Haemostatic Changes in Intermittent Claudication

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Statement of Originality

The work presented in this thesis is entirely original, and done by myself. Advice was received from Mr Tim Marshall, Senior Lecturer in Public Health and Epidemiology regarding the analysis of the data in Chapter 9. This thesis has not been presented in candidature for any other degree, postgraduate diploma or professional qualification.

Signed

Date 6 July 2004

Acknowledgments

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Both Ruth Penfold and Ellen Drew were essential to the recruitment of patients and the conduct of the study on a day to day basis.

Finally, I cannot overstate the help from Professor Andrew Bradbury during every stage of this work, from inception, through to writing up.

Dedication

I would like to dedicate this thesis to my wife, Hazel, without whose unstinting support this work would not have started, continued or finished.

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Published Work

The following papers arose from research carried out for the purpose of this thesis. Reprints of the papers are included in Appendix 3.

- Burns PJ, Wilmink ABM, Fegan C, Bradbury AW. Exercise in claudicants is accompanied by excessive thrombin generation.
  The European Journal of Vascular and Endovascular Surgery.
  2003;26(2):150-155
- Burns PJ, Gough S, Bradbury AW. The management of peripheral arterial disease in primary care.
  The British Medical Journal. 2003;324:484-488
- Burns PJ, Lima E, Bradbury AW. Second Best Medical Therapy.
  The European Journal of Vascular and Endovascular Surgery.
  2002;24(5):400-404
- Hill DM, Johnson LJ, Burns PJ, Neale AM, Harmening AM, Kenney AC. Effects of temperature on stabilization of blood homocysteine in commercially available evacuated collection tubes containing 3-deazaadenosine.
  Clinical Chemistry. 2002;48(11):2017-2022
- Burns PJ, Lima E, Bradbury AW. What constitutes best medical therapy for peripheral arterial disease?
  The European Journal of Vascular and Endovascular Surgery. 2002; 24(1):6-12

- Burns PJ, Mosquera DA, Bradbury AW. The prevalence and significance of thrombophilia in peripheral arterial occlusive disease.

  European Journal of Vascular and Endovascular Surgery. 2001; 22: 98-106

Abstract

Introduction

Intermittent claudication (IC) is the commonest manifestation of atherosclerosis affecting the lower limbs. Due to the systemic nature of atherosclerosis, it is not unexpected that patients with IC have a high risk of thrombotic cardiac and cerebrovascular events. However, there is some research to suggest that this may represent more than simply associated atheroma in the coronary and cerebral arteries - this excess risk could be provided by disturbance of the coagulation system. Numerous studies have demonstrated an association between thrombophilic states and peripheral arterial disease (PAD), and these seem to play a significant role in the severity, and progression of PAD, as well as the likelihood of success of any interventions. As IC is known to be accompanied by a well characterised systemic response – probably secondary to the repeated ischaemia-reperfusion seen during exercise in claudicants, this could putatively affect the coagulation system, leading to the increased risk of cardiovascular thrombotic events which these patients suffer from. We aimed therefore, to study the effect of exercise on the coagulation system in patients with IC.

Methods

Forty subjects were studied, 20 claudicants, and 20 age and sex-matched controls. All claudicants were smokers, 10 of the controls were smokers. Subjects had measurement of a wide variety of coagulation parameters before and after standard treadmill exercise in order to measure thrombin generation, thrombin turnover, and fibrinolysis.

Results

All subjects had similar levels of thrombin production at baseline, but following exercise, this was significantly higher in the subjects with IC. This was not accompanied by an corresponding increase in fibrinolysis when compared to the controls, resulting in probable prothrombotic state. There was increased fibrin turnover in the claudicants at baseline, but appreciable effect of exercise. The claudicants who could walk further seemed to have a better fibrinolytic response than the claudicants with poorer walking distances.

Platelet activation (measured by flow cytometry) following exercise was less in the claudicants than in the controls – probably reflecting the increased use of aspirin in these subjects.

All groups have an increase in neutrophil activation (measured by flow cytometry) following exercise, but this was more in the controls groups. This may reflect sequestration of neutrophils in the peripheral (or pulmonary) circulation in the claudicants.

Conclusion

Exercise in claudicants can lead to a prothrombotic state, characterised by increased thrombin generation. This needs to be further evaluated to determine whether this has clinical consequences, and can be ameliorated with treatment.

Abbreviations

AA	Arachidonic acid
AAA	Abdominal aortic aneurysm
ABPI	Ankle-brachial pressure index
ACEI	Angiotensin converting enzyme inhibitor
aCL	Anticardiolipin antibodies
ADP	Adenosine diphosphate
APA	Antiplatelet agent
APC	Activated protein C
APC-PCI	Activated protein C – Protein C Inhibitor
aPL	Antiphospholipid antibodies
APTC	Antiplatelet trialists' collaboration
APTT	Activated partial thromboplastin time
AT (III)	Antithrombin (III)
AUC	Area under the curve
BM	Basement membrane
BMT	Best medical therapy
cAMP	Cyclic adenosine monophosphate
CLI	Critical limb ischaemia
EC	Endothelial cell
ESR	Erythrocyte sedimentation rate
EXACT	Exercise versus angioplasty in claudication trial
FbDP's	Fibrin degradation products
FVL	Factor V Leiden
GP	Glycoprotein

Hcy	Homocysteine
HDL	High density lipoproteins
IC	Intermittent claudication
IHD	Ischaemic heart disease
IL-1	Interleukin-1
IRI	Ischaemia-reperfusion injury
LAC	Lupus anticoagulant
LTB_4	Leukotreine B_4
NO	Nitric oxide (Endothelium-derived relaxation factor)
NRT	Nicotine replacement therapy
ODFR	Oxygen-derived free radicals
PAD	Peripheral arterial disease
PAI-1	Plasminogen activator inhibitor -1
PC	Protein C
PF1+2	Prothrombin fragments 1+2
PGI_2	Prostacyclin (Prostaglandin I_2)
PS	Protein S
PT	Prothrombin Time
TAFI	Thrombin-activable fibrinolysis inhibitor
TAT	Thrombin-antithrombin complexes
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TNF	Tissue necrosis factor
tPA	Tissue plasminogen activator

TXA_2	Thromboxane A_2
UPA	Urokinase-type plasminogen activator
vWF	Von Willebrand factor

Chapter 1

Intermittent Claudication

Epidemiology

Peripheral arterial disease (PAD) is common, with over 20% of the population having asymptomatic disease, and up to 5% having lower limb symptoms; most commonly, intermittent claudication (IC).[1] Although IC is relatively benign in terms of limb-loss (1-2% per year), it is associated with a vascular mortality (5-10% per year) 2-4 times greater than that of an age and sex matched non-claudicant population (Figures 1.1 and 1.2); a risk that is, in fact, greater than that experienced by patients with angina.[2] There are several reasons for this.

- PAD is a marker for severe, multi-system atherosclerosis affecting the cerebral, visceral and coronary arteries.(Figure 1.3)[3]
- In the presence of exercise-limiting IC, even severe ischaemic heart disease (IHD) may be asymptomatic and thus go unrecognised and untreated.
- There is a suggestion that repeated ischaemia-reperfusion of leg muscles may lead to a systemic inflammatory response that accelerates atherosclerosis and may promote thrombotic events.[4]

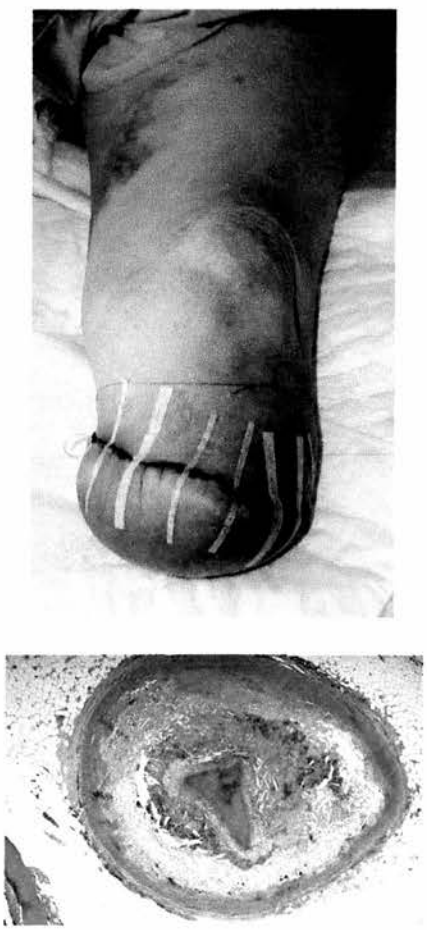
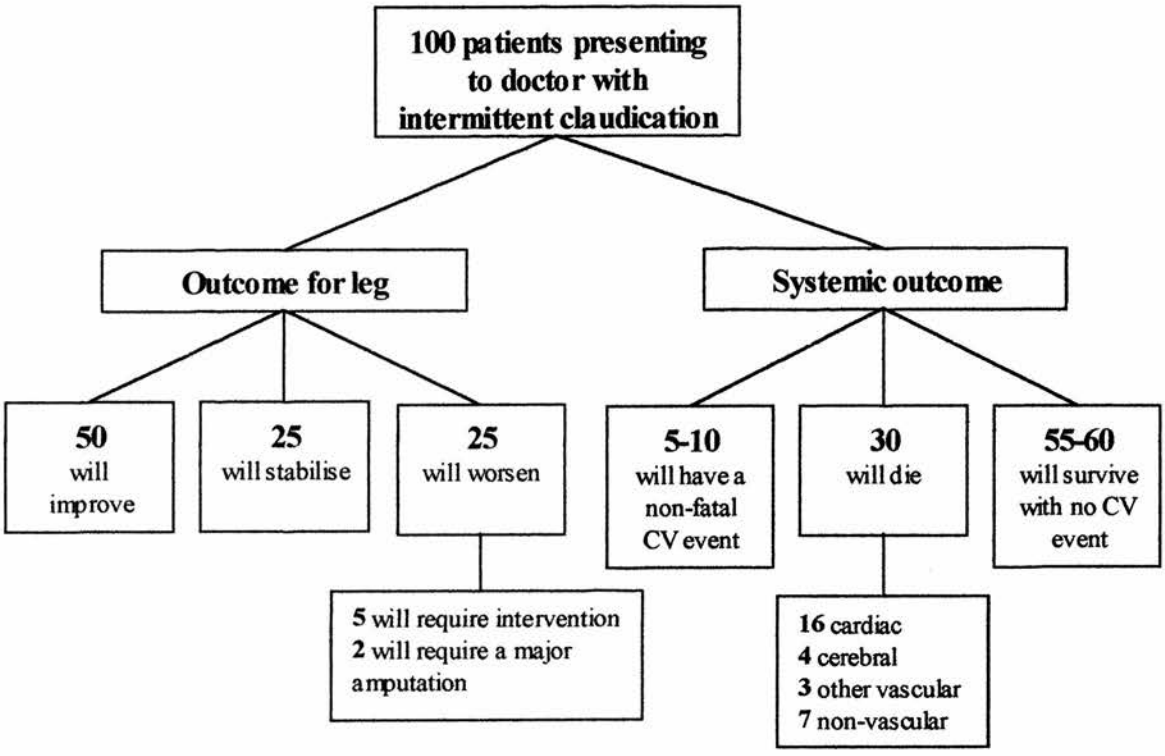


Figure 1.1 Amputation and coronary thrombosis. The rare and more common complications of IC.

Figure 1.2 The outcome for patients with intermittent claudication over 5 years.

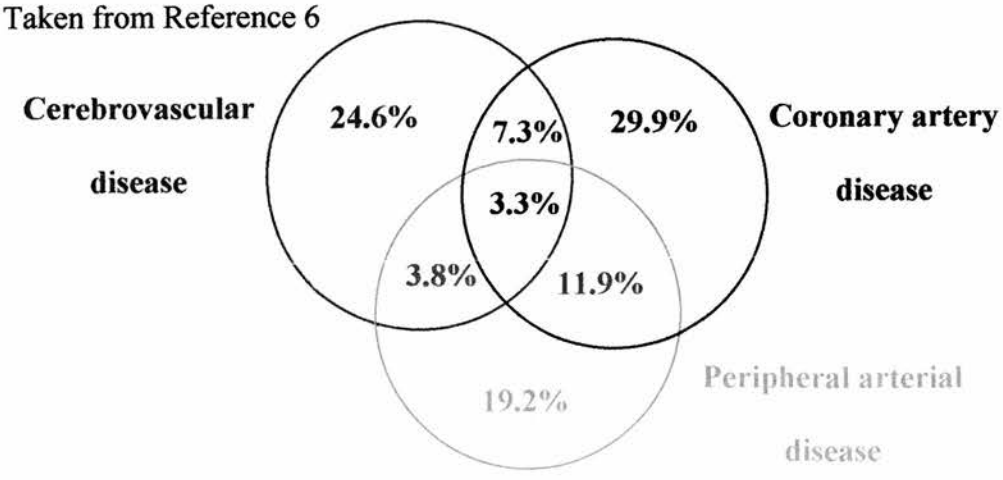
Taken from Reference 3.



CV=cardiovascular (coronary or cerebrovascular)

Figure 1.3 The systemic nature of atherosclerosis.

Taken from Reference 6



Diagnosis and investigation

A diagnosis of IC can usually be made on the basis of the patient's history. The Edinburgh Claudication Questionnaire is highly specific (91%) and sensitive (99%) for the condition.(Figure 1.4)[5] The differential diagnosis includes both venous and neurogenic claudication (Table 1.1). Examination usually reveals weak or absent pulses and further investigations (duplex ultrasound, angiography) are usually reserved for the small minority of patients in whom intervention is being considered.(Figure 1.5)

Table 1.1 Differential diagnosis of intermittent claudication

	Intermittent Claudication	**Venous Claudication**	**Nerve root pain**
Quality of pain	Cramping	'Bursting'	Electric shock-like
Onset	Gradual, consistent	Gradual, can be immediate	Can be immediate, Inconsistent
Relieved by	Standing still	Elevation of leg	Sitting down, bending forward
Location	Muscle groups (buttock, thigh, calf)	Whole leg	Poorly localised. Can affect whole leg
Legs affected	Usually one	Usually one	Often both

Figure 1.4 The Edinburgh Claudication Questionnaire

1	Do you get a pain or discomfort in your leg(s) when you walk?	Yes ☐	No ☐
If you answered "Yes" to question (1), please answer the following questions. Otherwise you need not continue			
2	Does this pain ever begin when you are standing still or sitting?	Yes ☐	No ☐
3	Do you get it if you walk uphill or hurry?	Yes ☐	No ☐
4	Do you get it if you walk at an ordinary pace on the level?	Yes ☐	No ☐
5	What happens to it if you stand still? a) usually continues for more than 10 minutes b) usually disappears in 10 minutes or less	 ☐ ☐	
6	Where do you get this pain or discomfort? Mark the place(s) with an 'X' on the diagrams below	Front	Back
Definition of positive classification requires all of the following responses: "Yes" to (1), "No" to (2), "Yes" to (3), grade 1 "No" to (4), grade 2 "Yes" to (4). If these criteria are fulfilled, a definite claudicant is one who indicates the pain is in the calf, regardless of whether pain is also marked at other sites; a diagnosis of atypical claudication is made if the pain is marked in the thigh or buttock, in the absence of any calf pain. Subjects should not be considered to have claudication if pain is indicated in the hamstrings, feet, shins, joints or appears to radiate in the absence of any pain in the calf			

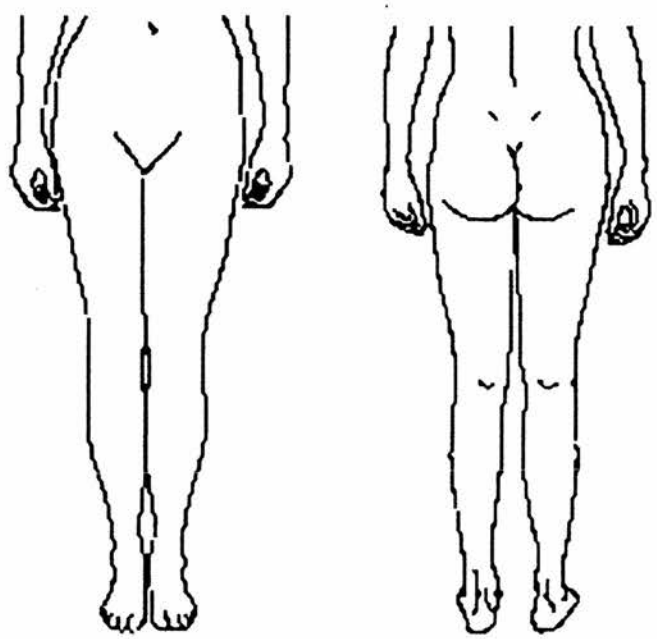
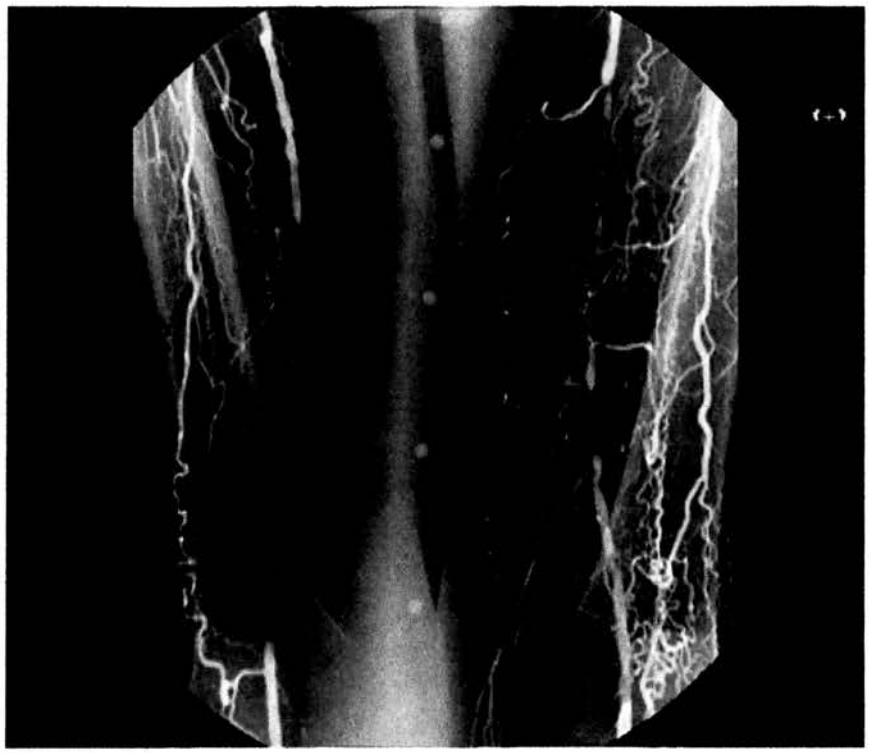


Figure 1.5 Angiogram showing bilateral femoral artery occlusions in a patient suffering from claudication.



Treatment

Since the main danger to patients with claudication is from general cardiovascular disease, initial management should comprise the modification of vascular risk factors and the implementation of Best Medical Therapy (BMT) in the expectation that this will extend life, lower still further the risk of critical limb ischaemia and improve the patients' functional status. Only once BMT has been instituted and given sufficient time to take effect should any consideration be given to intervention as most patients' symptoms improve with BMT to a point where intervention is no longer required.[6] Even in patients who eventually require invasive treatment, BMT is beneficial since the safety, immediate success and durability of intervention is greatly improved in patients who are compliant with BMT.[7; 8]

Traditionally, those treating PAD have tended to focus on the arterial lesion and its surgical or endovascular (angioplasty, stenting) treatment. Unfortunately, with the notable exception of carotid intervention for high-grade symptomatic disease,[9-11] there is little or no level 1 evidence to support intervention for PAD that is not immediately life or limb-threatening. What little data are available suggest that invasive intervention for claudication can lead to an early (1 year) improvement in symptoms, but there is no evidence this is sustained.[7; 8; 12; 13] Such interventions are expensive, potentially hazardous, usually of limited durability and do not impact upon the patients' high underling vascular risk.[7; 8] By contrast, there is increasing and compelling evidence that BMT comprising anti-smoking strategies, antiplatelet agents (APA), lipid lowering and exercise programmes dramatically reduce the vascular risk and significant increase functional status.[14; 15] BMT is also relatively inexpensive and virtually free from risk. With the release of data from the Heart Protection Study, which included over 6000 patients with PAD and confirmed the benefits of lipid lowering, it is timely to review what BMT should comprise and how it can be instituted universally in patients with PAD.[16]

Anti-smoking strategies

There is overwhelming evidence that smoking is the single most important risk factor for the development and progression of PAD and that it significantly increases the risk, and reduces the success, of peripheral arterial intervention.[14; 17-22] Despite the clear benefits of smoking cessation in PAD patients, only a minority (11-48%) of patients manage to quit.[23; 23] Simple oral advice is ineffective,[24] but more intensive counselling has been shown to be effective in unselected smokers, although not in PAD patients.[25-27] Nicotine

replacement therapy (NRT), whether delivered by patch, gum, intranasal spray, inhaler or sublingual tablet, is safe, and leads to significant improvements in smoking cessation (odds ratio 1.72, 95% confidence interval 1.60 to 1.84); at least in the short term.[28; 29] Bupropion is at least as effective as NRT, but appears to confer no additional benefit in combination with NRT.[30] The Cochrane group on tobacco addiction has found alternative therapies such as acupuncture, hypnotherapy, and 'aversive smoking', to be ineffective.[31-33]

Hypercholesterolaemia

Hypercholesterolaemia is clearly an independent risk factor for the development and progression of PAD.[2; 17; 18; 34] Cholesterol lowering has been shown to slow the progression of peripheral atherosclerosis in a number of large, including randomised, anatomical and pathological studies,[35; 36] although none have shown benefit with respect to PAD symptoms. The recently concluded Heart Protection Study has, for the first time, demonstrated a benefit of statins in PAD patients by reducing coronary events by 20%. Furthermore, this was achieved irrespective of starting total cholesterol.(Figure 1.6)[16] Whether there is benefit from raising high density lipoproteins (HDL), and reducing triglycerides levels is less clear.[37]

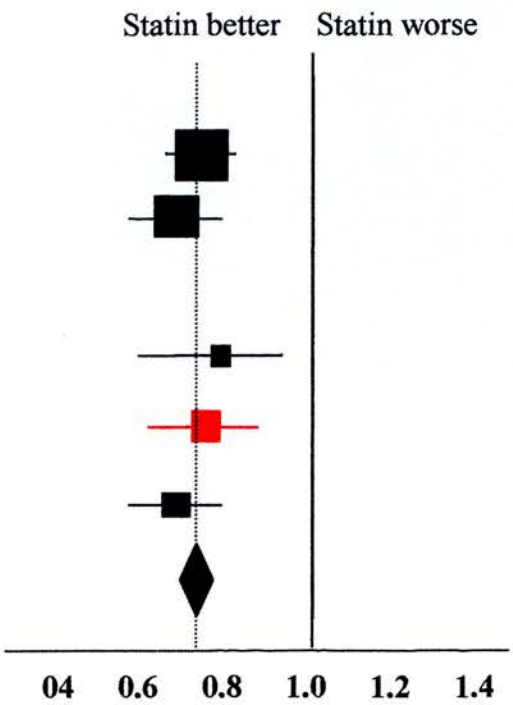
In summary, all patients with PAD should have their cholesterol reduced with aggressive statin therapy, regardless of starting total cholesterol. In the longer term it seems possible that statin therapy will be indicated for any patient with objective evidence of asymptomatic PAD; for example, as demonstrated by a reduced ankle:brachial pressure index. As patents expire, and generic drugs become available, the financial consequences of these massive changes in prescribing

practice will ease and, of course, the prevention of large numbers of vascular events will also help to offset the costs.

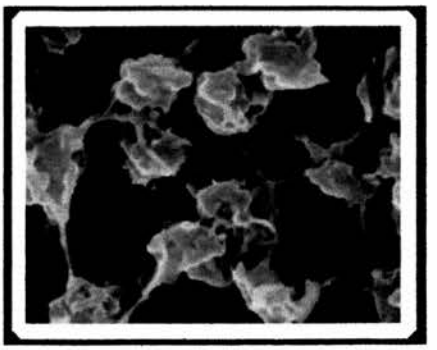
Figure 1.6 Benefit of 40mg simvastatin from The Heart Protection Study.

