-thrombin. In fact, there was a tendency to a down-regulation of response to agonists. Furthermore, the presence of peripheral vascular disease, whether on aspirin or not, had no effect on the expression of surface markers of activation, at rest or in response to agonists.

The negative outcome of this study may have several explanations. Firstly, there is no platelet activation in response to claudication. This agrees with several studies in the literature with negative outcomes. However, some studies report platelet activation in claudication, and some of our patients did show a response as individuals. It is possible there are sub-groups within the claudicant population in whom platelet hyper-responsiveness is a major part of their disease process. The small numbers in our study make it difficult to pick-up such groups, and furthermore, we selected stable claudicants who by definition may have more stable disease. It is possible we missed a difference because we chose to use the "wrong" markers and agonists. However, thrombosis is well known to involve platelet aggregation and degranulation, and as such the markers should have been appropriate. Shifts in platelet populations may

"hide" evidence of activation, i.e., activated platelets are taken up or bound to other cells, or dilutional effects may occur due to release of stored platelets. Platelets in PVD may be subjected to chronic, sub-maximal stimulation and hence be hypo-responsive. This effect maybe balanced by release of younger more reactive platelets. The flow cytometry technique relies on detection within a focused bit-map, as determined by the characteristics of individual platelets. Platelet aggregates are reported to occur in PVD and may represent the activated group, and may be missed by the bit-map.

Plasma markers

Thromboxane A_2 Reports detailing thromboxane release in claudication are varied. Not suprisingly patients receiving aspirin, a potent inhibitor of cyclo-oxygenase and hence thromboxane formation, had uniformly low levels of thromboxane throughout the study. Comparing controls and patients not taking aspirin reveals a similar pattern until the last time point, where patients show a marked increase in levels compared to controls. It could be argued this is due to sampling, sample six represents the third sample from one of the arms. However, sample five is also a third sample point and does not show a similar increase. If this "late" increase is a true finding, this may explain the differing results reported in the literature.

β-TG and PF 4 The patterns of release of β-TG and PF 4 were reassuringly similar over time and between groups. Furthermore, the ratio of β-TG values to PF4 values was between 1:3.5-4.5, which is in keeping with good sampling technique. However, control levels at rest would be expected to be around 40pg/ml, which illustrates

that all work using β-TG and PF 4 must be viewed critically because of artefact related to sampling. Overall, the patients not taking aspirin had higher levels of β-TG and PF 4, followed by controls and then patient receiving aspirin. This would suggest that thromboxane is involved in the release of β-TG and PF 4, however, the pattern of release is not matched with thromboxane levels. A particularly interesting point is that both β-TG and PF 4 increase after the first period of exercise, but fall following the second exercise. It is tempting to explain this remarkably similar pattern on sampling factors. Samples were taken from alternate arms but it would seem extremely unlikely that subjects should have a "high and low level" arm, or that we should sample the appropriate arm. All samples were treated the same and the pattern does not correlate with any aspect of sample handling or with time. The starting or resting levels are not significantly different from levels at the last time point. It may be that the pattern represents natural variation over time, although it seems unlikely it could occur so reproducibly between markers and between groups. Thus, the pattern may represent a response to exercise. It is difficult to explain the changes in marker levels based on physical changes occurring on exercise, when the first period shows an increase, and the second a decrease. If the pattern is to be explained in terms humoral factors or cell activation, it postulates that the first exercise period causes platelet degranulation, the products of which are rapidly cleared. A secondary activation occurs, presumably through different mechanism, which is "switched off" by the second exercise period, and thereafter gradually recovers. One possibility would be the first exercise period generates thrombin, which desensitises platelets to further activation and release[617]. There is no supporting evidence for such a course of events, either from our findings or from the reported literature. Interestingly, aspirin fails to block β-TG following the first exercise period, which may suggest an

alternative release mechanism, possibly related to thrombin generation. A simpler explanation for the pattern over time is the biphasic excretion of both β-TG and PF 4, with peaks around 10-15 minutes and 40-60 minutes. However, such an explanation makes interpretation of results after the initial exercise period virtually impossible.

Platelet Aggregation

Studies of platelet aggregation are confounded by the extensive preparation necessary. The process of centrifugation and separation is reported to activate platelets to a variable degree and this makes it a crude tool for assessing what are likely to be small changes in platelet activation in peripheral vascular disease. Not suprisingly, the inter-subject variability was so high it was difficult to draw any conclusions from the data.

Aggregation in response to ADP showed patients receiving aspirin had lower levels of aggregation at both concentrations and minimal response to exercise. The patients not on aspirin showed increased aggregation with exercise but down-regulation between exercise periods. The controls tended to show increased aggregation with the first exercise period, but reduction with the second. It could be suggested that it is in the response to the second exercise period that controls and patients are different, and that aspirin converts patient responses towards normal. Aggregation responses to adrenaline were more clearly different. Aspirin produced lower aggregation at both concentration, although no changes over time or in response to exercise. The patients not taking aspirin showed reduced aggregation on the first exercise, but increased with the second. The responses to α-thrombin tended to be higher in the control group, with the patients not on aspirin having slightly higher aggregation than those receiving aspirin. Other than that generalisation it is very difficult to find any pattern of responses in the data. Although,

there is some data to support down-regulation of aggregation between exercise periods, the variability of the responses and the lack of statistical significance within the groups supports the conclusion that no real changes in aggregation were found in response to α-thrombin. Hence, apart from the reduced aggregation in response to adrenaline seen in patients receiving aspirin, there were no effects of claudication on platelet aggregation.

v. Markers of Coagulation Activation

The involvement of coagulation and fibrinolysis in PVD can be considered from two angles. Firstly, a primary abnormality in either system predisposes the individual to the formation of atherosclerosis and the development of complications. Alternatively, but not exclusively, the presence of PVD produces a secondary abnormality in coagulation or fibrinolysis, which enhances disease progression and complications. Such a secondary abnormality may arise due to the presence of a large area of atherosclerosis and interactions between it and the circulating blood. We investigated another possibility, namely, that the episodes of repeated ischaemia and reperfusion of the skeletal muscle of the lower limbs cause an abnormality in the coagulation/ fibrinolysis axis which may either predispose the subject to the complications of atherosclerosis, or extend the effect.

We set out to assess activation of the coagulation/fibrinolytic system in response to claudication, and this coupled with local availability, determined our choice of markers.

Activated Partial Thromboplastin Time

APTT is a global indicator of the intrinsic pathway of blood coagulation. There is very little data on APTT values in PVD or thrombotic events. Shortening of the APTT has been reported after exercise such as triathlon, long-distance running, exhaustive running and maximal cycling[395]. Our results show that controls have higher APTT ratio at rest, with no change over time or following exercise. Both patient groups show a progressive fall in APTT ratio over time, suggesting an increasingly hypercoagulable state. There was a slightly more noticeable fall in ratio immediately following exercise. There was little difference between patient groups, and hence no effect of aspirin. Exercise in claudicants is to the point of ischaemic pain, and hence anaerobic metabolism in the muscles. This could be compared with the strenuous exercise causing a fall in APTT in normal healthy subjects. However, these changes are merely descriptive and no statistically significant differences were found.

Thrombin Generation

F1 & 2 fragments are generated when prothrombin is lysed by the prothrombinase complex to form thrombin[618]. F1 & 2 fragments are formed in equivalent amounts to thrombin, and hence act as a marker of thrombin formation and activation of the coagulation cascade[446]. Determination of F1 & 2 fragments is affected by venepuncture, different anticoagulants and the measurement technique[620-623]. Therefore, samples were all collected into citrate and assayed using the same ELISA technique to reduce variability. Venepuncture was performed with no tourniquet, minimal trauma and at sequentially more distal sites to reduce the influence of sampling. Furthermore, subjects were chosen with "good" veins to facilitate venepuncture, and the same investigator performed all sampling.

The levels of F1 & 2 fragments demonstrate thrombin generation, but not activity. The rapid action of natural inhibitors such as anti-thrombin III and α_2-antitrypsin neutralises the effect of thrombin. Thus, levels of thrombin-antithrombin (TAT) complexes also provide a marker of thrombin generation[623]. Although levels of antithrombin III are three times that required to neutralise the maximum amount of thrombin which could be generated, a reduction in antithrombin III levels by 40-50% is associated with an increased risk of thrombosis[618], and may influence TAT levels.

Increased levels of F1 & 2[568] fragments and TAT[559,568,621-625] have been found in patients with stable PVD, and TAT levels have been shown to correlate positively with the degree of atherosclerosis as assessed by angiography[559]. Several investigators have demonstrated significantly increased F1 & 2 fragment levels, and associated raised levels of TAT complexes, following exercise in normal subjects[626-628]. In addition, raised levels of TAT complexes have been reported after long distance running[629] and a graded maximal cycling test[630]. The exercise challenges in these studies have been severe, including a 2 hour triathlon and 1 hour of cycling, and, hence, bordering on the threshold of anaerobic exercise[631]. However, the evidence that thrombin generation in these circumstances causes fibrin formation is conflicting. Whilst some studies have demonstrated an increase in FpA (a product released during fibrin formation) following triathlon or marathon races[626,631], other work has found no increases after graded exercise, steady state cycling, maximal cycling, long or short distance running[627,629,632]. Whilst exercise intensity may influence fibrin formation, it seems reasonable to assume that, in young healthy subjects, thrombin generated by exercise is easily neutralised by natural inhibitors. The capacity of this system is evidenced by studies showing little or no reduction in antithrombin III levels

following maximal exercise[627,629,633,634], although a fall following a marathon has been reported[635].

Miller et al reported increased F1 & 2 fragment levels in men at high risk of myocardial infarction[506]. Patients deficient in antithrombin III, protein C or protein S are at increased risk of thrombotic events. However, only 25% demonstrate raised levels of F1 & 2 fragments. Furthermore, the levels were not different between those with and without a history of thrombosis suggesting raised F1 & 2 fragment levels do not predict those at risk of a thrombotic event[636,637]. A similar finding was reported in patients at risk of thrombosis from autoimmune disease[638]. However, prospective studies are required to see whether raised F1 & 2 fragment levels are linked to primary or recurrent thrombotic events[639]. Although Merlini et al have reported raised F1 & 2 fragments in patients with unstable angina and myocardial infarction[640], most of the increases in F1 & 2 fragments and TAT complexes have been reported in venous thrombotic events. Furthermore, they are less sensitive markers of acute thrombotic events compared with products of fibrin breakdown, such as D-dimer[639]. This appears to be at odds with the obvious thrombin generation, which occurs during thrombosis.

Our results shown very little difference in F1 & 2 fragment levels between controls and patients (receiving or not receiving aspirin), at rest or following either exercise period. Patients not receiving aspirin appeared to have more variable results but differences failed to reach statistical significance. This would suggest, firstly, no evidence of increased thrombin generation in patients with PVD. Secondly, no effect

of claudication in patients (or minimal exercise in controls), and finally no effect of aspirin on thrombin generation.

Thrombin generation as measured by TAT levels shows no differences between either patient group at rest, and no changes over time or following exercise. These results are in accordance with thrombin generation as determined by F1 & 2 fragment levels. However, in contrast, controls showed consistently higher levels of TAT complexes, although again there was no change over time or with exercise. Thus, controls and patients appear to generate similar amounts of thrombin, based on F1 & 2 fragment levels, but patients have markedly lower resulting levels of TAT. This could be explained by the patient group having members with markedly deficient AT III levels. However, the patient results are fairly consistent, requiring all patients to have deficient levels; which is not supported by studies[548]. Some or all of the control group may have raised AT III levels. Over 40% of the control group show levels of TAT complexes well above the patient average, but they don't correlate with those with higher F1 & 2 fragment levels. The patient group may rely more on α_2-macroglobulin to neutralise thrombin or have increased excretion or breakdown of TAT complexes. The action of antithrombin III is known to be enhanced by heparin and heparin-like molecules on the vascular endothelium[641]. There is evidence that the complexes maybe released from the endothelium into the circulation[642] and cleared by the liver[643], or they maybe cleared by the endothelium itself[644]. If clearance were enhanced in PVD it could explain the lower levels of TAT. Alternatively, with thrombin generation being similar in all groups, it could be postulated that in patients the thrombin is in some way bound, or taken up, from the circulation. Possible candidates would be circulating or bound platelets, atherosclerotic plaques or white

cells. Inhibition of some platelet functions by aspirin had little or no effect on measurements in the patient group. However, platelets can still express fibrinogen, the primary substrate for thrombin, in response to stimulation in the presence of aspirin[645]. Further work to assess thrombin metabolism in PVD patients compared to controls would be necessary to investigate this theory. In contrast to our results, De Buyzere et al reported raised TAT and F1 & 2 fragments in PVD patients compared to controls. They also found a treadmill exercise test had no effect on measurements[568]. We have confirmed this later finding and demonstrated no evidence of thrombin generation on repeated exercise.

This data does not support the theory that patients with PVD have increased thrombin generation. Nor does it support the hypothesis that claudication is associated with activation of the coagulation system as measured by thrombin generation. However, the reduced TAT levels in patients could suggest impaired inhibitor function, although not necessarily inhibitor levels, or a difference in the handling of thrombin in PVD patients.

Fibrin Formation And Fibrinolysis

D-dimer is a plasmin induced degradation product of cross-linked fibrin. Consequently, it provides a measure of fibrinolysis, as well as, an indirect measure of fibrin formation. There have been several reports of increased D-dimer levels in patients with PVD compared to controls[169,568-571,624,625]. Lassila et al reported a positive correlation between D-dimer levels and PVD severity, assessed using ABPI, duplex and angiography[559]. This has been supported by Woodburn et al[169], and others[570,571], who found a correlation between D-dimer and the angiographic extent of

PVD. An increase in D-dimer levels has been observed after short term maximal exercise[646], a triathlon[633], a marathon[646] and long distance running[629], indicating vigorous exercise causes increased fibrinolysis. However, other studies have reported no change in fibrin/fibrinogen degradation products (Fb/FDP) following moderate or maximal exercise[647,648] although these were earlier studies using less sensitive techniques. Thus, the effect of exercise on fibrin degradation in unclear, but elevated levels of D-dimer are reported. D-dimer levels have been demonstrated to increase in deep vein thrombosis[649], pulmonary embolism[650], acute limb ischaemia[651], stroke[652] and myocardial infarction[653,654]. Furthermore, raised levels have been found in unstable angina, which carries a high risk of infarction, and may act as a marker for increased risk of thrombosis[655]. This is supported by studies showing elevated D-dimer levels to be associated with ischaemic events in patients with PVD[562,572].

We found both patient groups have higher D-dimer levels at rest compared to controls, which agrees with other studies[169,568-571,624,625]. This suggests patients with PVD have increased fibrinolysis, with breakdown of cross-linked fibrin, and indirectly, increased fibrin formation. However, there is little evidence in the literature for increased fibrinolysis in PVD; and that which does support it is contradictory. Early work using global, non-specific tests of fibrinolysis suggested reduced function and this was supported by work on atherosclerotic vessels reporting reduced fibrinolytic potential[508]. More recent work tends to support these earlier reports. Speiser et al[625] found increased PAI-1 in PVD patients compared to controls, a finding supported by others[169,551,568,624]. Strano[624] and Blann[574] reported increased t-PA antigen, but Woodburn[169] and Speiser[625] found no difference. De Buyzere[568] reported increased Urinary Plasminogen Activator (u-PA) antigen, but normal t-PA antigen and activity and normal PAI-1. Hence, support

for the idea of increased fibrinolysis is limited. Increased fibrin formation is difficult to reconcile with our failure to find evidence of increased thrombin generation, in contrast to reports of increased D-dimer levels in PVD patients associated with increased F1 & 2 fragments[568] and TAT complexes[559,568]. The failure to detect increased F1 & 2 fragments maybe explained by a less sensitive assay compared to that for D-dimer, which is known to be very sensitive. Alternatively, the low levels of TAT complexes in the patient group may indicate a preferential role for α_2-macroglobulin as an inhibitor of thrombin. This molecule binds thrombin, but may not completely neutralise it[618], allowing continued action and fibrin formation. However, there are no reports of increased α_2-macroglobulin levels or activity in the literature, and further work would be required to confirm this idea. Fibrin generation is known to occur within atherosclerotic plaques, and this may happen without intravascular thrombin generation. The thrombin being generated in vessel wall in response to tissue factor released by inflammatory cells[559]. Thus, D-dimer maybe formed in the vessel wall, as opposed to the lumen, and then released into the circulation. D-dimer would then represent a marker of on-going atherosclerotic process. Raised levels may indicate a more aggressive atherosclerotic process, with an increased risk of thrombotic events. Finally, raised D-dimer levels may relate to the disease process of anyone of the risk factors for atherosclerosis. However, Speiser et al[625] found no association between smoking, hypertension, diabetes or hyperlipidaemia and plasma levels of D-dimer or TAT.

Our lack of evidence of intravascular thrombin formation may relate to the small number of subjects, and represent a type II error. Consequently, increased D-dimer levels may indicate increased intravascular fibrin formation and breakdown due to activation process

on the arterial wall defects[625]. As patients in our, and other, studies have stable PVD, the activation cannot be due to major thrombotic events. The concept of multiple, recurrent microthrombosis occurring on the damaged, atherosclerotic vessel wall has been proposed. Speiser et al arguing that increased D-dimer is representative of the area, i.e., extent, of atherosclerosis[625].

There is a major conflict within current evidence. There have been repeated demonstrations of increased fibrin degradation products, e.g., D-dimer, in PVD suggesting increased fibrinolysis. However, it has not been possible to consistently demonstrate such an increase. Possible reasons for this confusion could be, the assays for fibrinolysis are not sensitive enough or there is cross-reactivity of the assays between antigen/ activity/ inactivation compexes[576]. Another possibility is that there is a reduced fibrinolytic capacity (for whatever cause, e.g., primary disease, genetic or secondary to atherosclerosis itself), but in the face of increased substrate, i.e., fibrin, there is an absolute increase in D-dimer formation, but a fall in the proportion metabolised.