Number of vascular events by prior disease. Data taken from www.hpsinfo.org

Baseline Feature	Simvastatin (n=10 269)	Placebo (n=10 267)
Previous MI	1007	1225
Other CHD	452	597
No Prior CHD:		
CVD	182	215
PVD	**332**	**427**
Diabetes	279	369
All patients	**2042**	2606



Antiplatelet Therapy



Early studies suggested APA could produce angiographic improvement,[38] increase walking distance,[39, 40] and reduce the requirement for vascular intervention.[41] There is overwhelming evidence from the Antiplatelet Trialists' Collaboration that the prescription of an APA, usually aspirin, reduces vascular death in patients with symptomatic atherosclerotic disease by about 25%.[42] Most of the studies were in patients with IHD and, when taken in isolation, data from the few studies looking specifically at patients with PAD were not conclusive. However, more recently, a review of 24 trials has shown that, when compared with placebo, APA treatment reduced the risk of death by about a quarter in patients with PAD.[43] In summary, all patients with PAD should be on an APA because it reduces vascular events and death, improves the patency rates of surgery and endovascular interventions and may improve walking distance. For reasons of cost, non-enteric aspirin (75mg) is a reasonable first-line choice as there is no clear evidence that a higher dose is more effective (but will cause more adverse events) or that enteric coating is associated with less gastric upset (and is more expensive). Patients who cannot take aspirin should be considered for clopidogrel.

Exercise

There is little doubt that exercise leads to a significant improvement in exercise tolerance (most studies show at least a doubling in walking distance) in patients with PAD.[44-46] It is also likely, though not specifically proved, that exercise will reduce vascular risk. However, clinicians and academics

alike have largely neglected this simple, inexpensive and effective therapy; and as such, many important questions remain unanswered.

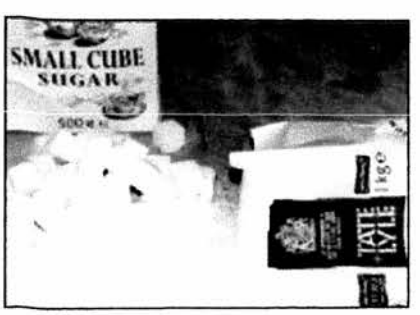
- How does exercise work? Whilst early animal studies suggested that exercise may improve blood flow by the development of collaterals, studies in humans using venous occlusion plethysmography, Xenon-133 clearance and duplex ultrasonography have not confirmed this.[47] Despite this, exercise training can lead to increased clearance of Xenon-133 injected into calf muscles, possibly indicating that blood is being diverted towards more active muscles. Exercise training in claudicants leads to increases in oxidative enzymes, and enhanced utilization of fatty acids in the calf muscles, maximising the use of oxygen delivered to the tissues. Improvements in walking distance may also be due to improvements in walking biomechanics[48] and blood rheology.[49]
- What is the best form of exercise? It has generally been thought to be walking but recent data have suggested that arm exercise, may be at least as beneficial, which further questions the mechanism by which exercise achieves its benefit.[50]
- Does exercise have beneficial effects on risk factor profile? A small non-randomised controlled trail showed that exercise training for claudicants, can lead to modest reductions in blood pressure, cholesterol and glucose levels.[51] Whether this translates to a significant improvement in cardiovascular risk, has not been specifically determined in claudicants, but data from IHD patients suggests that it may.[52]
- Should exercise be supervised and, if so, how and for how long? Supervised exercise programmes would seem intuitively to be better, but there is little evidence to support this. Gardner and Poehlman reviewed 21 studies of

exercise therapy in PAD, and found that supervised exercise programmes were no better than unsupervised.[44] A small randomised study of 54 patients, comparing a 12 week supervised exercise programme and unsupervised exercise, did suggest that the supervised programme was superior (improvement in maximum walking distance 207% vs 70% at 6 months).[46] What is unclear, is the durability of any benefit. It might be speculated that any advantage of supervised exercise will diminish with time, although there is no evidence to support this.

Until these issues are addressed one must approach this aspect of care in a pragmatic way based upon local resources. PAD patients should certainly be repeatedly and specifically informed that exercise is beneficial and that it is not (as far as we know) harmful to try to 'walk through' their claudication pain. Written advice may be a useful adjunct although this suggestion is not evidence-based. Although supervised programmes may be superior, at least in the UK, such programmes are not widely available. The ongoing, UK-based, Heath Technology Assessment funded Exercise versus angioplasty in claudication trial (EXACT) will provide more information about the relative benefits of exercise and angioplasty when it reports.

Diabetes

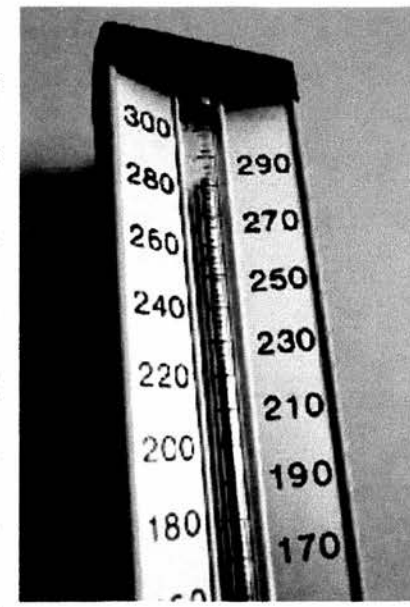
Diabetics have a 3 to 5 fold increased risk of PAD, and are at increased risk of progression from IC to critical limb ischaemia (CLI).[53; 54] The UK Prospective Diabetes Study has shown that intensive control decreased the risk of microvascular but not macrovascular vascular complications of the disease.[55] However, it is extremely important that the diagnosis of diabetes be specifically confirmed



or excluded in patients with PAD because it will affect other areas of their treatment, such as blood pressure and lipid control.[56; 57]

Blood pressure control.

The benefit of treating hypertension in terms of reducing stroke and coronary events is well-accepted with current data suggesting a target of less than 140/85 for non-diabetic and 140/80 for patients with type 2 diabetes.[58] However, in the short-term a reduction of blood pressure may worsen IC. This is true of whatever drug therapy has been instituted and there is no evidence that ß-blockers are any more culpable.[59] The HOPE study has shown that Ramipril, an angiotensin converting enzyme inhibitor (ACEI), reduces cardiovascular morbidity and mortality in patients with PAD by around 25%.[60; 61] Subjects did not need to be hypertensive to be included in the HOPE study and the observed risk reduction could not be accounted for by the relatively modest reduction in blood pressure. The implication of the HOPE study is that most patients with PAD would benefit from an ACEI provided that therapy is not associated with a reduction in renal function.



Other risk factors.

Hyperhomocysteinaemia is becoming increasingly recognised as an important risk factor for development of atherosclerosis, and cross-sectional studies have linked it specifically to PAD.[62] However, the effect of reducing homocysteine (hcy) levels has yet to defined, but should be answered by several ongoing trials. Observational studies have suggested that low levels of anti-oxidant vitamins are associated with

PAD, although no studies, including the Heart Protection Study, have yet shown any benefit from vitamin supplementation.[63-65]

The relationship between alcohol and PAD appears to be J shaped, with minimal risk occurring at around 2 units of alcohol per day.[66] Excess alcohol consumption is clearly associated with an increased vascular risk. Oestrogen has been proposed as being cardio protective, on the basis of reduced cardiovascular morbidity and mortality in women taking hormone replacement therapy, but a recent randomised controlled trials showed no difference in cardiovascular events between groups randomised to oestrogen/progestogen, and placebo.[67] In summary, these factors may represent important risk factors in PAD, but at present there is insufficient evidence to justify targeting them for treatment.

Drug therapy

Cilostazol has been shown to significantly increase (35-109%) walking distance in people with claudication in several large double-blind, placebo-controlled, randomised trials.[68-70] The precise role of Cilostazol remains to be defined but a trial of the drug is probably indicated in patients who remain unacceptably symptomatic despite 3-6 months of compliance with BMT.

Prevalence of BMT use in PAD.

Despite the overwhelming evidence for BMT in patients with PAD, clinical experience and the literature indicated that it has been poorly applied in the past (Table 1.2). The proportion of patients taking any kind of anti-thrombotic therapy (APA or warfarin) ranges from 39 to 66%, and prevalence of cholesterol lowering therapy ranges from 5 to 46%. Work by our group has confirmed that patients with PAD, and are poorly treated in terms of BMT.(Table 1.3) Patients who also have symptomatic IHD seem more likely to be treated but, in general there seems little or no relationship between the prevalence of treatment, the severity of the underlying disease and thus the potential benefits of BMT.[71-75] In other words, treatment is haphazard rather that the result of evidence.

The current situation is unacceptable, and clearly strategies need to be put in place to ensure that PAD patients do not miss out on evidence-based life saving treatment. The initial step needs to be the education of health professionals working with PAD patients about the increased cardiovascular risk of these patients, and the benefits of BMT.

One strategy to increase the institution of BMT is the use of record cards.(Figure 1.7) These chart the level of individual risk factors over time, and allow easy recognition for healthcare professionals of untreated, or inadequately treated risk factors. These charts could be held in the case notes, or by the patient. Another possibility for increasing BMT use is to have dedicated staff in out-patient clinics. This is an ideal role for clinical nurse specialists, who are increasing in number. Whatever technique is employed, it is important to co-ordinate the patient's care with primary care.

Table 1.2 The use of cholesterol-lowering and antithrombotic therapy in patients with PAD

Study	N=	Patient population	N with IHD (%)	N receiving	
				Cholesterol-lowering therapy (%)	Anti-thrombotic therapy (%)
Clark (1999)[71]	299	Admitted for angiography	106 (36%)	26 (9%)	140 (47%)
Anand (1999)[73]	195	Admitted for peripheral arterial surgery	106 (54%)	31 (16%)	94 (49%)
Bismuth (2000)[72]	147	Critical limb ischaemia	66 (45%)	8 (5%)	58 (39%)
McDermott (1997)[74]	202	ABPI < 0.9 or abnormal Doppler waveform	103 (51%)	93 (46%)	133 (66%)

Table 1.3 Proportion of patients with peripheral arterial disease receiving appropriate BMT. Data taken from Reference 77

Aspect of BMT	Percentage of patients receiving BMT
Smoking cessation (% smokers)	14%
Antiplatelet agent use (% those suitable)	83%
Blood pressure controlled	64%
Screened for diabetes (% non-diabetics)	53%
Exercise advice given	7%
Cholesterol controlled (<5mmol/l)	37%

Summary

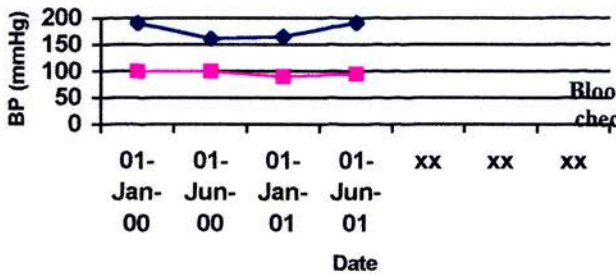
There is overwhelming evidence for the efficacy of BMT in PAD, in terms of cardiovascular risk reduction, and improvement in PAD symptoms. Recommendations for the use of BMT, based on the best evidence available to date are presented in Table 1.4. Despite the evidence of benefit, BMT is grossly underused in PAD patients. If BMT use increases, this will lead to a decrease in cardiovascular morbidity and mortality, a reduction in the requirement for peripheral vascular intervention, and an improvement in outcome for those interventions that are required. It is imperative that those involved in the care of patients with PAD are aware of the benefit of BMT, and develop strategies to help improve its implementation.

Figure 1.7 Best Medical Therapy chart.

Vascular Surgery

Best Medical Therapy

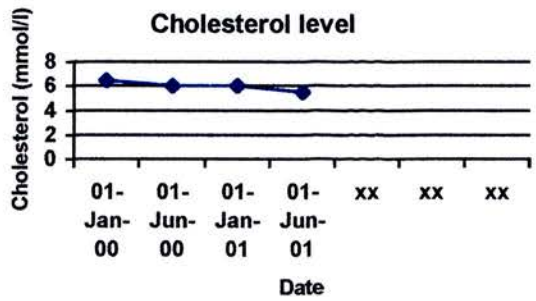
Name:_____-



Exercise advice sheet given:_____
Date

Blood glucose checked:

_____ _____
Date **Result**



	01-Jan-00	01-Jun-00	01-Jan-01	01-Jun-01	xx	xx	xx
Antiplatelet agent							
Cholesterol							

Drug Therapy

Table 1.4 Recommendations for BMT in patients with PAD.

Component of BMT	Recommendation
Smoking cessation	Repeated advice
	Nicotine replacement therapy
	Behavioural therapy (Smoking cessation classes)
Cholesterol	Cholesterol checked yearly.
reduction	Statin therapy to lower cholesterol by >25%.
	Additional treatment will be required if HDL low, or triglycerides high (Referral to lipid clinic)
Antiplatelet agent	Aspirin
	Clopidogrel if aspirin intolerant
Diabetes mellitus	Screen for diabetes mellitus
Blood pressure	Reduce blood pressure to <140/80 mmHg
Exercise	Patients with lower limb disease should be prescribed a supervised exercise programme.

Chapter 2

Overview of thrombosis and fibrinolysis

Introduction

The haemostatic process has evolved to provide a mechanism of arresting haemorrhage, whilst preventing unnecessary activation, or extension of the coagulation process. The delicate balance of the system is illustrated by the fact that 10ml of plasma contains sufficient thrombin to clot all the fibrinogen of the body in 30 seconds. This balance is achieved by the interaction of the five components of the haemostatic system: blood vessels, platelets, coagulation factors, coagulation factor inhibitors and fibrinolysis. Whilst it is convenient to consider these separately, it should be borne in mind that there are numerous complex interactions between each of these components, at many levels.

Blood Vessels

A number of blood vessel constituents are involved in haemostasis, (Table 2.1) but it is the endothelial cells (EC's) which play the major regulatory role.

Endothelial cells.

EC's have a multitude of functions in addition to the regulation of coagulation, but this discussion will be confined to haemostasis (Figure 2.1). EC influence haemostasis via platelets, coagulation factors and inhibitors of the coagulation cascade.

Table 2.1 Constituents of blood vessels involved in haemostasis

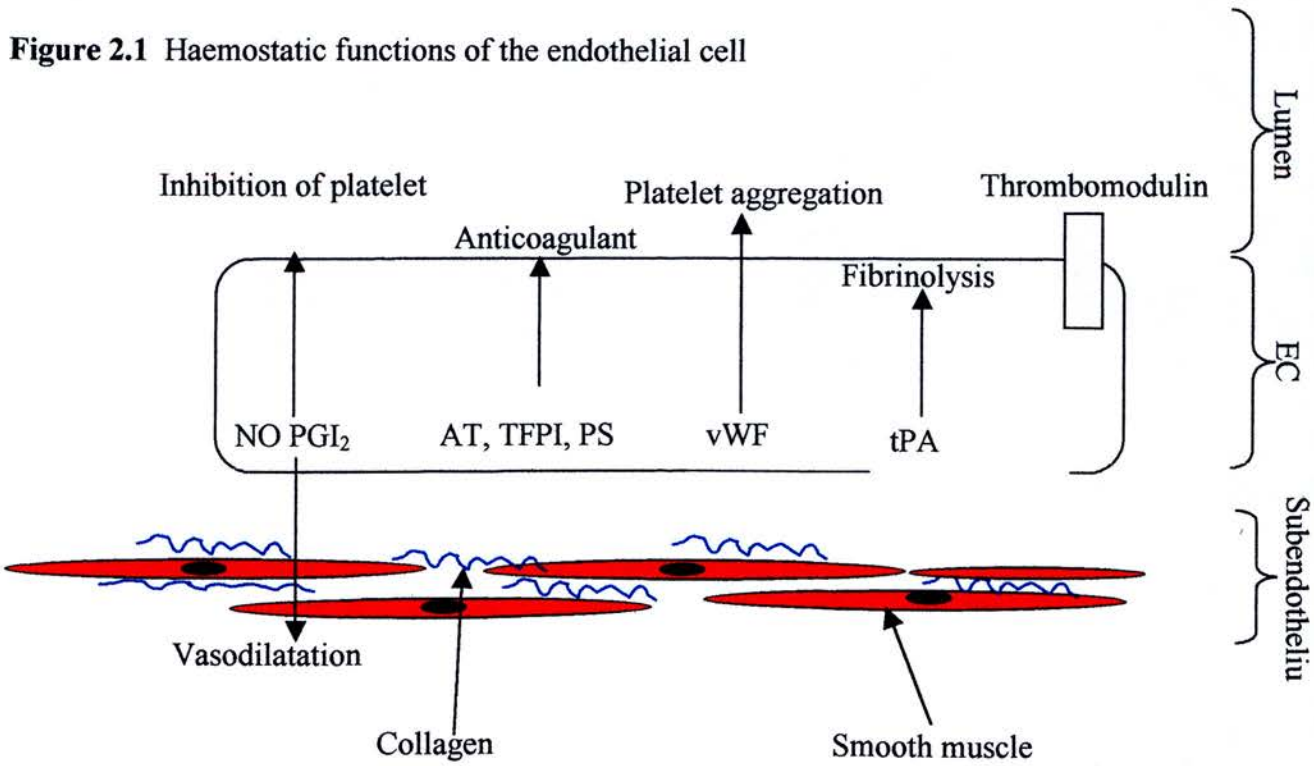
Blood vessel constituent	**Function**
Smooth muscle	Vessel constriction after trauma
Collagen	Platelet adhesion (via von Willebrand factor)
Elastin	Contributes to vessel resealing after minor trauma
Endothelial cell	Procoagulant, anticoagulant and antifibrinolytic functions (see text and Figure 2.1)

EC – platelet interactions.

Both prostacyclin (PGI_2), and nitric oxide (NO), produced by EC's, inhibit platelet aggregation by increasing platelet intra-cellular cyclic adenosine monophosphate (cAMP). Increased cAMP levels within platelets lead to increased uptake of calcium into the dense tubular system, effectively lowering intracytoplasmic calcium levels, preventing platelet aggregation and activation.

Von Willebrand factor (vWF) is produced by both endothelial cells and megakaryocytes (and therefore present in platelets), and is an important mediator of platelet-EC adhesion. Although secreted constitutively, its release is increased in response to a number of stimuli, including thrombin, interleukin-1 (IL-1), and adrenaline.

Figure 2.1 Haemostatic functions of the endothelial cell



AT=antithrombin, TFPI=tissue factor pathway inhibitor, PS=Protein S, tPA=tissue plasminogen activator,

EC – procoagulant.

Tissue factor (TF) is not released from resting EC's but it can be induced in response to endotoxin, IL-1 and TNF leading to localised production of thrombin.

EC – anticoagulant.

EC's are associated with a number of anticoagulant substances. Tissue factor pathway inhibitor (TFPI) is synthesised, and released by EC's. This anticoagulant effect is enhanced by the presence of antithrombin (AT) on and protein S (PS) on the EC surface. The membrane of EC's binds heparin and a number of endogenous heparin-like molecules – all capable of increasing the anticoagulant effect of AT.

A further, important component of the anticoagulant effect of EC's is thrombomodulin (TM). This is exclusively synthesised, and expressed by EC's. It binds thrombin, changing the function of thrombin from procoagulant, to anticoagulant. This helps to ensure that any coagulation event remains localised to the site of disrupted endothelium.

EC – fibrinolysis

EC's synthesise both tissue plasminogen activator (tPA) and its specific inhibitor plasminogen activator inhibitor 1 (PAI-1). In the resting EC, release of these factors is minimal, but is increased in response to specific stimuli. These will be discussed in detail in the section on fibrinolysis.

In summary, endothelial cells function mainly as a functional antithrombotic barrier to the procoagulant subendothelium.

Platelets

Platelets are normally prevented from adhering to intact EC's by high local concentrations of NO and PGI_2. Removal of EC's exposes subendothelial components such as collagen, which binds to the platelet glycoprotein (GP) Ia-Iia complex, leading to the activation of platelets and release of dense and alpha granules. Subendothelial collagen also binds vWF, which may help the adhesion of platelets. Activated platelets are then able to bind vWF with the GPIIb-IIIa complex. The GPIIb-IIIa complex is also capable of binding fibrinogen, which is important for platelet-platelet binding (aggregation). The activation of platelets is a complex, multistep process, which includes the release of thromboxane A_2 from platelet surface (the step inhibited by aspirin), the binding of adenosine diphosphate(ADP) by a specific receptor (inhibited by clopidogrel and ticlodipine), and the activation of the GPIIb-IIIa complex to allow the binding of fibrinogen (directly inhibited by GPIIb-IIIa inhibitors).

Although aggregation of platelets is sufficient to arrest haemorrhage from very small vessels (capillaries), bleeding from larger vessels requires reinforcement of the platelet plug with cross-linked fibrinogen. Activation of the coagulation cascade may occur independently of platelet aggregation, via TF, but platelets contribute to coagulation by increasing the expression of TF on EC's, and by the specific binding of coagulation factors on the platelet surface (factors II, V, VII, VIII, IX, X and XI). This assembly of the coagulation factors on the platelet surface, raises their local concentration, protects them from specific inhibitors such as antithrombin (AT) and protein C (PC), allowing propagation of the coagulation cascade, and the production of reinforcing, cross linked fibrinogen.

Coagulation factors

The coagulation cascade is a series of reactions, which lead to the rapid production of cross-linked fibrin. The substrates for the reactions, are soluble, inactive precursors (zymogen), which when cleaved (or activated) is transformed into an enzyme capable of acting upon the next protein in the cascade. This series of reactions results in a rapid increase in the number of active factors, which is compounded by a number of positive feedback loops, ie factor Xa and thrombin increase the activity of factors V and VIII. Original models of the coagulation cascade identified two separate initiators:

- The 'intrinsic' pathway, activated by contact with a negatively-charged surface (ie, glass or dextran sulphate). This leads to the activation of FXII, which then activates FXI, which in turn actives FX. This pathways was termed the intrinsic pathway, as all of the components of it are present in plasma.
- The 'extrinisic' pathway, was identified by recognising that coagulation may be triggered by the addition to plasma of certain tissue extracts (eventually identified as TF). TF, binds to, and activates FVII. This TF-FVIIa complex activates FIX, which then joins the pathway of the intrinsic system by activating FX.

However, individuals with a deficiency of factors specific to the intrinsic pathway do not suffer from a clinical bleeding tendency, leading to the belief, that in-vivo, it is the so-called 'extrinsic pathway' which predominates.

In reality, the process is not such a neat, stepwise cascade, but involves the production of complexes of factors on the surface of platelets, with numerous feedback loops (both positive and negative). For instance, the TF-FVIIa complex initially activates small

amounts of FX directly, bypassing FIX. This allows the production of a small amount of thrombin, which can activate factors V and VIII. The presence of FVIIIa, allows far greater amounts of FXa to be produced by FIXa when it is activated by TF-FVIIa. FXa is assembled in a complex on the platelet surface with calcium ions, and FVa, which converts FII (prothrombin) to FIIa (thrombin). Thrombin then converts soluble fibrinogen, to insoluble fibrin monomers, which then self-assemble into a finrin clot. Factor XIIIa (activated by thrombin) completes the process by chemically cross-linking the fibrin strands.

Coagulation inhibitors

If left unchecked, once activated, the coagulation process would quickly lead to clot forming throughout the circulation – a defect which evolution was presumably quick to rectify. A number of mechanisms exist to prevent this unwanted propagation.

TFPI.

Following initial activation of the cascade by TF, TFPI dampens down further activation. Production of the FVIIIa-FIXa complex continues, possibly via thrombin activation of the 'intrinsic' pathway.

Protein C pathway.

This is an important means of preventing extension of clot formation to areas it is not required. Following the formation of thrombin, in the presence of intact EC's, it is bound to EC surface TM. This alters the configuration of thrombin, such that it is unable to catalyse the conversion of soluble fibrinogen, but instead, binds and activates PC. Activated PC (APC) is an inhibitor of FVa and FVIIIa, its action being potentiated 10-fold by Protein S (PS) (also found on the EC surface). APC inactivates FV, by

cleaving between amino acids 506 and 507. When the amino acid at 506 (arginine) is replaced by lysine (factor V Leiden mutation), it is rendered resistant to cleavage by APC – explaining the increased thrombotic risk in this condition.