We found no difference between D-dimer levels in patients receiving or not receiving aspirin, in contrast to reports by Speiser et al[625]. This would suggest that prostaglandin metabolism is not involved in the increased fibrin formation and breakdown in PVD patients. It would seem that if increased D-dimer levels are associated with increased thrombotic events, and aspirin reduces the incidence of thrombotic events in arteriopathic patients, then their effects are via different mechanisms. It was not possible to determine any effect from aspirin consumption on levels of D-dimer, although values were more variable in the non-aspirin group.

Our patient groups appear to show an overall increase in D-dimer levels throughout the study, whereas the controls showed no changes. This may be due to exercise, although it is difficult to explain the changes following each period of exercise. Both patient groups show an increase in levels after the first exercise period, but the no-aspirin group falls after the second exercise period while the aspirin group increases further. Repeated exercise appears to have an accumulatory effect on the level of D-dimer. To confirm the overall increase was related to exercise would require non-exercising patient group. However, it is supported by work from Al-Zahrani et al, who noted very little change in D-dimer levels in PVD patients and controls measured serially over 6 weeks[569]. De Buyzere et al found no increase in D-dimer following treadmill exercise. However, they measured levels immediately post-exercise and only used a single exercise test[568].

Our results show claudicants have an increase, although not significant, in D-dimer levels following exercise. Repetitive episodes of claudication during an average day would cause a progressive increase in D-dimer levels. Such a rise, and the association of increased D-dimer levels with thrombotic events, would support the theory that claudication may contribute to the thrombotic tendency in patients with PVD. However, normal healthy subjects performing maximal exercise also show an increase in D-dimer levels[629,633,646]. Levels in healthy subjects return to normal after intense exercise, and although claudicant levels may return to baseline (as suggested by Al-Zahrani et al[569]), they are still chronically raised. As normal healthy subjects do not have atherosclerosis and are generally not prone to thrombotic events, it could be implied that it is only the chronic elevation of D-dimer, which indicates risk. However, the dynamics of the D-dimer changes in these two groups has not been studied comparatively.

Whilst, both normal healthy subjects and claudicants may have a similar response to intense or near-anaerobic exercise, the mechanism for the increase in D-dimer maybe the same or different. A mechanical activation due to increased blood flow in the muscles could be postulated. However, claudication occurs because of a failure to increase flow during exercise. Similarly, increased flow over atherosclerotic vessels is not seen in healthy subjects. Both groups would have a humoral response to exercise, and adrenergic mechanisms may stimulate fibrin formation and/or breakdown[417]. The acidosis, increased lactate, low pO2, etc, found in near anaerobic conditions, or the resulting ischaemia-reperfusion, may stimulate fibrin formation and/or breakdown, due to an effect on inflammatory cells, platelets or the endothelium[656]. Alternatively, whilst one of these mechanism may act in healthy athletes, the increased endothelial permeability found in PVD patients upon claudication[141] may result in an increased release of D-dimer formed by pathological processes in the vessel wall.

Increased fibrin generation during exercise could be normal, but usually the increase in fibrinolysis compensates. PVD patients may have an impaired fibrinolytic response to exercise. This is supported by evidence of impaired fibrinolytic response to exercise reported in coronary heart disease[657], hypertension[658], diabetes[659], and hypercholesterolaemia[658,660], which are major risk factors for atherosclerosis.

CONCLUSIONS

Using a combination of traditional and newer, more sensitive measures of platelet activation we have been unable to demonstrate any firm evidence linking platelet activation or down-regulation with the process of claudication. Thus, the increased

cardiovascular morbidity and mortality seen in claudicants cannot be explained by increased platelet reactivity, as measured in our work. Contrary to this is the well reported decrease in cardiovascular mortality seen in patients taking aspirin. Many of the effects of platelets are inhibited by factors present or released from the endothelium. It is conceivable that platelets do play a role in the increase mortality, but that they act normally in the presence of impaired endothelial inhibitory function.

Ultimately, we were trying to determine whether claudication causes activation of the coagulation cascade, and changes in the blood, which might enhance the thrombotic response. This work has shown increased breakdown of cross-linked fibrin in claudicants at rest. We also found a tendency to further increases after exercise, and to a possible cumulative effect of exercise, but these changes were not significant. Although this suggests, indirectly, claudicants have increased formation of fibrin and fibrinolysis, the exact mechanisms behind these results is unclear. Thus, although we have found increase fibrin breakdown in claudicants at rest, we have found no other evidence to support the idea of increased activation of the coagulation system in claudicants, either at rest or following exercise. This does not support the theory that claudication increases the mortality and morbidity of patients, from the complications of atherosclerosis, through exercise induced activation of the coagulation system.

CHAPTER FOUR: DISCUSSION, CONCLUSIONS AND FUTURE WORK

DISCUSSION

The association of intermittent claudication with increased cardiovascular morbidity and mortality, independent of existing levels of cardiac and cerebral atherosclerosis and known risk factors, raised the question as to whether the process of claudication itself contributes to this increased risk. From the literature we thought claudication might exert any effect through three important components of cardiovascular thrombosis and infarction; namely platelets, coagulation and neutrophils. In designing our studies we considered several points, accepting no single design was likely to deal with all possible confounding factors.

We assumed any effect would need to exist over a reasonable period of time, as opposed to a few minutes post-claudication. This reasoning was based in part on the knowledge that not all infarction, cardiac or cerebral, occurs following exercise. In addition, studies have shown myocardial infarction occurs often in the early hours of the morning before any significant exercise has been performed. Based on these deliberations we opted to measures changes over extended periods of time. Our initial study considered neutrophil function. In particular we wanted to assess whether neutrophils developed and maintained an increased capacity for damage, as a response to claudication. Studies of markers, such as elastase and even neutrophil filterability, may be influenced by clearance or sequestration. This would mean a finding of activation or increased levels

immediately post exercise, whilst demonstrating a local response, may mean nothing after a few minutes. With this idea in mind we monitored potential neutrophil free radical production over 2 hours, and demonstrated increased responses for up 120 minutes, whilst elastase returned to normal within 5 - 10 minutes. We changed the study design for our investigation of platelet and coagulation function for several reasons. Firstly, claudication in the clinical setting involves repetitive episodes of temporary ischaemia in the lower limbs, and a repeated exercise test would be closer to the situation in real life. Secondly, platelet and coagulation studies in claudication had produced variable results, in part due to insensitive techniques and preparation artefact. A repeated exercise test was thought more likely to produce a definitive answer. Finally, one exercise period may only produce a marginal effect, and a second episode may result in greater responses, suggesting priming of the systems.

Following the idea of an extended period of hyper-responsiveness or priming, we thought any changes would need to be detectable in systemic blood, as opposed to the blood draining the effected limb. For this reason we assessed peripheral venous blood, acknowledging the likely dilutional effect. In addition, any changes were unlikely to be large, since patients do not infarct after every episode of claudication. Consequently, we aimed to use as sensitive technique as possible to look for an effect.

Inherent in this design were certain problems, some of which became more apparent towards the end of the study. We measured a series of factors over time in response to exercise. Several difficulties may arise from this. Firstly, although we had a control group in each study, we did not control for the effect of time alone, i.e., variations in the measure during the day in resting subjects. Thus, changes over time superimposed upon

changes from exercise may confound results. However, this is the normal situation for the patients and the measurements remain valid. Another area of possible confusion comes from clearance of the studied markers. Humoral markers maybe cleared from the body, by the kidneys or liver, at different rates. βTG and PF4 are both cleared in a bimodal fashion, which might explain the confusing variation in values over time[413]. Activated or primed cells may also be cleared, or sequestered, from the circulation at different rates. When this factor is combined with possible destruction of effete cells the resulting picture is further confused.

From this work we have demonstrated that claudication is associated with increased neutrophil activation/ potential. However, results from the platelet and coagulation studies have been unclear, the platelet study results in particular have been very difficult to interpret.

Neutrophils Our finding of neutrophil priming agrees in some respects with other work showing neutrophil activation, as decreased filterability[139,141,235-238] or increased CD11b expression[661], in exercising claudicants. However, these studies show cellular activation has occurred whereas our work only found an effect when neutrophils were stimulated. This difference may represent mixed cell populations, i.e., some neutrophils have been full stimulated and are now refractory or show decreased activity, others are activated, whilst others are primed. The balance of these groups may be such that increased activation was only found in response to stimulation.

Neutrophil priming may occur in response to humoral triggers or by cellular interaction, e.g., neutrophil to neutrophil or neutrophil to platelet interactions. One possible humoral agent is thromboxane A_2 that is released from both platelets and neutrophils. Recent work has linked thromboxane levels with the degree of neutrophil activation[593], but a priming role could be possible. We found increased thromboxane levels in claudicants and although the temporal relationship was not exact it could still have acted as a trigger for neutrophil priming.

The important finding from our work is that essentially normal neutrophils have the capacity, following claudication, to generate an increased activation response to stimulation. Furthermore, this priming lasts for up to 2 hours, and may be longer. Hence, claudicants may have an exaggerated response to infarction and thrombosis, through interaction with platelets, resulting in increased tissue damage and clinical effect.

Platelets Our failure to demonstrate clear evidence of platelet activation with claudication is in disagreement with most of other studies. Increased beta-thromboglobulin and platelet factor four levels[410,413,422] and thromboxane[218,421,422] have been reported post-exercise in claudicants. However, others have found the contrary[423]. Whilst preparation artefact may explain some of the differences, the marked temporal variations we found in βTG and PF4 levels following exercise illustrates the importance of sample timing and pre-test exercise. Thromboxane remains a difficult marker to assess. Our earlier studies showed an increase in levels at 15 - 35 minutes post-exercise, but subsequent studies showed only a late rise, following the second period of exercise. The sampling technique used in the platelet

studies was more precise and less prone to activation effects. Hence, the differences may have arisen because of the second exercise period. Our aggregation results were as confusing and contradictory as other have reported[314,388,389,394-397]. Although recent work has proposed SPAA type aggregation as a more effective measure[662].

We had hoped that whole blood flow cytometric measurement of surface glycoprotein markers of activation would overcome the difficulties of measuring platelet activation. However, we found no clear evidence of platelet activation at rest, or priming or activation following exercise in claudicants. This agrees with the work of Galt et al who found no evidence of platelet activation in claudicants at rest based on a large bank of surface markers[397], but is contrary to other work showing increased expression of platelet surface markers [414-416]. To our knowledge our study remains the only one measuring surface markers pre- and post-exercise in claudicants.

There are several possible reasons for our failure to find any significant differences, either between patient groups or with respect to controls, in the platelet studies. One feature of almost all the results from the platelet study has been the large amount of variation between the subjects, including the controls. Analysis has demonstrated significant differences about the mean in all subject groups. This has led to considerable overlap between the groups and contributed to the lack of any significant findings. Reasons for such variability are unclear but several factors may play a role. Intermittent claudication is a symptom of peripheral vascular disease, and is most commonly due to atherosclerosis. However, the pathological process of atheroma formation is more complex. Many diseases and factors influence the development of atherosclerosis and can have an independent effect on cellular and humoral function;

particularly platelet physiology. Diabetes, hypertension, smoking, stress and hypercholesterolaemia are all known to influence platelet function. Furthermore, medications such as β-blockers, non-steroidal anti-inflammatories and some newer cardiac drugs can all influence platelet behaviour. Unfortunately, due to practical and financial constraints, large scale testing of patients was not possible. Hence, analysis of sub-groups of diseases, drugs or outside factors was not possible in our study.

Platelet release and sequestration associated with exercise is uncertain with conflicting reports in the literature[611,614-616]. It is difficult to assess the potential effect on our results as there is little or no data on the behaviour of platelet populations with exercise in vascular patients. To measure platelet function we used established techniques as well as newer more sensitive methods, with minimal preparation. Despite this the effect of systemic dilution of an already small effect may not be detectable using these techniques. Alternatively, we used the wrong markers and/or agonists to detect any changes. However, we believe the markers and agonist are a good representation of elements likely to be involved in thrombosis. Finally, statistical analysis of repetitive measurements is complex and associated with difficulties in interpretation. This coupled with the comparatively small numbers in our study may have contributed to the lack of findings.

However, despite the complex disease processes, numerous confounding factors and difficult statistical interpretation, the ultimate result is that we found no evidence to support a role for claudication causing platelet activation and hence contributing to increased cardiovascular morbidity and mortality. A possible conclusion is that there

is no effect of claudication on platelet behaviour, hence, disproving this part of the theory.

Coagulation system Using indirect measures we found no evidence of activation of the coagulation system following exercise in claudicants. The levels of markers stayed constant over the study period with no response to either exercise test. Interestingly, although not significant, the pattern of D-dimer levels was identical to that of βTG and PF4. This would suggest some link with platelet release/ activation. Aspirin caused a loss of this pattern but no real change in the absolute values. This failure to find exercise induced activation of the coagulation system in claudicants agrees with the work of De Buyzere et al[568].

We found increased levels of D-dimer in patients suggesting increased fibrin degradation. This agrees with other published reports[169,569,662,663]. However, we found contradictory evidence of thrombin generation, with no real difference in F1 + 2 fragments between patients and controls and reduced TAT levels in patients. These findings are at odds with those of De Buyzere et al[568] and others[551,559,662-664].

Studying activation, particularly potential to be activated, in the coagulation system is extremely difficult. Essentially, the system is a dynamic, micro-system involving numerous local and systemic factors and accurate *in-vitro* or *ex-vivo* studies are virtually impossible. The only option available is measurement of factors released during coagulation or levels of precursors. We used markers for formation of thrombin, its neutralisation and the breakdown of fibrin, to provide an indirect measure of activation and possibly potential to be activated. Again there was

considerable variation between subjects in each of the groups, but differences were detected between patients and controls for D-dimer and TAT complexes. However, the differences were "static" with no effect of time or exercise. Furthermore, no difference was found from taking aspirin. These results have interesting possible connotations. Exercise has no effect on the coagulation system, but the disease itself does. Furthermore, aspirin, which is known to improve cardiovascular mortality, has no effect on the coagulation markers. Thus, there may be potential for anti-thrombotic agents, in addition to aspirin, in the management of chronic peripheral vascular disease.

We may have missed an effect due to exercise because of intra-group subject variability, small study numbers, complex statistical analysis or inadequate techniques to assess such a complex system. Again our failure to find an effect maybe that there is no effect.

Interaction We found no clear, statistically significant evidence of any interaction between neutrophils, platelets and/or the coagulation system. However, there were some suggestive findings. In particular, the increase levels of thromboxane in patients prior to the neutrophil priming effect. This is in keeping with recent evidence[593] and suggests a possible link with platelet activation. In addition, similar patterns of release for βTG and PF4, and D-dimer raises questions about links between platelet activation and fibrin breakdown.

CONCLUSION

The aim of this work was to assess possible mechanisms by which the process of claudication might contribute to the increased cardiovascular morbidity and mortality seen in these patients. Review of the literature relating to the mechanisms of atherogenesis, thrombotic complications of atherosclerosis and infarction of tissue, has confirmed a possible role for platelets, neutrophils and the coagulation system. We chose to evaluate each of these factors as a potential contributor to cardiovascular events. We have made the assumption that the presence of enhanced activation or potential for activation, in the above elements would translate into a more vigorous response to plaque rupture, thrombosis or infarction, and increase the risk and severity of clinical events.

With this in mind, we have found evidence that claudication is associated with increased neutrophil response to stimulation. In the presence of thrombosis and tissue infarction neutrophils may "over-react" and increase the inflammatory response in the peri-infarct tissue. This could increase the size of the infarct and hence the clinical effects.

Thus, conclude that we have found evidence to support claudication as a cause of increased cardiovascular morbidity and mortality, through the mechanism of neutrophil activation. However, we have no evidence for a similar role for platelets or the coagulation system.

FUTURE WORK

Atherosclerosis is a heterogeneous disease both in cause and pathophysiology. Consequently, some sub-groups of patients may demonstrate activation is response to claudication, whereas others show little effect. Further studies, initially a population screening or a simple pre- and post-exercise design, may help pick out those groups more likely to respond with exercise.

Neutrophils Although we have demonstrated a significant increase in the potential for activation of neutrophils following claudication, this work has raised several questions which require further investigation before the true role of neutrophils in claudication and its clinical course can be stated. There is little data on the pattern of leukocyte shifts in patients with peripheral vascular disease. Normal shifts are thought to arise from changes in cardiac output, catecholamine levels and cortisol release. PVD is associated with impaired cardiac function, increased catecholamine and cortisol levels due to the pain of claudication and the atherosclerotic changes in the vessel walls may have effect on leukocyte margination seen in normal flow patterns. Regular exercise is associated with better prognosis, partly due to improved rheology and conditioning effects. Studies on the effect of training on neutrophil function, white cell count and the increases associated with exercise may help guide treatment. The importance of cell trapping is uncertain. We found a relative neutropenia following exercise, which could be interpreted as due to neutrophil trapping. The dynamics of such trapped neutrophils, particularly of their washout phase from the muscle, are unknown. Studies to determine if this, or humoral agents released by trapped neutrophils and acting on circulating cells, might explain the extended duration of our results. Following on from this is further work

on the dynamics of different neutrophil sub-populations. There is little data on the turnover of neutrophils in peripheral vascular disease. Different sub-populations have different cellular, surface or adhesion markers. Their response to the stress of claudication may vary, furthermore, the levels of different sub-populations may vary in claudicants at rest. Flow cytometry is the ideal tool to study changes in cellular surface markers and may provide useful insights in the future. In addition, claudication is a dynamic and repetitive process and further work is required to determine the effect of further exercise challenges on neutrophil parameters.