AT.

AT (formerly ATIII) is produced by the liver, but is bound to EC surface. It acts by forming a stable complex with several coagulation factors, including thrombin and FXa. Its activity is enhanced 2000-fold by the presence of heparin.

Fibrinolysis

Plasmin

The final component of the haemostatic system to be discussed is the fibrinolytic system. Plasmin is activated from plasminogen by one of its activators - tPA or urokinase plasminogen activator (uPA). Activated plasmin degrades fibrin clot to fibrin degradadation products (FbDP). However, plasminogen can only be activated, when it has been incorporated into a fibrin clot, and 'protected' from the various fibrinolysis inhibitors.

tPA

tPA is synthesised by EC's, but is rapidly inactivated by circulating inhibitors. As mentioned previously, a number of stimuli, including thrombin, and exercise increase the amount of tPA released by EC's. Because of the excess of tPA inhibitors which rapidly bind tPA, circulating tPA is largely inactive. This is altered when it is bound to fibrinogen, which increases its affinity for plasmin and its resistance to inactivation.

uPA

This is so named because it was first extracted from urine. Although capable of activating plasminogen, its primary role is thought to be in cell-signalling rather than fibrinolysis.

Plasminogen activator inhibitor-1

This fast, specific inhibitor of tPA is also synthesised and secreted by EC's. It mostly circulates in an inactive form bound to tPA

Plasminogen activator inhibitor-2

This is produced by the placenta from the eighth week of pregnancy onwards, and may contribute to the inhibition of fibrinolysis seen during this period. It is not thought to play a role in haemostasis outside this time.

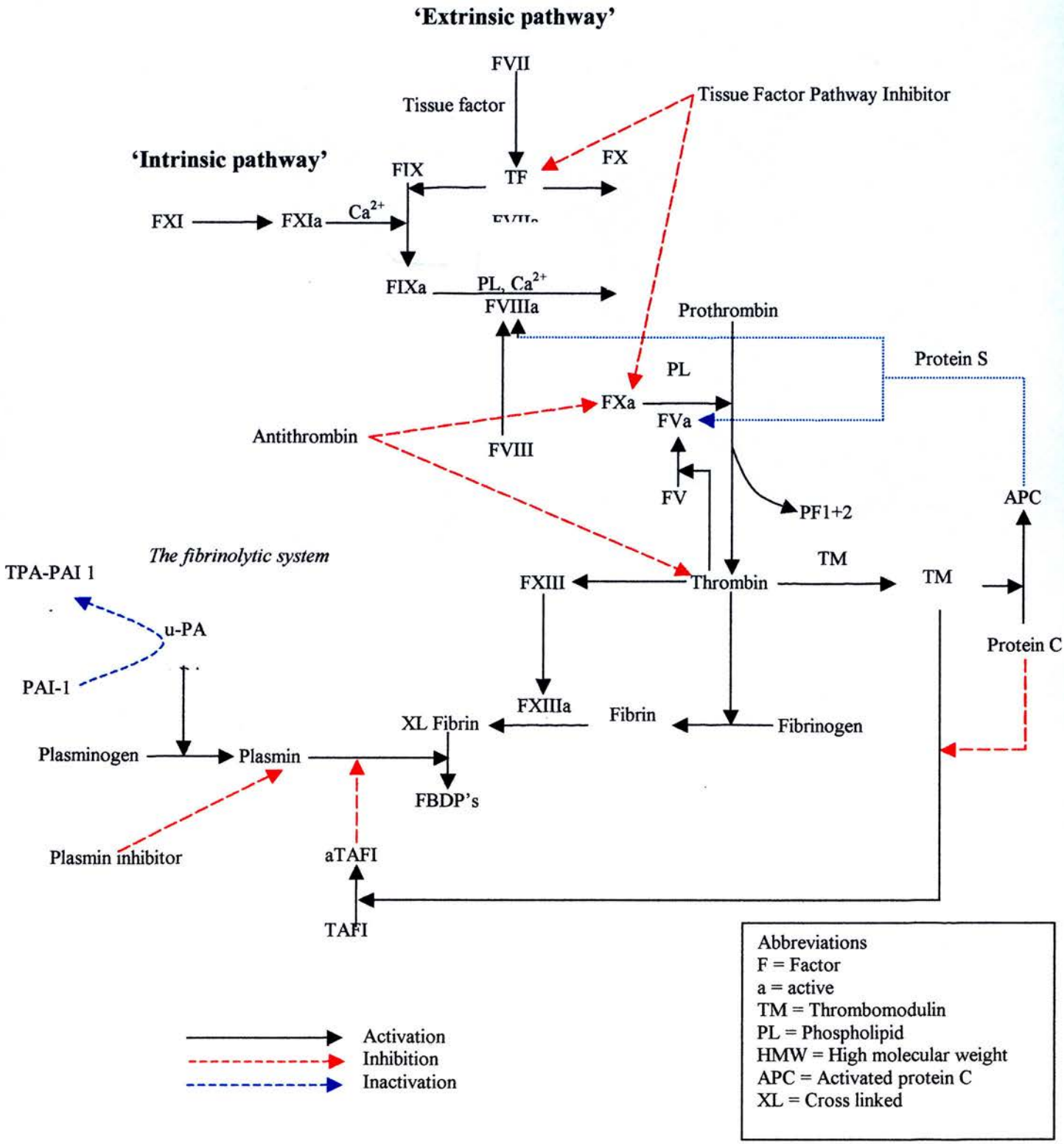
Thrombin activable fibrinolysis inhibitor

Thrombin activable fibrinolysis inhibitor (TAFI) also known as procarboxypeptidase B, is activated by thrombomodulin-bound thrombin, inhibits the activation of plasmin, possibly by downregulating the co-factor effect of fibrin. The physiological role of the connection between thrombin generation and fibrinolysis that TAFI provides is not clear.

Summary

Figure 2.2, provides an overview of the preceding discussion. Despite its complexity, this model has been reproduced in-vitro successfully, which would seem to confirm its veracity.

Figure 2.2 Coagulation cascade



Chapter 3

The systemic response in intermittent claudication

Introduction

The last 15 years have seen a realisation that IC is accompanied by a complex systemic response. This is secondary to the repeated ischaemia-reperfusion of the muscles in the leg when patients with IC walk. The full clinical relevance of this repeated ischaemia-reperfusion injury (IRI) is not fully understood, but there are theoretical reasons why it may be harmful – both locally in the muscles which are repeatedly ischaemic and reperfused, and systemically. The situation is complicated by the systemic atherosclerosis which is almost universally present in patients with IC, and the effect this has on EC function. The effects of IRI in claudication can be divided into two types – cellular effects and non-cellular effects, although this distinction is somewhat artificial, as there are complex interactions between the two.(Table 3.1)

Cellular effects

Neutrophil activation.

Neumann showed that exercise in claudicants leads to a rise in neutrophil count in the femoral vein of the leg affected by IC.[76] Initially, this change is not seen in the femoral artery of the same limb (systemic blood), but after 10 minutes, similar neutrophil counts are seen systemically. There is also a decrease in the proportion of neutrophil able to pass through a 8μm pore filter, indicating an increase in neutrophil activation. Other, indirect indicators of increased neutrophil activation including serum lysozyme levels

have been shown to rise in claudicants following exercise.[77] Recently, more specific markers of neutrophil activation have become available such as flow cytometry. This is able to detect neutrophil expression of activation-specific surface markers (CD11b). Using this technique, Turton showed that exercise in patients with IC is associated with an increase in CD11b expression on neutrophils, which is not seen in healthy controls – even with exhaustive exercise.[78]

Table 3.1 The systemic consequences of exercise in claudicants.

Taken from references [76; 79; 80]

Rise in neutrophil count
Neutrophil activation
Rise in thromboxane
Rise in von Willebrand Factor
Rise in microalbuminuria (glomerular endothelial cell injury)
Fall in antioxidants

In addition to the activation of neutrophils directly by the ischaemic/reperfused muscle, there is also indirect evidence of systemic activation of neutrophils. Patterson used plasma taken from patients undergoing aortic surgery following clamp release, and showed that it was capable of activating neutrophils.[81] This 'circulating neutrophil activator' has been proposed to be Interleukin 8, but has not been confirmed.

In summary then, it appears that exercise in claudicants, leads to an increase in the number of activated neutrophils, originating in the affected limb, and in response a number of systemic effects.

Platelets.

Platelets may also be affected by exercise in claudicants, but the data regarding this are conflicting depending on the methods used to measure platelet activation. Kirkpatrick, showed that exercise in claudicants was associated with a rise in soluble P selectin levels (a putative marker of platelet activation) when compared to controls.[82] However, Lewis, using flow cytometry, did not show any difference in platelet activation between claudicants and controls.[83] These studies, and the methods of assessing platelet activation are discussed in more detail in a subsequent chapter. As yet therefore, it is not clear, if and how platelets are affected by the IRI seen when patients with IC exercise.

Non-cellular effects

Aside from the activation of neutrophils, there are marked biochemical effects of ischaemia-reperfusion.(Figure 3.1) In the presence of ischaemia, xanthine dehydrogenasse is converted to xanthine oxidase. Reperfusion provides the oxygen that xanthine oxidase requires as a co-factor for the metabolism of hypoxanthine (which accumulates in muscle ischaemia) to xanthine, with the generation of superoxide ion – the source of oxygen derived free radicals (ODFR). These ODFR, which can also be produced by activated neutrophils, result in numerous harmful effects which are discussed below.

There is a substantial amount of data to suggest that ODFR are produced during exercise in claudicants. Because of the very short half-lives of ODFR, they are difficult

to measure, but their levels can be assessed by measuring their stable metabolites, such as malondialdehyde (MDA), and/or a decrease in the amount of free-radical scavengers, such as glutathione peroxidase, and selenium. Hickman et al showed that exercise in claudicants results in an increase in MDA,[84] while Edwards et al showed that at rest, claudicants have reduced free-radical scavenging.[85]

Consequences of ischaemia reperfusion in IC

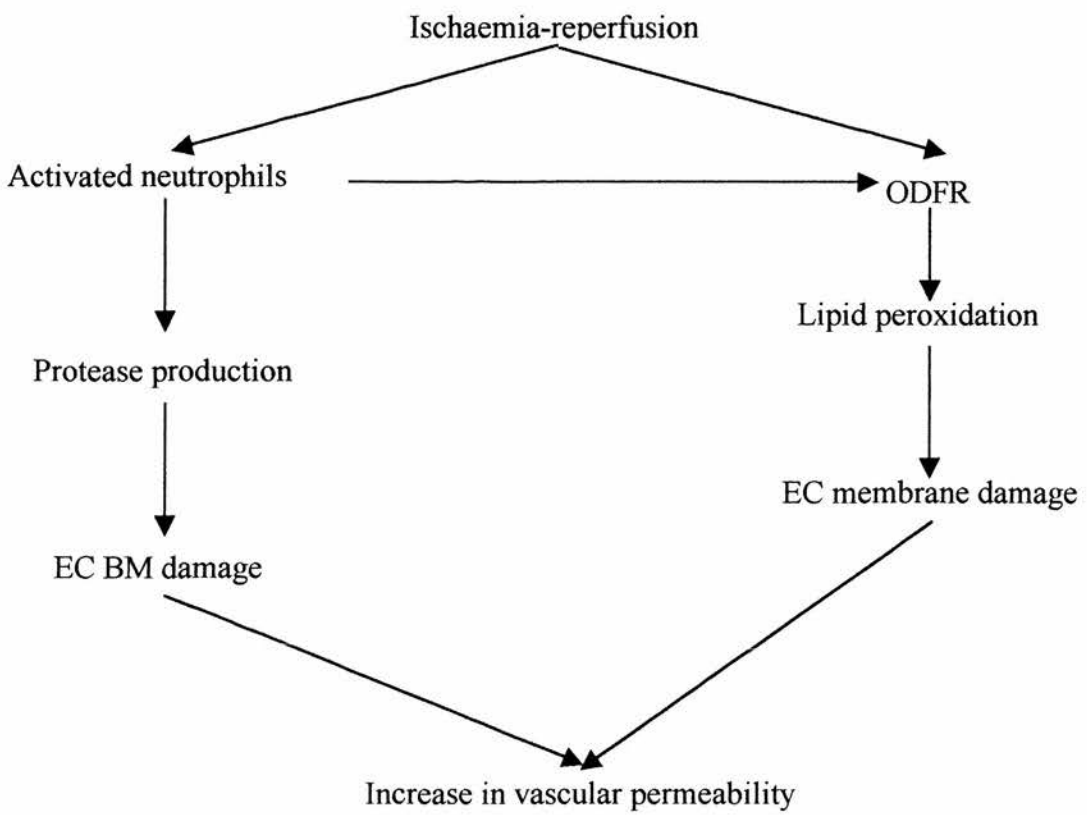
The production of activated neutrophils and ODFR in the exercising claudicant has a number of potentially harmful effects.(Figure 3.1)

- Lipid peroxidation. ODFR lead to peroxidation of cell membrane phospholipids, damaging vascular EC.
- EC basement membrane damage. This is caused by elastase from activated neutrophils, leading to an increase in vascular permeability.
- Neutrophil chemotaxis. ODFR activate phospholipase A_2 in the EC membrane, which produces arachidonic acid (AA) from cell membrane phospholipids. AA is the precursor of important eicosanoids such as Thromboxane A_2 (TXA_2), Leukotreine B_4 (LTB_4) and PGI_2. Both TXA_2 and LTB_4 are chemotactic for neutrophils, further increasing the inflammatory, and potentially damaging process.

There is evidence of EC injury following exercise in claudicants from numerous studies. The majority of these use a measurement of microalbuminuria as an indication of EC damage, which has been validated by experimental work in animals. This has shown that following exercise, claudicants have an increase in urinary microalbuminuria, which is not seen in non-claudicants.[86] Further evidence of systemic cellular damage was seen in a study by Iwata et al, who assessed intestinal permeability pre and post-

exercise by measuring the differential absorption of lactulose and mannitol. Mannitol is relatively well absorbed from the intestine, while in normal conditions, lactulose remains largely unabsorbed (the basis for its use as a laxative). However, if the intestinal mucosal cells are damaged, then lactulose may be absorbed across widened cellular tight junctions. Therefore, systemic damage is seen as a rise in lactulose absorption, with respect to mannitol absorption. In Iwata et al's study of 11 claudicants, lactulose absorption rose significantly following exercise in claudicants, an effect not seen in a control group.[87]

Figure 3.0 The consequences of ischaemia-reperfusion



Edwards, in the study mentioned previously, also assessed EC damage, using vWF. This EC product is thought to be released into the circulation following EC damage, and has been widely used as a marker of EC disruption – although this is not universally accepted since it is also released from platelets. Edwards seemed to confirm that exercise in claudicants, unlike in controls, is accompanied by EC damage as evidenced by a significant rise in serum vWF levels.[85]

What, if any, are the potential consequences of such systemic cellular damage? One possibility is the progression of atherosclerosis. EC damage is one of the first events in the development of atherosclerotic plaque. EC function (as indicated by decreased NO production) is known to be poorer in patients with atherosclerotic risk factors but no overt signs of vascular disease. To date however, there are no published studies which have directly studied the effect of IRI in claudication on EC function, or the progression of atherosclerosis, so this remains as a hypothesis.

It may also be speculated, that with the central role of the EC in haemostasis (Chapter 2), IRI could potentially disrupt the coagulation system, but this too remains to be answered.

Chapter 4

Thrombophilia in Peripheral Arterial Disease

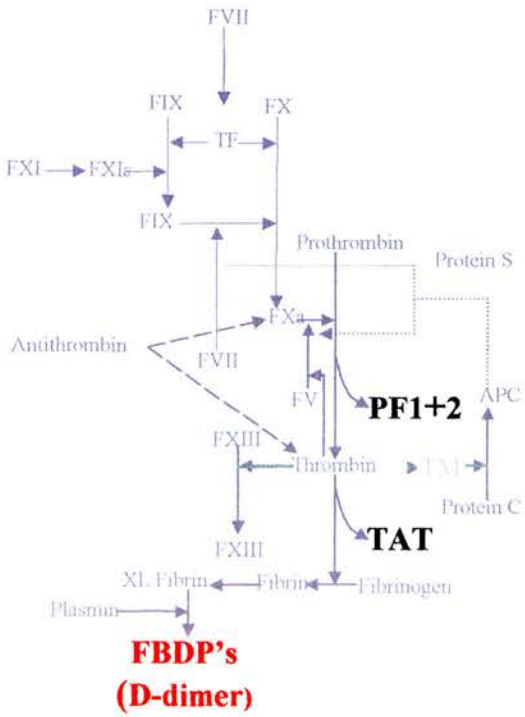
Introduction

In addition to well-established risk factors such as smoking, diabetes, hypercholesterolaemia and hypertension, an increasing number of novel humoral and endothelial factors have recently been implicated in the aetiology and progression of vascular disease. Thrombophilia may be defined as a propensity to thrombosis secondary to abnormalities in haemostasis.[88] Thrombophilia has long been recognised as contributing to venous thrombosis, but is increasingly associated with arterial disease. It is important because screening may identify patients at high risk of thrombosis who may then be offered prophylaxis. This chapter will focus on the prevalence and significance of thrombophilic states associated with PAD and discuss possible strategies for screening and treatment.

Prevalence of thrombophilia

General coagulation activation

If thrombophilia is important in PAD then there should be evidence of activation of coagulation in affected patients. Thrombin and fibrinogen, and products of their metabolism, including thrombin-antithrombin (TAT) complexes, prothrombin fragments (PF) 1+2 and fibrin degradation products (FbDPs) can be used to measure coagulation activation. Cross-sectional[89-92] and



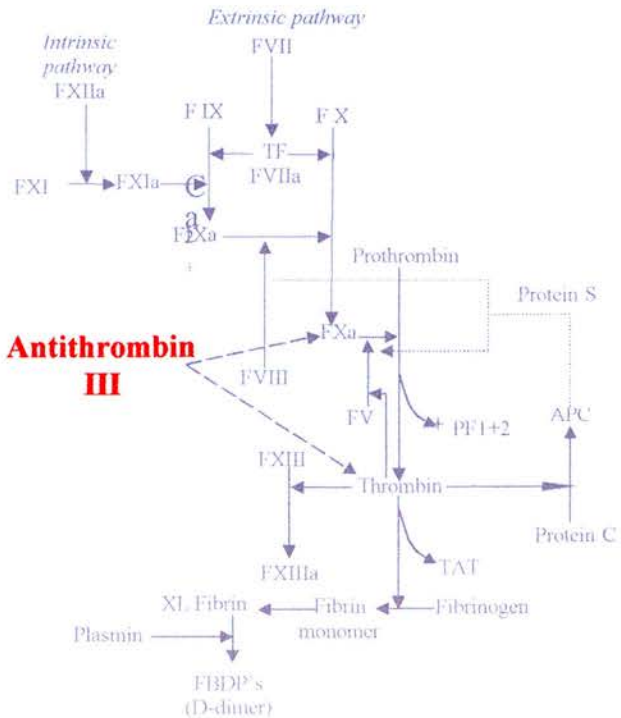
longitudinal[93] epidemiological studies have demonstrated an association between activation of coagulation and PAD. Furthermore, in 1988, Boneu showed that PAD was associated with inhibition of fibrinolysis.[94] In young patients (<51 years old) undergoing lower limb revascularisation, as many as 76% may have a hypercoagulable state (increased platelet aggregation or coagulation abnormality).[95]

Hcy

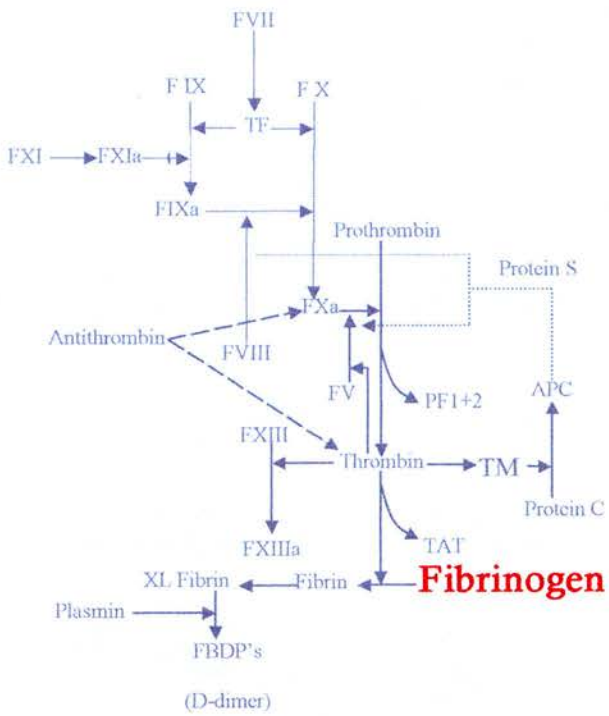
A mild elevation of hcy levels (hyperhomocysteinaemia) affects 5% or more of the population and is increasingly recognised as an independent risk factor for atherosclerosis and thrombosis.[96] Hyperhomocysteinaemia can cause increased Factor V activity, possibly via a decrease in thrombomodulin cell surface activity and a corresponding decrease in APC.[97-100] The prevalence of hyperhomocysteinaemia in PAD may be between 50 and 60%[101-103]and many cross-sectional studies have demonstrated a clear association between plasma homocysteine levels and PAD.[104]

AT

AT is an endogenous anti-coagulant, produced by the liver, which inactivates thrombin and Factor Xa. Deficiency of AT is inherited in an autosomal dominant fashion. In a population-based study of 7983 subjects over 55 years old 3.1% had deficiency of AT, dcfincd as <75% activity.[105]

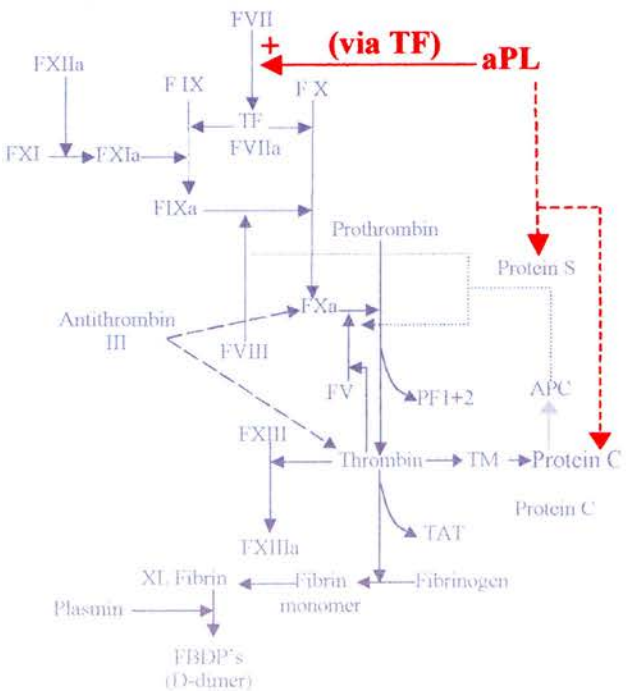


Fibrinogen



Fibrinogen is the substrate on which the end-product of the coagulation cascade, thrombin, acts to produce fibrin, and ultimately, a blood clot. Its effects are diverse and include increases in blood viscosity, red cell aggregation, platelet aggregation and activation.[106] Fibrinogen deposited in the arterial intima may also lead to smooth muscle cell proliferation and leukocyte migration.[107-110] Hyperfibrinogenaemia has long been associated with cardiovascular disease and is present in more than 50% of patients with PAD.[111-113]

Antiphospholipid antibodies



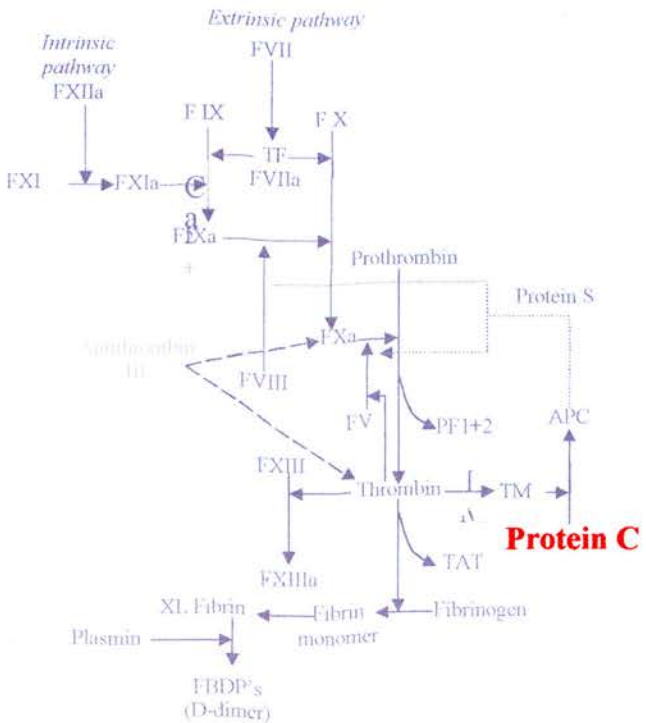
Antiphospholipid antibodies (aPL) are a group of auto antibodies originally thought to be targeted towards negatively charged phospholipid, although recent work suggests that they are directed against β_2-glycoprotein I.[114] aPLs are of two types, detected by different laboratory methods: the anticardiolipin antibody (aCL) enzyme linked immunoassay and the lupus

anticoagulant (LAC) coagulation assay. Although the lupus assay relies on the *in-vitro* effect of aPL to prolong coagulation assays, the *in-vivo* effect is procoagulant, the mechanism for which is uncertain. Antiphospholipid antibodies may inhibit protein C and protein S, and have prothrombotic effects via enhanced platelet reactivity or endothelial cell surface molecules such as heparan sulphate and tissue factor.[114]

Cross-sectional studies have shown that the prevalence of aPL amongst patients with PAD requiring intervention varies between 26% and 45%.[115-117] This mostly comprises patients with aCL who constitute 84%-94% the total. A small proportion of patients with aPL have both LAC and aCL (2%-3%).[115; 117; 118] No studies have yet compared the prevalence of aPL in PAD with the prevalence in the general population, and no large cross-sectional studies have been performed to give a population prevalence of aPL.