Platelets There are few studies using flow cytometric technique to assess platelet function in peripheral vascular disease, and further studies of surface markers using the minimal preparation, whole blood technique may give some idea as to the most appropriate glycoproteins to study. Further patient screening-type studies may help determine those patients in whom platelet dysfunction may play a significant role. Results from such work could then be used to target suitable groups to investigate the effect of exercise. The sensitivity of flow cytometric techniques means "normal" variations in platelet responsiveness maybe more pronounced and work is required to determine daily variations in the expression of surface markers. In addition, flow cytometry and radiolabelled studies could be used to investigate platelet population dynamics, especially the effect of surface glycoprotein expression on release and sequestration.

Coagulation system We have shown increased D-dimer levels at rest and a tendency to increases upon exercise, but the mechanisms behind these findings are unclear, and require further investigation. In particular, studies of fibrinolysis and

fibrin breakdown pre- and post-exercise may help explain these findings. In the static situation it would be of value to look at thrombin generation more closely, and with greater numbers of subjects. Of particular interest would be thrombin inhibition and the proportion of activity due to antithrombin III compared to α^2-macroglobulin. Investigation of intra-plaque thrombin generation and fibrin formation/breakdown, compared to intravascular events could provide insight into atherogenesis, disease progression and thrombotic complications. Measuring the balance, and imbalance, of two dynamic systems such as coagulation and fibrinolysis would be complicated, but perhaps vital to our understanding of the significance of plasma markers, as well as the biology of vascular diseases.

Neutrophils, platelets and the coagulation system are known to interact at the site of endothelial damage[665,666] thrombosis[667] and infarction. Furthermore, platelets and neutrophils have been shown to interact in symptomatic ischaemic heart disease[668]. Using different markers and altering the "gate" settings of the flow cytometer may allow investigation of the interaction of the components of thrombosis[669]. However, although techniques to study the various components of blood are becoming increasingly sensitive, there remains a fundamental problem. These methods look at components of circulating blood and thrombosis in isolation. In reality the process of atherosclerotic vessel thrombosis is a complex interaction of all the components we have studied, and more. An animal model of atherosclerotic plaque rupture, using balloon angioplasty or an atherectomy device, could be developed. Superimposing an animal model of claudication over this would allow assessment of the response to plaque rupture in those animals which claudicate and those, which don't. A myocardial infarction model could also be used to assess the

effect of claudication on the size and significance of a standard infarction injury. Further investigation using these models would enable the mechanism behind any cellular or humoral activation to be determined. Both models could then be studied to determine the effect of pharmacological manipulation on the response, and hence, potential future treatments.

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APPENDIX ONE: DATA

NEUTROPHIL DATA

	0	5	10	15	20	35	65	125
Controls	6.08 (5.49-7.1)	7.71 (6.84-8.85)	6.86 (6.08-7.89)	6.34 (5.73-7.27)	6.09 (5.54-7.06)	5.93 (5.36-6.96)	6.16 (5.61-7.06)	6.49 (5.86-7.14)
Patients	7.42 (6.18-8.66)	9.79 (8.04-11.5)	8.86 (7.18-10.5)	8.18 (6.64-9.71)	7.8 (6.36-9.24)	7.87 (6.53-9.19)	8.24 (7.03-9.44)	7.94 (7.03-9.44)

Table 1 Total white cell counts for controls and patients with 95% confidence intervals.

	0	5	10	15	20	35	65	125
Controls	3.71 (3.11-4.66)	4.28 (3.57-5.21)	4 (3.37-4.79)	3.8 (3.26-4.61)	3.77 (3.23-4.55)	3.68 (3.13-4.54)	3.84 (3.29-4.59)	3.9 (3.38-4.51)
Patients	4.78 (3.75-5.81)	5.63 (4.48-6.78)	5.39 (4.2-6.58)	5.12 (3.97-6.26)	4.95 (3.79-6.09)	5 (3.95-6.05)	5.34 (4.31-6.37)	5.19 (3.7-6.68)

Table 2 Neutrophil counts for controls and patients with 95% confidence intervals.

	0	5	10	15	20	35	65	125
Controls	0.62 (0.59-0.63)	0.56 (0.5 - 0.61)	0.59 (0.53-0.64)	0.61 (0.55-0.66)	0.62 (0.59-0.65)	0.63 (0.6 - 0.65)	0.63 (0.6 - 0.65)	0.61 (0.58-0.64)
Patients	0.63 (0.61-0.66)	0.57 (0.52-0.62)	0.61 (0.57-0.64)	0.62 (0.6 - 0.64)	0.62 (0.59-0.65)	0.63 (0.58-0.66)	0.64 (0.6 - 0.67)	0.64 (0.62-0.67)

Table 3 Neutrophil count as a fraction of total white cell count in controls and patients over time, with 95% CI

	0	5	10	15	20	35	65	125
Controls	1.69 (1.53-1.85)	2.49 (2.21-2.77)	1.99 (1.76-2.22)	1.8 (1.63-1.97)	1.69 (1.54-1.84)	1.5 (1.36-1.64)	1.66 (1.51-1.81)	1.9 (1.72-2.08)
Patients	1.79 (1.49-2.09)	2.76 (2.28-3.24)	2.38 (1.94-2.82)	2.02 (1.64-2.39)	1.92 (1.56-2.28)	1.86 (1.55-2.17)	1.97 (1.68-2.26)	1.88 (1.52-2.24)

Table 4 Lymphocyte counts in controls and patients over time, with 95% CI

	0	5	10	15	20	35	65	125
Controls	0.49 (0.44-0.54)	0.73 (0.65-0.81)	0.57 (0.51-0.64)	0.49 (0.44-0.54)	0.47 (0.43-0.5)	0.5 (0.45-0.55)	0.44 (0.4-0.48)	0.53 (0.48-0.59)
Patients	0.59 (0.49-0.69)	0.84 (0.69-0.99)	0.76 (0.62-0.9)	0.69 (0.56-0.82)	0.64 (0.52-0.76)	0.68 (0.57-0.79)	0.62 (0.53-0.71)	0.61 (0.49-0.73)

Table 5 Monocyte counts in controls and patients over time, with 95% CI

	0	5	10	15	20	35	65	125
Controls	0.287 (0.27-0.31)	0.322 (0.29-0.35)	0.289 (0.26-0.32)	0.287 (0.25-0.32)	0.28 (0.26-0.3)	0.265 (0.25-0.28)	0.27 (0.26-0.287)	0.29 (0.27-0.3)
Patients	0.25 (0.24-0.26)	0.29 (0.27-0.31)	0.273 (0.25-0.29)	0.254 (0.24-0.27)	0.253 (0.24-0.27)	0.244 (0.23-0.26)	0.25 (0.23-0.27)	0.246 (0.22-0.27)

Table 6 Lymphocyte counts as a fraction of total white cell count in controls and patients over time, with 95% CI

	0	5	10	15	20	35	65	125
Controls	0.085 (0.08-0.09)	0.099 (0.087-0.11)	0.088 (0.095-0.138)	0.083 (0.077-0.089)	0.082 (0.078-0.086)	0.089 (0.082-0.096)	0.075 (0.068-0.082)	0.083 (0.076-0.09)
Patients	0.081 (0.075-0.087)	0.089 (0.083-0.095)	0.138 (0.098-0.178)	0.183 (0.153-0.213)	0.127 (0.09-0.164)	0.088 (0.081-0.097)	0.076 (0.064-0.088)	0.08 (0.071-0.089)

Table 7 Monocyte counts as a fraction of total white cell count in controls and patients over time, with 95% CI

	0	5	10	15	20	35	65	125
Controls	17.23 (13.7-20.8)	22.43 (18-26.8)	18.25 (14.4-22)	19.35 (14.9-23.7)	18.58 (13.9-23.3)	20.1 (14.6-25.5)	18.6 (14.7-22.5)	20.13 (16.3-23.9)
Patients	18.11 (14.1-22.1)	23.41 (18.7-28.1)	18.31 (14.5-22.1)	19.11 (15.3-22.9)	18.84 (14.6-23.1)	18.87 (14.4-23.3)	18.87 (14.9-22.8)	20.98 (16.9-25)

Table 8 Plasma elastase values for controls and patients with 95% confidence intervals.

	0	5	10	15	20	35	65	125
Controls	2.82 (2.27-3.37)	3.03 (2.45-3.61)	2.78 (2.27-3.29)	3.14 (2.46-3.82)	3.12 (2.4-3.84)	3.46 (2.89-4.03)	3.06 (2.38-3.74)	3.2 (2.52-3.88)
Patients	2.72 (2.42-3.02)	2.69 (2.3-3.08)	2.37 (2.14-2.6)	2.69 (2.27-3.11)	2.8 (2.44-3.16)	2.7 (2.22-3.18)	2.54 (2.04-3.04)	2.85 (2.39-3.31)

Table 9 Elastase production standardised for white cell count in controls and patients over time, with 95% CI

	0	5	10	15	20	35	65	125
Controls	4.75 (4.48-5.02)	5.54 (5.3-5.78)	4.88 (4.55-5.21)	5.31 (4.94-5.68)	5.13 (4.73-5.53)	5.84 (5.37-6.31)	5.05 (4.63-5.47)	5.34 (4.97-5.71)
Patients	4.42 (4.12-4.72)	4.77 (4.35-5.19)	3.95 (3.42-4.48)	4.38 (3.97-4.79)	4.54 (4.18-4.9)	4.31 (4.01-4.61)	4.01 (3.44-4.58)	4.53 (4.21-4.85)

Table 10 Elastase production standardised for neutrophil count in controls and patients over time, with 95% CI

	0	5	10	15	20	35	65	125
Controls	0 (-12 -11)	0.3 (-8.4-0.6)	-0.8 (-14.5-2.7)	0 (-12.1 - 12)	-2.1 (-14.6-0.6)	-0.6 (12.6-11.7)	0.2 (-12.2-12.4)	-0.1 (-12.7-12.4)
Patients	0 (-11.4-12.7)	-5.2 (-14.2-16.1)	3.6 (-8.8- 16.2)	2.7 (-10.2-15.6)	6.3 (-6.2- 18.8)	10.5 (-3.5- 24.3)	17 (3.8 - 30.2)	19.8 (8.6 - 31)

Table 11 Neutrophil superoxide release, as a percentage of resting value, for controls and patients with 95% confidence intervals.

	0	5	10	15	20	35	65	125
Controls	0.202 (0.136-0.273)	0.244 (0.045-0.443)	0.162 (0.077-0.248)	0.173 (0.103-0.243)	0.212 (0.095-0.328)	0.196 (0.146-0.245)	0.212 (0.106-0.295)	0.208 (0.115-0.286)
Patients	0.335 (0.233-0.436)	0.375 (0.227-0.524)	0.457 (0.301-0.614)	0.525 (0.361-0.689)	0.509 (0.368-0.651)	0.596 (0.409-0.783)	0.517 (0.365-0.668)	0.42 (0.23-0.61)

Table 12 Neutrophil hydrogen peroxide generation for controls and patients with 95% confidence intervals.

	0	5	10	15	20	35	65	125
Controls	15.5 (7.3-23.5)	25.6 (13.9-36.8)	19.5 (12.1-26.5)	26.7 (13.4-37.6)	27.3 (16.8-35.7)	32.2 (16.6-44.8)	23.4 (12.1-34.1)	19.4 (10.9-26.6)
Patients	12 (9-14.7)	28.5 (9.8-45.1)	29.8 (11.1-46.1)	43.2 (11.5-71)	52.8 (13.7-86.7)	42.2 (13.9-66.7)	32.4 (13.9-48.1)	31.2 (13.9-48.1)

Table 13 Thromboxane B_2 levels for controls and patients with 95% confidence intervals.

PLATELET DATA

a) Flow Cytometry set-up data

Volume of MoAb	0	5(1/20)	5(1/10)	5(1/5)	5(1/2)	5	10	15	20
Dilution factor	0	0.0036	0.0071	0.0143	0.0357	0.0714	0.1429	0.2143	0.2857
Negative MFI	0.163	3.08	5.55	12.59	20.4	30.47	37.17	40.54	39.78
ADP MFI	0.165	3	6.27	12.18	20.6	32.44	37.62	41	42.93
α-thrombin MFI	0.184	2.75	5.67	11.41	19.96	30.64	39.4	41.15	40.89

Table 14 Mean fluorescence intensity for each group plotted against 'concentration' (dilution factor) of GPIb MoAb. (Values in parenthesis indicate dilution of neat MoAb)

	0	0.5	1	2	5	7.5	10
Volume of Ab	0ul	5ul(1:20)	5ul(1:10)	5ul(1:5)	5ul(1:2)	5ul	10ul
Dilution factor	0	0.00357	0.00714	0.01429	0.03571	0.07143	0.14286
Negative % +ve cells	2.45	10.85	12.6	16.12	76.55	81.95	72.5
ADP % +ve cells	3.14	45.1	60.25	78.21	96.6	100	100
a-TH % +ve cells	3.61	78.26	86.46	91.47	97.65	100	100

Table 15 Results and graph of the percentage positive cells against the dilution of Fb antibody, with and without agonist.

Volume of MoAb	0ul	5ul (1/5)	5ul (1/2)	5ul	10ul	15ul	20ul	30ul
Dilution factor	0	0.01429	0.03571	0.07143	0.14286	0.21429	0.28571	0.42857
Neg	0.1588			0.2566	0.3041	0.3576	0.4104	0.491
ADP				1.712	2.1271	2.64	3.096	3.653
a-TH		15.84	17.24	18.12	21.9	22.52	23.05	22.766

Table 16 Results and graph of the percentage positive cells against the dilution of P-selectin MoAb, with and without agonist.

	1E-08	3E-08	1E-07	3E-07	0.000001	0.000003	0.00001	0.00003
Fb	7.08222	7.0906	17.765	37.699	68.525	77.385	84.742	82.10429
P-sel	6.47	6.73	7.055	10.215	21.42222	30.201	34.554	35.422

Table 17 Expression of Fibrinogen binding and P-selectin expression in response to varying concentrations of ADP.

[α- thrombin]	0.0025	0.005	0.01	0.025	0.05	0.1	0.2	0.4	0.8
Fb	13.048	13.263	19.454	67.275	87.215	90.985	94.308	96.334	95.653
P-sel	5.9722	6.2644	7.3767	36.82	67.49	83.475	90.409	89.861	88.871

Table 18 Expression of Fibrinogen binding and P-selectin in response to varying concentrations of α-thrombin.

b) Patient results data

	0 mins	10 mins	20 mins	35 mins	45 mins	55 mins
Controls	212 (196 - 229)	220 (202 - 236)	216 (199 - 233)	213 (197 - 231)	216 (199 - 233)	214 (198 - 232)
Patients: Aspirin	216 (190 - 252)	234 (199 - 260)	227 (196 - 256)	224 (194 - 255)	222 (194 - 254)	220 (192 - 252)
Patients: No aspirin	212 (186 - 238)	224 (198 - 250)	218 (192 - 244)	215 (188 - 242)	227 (200 - 254)	220 (194 - 248)

Table 19. Platelet count values in patients and controls over time, with 95% CI

	0 mins	10 mins	20 mins	35 mins	45 mins	55 mins
Controls	8.84 (8.36 - 9.24)	8.83 (8.36 - 9.28)	8.61 (8.16 - 9.04)	8.51 (8.08 - 8.96)	8.45 (8.00 - 8.88)	8.28 (7.84 - 8.72)
Patients: Aspirin	9.66 (9.00-10.30)	9.60 (8.95-10.25)	9.71 (9.05-10.35)	9.48 (8.85-10.15)	9.51 (8.85-10.15)	9.22 (8.55 - 9.85)
Patients: No aspirin	9.32 (8.58-10.26)	9.42 (8.58-10.26)	9.55 (8.76-10.50)	9.32 (8.46-10.20)	9.17 (8.34-10.02)	9.05 (8.28-10.02)

Table 20. Platelet volumes in patients and controls over time, with 95% CI

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	2.39 (2.18-2.61)	2.2 (1.89-2.52)	2.54 (1.77-3.32)	1.81 (1.33-2.29)
Patients: Aspirin	2.37 (1.61-3.13)	2.76 (1.73-3.79)	3.72 (1.78-5.67)	4.23 (0.74-7.73)
Patients: No aspirin	2.56 (1.91-3.2)	3.72 (1.78-5.67)	4.23 (0.74-7.7)	2.56 (1.91-3.2)

Table 21 Fibrinogen binding, expressed as % positive cells, in unstimulated platelets, in patients and controls over time, with 95% CI.

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	2.49 (2.12-2.87)	2.09 (1.72-2.46)	2.29 (1.64-2.94)	2.27 (1.47-3.07)
Patients: Aspirin	2.2 (1.89-2.5)	1.74 (1.44-2.04)	1.72 (1.32-2.12)	1.6 (1.17-2.04)
Patients: No aspirin	2.49 (2.25-2.75)	2.15 (1.83-2.46)	2.43 (1.6-3.25)	2.06 (1.49-2.62)

Table 22 P-selectin binding, expressed as % positive cells, in unstimulated platelets, in patients and controls over time, with 95% CI.

	0 mins (Pre-Ex 1)	10mins (Post-Ex 1)	35mins (Pre-Ex 2)	45mins (Post-Ex 2)
Controls	67.3 (60.1-74.4)	67.2 (59.6-74.8)	63.8 (54.6-73.1)	63.4 (55.9-70.9)
Patients: Aspirin	66.4 (58.9-73.9)	68.3 (61.4-75.3)	63.5 (53.2-73.9)	64.7 (54.9-74.5)
Patients: No aspirin	62.6 (54.9-70.2)	65.5 (59.1-71.9)	62.5 (47.1-77.8)	60.3 (51.3-69.3)

Table 23 Fibrinogen binding, expressed as % positive cells, with 1 x 10^{-6}mol/l ADP in patients and controls over time, with 95% CI.