PC deficiency

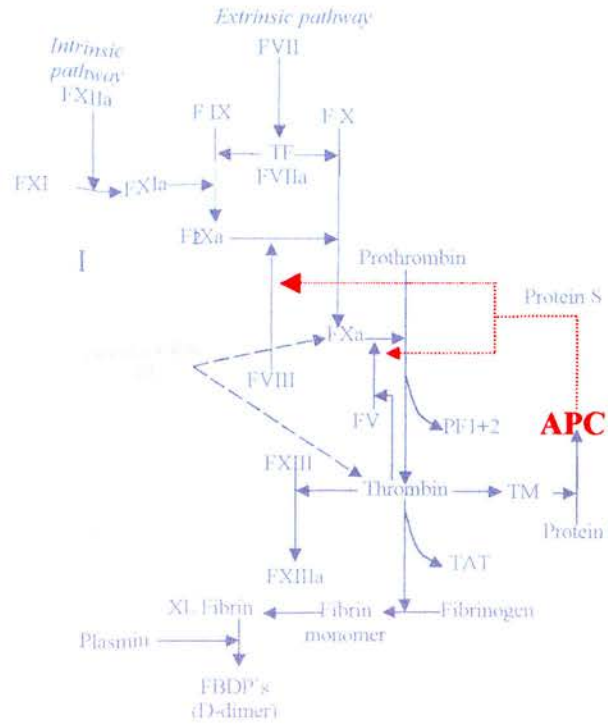
PC is a vitamin K dependent protein which, when activated by the thrombin-thrombomodulin complex, inactivates factors Va and VIIIa. PC deficiency is established as a risk factor for venous thrombosis, but its role in arterial pathology is less clear. Few studies have investigated the prevalence of PC deficiency in PAD. In a recent study of 116



claudicants, deficiency of PC was found in 2 (1.7%).[118] Other studies have shown the prevalence of PC deficiency to be between 2.5 and 15% in PAD patients, but no comparisons were made with control groups.[95; 119-121]

APC Resistance / Factor V Leiden

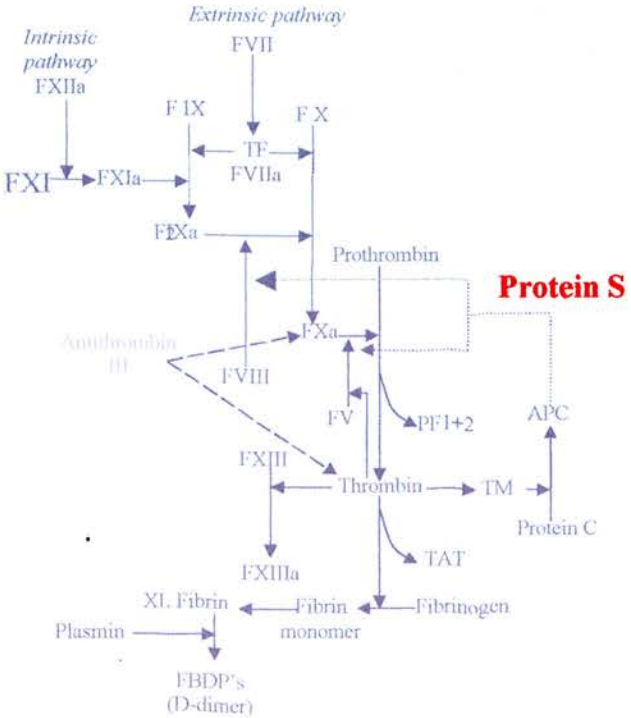
APC resistance is the most common inherited risk factor for thrombosis. The prevalence varies in different ethnic populations; in UK it is 3.5-4.9%, Africans 0% and in Cyprus 13%.[122; 123] The most common cause of APC resistance is a mutation in the factor V gene leading to the replacement of Arginine 506 with Glutamine, (Factor V Leiden, [FVL]) which renders it more resistant to degradation by PC. This is responsible for 90-95% of APC resistance, the remainder of which is made up of acquired conditions such as aPL, pregnancy and the oral contraceptive pill.[124-127] APC resistance is measured using a plasma assay and exogenous APC, and is indicated by a lowering of the APC ratio (normal range 2.2 to 2.6). This will identify the majority, but not all of patients with FVL. FVL may also be identified directly using genomic analysis, but not all mutations lead to lowering of the APC ratio. FVL is thought to underlie 18%-30% of venous thromboses, but its importance in arterial disease is less well defined.



Both APC resistance and FVL have been demonstrated to be more common in patients with PAD compared with the general population. Sampram found the prevalence of

FVL and APC resistance (defined as ratio <2.6) to be higher (26.4%) in 359 patients with PAD than in 278 controls (12.2%).[128] A smaller study by Foley in patients who had undergone lower limb arterial bypass surgery reported a 17.8% prevalence of FVL, compared with a local population prevalence of 3.5%.[122] Evans only reported one positive APC resistant patient in 116 claudicants[118]. Variations in these reported figures may be explained by the preferential use of DNA analysis or APC ratio to define FVL; variations in the lower end of the normal range for defining the normal APC ratio and the severity of the presenting PAD.

PS Deficiency

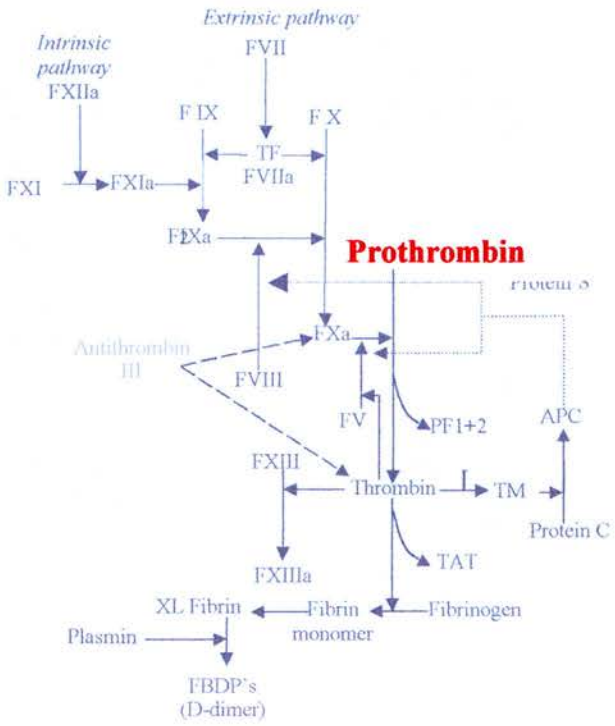


PS is a vitamin K dependent plasma protein and an essential co-factor for the anticoagulant and profibrinolytic effect of APC.[129] PS deficiency has been identified as a cause of venous thrombosis, and more recently has been proposed as a factor in arterial disease. The prevalence of PS deficiency in the general population is thought to be around 0.7%.[130]There are only a few small studies investigating the prevalence of PS deficiency in PAD. Allart in 1990 showed PS deficiency to be present in 3 out of 45 patients (8%) less than 45 years old who required surgical treatment for PAD.[129]. A study of 33 patients undergoing arterial surgery, and 10 controls found a prevalence of PS deficiency in PAD patients of 15%. Although no statistical difference was shown

between patients and healthy controls, all five subjects with PS levels less than normal were PAD patients.[131]

Prothrombin 20210A

A G to A transition at position 20210A of the prothrombin gene is associated with an increased risk of venous thrombosis, although the underlying mechanism is not clear. The prevalence of this mutation is 1.2% to 4.3% in patients with venous thrombosis, 5.7% in patients with PAD, and 0.7% in controls.[132; 133] However, no specific studies have been performed investigating the association between prothrombin 20210A and arterial disease.



Despite studies screening for different states, using a variety of methods, in patients with a range of disease severity, it is clear that there is an increased prevalence of thrombophilic states in PAD, perhaps as high as 60%. Although common, the clinical relevance of thrombophilia in PAD is a more important issue, which will now be discussed.

Significance of thrombophilia

Studies of general coagulation activation

There is a correlation between the level of coagulation activation and the severity of PAD as determined by walking distance[91], ankle-brachial pressure index (ABPI)[134; 135], duplex ultrasonography, angiography[136] and clinical symptoms[112]. For example, Ray reported the prevalence of thrombophilia (PC deficiency, PS deficiency, AT deficiency, LAC) to be 11% in controls, 27% in claudicants and 40% in patients who had received a revascularisation.[121]

The importance of a hypercoagulable state in PAD has also been revealed through the association between coagulation abnormalities and the progression of PAD. In the Edinburgh Artery Study, whole blood viscosity, plasma viscosity and fibrinogen levels were predictive of the requirement for vascular intervention,[137] or fall in ABPI,[93] over a six year follow-up period. Furthermore, whole blood viscosity, and fibrinogen levels have been shown to be predictive for the progression of PAD as determined by walking distance.[138]

Thrombophilic states may also be important causes of failure of arterial interventions. In 1994, Ray studied 124 patients undergoing arterial reconstruction and reported 75 graft occlusions after a mean follow up of 44 months.[121] Almost half (49%) of these were subsequently identified as having a thrombophilia, compared with 27% of patent reconstructions. Abnormalities identified in the graft occlusion group were: PC deficiency (21% of occlusions), PS deficiency (17%), LAC (25%) and multiple abnormalities (12%). A subsequent prospective study, investigated the presence of a thrombophilia prior to arterial reconstruction in 60 patients with one-year follow up.[139] A pre-operative thrombophilia was identified in 65% of patients whose graft

subsequently occluded within one year, compared with 20% of those with a patent graft ($P < .05$). The presence of thrombophilia was particularly significant in early graft failures, where 11 of the 12 occlusions within one month had a pre-operative hypercoagulable abnormality. A prospective study of 137 patients undergoing a mixture of arterial reconstructions identified 14 patients (10%) with a hypercoagulable state.[120]. Three of these patients (27%) suffered a graft thrombosis within 30 days, compared with two of 123 patients with a normal thrombophilia screen (1.6%). Eldrup Jorgensen studied 20 young (<51years old) patients undergoing aorto-iliac (7) or infra-inguinal (13) vascular surgery.[95] Four patients suffered an early (<30 days) post-operative thrombosis, all of who had thrombophilia identified pre-operatively. Patients with multiple coagulation abnormalities appear to be at special risk. Thus, of 124 patients undergoing revascularisation studied by Ray, 11 had multiple thrombophilias, all of whom had had a previous revascularisation. Nine of these patients had a further occlusion during the follow-up period.[121]

Hcy

Hyperhomocysteinaemic patients have an increased rate of vein graft stenosis and increased failure of bypass grafts and angioplasty. [103; 140] Patients undergoing peripheral arterial bypass surgery with elevated homocysteine have evidence of pre-existing intimal hyperplasia in saphenous vein biopsies.[102] A prospective study investigating hyperhomocysteinaemia and progression of PAD, with mean follow-up of 37 months found a trend towards an association, but this was not statistically significant.[141] This may, however, represent a type II error as only a relatively small number of patients (22) were judged to have progression of PAD during follow-up.

Fibrinogen

Fibrinogen levels correlate with the severity of PAD, higher levels being associated with more severe disease, as determined by claudication distance,[112] angiography, [142; 143] and ABPI.[134; 136; 144] Hyperfibrinogenaemia has been shown to be predictive for the progression of PAD, as measured by change in claudication distance,[138] or the requirement for intervention.[137]

Given that hyperfibrinogenaemia is associated with the development and progression of PAD, it is unsurprising that high levels of fibrinogen are predictive of failure of interventions for PAD.[145] Prospective studies have shown that hyperfibrinogenaemia is associated with failure of vein and prosthetic femoral popliteal bypass grafts. In additon, associations have been demonstrated between raised fibrinogen levels and graft stenosis, implying that it is not simply an increased thrombotic tendency underlying the failure of such interventions.[145-147] Data regarding fibrinogen and patency following percutaneous angioplasty are conflicting. Two prospective studies have shown that hyperfibrinogenaemia is associated with poorer patency rates, while another prospective study showed that high fibrinogen levels measured prior to angioplasty were associated with improved patency rates.[148-150]

At present there are no selective treatments available to lower fibrinogen and consequently no reported trials confirming benefit in treating hyperfibrinogenaemia. While there is a great deal of evidence associating fibrinogen levels and PAD, it is difficult to conclude that this relationship is causal until such trials are available. Fibrinogen is an acute phase protein, and its increased levels in arterial disease may merely be representative of an underlying low-grade inflammatory process.

aPL

No studies to date have demonstrated an association between the prevalence of aPL and the progression of PAD. However, aPL and the antiphospholipid syndrome are associated with an increased risk of thrombotic complications of vascular surgery, although the majority of these studies are retrospective.[151-154] Ahn retrospectively identified seven patients with aPL who underwent a total of 18 vascular procedures.[155] Three of these patients, none of whom were anticoagulated developed multiple post-operative thrombotic complications and all eventually required amputation. The remaining four vascular patients in this study were taking steroids, anticoagulants, or vitamin K at the time of the initial operation. A similar study found that 16 of 19 patients with aPL undergoing a vascular procedure suffered a thrombosis, 12 of who died.[156] In a retrospective report of 234 patients undergoing vascular surgery, aPLs were associated with a shorter bypass patency period (17 v 58 weeks) and a risk of occlusion that was 5.6 times greater than patients without aPLs.[152]

The only prospective study to investigate the association between aPL and the outcome of vascular intervention, showed a trend towards the presence of aPL and failure of arterial bypass surgery, but this did not reach statistical significance.[157] This result is unfortunately confounded by the fact that, significantly more of the aPL group were anticoagulated post-operatively thus diminishing any likely difference between the groups.

APC resistance / FVL

Ouriel prospectively monitored 76 patients who underwent lower limb revascularisation for a mean of 47 months. 60% of those with APC resistance (defined as APC ratio

<2.0) had an occlusion of their graft, while only 24% of those without APC resistance suffered a graft failure ($P < .02$).[158] A similar finding was seen in Sampram's study in which 32% of those with fVL and 49% of those with APC resistance suffered a graft occlusion (both $P < .001$).[159] A study from Foley et al reported no association between fVL and graft occlusion, but excluded patients whose graft occluded within six weeks of surgery, a time that others have reported as important in graft occlusion associated with thrombophilia[160].

PS deficiency

Although the prevalence of PS deficiency is higher in patients with PAD, its significance is unknown. Allart investigated the families of young (<45 years old) PAD patients who were found to be heterozygotes for PS deficiency, but no association was found between likelihood of PS deficiency, and arterial thrombosis.[161] This finding corroborated a previous study, which showed that relatives of PS deficient individuals did not have an increased incidence of arterial thrombosis.[162]

Deficiency of PS was identified in 4 of 20 patients (20%) whose arterial reconstruction failed compared with 6 of 40 (15%) of those with a successful reconstruction at 30 days post surgery, although this difference did not reach statistical significance.[161]

AT deficiency

In Van der Bom's population study, examination of 7983 subjects revealed a complex relationship between level of AT and ABPI.[163] In men, mild PAD was associated with a high level of AT, while severe PAD was associated with lower levels of AT. Whilst in women, there was in an inverse relationship between ABPI and AT level. The authors suggest that levels of AT rise in the presence of cardiovascular disease as a protective

mechanism, but as vascular disease progresses, AT becomes consumed, leading to lower levels. The reason for the difference between the sexes is not clear.

The poor results of intervention in patients with thrombophilias, in terms of intervention failure and mortality, reinforce the clinical importance of these states in patients with PAD. The presence of two or more co-existent thrombophilias, seems to have an additive effect, and be particularly dangerous clinically. However, many thrombophilic states may be asymptomatic for many years and the 'two-hit' hypothesis suggests that thrombophilic states only become apparent when a subject is exposed to some other thrombogenic trigger such as surgery, oestrogen-containing medication, dehydration or systemic upset.

Clinical implications

Screening

The British Committee for Standards in Haematology identified 10 groups of patients who should be screened for thrombophilia (Table 1).[164] The treatment of thrombophilic abnormalities is complex, and the decision for treatment, which may be lifelong anticoagulation, should only be made after careful consideration of the patient, the individual thrombophilia and any triggering factors that may have precipitated a previous thrombosis. It is our recommendation that such patients are referred to a haematologist.

Table 4.1 Patients to be investigated for thrombophilia.

1	Venous thromboembolism before the age of 40-45 years
2	Recurrent venous thrombosis or thrombophlebitis
3	Thrombosis in an unusual site, eg mesenteric vein, cerebral vein etc
4	Unexplained neonatal thrombosis
5	Skin necrosis, particularly if on coumarins
6	Arterial thrombosis before the age 30 years
7	Relatives of patients with thrombophilic abnormality
8	Patients with clear family history of venous thrombosis
9	Unexplained prolonged activated partial thromboplastin time
10	Patients with recurrent foetal loss, idiopathic thrombocytopaenia or SLE

For patients with PAD who do not fall into one of the groups in Table 4.1, thrombophilia screening is still likely to reveal an abnormality in approximately 30 - 60% of patients. In those who are not undergoing a vascular intervention, there is no evidence to suggest that treatment of the thrombophilia will alter the progression of arterial disease. There is evidence however, that patients with a thrombophilia undergoing a vascular intervention have a poor prognosis, with increased risk of graft occlusion, limb loss and death, and this can be partially offset by treatment. It is therefore recommended that all patients undergoing a vascular intervention should be screened for a thrombophilic tendency.

Testing for thrombophilia should depend on the individual abnormality. Antiphospholipid antibodies, activated protein C resistance, and hyperhomocysteinaemia are the commonest abnormalities, and should form the basis of a thrombophilia screen. Screening for PC and PS deficiency, prothrombin 20210A, and AT deficiency may be useful, but likely to yield less positive results, although no less significant.

Assays for hcy have previously been difficult to perform, due to the requirement for immediate cooling of the sample and separation within 1 hour. New techniques are being developed to improve the stability of blood samples for hcy analysis, increasing the ease by which homocysteine assays can be performed.[165; 166]

The cost of thrombophilia screening is used as an argument against its use. However, if screening were targeted to high-risk groups, such as those in Table 4.1, or those undergoing intervention, the cost of screening may be offset against the reduced risk of failure of vascular intervention. The treatment of intervention failure may include prolonged hospital stay, repeated intervention, or amputation, all with significant costs. A more detailed cost-benefit analysis is beyond the scope of this article, and would be difficult to perform given that the lack of trials in this area means the true benefit of screening and/or treatment cannot be quantified.

Treatment

Although numerous different treatments are available for thrombophilias, they have not been formally studied in patients with PAD to determine whether improved outcomes can be attained.

Hyperhomocysteinaemia.

Patients with hyperhomocysteinaemia, who are undergoing a vascular intervention, should be treated with hcy lowering therapy prior to surgery if there is sufficient time. If the surgery is urgent, consideration should be given to formally anticoagulating these patients until the level of hcy can be reduced. Hyperhomocysteinaemia may be corrected simply with folic acid, and vitamins B_{12} and/or B_6, although it has yet to be demonstrated whether such treatment will lead to a reduction in cardiovascular risk or improvement in patency rates. Trials are presently being undertaken to determine whether lowering homocysteine levels is beneficial in terms of outcome for vascular patients both in PAD and in cardiac and cerebrovascular disease. It seems sensible in the absence of current evidence however, to lower homocysteine levels in PAD patients undergoing intervention.

Anticoagulation.

Anticoagulation in non-thrombophilic patients is of benefit in femoro-popliteal bypass grafts when compared with no treatment, but when compared to aspirin, the data are conflicting. [167]. The largest study was a multicentre, randomised controlled trial investigating the effectiveness of oral anticoagulation (to maintain an INR 4.0-4.5) against aspirin (80mg daily) in 2690 patients undergoing infrainguinal bypass

surgery,[168] which showed no overall benefit of either treatment in preventing graft occlusion. Patients with aPL who are anticoagulated (with heparin and subsequently warfarin) when they underwent vascular surgery were noticed to suffer fewer complications[155]. No studies to date have prospectively investigated the use of anticoagulation in PAD patients with a thrombophilia. However, Khamashita et al retrospectively studied the effectiveness of anticoagulation in patients with antiphospholipid syndrome.[169] They showed that anticoagulation with warfarin to an international normalised ratio (INR) of >3 was significantly more effective in preventing recurrent thrombosis than anticoagulating to an INR < 3, or aspirin. This study was not confined to patients with PAD, but is significant in demonstrating a benefit of aggressive anticoagulation in thrombophilia.

Steroids.

Whilst the thrombophilias discussed previously are not thought to be associated with a vasculitis, patients with the lupus anticoagulant who are taking steroids seem to have a reduced thrombotic risk.[155] The protective effect of steroids in conjunction with aspirin has been demonstrated previously in obstetric patients, and leads to a decrease in lupus anticoagulant levels.[170] There are no data on the use of steroids for PAD patients with thrombophilia.

Anti-platelet agents.

Aspirin is beneficial in obstetric patients with the lupus anticoagulant.[171] The use of aspirin has not been investigated in PAD with a thrombophilia, but it is suggested that it be used in patients with aPL, with no history of thrombosis. Patients with aPL

undergoing surgery, or with a history of thrombosis should be formally anticoagulated, as these patients are at high risk of thrombosis.

Factor replacement.

An alternative treatment for patients with PC and PS deficiency undergoing surgery is the use of peri-operative fresh frozen plasma or PC concentrate. In the case of peripheral vascular surgery, patients will usually require formal anticoagulation to ensure the patency of the graft.

Nucleic acid therapy.

Recently, oligonucleotides have been shown to have in-vitro anticoagulant effects through specific protein binding.[172] It remains to be seen whether this will translate into improved outcomes in thrombophilias.

Summary

The evidence to date supports an association between certain thrombophilias and peripheral vascular disease. Hyperhomocysteinaemia, hyperfibrinogenaemia, APC resistance and aPL syndrome are more common in PAD, but there is no clear evidence for the other thrombophilias.

Thrombophilic states in general are associated with an increased failure rate of vascular reconstruction. This is particularly marked when considering patients with multiple thrombophilias, and early intervention failures. No conclusive evidence yet exists to show that treatment of these thrombophilic states can lead to an improvement in the course of PAD, or the results of intervention. While it may be appropriate to anticoagulate patients identified with a thrombophilia who are undergoing a vascular intervention, it cannot yet be justified to recommend screening of all patients with PAD

for thrombophilia. There is a pressing need for well-designed trials of therapeutic intervention in patients with thrombophilia to determine whether outcomes are genuinely improved.

Hypothesis

The excess number of deaths suffered by patients with IC can only be partly explained by the presence of widespread atherosclerotic disease.[2] Most of this higher than expected morbidity is due to the thrombotic complications of systemic atherosclerosis. It therefore seems plausible that claudicants may suffer from a prothrombotic tendency. As one of the main differentiating factors between patients with IC, and patients with other manifestations of atherosclerosis is the repeated ischaemia-reperfusion of a large muscle mass, this could be the source of any haemostatic disturbance.

From the literature reviewed in Chapter 4, it can be that patients with peripheral arterial disease have a higher prevalence of thrombophilia than the general population. However, the majority of these were inherited abnormalities, and are unlikely to be secondary to IR.

It would seem to be biologically plausible that IR in claudicants could lead to disturbances in haemostasis. The studies presented in Chapter 3 show that IC is accompanied by a significant systemic response, much of which leads to EC disturbance. As the EC plays such a pivotal role in haemostasis (Chapter 2), it is entirely probable that such a disturbance could have effects on the coagulation system. Further coagulation disturbance could occur via platelet involvement – which may also be affected by IR in claudication (Chapter 2).

In summary, therefore, the hypothesis is that the ischaemia-reperfusion seen during exercise in patients with IC, is associated with disturbances in haemostasis, which may contribute to the excess number of deaths seen in this population.

Chapter 5

Thrombin Production in Intermittent Claudication.

Introduction

In general terms, the haemostatic system can be thought of as a balance, with fibrin production on one side and fibrin breakdown (fibrinolysis) on the other. Thrombin is the enzyme which converts fibrinogen to fibrin, and therefore anything which increases thrombin production may contribute to the high number of thrombotic events claudicants are known to suffer.

As exercise in claudicants is associated with a well described systemic inflammatory response, it would perhaps be surprising if the coagulation system were unaffected. We therefore studied the effect of exercise in claudicants on the coagulation system. This chapter will examine thrombin production, and the next chapter will investigate the effect on fibrinolysis.

Hypothesis

Exercise in patients with IC, leads to an increase in the production of thrombin, and fibrin, when compared to a non-claudicant population.

Aim

To compare the production of thrombin, using several biomarkers, in claudicants, and age and sex matched controls, before and after exercise.

Subject and methods

Subjects

40 male subjects were recruited, 20 patients with intermittent claudication, all current cigarette smokers; 20 patients with no evidence of peripheral arterial disease, 10 smokers and 10 non-smokers. Every alternate claudicant was age matched +/- 3 years to a smoking, and non-smoking control. Inclusion and exclusion criteria are presented in Tables 5.1 and 5.2.

Table 5.1 Inclusion and exclusion criteria for claudicants.

Inclusion criteria	Exclusion criteria
Male	Diabetes mellitus
Claudicant, as defined by Edinburgh Claudication Questionnaire, for ≥ 6 months	Haemoglobin concentration < 11g/dl
Resting ankle-brachial pressure index <0.8	Current viral infection
Absolute claudication distance 25-300m	Current therapy with: warfarin
Walking unrestricted by co-morbidity other than claudication	
Smoker of equivalent of at least 20 cigarettes per day, for >10 years	

Table 5.2 Inclusion and exclusion criteria for smoking and non-smoking controls.

(* smoking controls only. † non-smoking controls only)

Inclusion criteria	Exclusion criteria
Male	Diabetes mellitus
No history of peripheral arterial disease	Haemoglobin concentration < 11g/dl
Palpable pedal pulses, and resting ankle-brachial pressure index ≥ 1.0	Current viral infection
Walking unrestricted by co-morbidity	Current therapy with: warfarin
*Smoker of equivalent of at least 20 cigarettes per day, for >10 years	
†Non-smoker for > 15 years	

Methods

The study was approved by the Local Research and Ethics Committee. All subjects signed a written consent form (Appendix 1).

Subjects attended the Vascular Studies Unit on two occasions: a screening visit and an assessment visit. All studies were conducted in a quiet, temperature controlled room, maintained at 25°C to 28°C.

Screening visit. Subjects were screened to ensure they fulfilled the inclusion and exclusion criteria. This consisted of:

- History and examination, including documentation of lower limb pulses.
- For claudicants, completion of the Edinburgh Claudication Questionnaire.[5]
- Calculation of ABPI as follows:
    - Measurement of systolic blood pressure in both arms.
    - Measurement of systolic blood pressure in posterior tibial, and dorsalis pedis arteries, in both feet.
    - ABPI was calculated for each leg by dividing the highest pressure for that limb, by the highest arm pressure.
- Treadmill test, with recording of maximum walking distance as follows:
    - Subjects walked on a motorised treadmill, at 3.5 km/h, at a gradient of 12°
- Blood sampling, for measurement of full blood count.

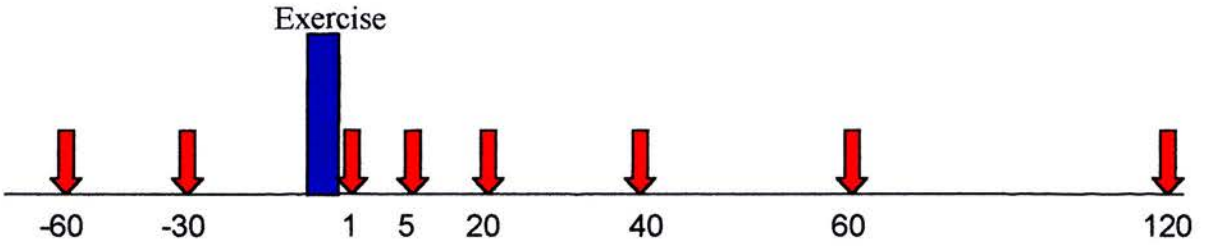
If subjects fulfilled all inclusion and exclusion criteria, they returned for an assessment visit , within 3 weeks of screening.

Assessment visit. Subjects were transported to the hospital by taxi, and transferred from the drop off-point to the vascular laboratory by wheelchair to avoid walking. During the whole visit, subjects did not exercise other than on the treadmill. Those requiring to visit the toilet were taken by wheelchair. Subjects were asked to abstain from smoking, or eating heavy meals for 1 hour prior to arrival, and were told to avoid unaccustomed exercise in the preceding 24 hours.

Upon arrival, the subject was seated in a quiet area, and an 18 gauge cannula inserted in the right ante-cubital fossa, which was kept patent with boluses of 0.9% saline. After

60 minutes, and 90 minutes, blood was drawn through the cannula. Details of blood collection at each time point are presented below (Table 5.3). After sitting for a total of 120 minutes, subjects underwent a standard treadmill exercise. Subjects walked at 3.5km/hr, at a gradient of 12° until maximum claudication distance (claudicants), or for 3 minutes (controls). The subject then returned to his seat, and blood was collected at the following times following the end of exercise: 1 minute, 5 minutes, 20 minutes, 40 minutes, 60 minutes and 120 minutes.(Figure 5.1) Following the last blood sample, subjects were given something to eat and drink, had their pulse and blood pressure checked, and were allowed to leave if well.

Figure 5.1 Outline of blood sampling protocol



Times refer to minutes prior to the start of exercise, and minutes post-cessation of exercise (not to scale).

Table 5.3 Details of blood collected at each time point

Sample No	**Tube(s)**	**Tube type**	**For measurement of:**
1	1x 1.8ml	0.105M citrate	Discard
2	1x 8.1ml	0.105M citrate	D-dimer, PF 1+2, Fibrinogen, PT, APTT
3	1x 4.5ml	Stabilyte	tPA activity/ antigen, PAI-1 activity/ antigen, APC-PCI
4	1x 10ml	SCAT-2	FbDP, PAP, TPA:PAI-1, TAT
5	1x 4.5ml	EDTA Tube	ESR

All blood was drawn without venous occlusion. PT = prothrombin time; APTT = Activated partial thromboplastin time; APC-PCI = Activated Protein C-Protein C Inhibitor Complexes; PAP = plasmin-antiplasmin complexes; ESR = erythrocyte sedimentation rate.

Blood Collection

Five samples were taken at each time point, they are detailed in Table 5.3. Each tube was gently inverted five times, and then placed immediately on ice. Subsequent handling was as follows;

- Tube 1. Discarded
- Tube 2. Kept in ice-water for 10 minutes, then centrifuged at 2000g for 15 minutes at 4°C. The supernatant (platelet-free plasma) was then aliquoted into cryovials for storage at -80°C and later batch analysis. 100µl of sample was used for measurement of prothrombin time (PT) and activated partial thromboplastin time (APTT) (detailed below).
- Tube 3. Kept in ice-water for 10 minutes, then centrifuged at 2000g for 15 minutes at 4°C. The supernatant was then aliquoted into cryovials for storage at -80°C and later batch analysis.
- Tube 4. Kept in ice-water for 10 minutes, then centrifuged at 2000g for 15 minutes at 4°C. The supernatant was then aliquoted into cryovials for storage at -80°C and later batch analysis.
- Tube 5. Kept at room temperature, for batch measurement of erythrocyte sedimentation rate (ESR).

Assays

Full blood count. These were performed in the Department of Haematology, Birmingham Heartlands Hospital using a Bayer Advia 120 (Bayer, UK).

PT and APTT. This was performed using near patient-testing equipment (Rapidpont Coag, Bayer, UK), within 30 minutes of sampling. Each analysis required the transfer of 50µl of citrated plasma onto the appropriate test card, placed in the analyser. Quality control specimens were run at the start and end of each day.

Fibrinogen, PF1+2, TAT. These assays were all performed using commercially available kit assays as detailed in Table 5.4.

Activated Protein C-Protein C Inhibitor (APC-PCI). This assay was performed using the method of Stenflo.[173]

ESR. Samples were batch analysed at the end of each day using a Starsted Automatic ESR analyser in the Department of Haematology, Birmingham Heartlands Hospital.

Table 5.4 Summary of assays.

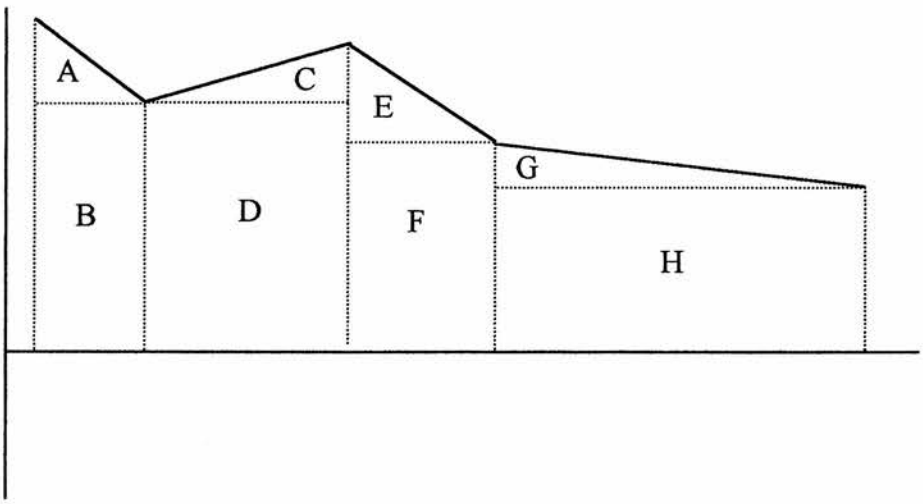
Biomarker	**Manufacturer**	**Reference range**	**Lower limit of quantification**
Fibrinogen	Dade-Behring (IL,USA)	180-400mg/dl	17 mg/dl
TAT	Dade-Behring (IL,USA)	0-5ng/ml	0 ng/ml
PF 1+2	Dade-Behring (IL,USA)	0.4-1.8nmol/l	0 nmol/l

Statistical methods

Summary variables for each biomarker were calculated for the periods pre and post-exercise by calculating the area under the curve (AUC).(Figure 5.2). Groups were compared pre, and post-exercise with the Kruskal-Wallis test. Where this returned a value of $P<0.05$, the Mann-Whitney U test was used to identify the groups which had significantly different values. A value of $P<0.05$ was taken to be statistically significant.

SPSS version 11.0.0 package (Statistical Package for Social Sciences Inc, Chicago, IL, USA) was used for all statistical analysis.

Figure 5.2 Calculation of the area under the curve (AUC).



Total AUC = area of A+B+C+D+E+F+G+H.

Results

All data are presented as median and interquartile range (IQR) unless otherwise stated. Preliminary analysis revealed subject 007 (claudicant) had very high levels of APC-PCI, D-dimer and FbDP. This individual was known to have a small abdominal aortic aneurysm (AAA), which was thought to be the cause for the coagulation abnormalities. Therefore, it was decided to exclude other subjects who were known to have an AAA (subject 103, and subject 111 – both smoking controls).

Subjects.

Details of the remaining subjects for analysis in the three groups are shown in Table 5.5. There was no significant differences between the groups with regards to age or body mass index. There was a higher prevalence of chronic obstructive airways disease in the claudicants compared to the control groups and claudicants were also more likely to be taking aspirin. There was no significant difference in cholesterol levels between the three groups.

Table 5.5 Subject details

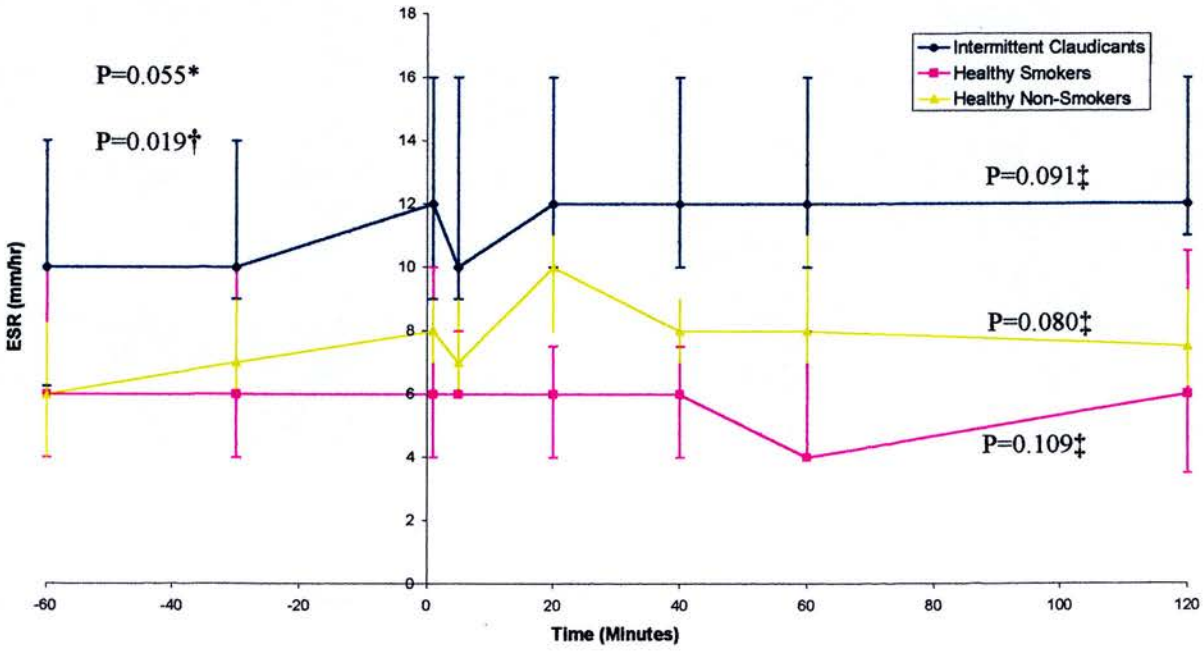
		Controls		
	Claudicants	**Smokers**	**Non-smokers**	**P=**
	n=19	**n=8**	**n=10**	
Age, years (IQR)	63 (57-70)	65 (60-70)	66 (58-70)	0.742*
Body mass index, kg/m^2	23.6	24.5	25.4	0.137*
(IQR)	(21.7-26.0)	(23.4-26.7)	(23.4-28.5)	
Smoking				
pack years	44	45	0	<0.001*
Medical history				
CAD	4	0	1	0.320†
CVD	0	0	0	
HT	4	2	0	0.260†
COAD	6	0	0	0.034†
Medication				
Aspirin	13	1	1	0.002†
ß-blocker	1	0	1	0.647†
Nitrate	1	0	0	0.615†
Ca blocker	3	0	0	0.213†
ACEI	2	1	1	0.984†
PAD				
Lowest ABPI	0.56	1.09	1.12	<0.001*
Walking distance, m,	82	174	174	<0.001*
(IQR)	(64-174)	(170-174)	(174-174)	
Cholesterol, mmol/l	4.7 (4.1-5.6)	5.2 (4.8-6.6)	4.5 (4.4-5.1)	0.274*
(IQR)				

CAD = coronary artery disease (angina, myocardial infarction or coronary bypass surgery); CVD = cerebrovascular disease (transient ischaemic attack or stroke); HT = hypertension; COAD = chronic obstructive airway disease; Ca blocker = calcium channel blocker. *=Kruskal-Wallis Test. †=Fisher's Exact Test.

ESR

(Figure 5.3) ESR was significantly higher in claudicants compared to controls before and after exercise.

Figure 5.3 Plot of ESR with time for the three subject groups

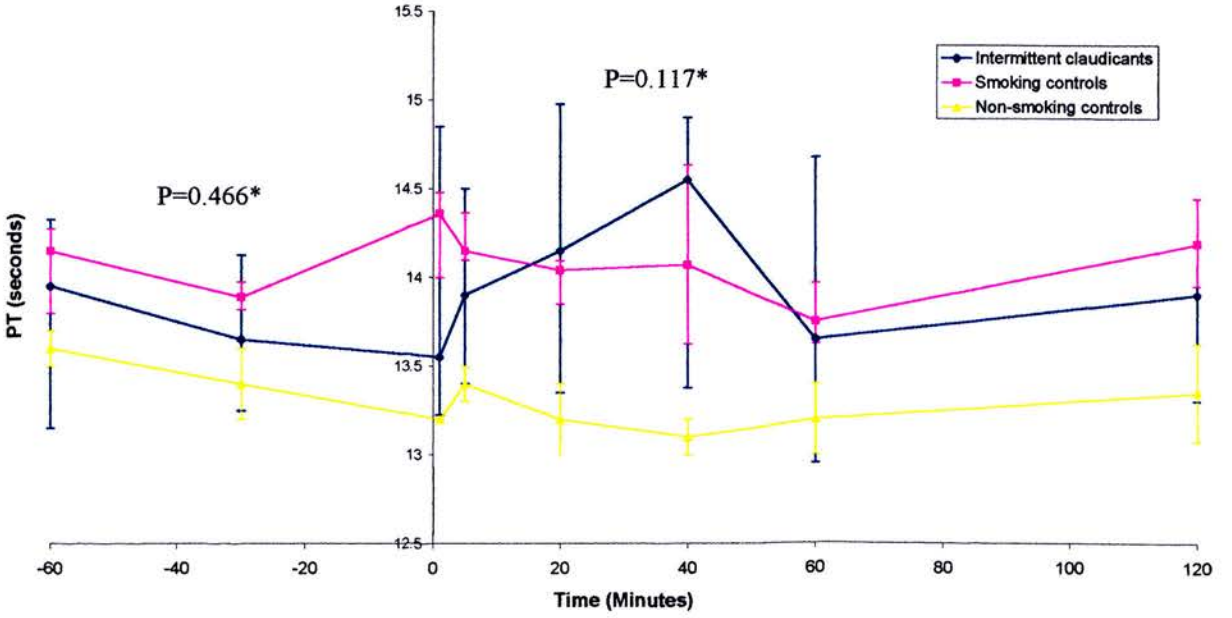


Data are presented as medians with interquartile ranges. *=Intermittent claudicants vs Smoking controls, †= Intermittent claudicants vs Non-smoking controls. Both Mann-Whitney U Test.

PT / APTT.

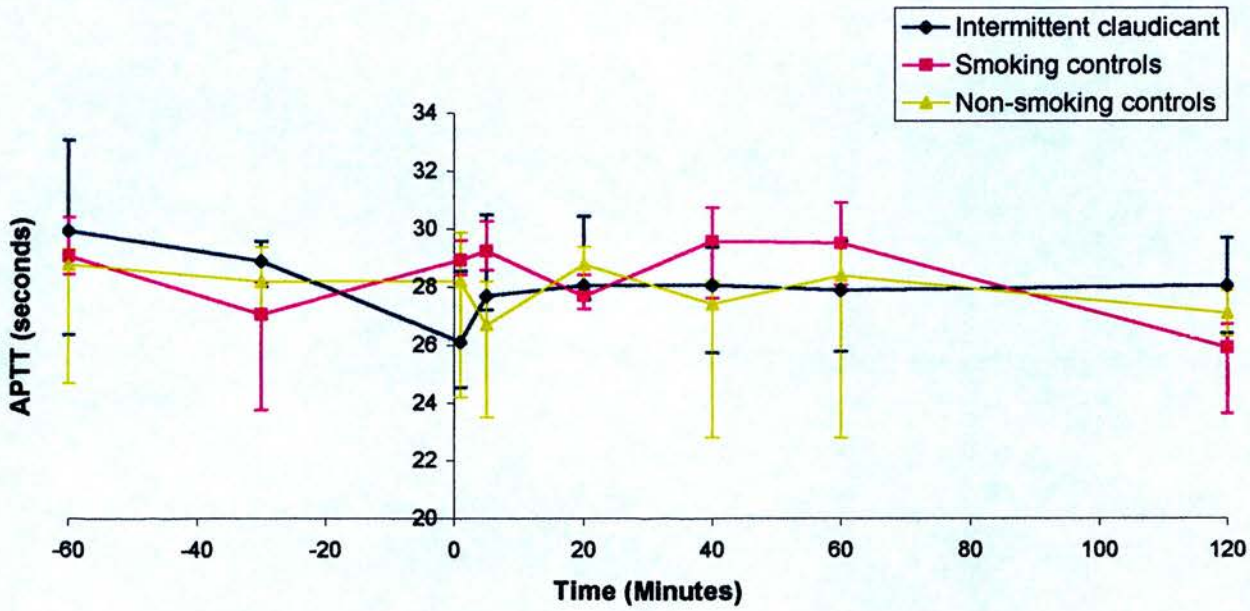
(Figures 5.4, 5.5) PT was slightly higher in both smoking groups (claudicants and smoking controls) than non-smokers. All groups showed a prolongation of the PT after exercise, but this was more marked, and lasted longer in the smokers. APTT was similar in all groups with no obvious effect of exercise.

Figure 5.4 Plot of PT with time for the three subject groups



Data are presented as medians with interquartile ranges. *=Kruskal-Wallis Test comparing all three groups.

Figure 5.5 Plot of APTT with time for the three subject groups

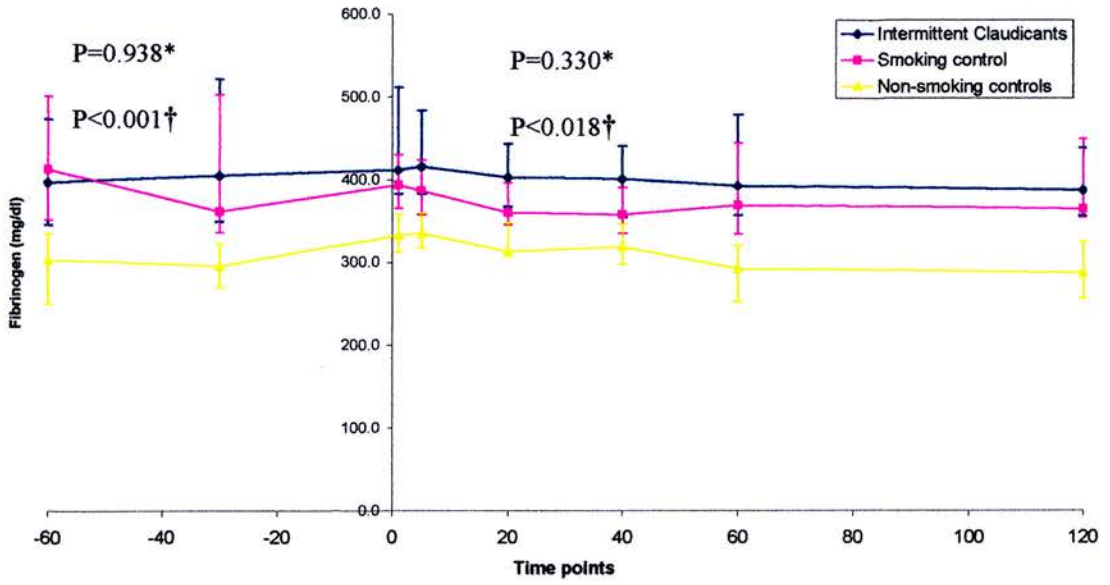


Data are presented as medians with interquartile ranges. *=Kruskal-Wallis Test comparing all three groups.