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	47.93 (38.5 - 57.4)	49.11 (39.2 - 58.8)	44.24 (35 - 53.9)	43.34 (33.6 - 53.6)
Patients: Aspirin	43.31 (35.4 - 51)	48.09 (40.2 - 55.8)	43.57 (35.4 - 51.6)	45.83 (37.8 - 54)
Patients: No aspirin	44.58 (36 - 52.8)	46.37 (39 - 54)	43.2 (35.4 - 51)	42.31 (33.6 - 51)

Table 24 Fibrinogen binding, as % positive cells, with 3 x 10^{-7}mol/l ADP in patients and controls over time, with 95% CI.

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	22.9 (12.3 - 33.5)	25.2 (14.7 - 35.7)	20.1 (14.2 - 25.9)	18.5 (10.5 - 26.6)
Patients: Aspirin	22.8 (16.8 - 28.8)	23.1 (18.6 - 27.6)	21.8 (14.6 - 28.9)	20.5 (13.8 - 27.2)
Patients: No aspirin	22.3 (15 - 29.5)	20.4 (15 - 25.7)	20.5 (13.9 - 27)	19.3 (11.3- 25.2)

Table 25 Fibrinogen binding, expressed as % positive cells, with 1 x 10^{-7}mol/l ADP in patients and controls over time, with 95% CI.

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	15.04 (11.4 - 18.7)	12.9 (10.2 - 15.6)	13.5 (10.2 - 16.9)	14.9 (12.2 - 17.7)
Patients: Aspirin	17.2 (12.7 - 21.7)	15.5 (11.8 - 19.3)	15.3 (11.2 - 19.4)	14.9 (10.1 - 19.8)
Patients: No aspirin	19.6 (13.8 - 25.3)	17.3 (12.5 - 22.2)	17.3 (10.5 - 24.2)	18.2 (12.5 - 24)

Table 26 P-selectin binding, expressed as % positive cells, with 1 x 10^{-6}mol/l ADP in patients and controls over time, with 95% CI.

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	6.92 (4.75 - 9.1)	5.8 (4.26 - 7.39)	5.91 (3.78 - 8.04)	6.45 (4.79 - 8.09)
Patients: Aspirin	7.73 (5.63 - 9.8)	6.83 (4.9 - 8.73)	6.29 (3.79 - 8.8)	5.85 (4.44 - 7.27)
Patients: No aspirin	9.04 (5.78 - 12.3)	7.82 (5.08 - 10.6)	7.35 (3.92 - 10.8)	7.64 (4.79 - 10.5)

Table 27 P-selectin binding, expressed as % positive cells, with 3 x 10^{-7}mol/l ADP in patients and controls over time, with 95% CI.

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	2.98 (2.41 - 3.55)	2.86 (2.01 - 3.72)	3.23 (1.32 - 5.13)	2.92 (1.96 - 3.88)
Patients: Aspirin	3.05 (2.46 - 3.63)	2.66 (2.09 - 23.23)	2.38 (1.76 - 2.99)	2.28 (1.73 - 2.83)
Patients: No aspirin	3.67 (2.75 - 4.58)	3.22 (2.35 - 4.09)	1.78 (1.99 - 4.39)	3.44 (2.37 - 4.51)

Table 28 P-selectin binding, expressed as % positive cells, with 1 x 10^{-7}mol/l ADP in patients and controls over time, with 95% CI.

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	72.89 (60 - 85.5)	58.48 (48 - 69)	50.1 (37.5 - 63)	59.82 (46.5 - 73.5)
Patients: Aspirin	74.75 (60 - 90)	68.7 (50 - 88)	45.2 (30 - 60)	52.5 (38 - 68)
Patients: No aspirin	68.95 (54 - 84)	69.6 (54 - 85.5)	56.3 (39 - 73.5)	59.5 (42 - 78)

Table 29 Fibrinogen binding, expressed as % positive cells, with 0.03units/ml α-thrombin in patients and controls over time, with 95% CI

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	43.1 (28.5 - 57)	40.1 (24 - 55.5)	27.5 (13.5 - 42)	40.8 (27 - 55.5)
Patients: Aspirin	49.8 (38 - 64)	49.1 (34 - 64)	32.6 (16 - 48)	19 (4 - 34)
Patients: No aspirin	47.3 (27 - 67.5)	45 (27 - 63)	41 (21 - 60)	33.8 (12 - 55.5)

Table 30 Fibrinogen binding, expressed as % positive cells, with 0.02units/ml α-thrombin in patients and controls over time, with 95% CI

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	8.87 (0.6 - 16.8)	5.72 (-1.8 - 13.2)	11.6 (3.6 - 19.2)	10.3 (2.4 - 18)
Patients: Aspirin	11.6 (6 - 17)	8.7 (3 - 14.5)	8.4 (2.5 - 14.5)	5.48 (0 - 11)
Patients: No aspirin	14.6 (7.8 - 21.6)	10.5 (3 - 18)	9.98 (3 - 16.8)	8.66 (1.8 - 15.6)

Table 31 Fibrinogen binding, expressed as % positive cells, with 0.01units/ml α-thrombin in patients and controls over time, with 95% CI

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	66.2 (55.1 - 77.2)	59 (47.6 - 70.4)	52.48 (34.7 - 70.2)	62.66 (48.5 - 76.9)
Patients: Aspirin	69.8 (59.9 - 79.7)	66.9 (53.6 - 80.3)	48.88 (30.4 - 67.4)	55.75 (39.8 - 71.7)
Patients: No aspirin	68.6 (56.1 - 81)	70.65 (58.8 - 82.4)	57.4 (39.9 - 74.9)	61.9 (44.9 - 78.9)

Table 32 P-selectin binding, expressed as % positive cells, with 0.05units/ml α-thrombin in patients and controls over time, with 95% CI.

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	30.3 (16.2-44.5)	28.9 (16.1-41.8)	19.1 (8.2-29.9)	27.3 (12.9-41.7)
Patients: Aspirin	30.48 (15.2-45.8)	32.4 (17.1-47.6)	17 (5.6-28.4)	18.8 (9.7-27.9)
Patients: No aspirin	36.9 (19.5-54.4)	36 (22.7-49.4)	24.4 (11-38.4)	26.9 (13.7-40.1)

Table 33 P-selectin binding, expressed as % positive cells, with 0.02units/ml α-thrombin in patients and controls over time, with 95% CI

	0 mins (Pre-Ex 1)	10mins(Post-Ex 1)	35mins (Pre-Ex 2)	45mins(Post-Ex 2)
Controls	5.95 (1.5 - 10.2)	4.76 (0.3 - 9)	4.2 (-0.3 - 8.7)	5.24 (0.6 - 9.9)
Patients: Aspirin	3.19 (2.4 - 3.92)	2.59 (1.76 - 3.44)	2.08 (1.2 - 2.88)	2.04 (1.2 - 2.88)
Patients: No aspirin	4.66 (3.48 - 5.88)	3.85 (2.64 - 5.04)	2.96 (1.8 - 4.2)	2.98 (1.68 - 4.2)

Table 34 P-selectin binding, expressed as % positive cells, with 0.01units/ml α-thrombin in patients and controls over time, with 95% CI

	0 mins	10 mins	20 mins	35 mins	45 mins	55 mins
Controls	34.7 (10.9 - 58.4)	28.5 (4.2 - 52.9)	31.7 (15.2 - 48.1)	46.6 (11.5 - 73.9)	49.6 (6.7 - 92.5)	147.5 (-10.7 - 305.6)
Patients: Aspirin	10.4 (9.9 - 10.9)	11.3 (9.7 - 12.8)	10 (10 - 10)	12.5 (9.3 - 15.8)	10.3 (9.6 - 11.1)	11 (9.9 - 12.1)
Patients: No aspirin	39 (-0.5 - 78.5)	30.9 (13.1 - 48.8)	29.8 (11.4 - 48.1)	34.9 (3.7 - 66.3)	50.9 (17.3 - 84.6)	104.3 (-28.4 - 237.1)

Table 35. Thromboxane B_2 values in patients and controls over time, with 95% CI

	0 mins	10 mins	20 mins	35 mins	45 mins	55 mins
Controls	170.3 (7.5 - 333)	256.7 (89.5 - 423.8)	56.7 (17.6 - 95.8)	211.4 (52.7 - 370.2)	136.9 (-21.6 - 295.4)	287.1 (64.1 - 510)
Patients: Aspirin	79 (42.2 - 115.9)	207.3 (111.3 - 303.3)	68.2 (-20.3 - 156.8)	82.1 (44.6 - 119.7)	51.8 (16.3 - 87.3)	102.7 (69.4 - 136.1)
Patients: No aspirin	332 (101.7 - 562.6)	443.8 (202 - 685.6)	232.9 (44.8 - 420.9)	378.6 (152.5 - 604.6)	212.2 (41.8 - 382.7)	326.4 (78.9 - 573.9)

Table 36. Beta thromboglobulin values in patients and controls over time, with 95% CI