Fibrinogen.

(Figure 5.6). This was significantly higher at baseline in claudicants compared to non-smokers, but similar to smoking controls. Smoking controls had significantly higher levels than non-smoking controls. Exercise had no perceptible effect on fibrinogen levels in claudicants or controls.

Figure 5.6 Plot of fibrinogen with time for the three subject groups.

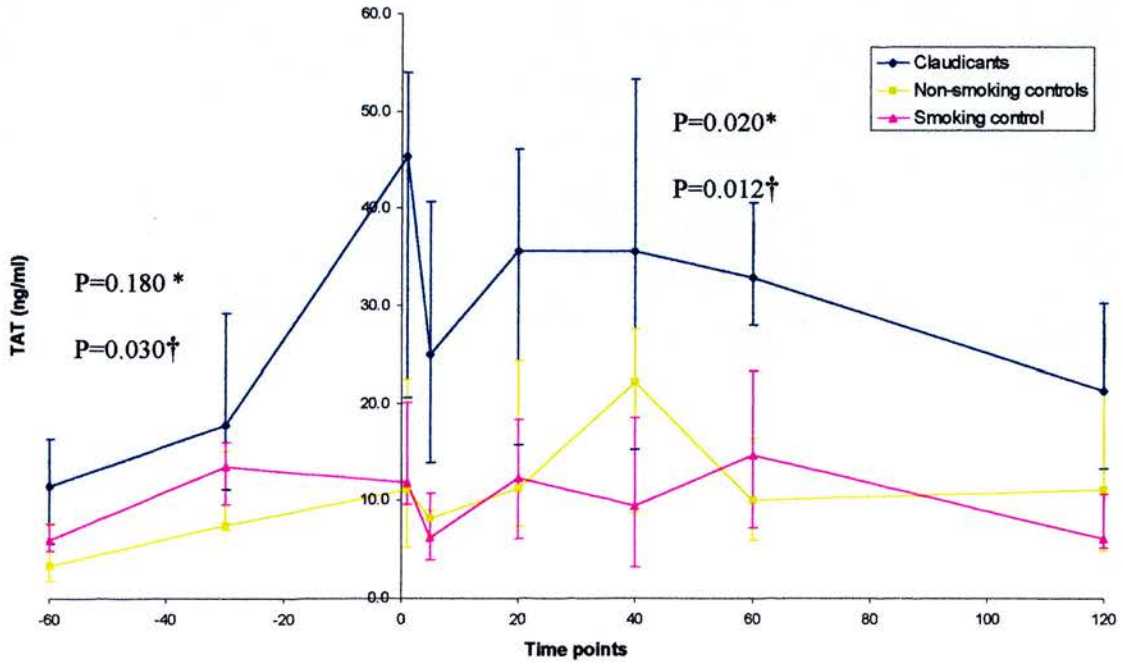


Data are presented as medians with interquartile ranges. *=Intermittent claudicants vs Smoking controls, †= Intermittent claudicants vs Non-smoking controls. Both Mann-Whitney U Test.

TAT / PF1+2.

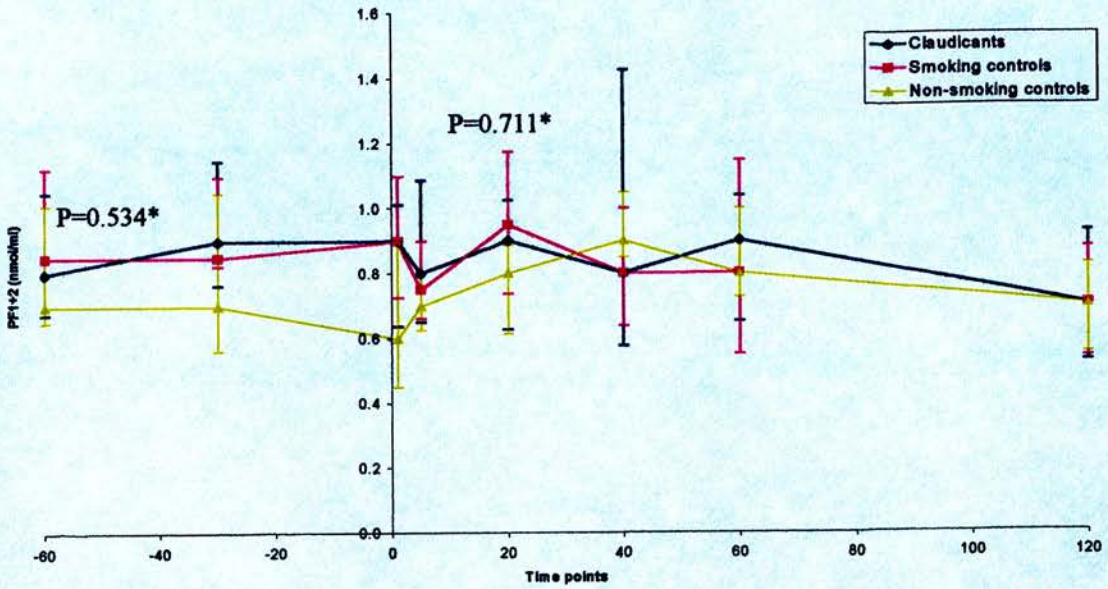
(Figures 5.7 and 5.8) TAT and PF 1+2 are formed when thrombin is produced. Levels were similar in all three groups prior to exercise, but following exercise, TAT was significantly higher in the claudicants compared to controls, although PF1+2 levels were not significantly different

Figure 5.7 Plot of TAT with time for the three subject groups



.Data are presented as medians with interquartile ranges. *=Intermittent claudicants vs Smoking controls, †= Intermittent claudicants vs Non-smoking controls. Both Mann-Whitney U Test.

Figure 5.8 Plot of PF1+2 with time for the three subject groups

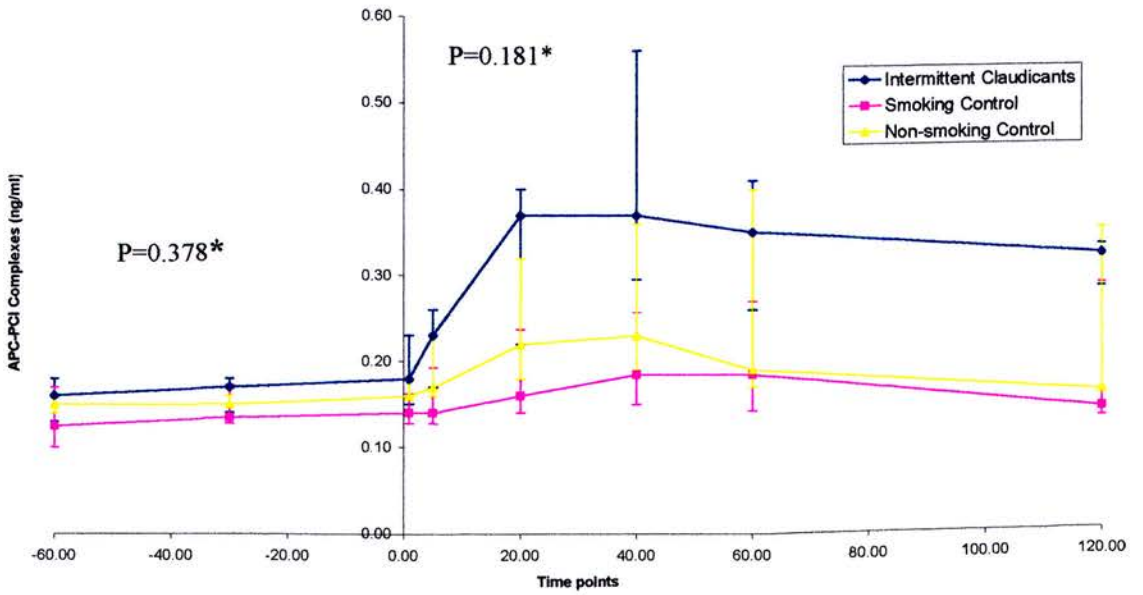


Data are presented as medians with interquartile ranges. *=Kruskal-Wallis comparing all three groups.

APC-PCI

(Figure 5.9) When formed, thrombin is bound to thrombomodulin on the endothelial cell surface. This complex activates Protein C, an important mediator in negative feedback in the coagulation cascade, and also the activator of thrombin-activable fibrinolysis inhibitor (TAFI). Protein C, is quickly bound by its specific inhibitor (Protein C inhibitor), and it is this complex which was assayed. Levels of APC-PCI were similar between the groups at baseline, but were higher in the claudicants following exercise, but this failed to reach statistical significance.

Figure 5.9 Plot of APC-PCI with time for the three subject groups.



Data are presented as medians with interquartile ranges. *=Kruskal-Wallis Test between all three groups.

Discussion

This study has, shown that exercise in claudicants is accompanied by increased generation of levels of TAT and APC-PCI when compared to matched controls, suggesting increased fibrin formation. We did not show a difference in PF1+2 levels between groups, before or after exercise, and we are unable to explain this. PF1+2 have a longer half life than TAT (90 minutes vs 15 minutes), so may be less responsive to acute events such as the exercise in this study. However, given the findings that claudicants seem to have increased levels of thrombin production, we might expect to have found elevated levels of PF1+2 in the claudicants at rest, which was not the case. Three previous studies have examined thrombin production in exercising claudicants (Table 5.6). Two of these studies showed that in contrast to our data, levels of thrombin generation (as indicated by TAT and PF 1+2) were higher at baseline in claudicants compared to controls.[90; 91] In addition, they did not demonstrate a response to exercise. The baseline differences in these two studies are probably accounted for by the controls and claudicants being poorly matched. De Buyzere does not mention smoking or diabetic status in his paper. Herren's claudicant and control groups were poorly matched for smoking status (50% current smokers vs 8%), hyperlipidaemia (36% vs 9%) and hypertension (50% vs 38%). As these conditions have all previously been shown to influence levels of PF 1+2 and TAT, this could explain the baseline differences between the two groups.[174-176] This is confirmed by the study by Mustonen, who compared claudicants and controls who were well matched for all these factors.[177]

He demonstrated that levels of thrombin generation were similar at baseline in claudicants and controls, and that claudicants, but not controls had a rise in thrombin generation following exercise – the same as our findings.

Table 5.6 Previous studies of thrombin generation in claudicants

Author and year	Subjects	Sampling time points	Baseline differences	Effect of exercise	Notes
De Buyzere 1993[91]	34 claudicants 30 age, sex-matched controls	30 minutes rest before sampling Sampled 'before and after'.	TAT, PF higher in claudicants	No change in either group with exercise	No mention made of number of diabetics, smokers, or hypertensives
Herren 1994[90]	22 claudicants 13 age, sex-matched controls	3 minutes before and after exercise. No mention of rest prior to sampling	TAT, PF higher in claudicants	No change in either group with exercise	Groups poorly matched for smoking, hypertension, hyperlipidaemia.
Mustonen 1997[177]	15 claudicants 15 age, sex matched controls	15 minute rest. Sampling before, and immediately after exercise.	TAT levels equal in claudicants and controls	Increase in TAT in claudicants, no change in controls	Matched for smoking, hypertension, hyperlipidaemia and diabetes mellitus

There is no clear reason why both the De Buyzere and Herren study did not show an effect of exercise on thrombin production. Our data have demonstrated that the increase in thrombin production can persist for up to 2 hours. De Buyzere's patients were only rested for 30 minutes prior to exercise, and no mention is made of the rest period in Herren's paper; compared to over two hours in our study. If patients are studied

without sufficient rest to allow a true ' baseline', then it will be difficult to detect a rise in thrombin production following exercise.

These findings support our hypothesis that exercise in claudicants is accompanied by a procoagulant state, however, they do not suggest why this occurs. A number of areas in the coagulation cascade might be susceptible to the systemic effects of claudication.

- Thrombomodulin (TM) is a protein bound to the surface of EC's. Following the production of thrombin, it binds to TM, and activates PC, which in turn inhibits the formation of further thrombin. EC disturbance is known to occur following exercise in claudicants,[178] and this could reduce the effectiveness of this feedback system, resulting in excessive thrombin formation.
- In addition, it is know that cytokines can result in the release of tissue factor from EC's. IL 1 and TNF, which are released in ischaemia-reperfusion, can lead to thrombin production via the extrinsic coagulation pathway.[179; 180]
- Work with cultured EC's has shown that in hypoxic conditions, certain membrane proteins are upregulated, including Factor X, which might also increase thrombin production.[181]
- Catecholamines have been shown to increase levels of TAT, possibly by platelet activation.[182] It may be that the pain of exercise in claudication induces catecholamine release, which leads to activation of the coagulation cascade. However, in Mustonen's study, catecholamine levels were similar in the claudicant and controls groups, despite differing responses to exercise.[177]

Our study does not point to any particular mechanism, and further work would be required to try and clarify this.

Conclusion

We have provided evidence that exercise in claudication is associated with increased production of thrombin. This state may contribute to the high cardiovascular morbidity and mortality in this patients group.

Chapter 6

Fibrinolysis in Intermittent Claudication.

Introduction

As discussed in the previous chapter, thrombin production is only one side of the balance when considering the coagulation system. If the excess thrombin production seen in claudicants is accompanied by an increase in fibrinolysis, then the net effect on thrombus formation could be cancelled out. In theory, fibrinolysis may be more susceptible to the effects of exercise in the claudicant. The main source of tissue Plasminogen Activator (tPA) is the endothelial cell – which is known to be affected in exercising claudicants.[80] It is known that the normal increase in fibrinolytic activity seen following exercise is reduced in both CAD[183] and diabetes mellitus.[184] Furthermore, a reduction in fibrinolytic activity is becoming increasingly recognised as a risk factor for coronary events,[185] which claudicants are known to be at increased risk of.[186]

Hypothesis

Exercise in patients with IC, leads to a reduced fibrinolytic response when compared to subjects without claudication.

Aim.

To compare fibrinolysis, using several biomarkers, in claudicants, and age and sex matched controls, before and after exercise.

Methods

The study was conducted as part of the study outlined in Chapter 5. Briefly, claudicants and controls underwent a standardised treadmill exercise in the middle of a four hour rest period. Blood samples were taken pre-exercise, and post-exercise to study the effect on fibrinolysis and fibrin turnover. The assays performed to study fibrinolysis, and fibrin turnover are detailed in Table 6.1.

Table 6.1 Fibrin turnover and fibrinolysis assays

Assay	Manufacturer	Reference range	Lower limit of quantification
FbDP	Organon Teknika (Belgium)	0-310 ng FE/ml	50 ng FE/ml
D-dimer	American Diagnostica (CT, USA)	0-100 ng/ml	32 ng/ml
tPA activity	Biopool (CA, USA)	0-1.2 IU/ml	0.2 IU/ml
tPA antigen	Biopool (CA, USA)	0.5-14 ng/ml	0 ng/ml
PAP	DRG Diagnostics, Germany	Not available	0 ng/ml
PAI-1 activity	Chromogenix, Italy	Not available	2 IU/ml
PAI-1 antigen	Biopool (CA, USA)	4-43 ng/ml	0 ng/ml
TAFI antigen	DRG Diagnostics, Germany	Not available	0.3 µg/ml

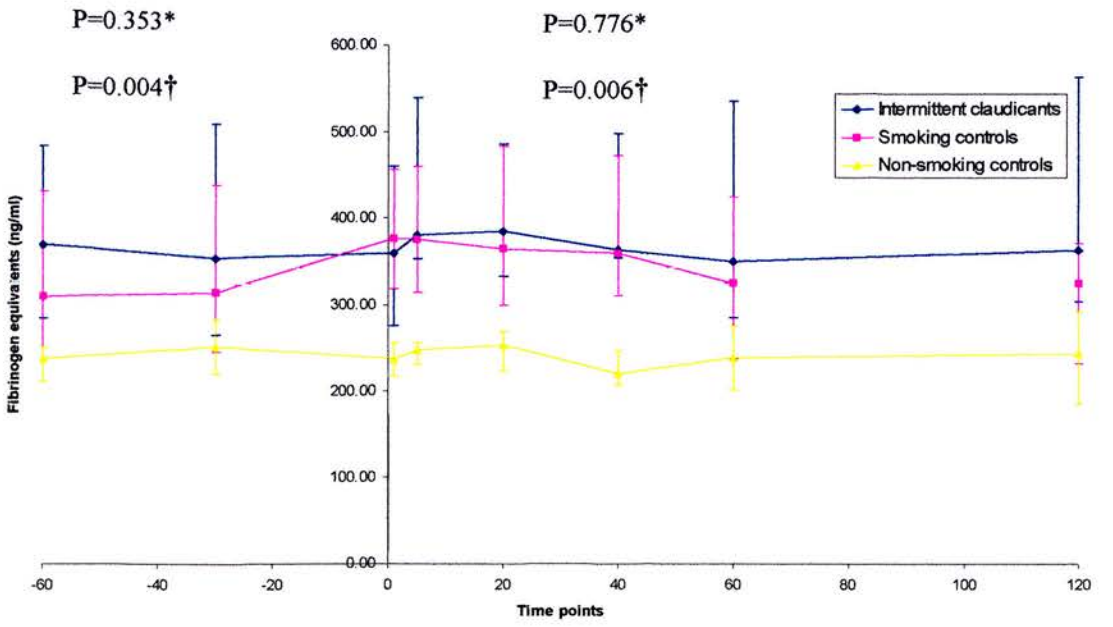
FbDP=Fibrin degradation products; FE=fibrinogen equivalents; tPA=tissue plasminogen activator; PAP=plasmin-antiplasmin complexes; PAI=plasminogen activator inihibitor; TAFI=thrombin activable fibrinolysis inhibitor.

Results

FbDP and D-dimer.

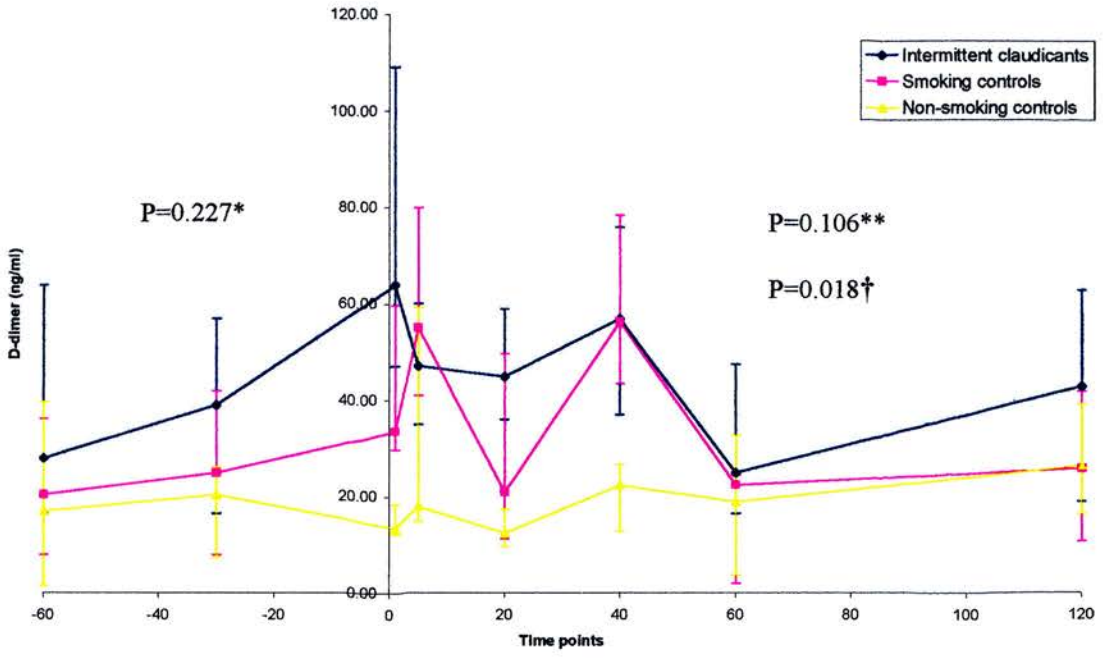
(Figures 6.1, 6.2) These are both products of the action of plasmin on fibrin. As such, they are a product of both thrombus formation, and fibrinolysis. Overall, levels of D-dimer and FbDP in claudicants were consistently higher than the control groups, but this did not reach statistical significance. D-dimer increased with exercise in claudicants and the smoking controls, with no appreciable rise in non-smoking controls.

Figure 6.1 Plot of FbDP with time for the three subject groups.



Data are presented as medians with interquartile ranges. *=Intermittent claudicants vs Smoking controls, †= Intermittent claudicants vs Non-smoking controls. Both Mann-Whitney U Test.

Figure 6.2 Plot of d-dimer with time for the three subject groups

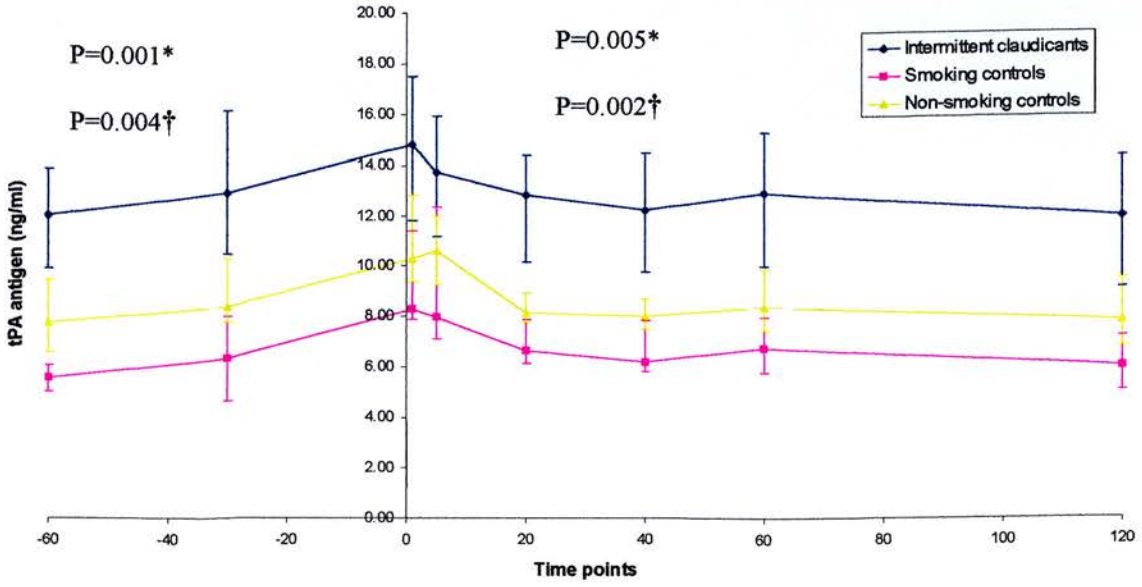


Data are presented as medians with interquartile ranges. *=Kruskal-Wallis Test comparing all three groups. **=Intermittent claudicants vs Smoking controls, †= Intermittent claudicants vs Non-smoking controls. Both Mann-Whitney U Test.

tPA antigen / activity

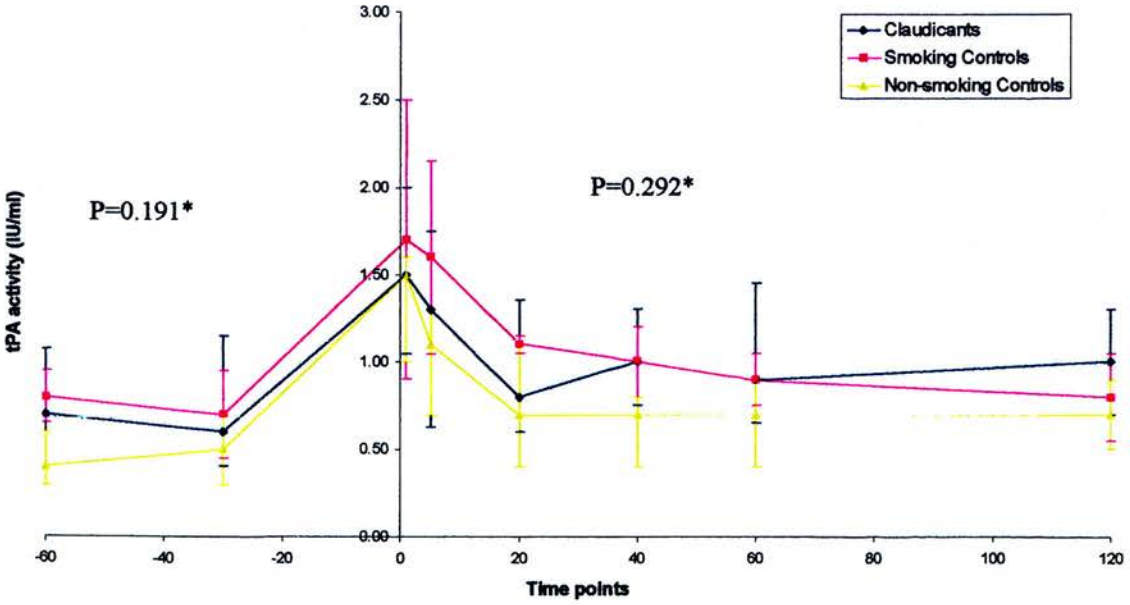
(Figures 6.3, 6.4) Claudicants had higher levels of tPA antigen at baseline, although the amount of tPA activity was similar in the three groups. All three groups had an increase in tPA antigen with exercise, which was similar in magnitude. All three groups showed a large rise in tPA activity following exercise, which reached the highest level in the smoking controls, although there was no overall difference in the groups post-exercise.

Figure 6.3 Plot of tPA antigen with time for the three subject groups



Data are presented as medians with interquartile ranges. *=Intermittent claudicants vs Smoking controls, †= Intermittent claudicants vs Non-smoking controls. Both Mann-Whitney U Test.

Figure 6.4 Plot of tPA activity with time for the three subject groups

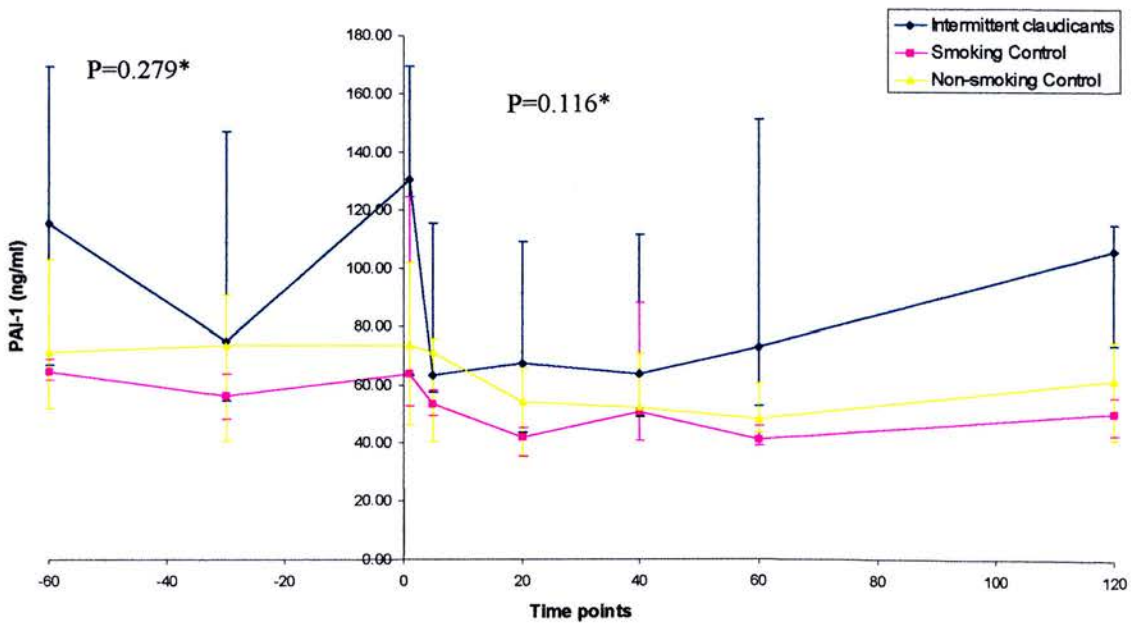


Data are presented as medians with interquartile ranges. *=Kruskal-Wallis Test comparing all three groups.

PAI-1 antigen.

(Figure 6.5) Claudicants had consistently higher levels of PAI-1 compared to controls. Claudicants seem to have an increase in levels of PAI-1 antigen with exercise, but this quickly returns to pre-exercise levels.

Figure 6.5 Plot of PAI antigen with time for the three subject groups

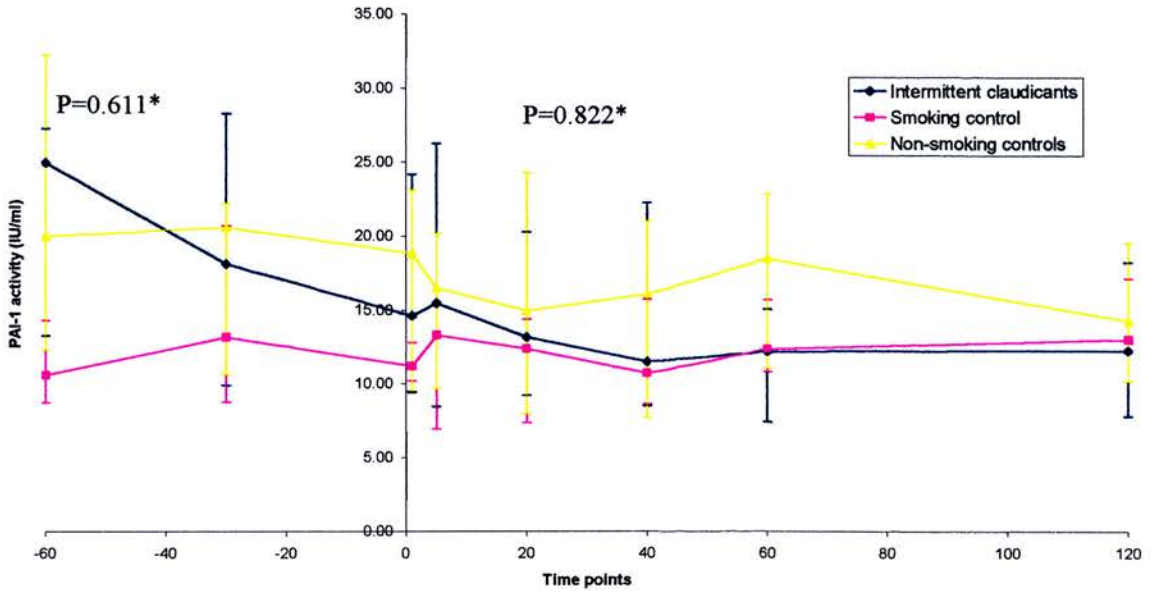


Data are presented as medians with interquartile ranges. *=Kruskal-Wallis Test comparing all three groups.

PAI-1 activity.

(Figure 6.6) No difference was detected in PAI-1 activity levels between the three groups, pre, or post-exercise. There is a trend for PAI-1 activity in the claudicants to decrease with exercise.

Figure 6.6 Plot of PAI-1 activity with time, for the three subject groups

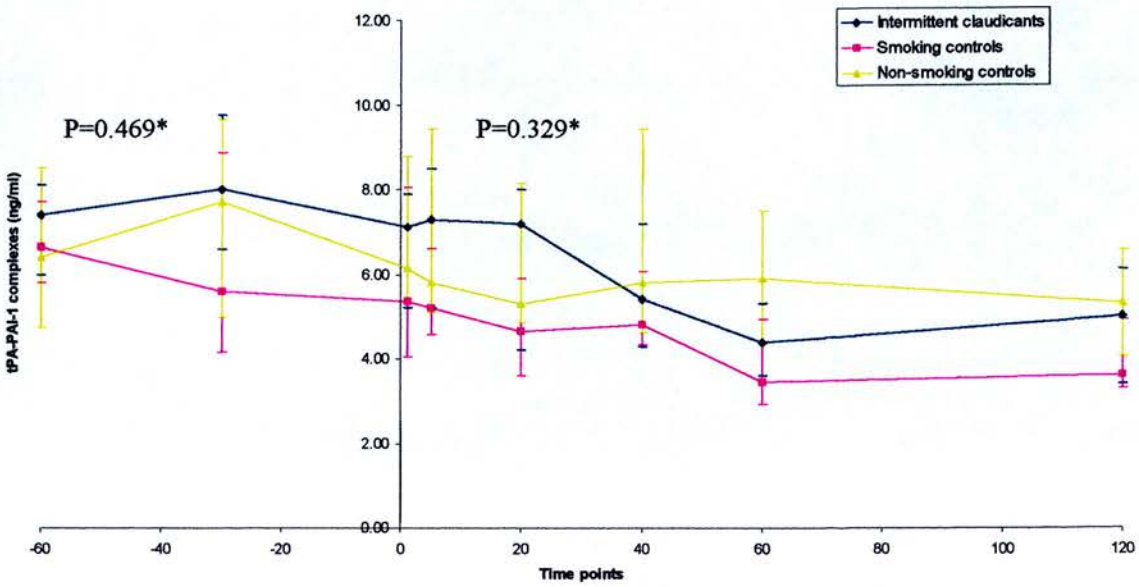


Data are presented as medians with interquartile ranges. *=Kruskal-Wallis Test comparing all three groups.

tPA-PAI-1 complexes

(Figure 6.7) Claudicants have slightly higher levels of tPA-PAI-1 complexes compared to the control groups pre-exercise, which persists until 40 minutes post-exercise.

Figure 6.7 Plot of tPA-PAI-1 complexes with time for the three subject groups

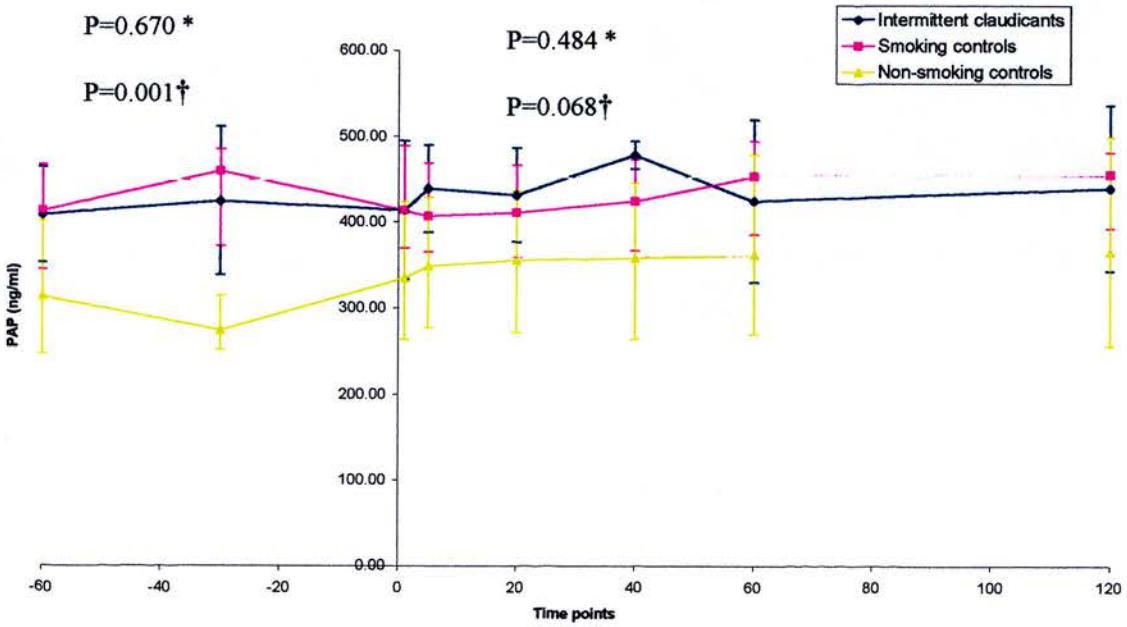


tPA-PAI-1 = tissue plasminogen activator – plasminogen activator inhibitor complexes. Data are presented as medians with interquartile ranges. *=Kruskal-Wallis Test comparing all three groups.

PAP

(Figure 6.8) Claudicants have similar levels of PAP, an indicator of plasmin production, as the smoking controls, but higher levels than the non-smokers. Claudicants and the non-smoking controls have a small rise with exercise.

Figure 6.8 . Plot of PAP with time for the three subject groups

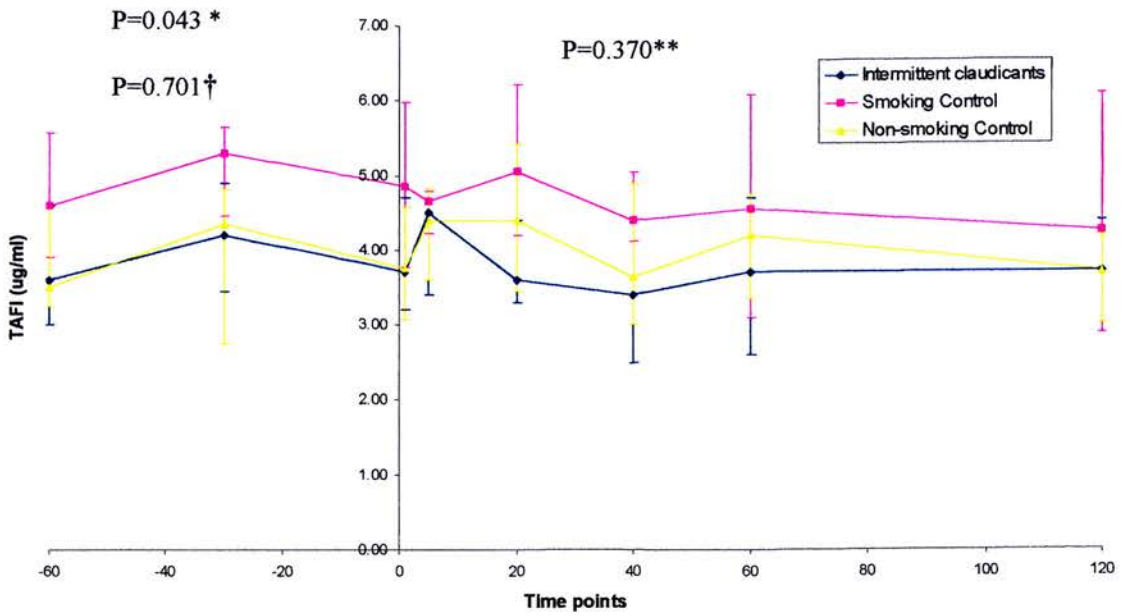


Data are presented as medians and interquartile ranges. *=Intermittent claudicants vs Smoking controls, †= Intermittent claudicants vs Non-smoking controls. Both Mann-Whitney U Test.

TAFI.

(Figure 6.9) TAFI antigen levels were higher at baseline in the smoking control group compared to the claudicants pre, but no significant differences were present between the groups post exercise.

Figure 6.9 Plot of TAFI antigen with time for the three subject groups



Data are presented as medians and interquartile ranges. *=Intermittent claudicants vs Smoking controls, †= Intermittent claudicants vs Non-smoking controls. Both Mann-Whitney U Test. **=Kruskal-Wallis Test comparing all three groups.

Discussion

Our data show that despite an increase in thrombin production, claudicants do not have a corresponding increase in fibrinolytic activity following exercise as shown by similar levels of PAP when compared to the smoking controls. Indeed, some of the results point to a possible reduction in fibrinolysis:

- Fibrin turnover, as measured by D-dimers and FbDP is a function of thrombin production and fibrinolysis. Given claudicants greater production of fibrin, if their fibrinolytic capacity were similar to controls, we may expect that they should have higher levels of FbDP and D-dimers. As these levels were similar in all three groups, we may speculate this reflects an actual deficiency in the fibrinolytic response of claudicants.
- Following exercise, tPA activity levels in claudicants are slightly less than in smoking controls, but the wide interquartile ranges of the data prevent this from becoming statistically significant.

Our data provide some explanation of why the fibrinolytic response may be blunted in the claudicant group:

- As mentioned above, there may be a reduction in tPA activity following exercise. This is despite there being significantly more tPA antigen present in the claudicant group
- This study shows that at baseline, claudicants have an excess of PAI-1 antigen compared to controls. It is likely that any tPA produced in claudicants is therefore more likely to be 'mopped up' by this excess PAI-1. This is supported by the fact that all three groups had similar rises in tPA antigen, but the controls

had greater rises in tPA activity. However, if this were the case, we might expect to see a greater rise in tPA-PAI-1 complexes in the claudicants, which we do not.

- As Protein C is the activator of TAFI, and given the increased levels of APC-PCI seen in the claudicants, we could speculate that fibrinolysis may be reduced by this pathway in the claudicants. Unfortunately, our assay for TAFI measures only TAFI antigen, not activity, and no differences were found in TAFI levels between the groups.
- The main source of inducible tPA release is from EC's. It may be that the EC's in claudicants are unable to respond to exercise in the same way as EC's in non-claudicants in terms of the magnitude of tPA release. This may be due to two reasons:
    - Patients with PAD are known to have chronic, systemic EC dysfunction, as determined by reduced brachial artery reactivity.[187] This simply reflects the systemic nature of atherosclerosis, and the involvement of EC's in the early development of atherosclerotic plaque. The data from patients with CAD are conflicting. One study which used venous occlusion to measure tPA release showed a reduced response in patients with CAD compared to controls. In studies measuring the fibrinolytic response to exercise, some have shown a significant 'blunting' of the response in CAD patients,[188-191] while another showed no significant difference between groups.[192] However, even in this final study, there was a marked trend towards a reduced tPA activity response in the CAD group compared to matched

controls, and this may represent a type II error, despite the author's assertion it was adequately powered.

- EC dysfunction may be exacerbated by ischaemia-reperfusion. As discussed earlier, exercise in claudicants leads to demonstrable systemic EC disturbance.[178]

A number of previous studies have examined different aspects of fibrinolysis in claudicants. In contrast to our findings, Killewich found baseline levels of tPA activity were lower in claudicants, but in this study, the claudicant groups (divided into mild and severe claudicants) had more diabetics ([mild claudicants vs severe claudicants vs controls] 49% vs 55% vs 0%) and smokers (44% vs 45% vs 0%), both of which are known to have reduced fibrinolytic activity.[185; 193; 194] Furthermore, no mention is made in the paper of how long the patients were rested prior to blood sampling. As the fibrinolytic response can last up 20 minutes, it is important to ensure subjects are sufficiently rested prior to baseline blood sampling. Womack examined the effect of exercise on fibrinolysis in claudicants, and concluded they had a substantial rise in fibrinolytic activity (tPA activity rose by 180%), but did not have a control group.[195]

Strengths and weaknesses.

The major strengths of this study are the uniform population, and the well-matched nature of the controls. All of our claudicants were male, smoking, non-diabetics. This was chosen to represent a 'typical' claudicant, while minimising variation. Claudicants were age matched, to a smoking, and non-smoking control each, again all non-diabetic. As might be expected, claudicants were more likely to have other co-morbidities, and be taking more medication, particularly aspirin. The issue of aspirin, and its influence on

the fibrinolytic response to exercise has been studied, and was not found to be significant.[196] However, it is impossible to exclude that the co-morbidities and concomitant medications did not have an effect, although we think this is unlikely to have a significant influence. Unfortunately, with the small number of subjects we studied, formal statistical modelling to control for these factors was not possible.

An inescapable drawback is the differing duration of exercise in the claudicants, and controls. Perhaps, if the control group had walked to exhaustion, then they may have had a different response. Studies in CAD patients have tried to standardise exercise between subjects and controls using VO_2, heart rate rises, or inability to keep up with a graded treadmill test, but in each of these, the CAD patients walked less far than the controls.

Conclusion

In conclusion, this study has shown that despite the excess thrombin that is produced when patients with intermittent claudication exercise, this is not accompanied by an enhanced fibrinolytic response.

Chapter 7

Cellular Responses to Exercise in Claudicants

Introduction

The previous chapters have demonstrated how exercise in patients with claudication affects the non-cellular components of the coagulation system. However, cells within the blood may also be affected by exercise in claudicants. Platelets and neutrophils, which have both inflammatory and prothrombotic influences, are possible candidates which may be involved.

Platelet activation.

Platelets play a pivotal role in haemostasis, by helping to promote, and localise the coagulation cascade. Platelet activation is one of the initial events in the coagulation process, and some of the subsequent coagulation reactions take place on the platelet surface. (Figure 7.1) Furthermore, because of the inflammatory mediators which can be released by platelets (such as thromboxane A_2), they may be an important link between the low-grade inflammatory reaction seen following exercise in claudicants, and any coagulation changes seen.

There are a large number of methods of measuring platelet activation, most of which suffer from significant shortcomings. (Table 7.1) Plasma separation prior to assays of secreted proteins can lead to ex-vivo platelet activation. P-selectin is commonly used as a marker of platelet activation, but it is also released from EC – cells which are known to be affected by atherosclerosis and ischaemia-reperfusion raising further doubts about its usefulness.[197]

Figure 7.1 Role of platelets in coagulation.

Information from Ref 200

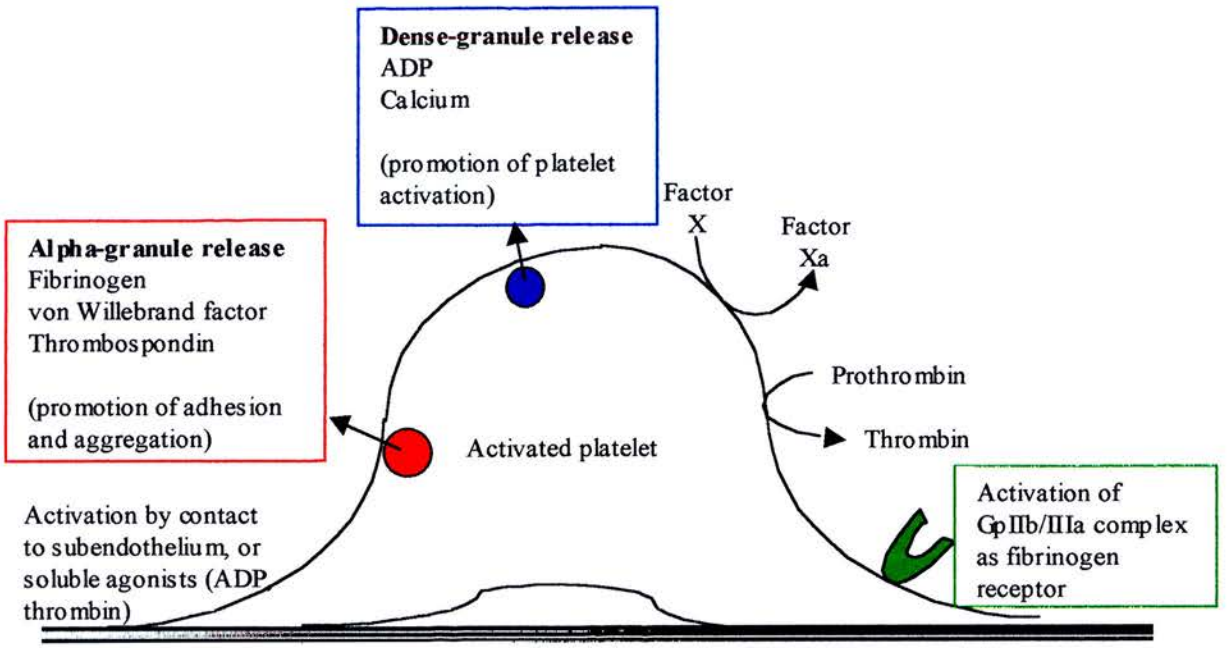


Table 7.1 Common methods for assessing platelet function

Information from Ref 201

- Bleeding time
- Platelet aggregation studies
- Expressed / secreted platelet proteins
  - Plasma P-selectin
  - Plasma ß-thromboglobulin
  - Plasma Platelet Factor 4
- Flow cytometry
  - Measurement of platelet-bound activation-specific proteins
  - Measurement of platelet-leukocyte complexes

Measurement of the bleeding time can detect platelet abnormalities, however the test is difficult to standardise and is not sensitive enough to detect small changes in platelet function. Platelet aggregation studies, monitoring the formation of platelet aggregates following deliberate stimulation, is also subject to standardisation difficulties, and are not widely used.[198]

Flow cytometry.