	0 mins	10 mins	20 mins	35 mins	45 mins	55 mins
Controls	31.6 (-13.7 - 76.9)	88.1 (6.8 - 169.4)	33.1 (-27.4 - 93.7)	65.7 (-8.5 - 139.9)	17.9 (-1.7 - 37.6)	105.2 (2.6 - 207.8)
Patients: Aspirin	10 (1.67 - 18.4)	37.2 (19.2 - 55.3)	7.8 (-4.5 - 20.1)	11.9 (2.68 - 21.2)	7.68 (-4.8 - 20.2)	16.5 (5.1 - 27.9)
Patients: No aspirin	88.3 (6.7 - 169.8)	137.6 (45.6 - 229.5)	63.2 (-6.1 - 132.5)	106.5 (16.5 - 196.5)	44.8 (8.1 - 81.4)	149.3 (38.2 - 260.4)

Table 37. Platelet Factor 4 values in patients and controls over time, with 95% CI

COAGULATION DATA

	0 mins	10 mins	20 mins	35 mins	45 mins	55 mins
Controls	0.878 (0.843 - .912)	0.88 (0.847 - 0.913)	0.882 (0.849 - 0.915)	0.886 (0.845 - 0.926)	0.878 (0.843 - 0.912)	0.882 (0.839 - 0.925)
Patients: Aspirin	0.859 (0.823 - 0.895)	0.851 (0.814 - 0.888)	0.847 (0.809 - 0.885)	0.847 (0.81 - 0.883)	0.842 (0.808 - 0.875)	0.829 (0.785 - 0.873)
Patients: No aspirin	0.862 (0.809 - 0.914)	0.85 (0.801 - 0.899)	0.846 (0.801 - 0.889)	0.844 (0.799 - 0.889)	0.83 (0.788 - 0.872)	0.825 (0.774 - 0.876)

Table 38. APTT values in patients and controls over time, with 95% CI

	0 mins	10 mins	20 mins	35 mins	45 mins	55 mins
Controls	96.7 (72 - 122)	81.7 (56 - 106)	82.5 (58 - 108)	90.8 (66 - 116)	92.5 (68 - 118)	81.7 (56 - 106)
Patients: Aspirin	269 (-125 - 675)	309 (-100 - 700)	318 (-75 - 725)	304 (-100 - 700)	385 (-25 - 775)	331 (-75 - 725)
Patients: No aspirin	245 (-75 - 575)	318 (0 - 650)	222 (-100 - 550)	354 (25 - 675)	271 (-50 - 600)	325 (0 - 650)

Table 39. D-dimer levels in patients and controls over time, with 95% CI

	0 mins	10 mins	20 mins	35 mins	45 mins	55 mins
Controls	1.95 (1.51 - 2.39)	2.1 (1.61 - 2.59)	2.09 (1.51 - 2.67)	1.94 (1.32 - 2.57)	1.96 (1.54 - 2.38)	2.23 (1.77 -2.68)
Patients: Aspirin	1.93 (1.57 -2.28)	2.02 (1.62 - 2.42)	2.45 (1.47 - 3.43)	2.33 (1.44 - 3.23)	2.15 (1.53 - 2.77)	2.26 (1.61 - 2.9)
Patients: No aspirin	2.27 (1.63 - 2.9)	2.5 (1.81 - 3.19)	2.39 (1.53 - 3.26)	2.25 (1.57 - 2.93)	2.4 (1.72 - 3.08)	2.29 (1.53 - 3.06)

Table 40. F1 & 2 Fragment levels in patients and controls over time, with 95% CI

	0 mins	10 mins	20 mins	35 mins	45 mins	55 mins
Controls	4.23 (1.57 - 6.88)	4.37 (1.65 - 7.09)	4.28 (1.68 - 6.87)	4.9 (1.43 - 7.55)	5 (1.36 - 8.64)	3.97 (1.57 - 6.36)
Patients: Aspirin	1.25 (0.9 - 1.59)	1.31 (1.09 - 1.52)	1.37 (0.69 - 2.04)	1.18 (0.98 - 1.37)	1.38 (0.89 - 1.87)	1.94 (0.68 - 3.21)
Patients: No aspirin	1.29 (0.82 - 1.76)	1.6 (0.87 - 2.34)	1.37 (0.69 - 2.04)	1.18 (0.98 - 1.37)	1.29 (0.93 - 1.65)	1.64 (0.93 - 2.35)

Table 41. TAT levels in patients and controls over time, with 95% CI

APPENDIX TWO: PATIENT DATA

1. NEUTROPHIL STUDY

Patients	Age	Sex	Pre-Ex ABPI	Post-Ex ABPI	ECG	Routine Bloods*	Blood Pressure	Exercise Test***
1	57	M	0.72	0.36	✓	Normal	125/60	Y
2	61	M	0.66	0.4	✓	Normal	115/60	Y
3	62	M	0.68	0.27	✓	Normal	165/85	Y
4	59	M	0.58	0.24	✓	Normal	175/75	Y
5	62	M	0.78	0.58	✓	Normal	140/80	Y
6	65	M	0.65	0.39	✓	Normal	135/70	Y
7	72	F	0.7	0.53	✓	Normal	110/58	Y
8	60	M	0.32	0	✓	Normal	120/85	Y
9	66	M	0.67	0.45	✓	Normal	145/80	Y
10	70	M	0.54	0.22	✓	Normal	155/65	Y
11	68	M	0.6	0.31	✓**	Normal	150/70	Y
12	62	M	0.5	0.15	✓	Normal	130/85	Y
13	66	M	0.57	0.27	✓	Normal	140/85	Y
14	68	M	0.29	0.1	✓	Normal	165/80	Y
15	64	M	0.76	0.32	✓	Normal	170/90	Y
16	62	M	0.8	0.42	✓	Normal	150/65	Y
Controls								
1	58	M	1.1	1	✓	Normal	140/80	N
2	67	M	1	1.06	✓	Normal	135/76	N
3	64	M	1.02	1.1	✓	Normal	145/88	N
4	63	M	1	1.2	✓	Normal	110/65	N
5	62	M	1.05	1.05	✓	Normal	120/70	N
6	66	M	1.04	1.08	✓	Normal	120/80	N
7	77	M	1	1.1	✓	Normal	135/60	N
8	75	M	0.98	1.15	✓	Normal	115/70	N
9	66	F	1	1.2	✓	Normal	108/58	N
10	69	M	1.2	1.2	✓	Normal	130/75	N
11	67	M	1.05	1.1	✓	Normal	120/75	N
12	70	M	1	1	✓	Normal	125/80	N

* Including: Haemoglobin, White cell count, Platelet count, Urea, Creatinine & Electrolytes, ESR, Glucose

** ? First degree heart block, assessed by cardiologist and thought to have no significant ischaemic disease

*** Limited by claudication

2. PLATELET/COAGULATION STUDY

Patients	Age	Sex	Pre-Ex ABPI	Post-Ex ABPI	ECG	Routine Bloods*	Blood Pressure	Exercise Test***
1	52	M	0.55	0	✓	Normal	110/65	Y
2	58	M	0.56	0.26	✓	Normal	160/80	Y
3	67	M	0.58	0.2	✓	Normal	165/85	Y
4	59	M	0.61	0.26	✓	Normal	145/88	Y
5	65	M	0.4	0.13	✓	Normal	190/78	Y
6	66	M	0.6	0.39	✓	Normal	120/70	Y
7	79	M	0.6	0.26	✓	Normal+	150/85	Y

8	68	M	0.54	0.23	✓	Normal	125/60	Y
9	75	M	0.59	0.21	✓	Normal++	130/70	Y
10	67	M	0.29	0	✓	Normal	165/90	Y
11	72	M	0.38	0.18	✓	Normal	145/80	Y
12	69	M	0.96	0.7	✓	Normal	120/80	Y
Controls								
1	58	M	1.08	1.25	✓	Normal	105/50	N
2	63	M	1.02	1.1	✓	Normal	115/70	N
3	59	M	1.06	1.09	✓†	Normal	150/80	N
4	69	M	1.07	1.15	✓	Normal	160/70	N
5	67	M	1.09	1.2	✓	Normal	135/75	N
6	64	M	1.23	1.54	✓	Normal	140/80	N
7	74	M	1.1	1.2	✓	Normal+	135/60	N
8	68	M	1.06	1.06	✓	Normal	120/67	N
9	77	M	1.12	1.6	✓	Normal	175/78	N
10	70	M	1	1	✓	Normal	160/60	N
11	68	M	1	1.1	✓	Normal	140/80	N
12	71	M	1.02	1.1	✓	Normal	135/85	N

* Including: Haemoglobin, White cell count, Platelet count, Urea, Creatinine & Electrolytes, ESR, Glucose

*** Limited by claudication

† Occasional ventricular ectopics, assessed by cardiologist and thought to have no significant ischaemic disease

\+ Urea 0.1-0.3 above lab upper limit, creatinine normal

++ Glucose 0.6mmol/l above normal range for random glucose. Fasting result normal