This technique passes single particles through a laser beam, and can measure a variety of parameters based on particle size and granularity. In addition, by detecting the binding of fluorochrome-bound monoclonal antibodies to individual cells, the expression of specific cell markers can be assessed. In the assessment of platelets, flow

cytometry has the advantage that it is performed in the more physiologic environment of whole blood, with less pre-assay manipulation. Flow cytometry can measure platelet activation in two ways:

- Measurement of the number of platelets which are expressing activation-specific proteins (ie P-selectin [CD 62P]).
- Measurement of leukocyte-platelet complexes. Platelet activation in-vivo leads to the formation of platelet-monocyte, and platelet-neutrophil complexes and measurements of these can be used to assess in-vivo platelet activation.[198] Previous studies have shown that measuring the number of monocyte-platelet complexes is a more sensitive marker of in-vivo platelet activation than other flow-cytometry methods.[199; 200]

Platelet activation in intermittent claudication.

A number of studies have demonstrated that at rest, subjects with intermittent claudication have more activated platelets, and their unactivated platelets are more reactive when compared to healthy controls.[201-203]

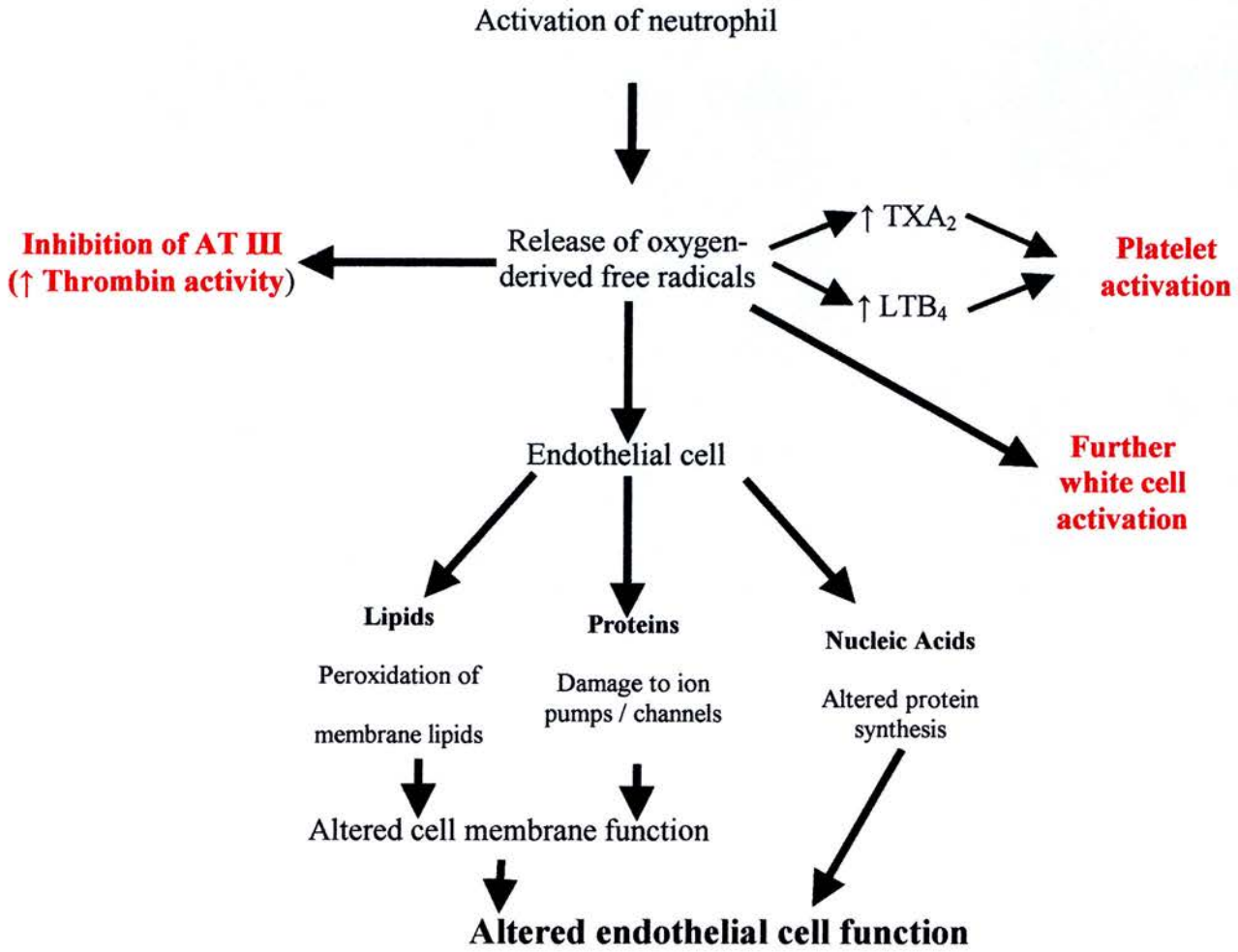
The evidence regarding the effect of exercise on platelet activation is more equivocal. In a comparison of exercise in 34 claudicants and 12 matched controls, it was found that soluble P-selectin levels rose after exercise in the claudicant group, but not in the control group.[82] However, the rise only reached statistical significance at one time point, and only after a second period of exercise. A similar study, using flow cytometry to assess platelet-bound P-selectin expression found no change with exercise in 16 claudicants.[83] Therefore, the question of the effect of exercise on platelet activation in patients with claudication remains unanswered.

Neutrophil activation.

The white cell count (WCC) has been shown to be predictive of future coronary, cerebrovascular and peripheral vascular events.[204] Neutrophils are the commonest white cell type, whose primary function is the removal of bacteria and dead tissue. Previous research has shown that ischaemia-reperfusion leads to neutrophil activation, which in turn can have significant systemic effects on the circulation:[204]

- Activation of neutrophils increases their stiffness, slowing their travel through capillaries, and possibly blocking them.
- Activated neutrophils increase their adhesion to EC, and other blood cells, further increasing the chance of becoming trapped in the microcirculation.
- Following activation, neutrophils release a number of toxic agents, including highly reactive oxygen-derived free radicals. These can interact with EC, having deleterious effects on their function. (Figure 7.2)

Figure 7.2 Prothrombotic effects of neutrophil activation



(TXA_2=Thromboxane A_2, LTB_4=Leukotreine B_4) Data taken from Ref 207

Claudicants are known to have higher neutrophil counts than matched controls,[85] and exercise in claudicants leads to further rise in neutrophil counts, while even with exhaustive exercise, no such rise is seen in non-claudicants.[78; 205] The rise in neutrophil numbers is thought to be due to the release of neutrophils, from ischaemic muscle, into the draining blood, as demonstrated by comparing neutrophil counts in the femoral artery and vein of exercising claudicants.[76] This release of inflammatory cells is accompanied by a decrease in plasma antioxidant levels (indicating release of free radicals), and EC damage.[84] As discussed in the previous chapters, it may be EC damage that mediates the coagulation changes associated with exercise in claudication. A number of indirect markers of neutrophil activation have been used previously (Table 7.2), but more recently, flow-cytometry detection of CD11b expression has been used as a more direct marker of neutrophil activation.[78; 83] CD11b is an adhesion molecule with is uniquely expressed on the surface of activated neutrophils,[206] and can be targeted by fluorochrome-linked monoclonal antibodies.

Table 7.2 Methods used for measuring neutrophil activation in patients with PAD

Neutrophil filterability[76]
Plasma elastase concentrations[78]
Neutrophil transit time[205]
Plasma lysozyme concentrations[77]

Hypothesis

Exercise in claudicants is associated with activation of platelets and neutrophils, which may mediate, and contribute to the coagulation changes seen in these patients.

Aim.

The aim of our study was to assess platelet and neutrophil activation following exercise in claudicants against well matched control groups, using the specific and sensitive method of flow-cytometry.

Subjects and Methods

Subjects.

The first 21 subjects recruited into the study detailed in Chapter 5 were recruited into this sub-study, consisting of 10 claudicants, and 11 control subjects (6 smokers and 5 non-smokers). As we had no previous data regarding platelet or neutrophil activation, we were unable to perform a power calculation to determine sample size.

Methods.

Full details of the methodology are presented in Chapter 5. Briefly, subjects (claudicants, or controls) undertook a standard treadmill exercise, with blood sampling prior to, and following the exercise. Blood was taken 60 and 30minutes prior to exercise, and 1, 5, 20, 40, 60 and 120 minutes following exercise. For this sub-study, an additional 2 blood samples were taken at each time point. (Table 7.3)

Table 7.3 Additional blood samples taken for flow cytometry analysis

Sample No	Tube(s)	Tube type	For measurement of:
1	**1x 1.8ml**	**0.105M citrate**	**Platelet activation**
2	**1x 3ml**	**ACD-B**	**CD11b expression**

Samples were prepared as follows:

- Tube 1 (platelet analysis). 1ml of blood immediately transferred to a chilled cryovial containing 1 ml of 2% paraformaldehyde (Esoterix Cell Oncology, Holland), and gently inverted five times. Samples were then incubated with the following fluorochrome-bound monoclonal antibodies:
    - Anti-CD41 (GP IIb) which allows unique identification of platelets.
    - Anti-CD62P (P-selectin) to identify activated platelets.
- Tube 2 (neutrophil analysis). Kept in ice-water for 30 minutes. 100µl of chilled blood was added to 50µl of anti-CD11b antibody (Esoterix Cell Oncology, Holland), and 100µl of blood added to 50µl of control antibody (Esoterix Cell Oncology, Holland). Following incubation for 2 hours in ice water, 1 ml of 2% paraformaldehyde was added to each vial to fix the samples.

Flow cytometry.

Flow cytometry was performed within 24 hours on a Becton Dickinson FACscaliber (Becton Dickinson Biosciences, CA, USA). Cell types were identified on the basis of forward and side scatter and further analysed for the binding of fluorochrome-bound monoclonal antibodies.

Statistical methods

Pre-exercise values were calculated by taking the average of the results of the two pre-exercise samples. For samples taken post-exercise, the area under the curve (AUC) was calculated for each individual. Groups were compared pre, and post-exercise with the Kruskal-Wallis test, or ANOVA where the data were distributed normally. Where this returned a value of $P<0.05$, the Mann-Whitney U test (or T-test) was used to identify the groups which had significantly different values. A value of $P<0.05$ was taken to be statistically significant.

SPSS version 11.0.0 (Statistical Package for Social Sciences Inc, Chicago, IL, USA) was used for all statistical analysis.

Results

All results are presented as median (interquartile range) unless otherwise stated. Details of the patients recruited into this sub-study are detailed in Table 7.4. The blood cell counts at screening were similar for the three groups, (Table 7.5) although non-smoking controls had a higher platelet count than the smoking controls. (P=0.004 Mann-Whitney U).

Table 7.4 Details of subjects in the cytometry sub-study

		Controls		
	Claudicants	**Smokers**	**Non-smokers**	**P=**
	n=10	**n=46**	**n=5**	
Age (median), years (IQR)	61	69	69	0.408*
	(58-69)	(60-70)	(58-70)	
Body mass index, kg/m^2	23.5	24.5	27.4	0.214*
(IQR)	(21.8-25.0)	(23.4-26.7)	(23.5-31.7)	
Smoking				0.875†
pack years	42	45	0	
Medical history				
CAD	0	0	1	
CVD	0	0	0	
HT	0	0	0	
COAD	0	0	0	
Medication				
Aspirin	9	2	1	
ß-blocker	0	0	1	
PAD				
Lowest ABPI (median)	0.72	1.09	1.12	0.232††
Walking distance				
(median) ,m	169	174	174	1.0††
Cholesterol (mmol/l)	187	204	180	0.875*

*=Kruskal-Wallis between all 3 groups. †=Mann-Whitney U claudicants v smoking controls. ††=Mann-Whitney U smoking v non-smoking controls.

CVD=cerebrovascular disease, COAD=chronic obstructive airways disease, HT=hypertension, ABPI=ankle-brachial pressure index.

Table 7.5 Blood cell counts at screening

		Controls		
	Claudicants	**Smokers**	**Non-smokers**	**P=**
	n=10	**n=6**	**n=5**	
White cell count ($x10^9$/l)	9.8	8.4	7.7	0.111*
(IQR)	(8.8-10.7)	(7.6-9.7)	(7.0-9.3)	
Neutrophils ($x10^9$/l)	6.8	5.1	5.1	0.111*
	(5.0-7.8)	(4.7-6.2)	(3.8-5.6)	
Lymphocytes ($x10^9$/l)	2.0	2.6	2.4	0.695*
	(1.7-3.2)	(2.2-2.8)	(1.6-3.3)	
Platelet count ($x10^9$/l)	238	245	316	.046*
	(203-292)	(141-263)	(274-3250	

*=Kruskal-Wallis Test.

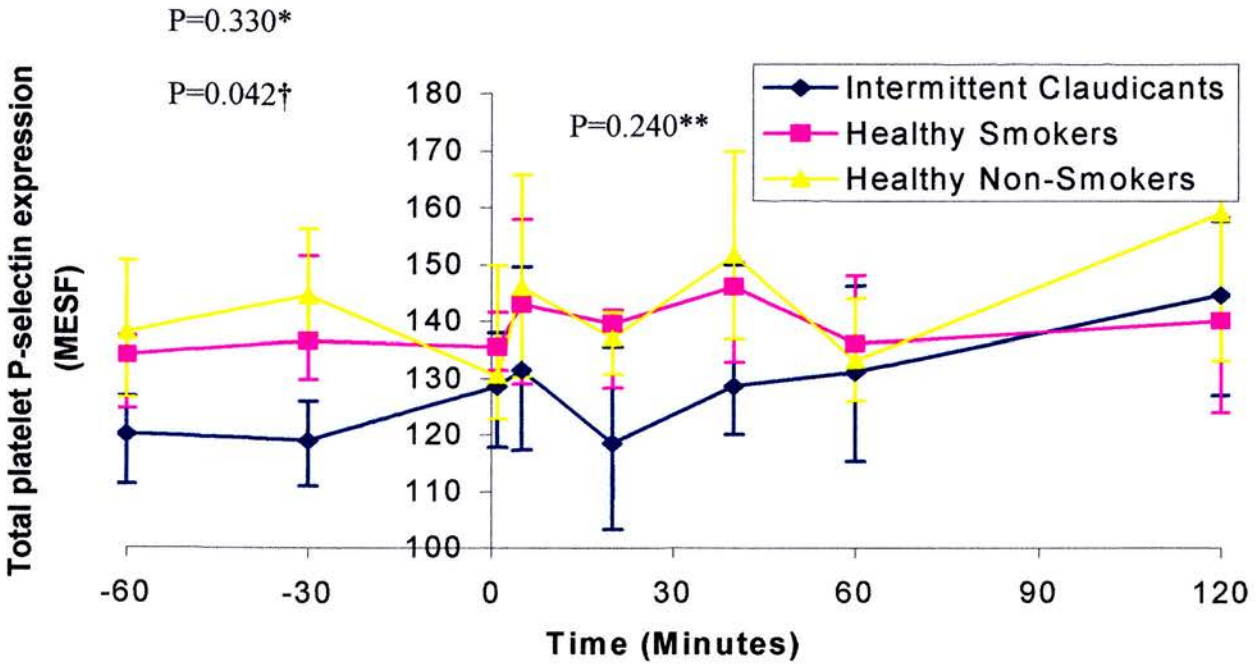
Platelet activation

Two measures of platelet activation were used, total P-selectin expression (expressed as molecules of equivalent soluble fluorochrome [MESF]), and monocyte-platelet complexes (% monocytes bound to platelets).

P-selectin expression.

(Figure 7.3) At baseline, the overall P-selectin expression was greater in the control groups compared to the claudicants. Following exercise, all groups showed a rise in platelet P-selectin expression, but overall there was no significant difference between the groups post-exercise.

Figure 7.3 P-selectin expression on platelets before and after exercise (MESF).

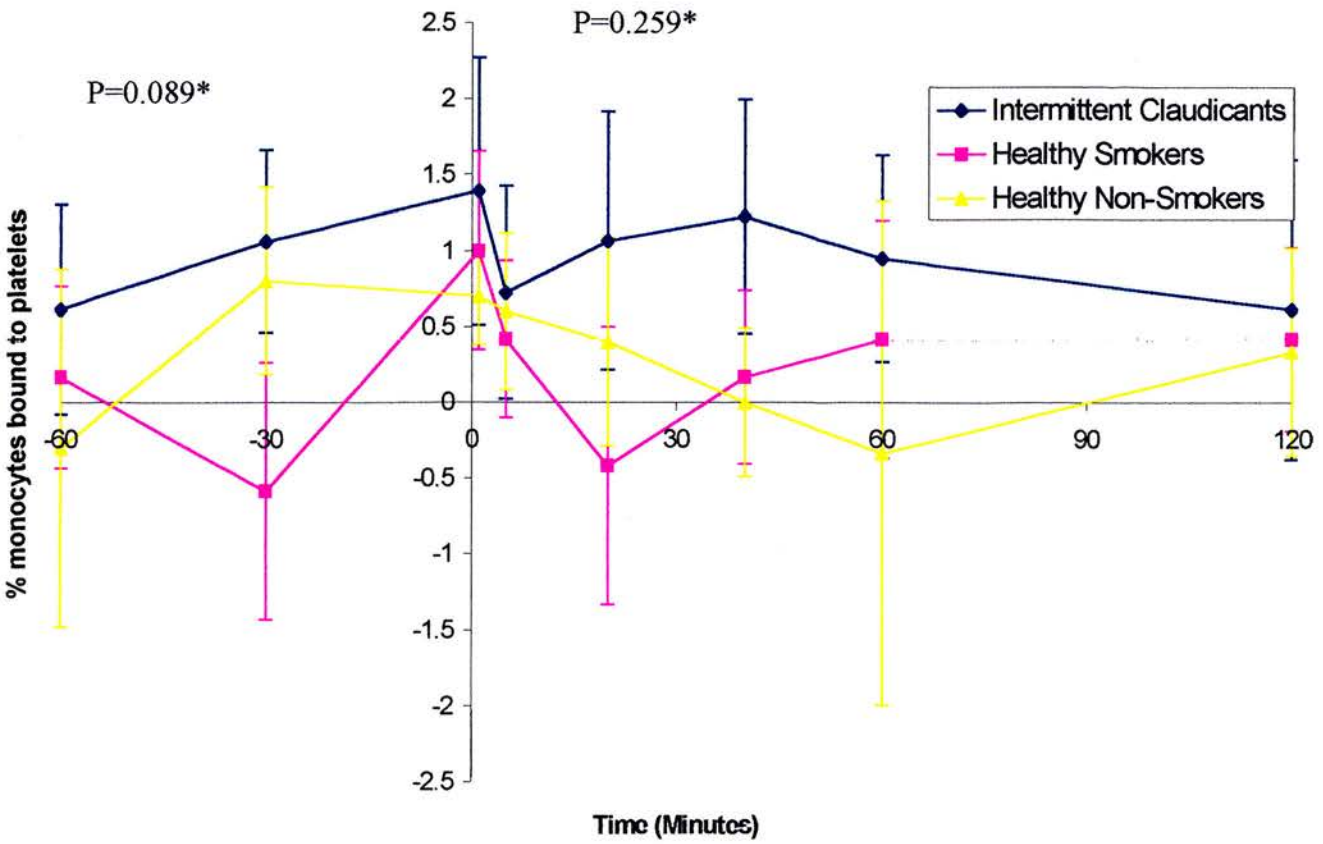


Points represent median values. Error bars show interquartile ranges. *=Intermittent claudicants vs Smoking controls, †= Intermittent claudicants vs Non-smoking controls. Both Mann-Whitney U Test. **=Kruskal-Wallis comparing all three groups.

Monocyte-platelet complexes (MPC).

(Figure 7.4) Results are expressed as the percentage of monocytes bound to platelets. These data were normally distributed, therefore means, standard errors and parametric significance tests were used. Some negative values were obtained because of the compensation used by the flow cytometer. At all time points, the % of MPC's was highest in the claudicant group, although this did not reach statistical significance

Figure 7.4 Monocyte platelet complexes before and after exercise (%MPC)

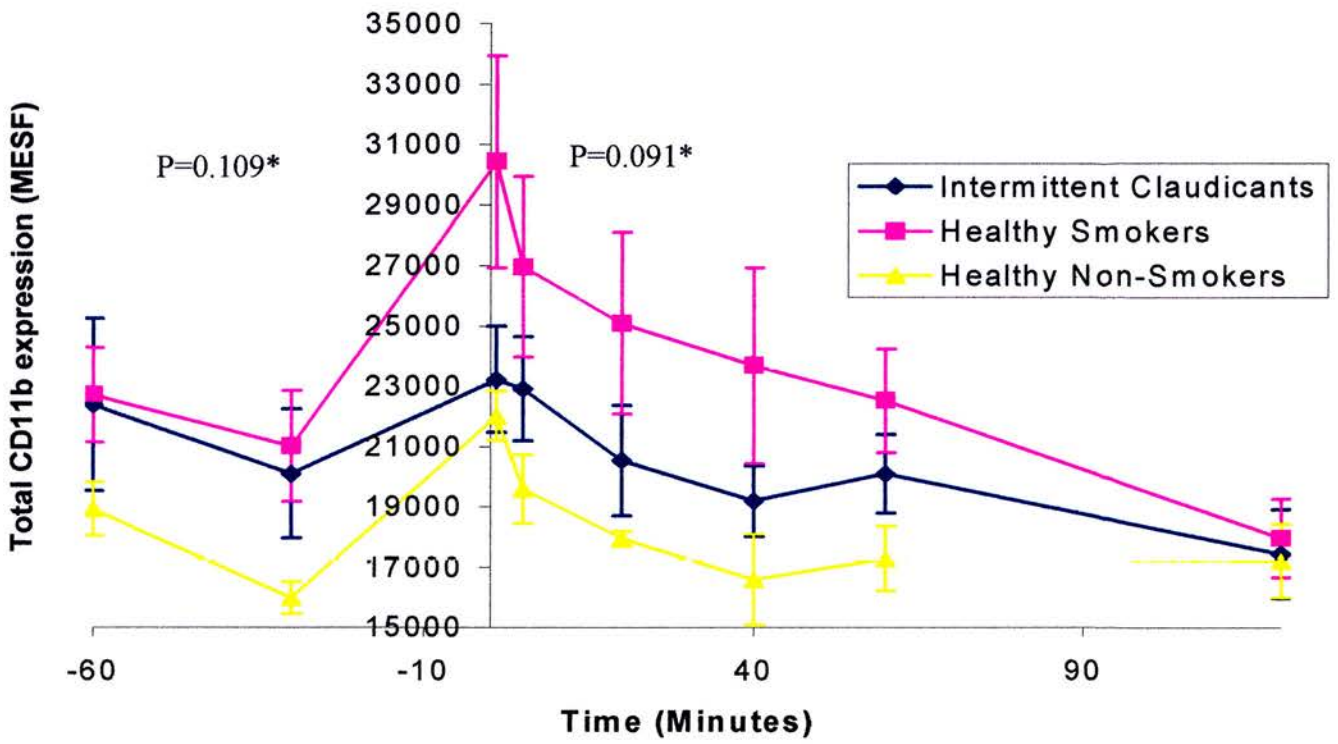


Points represent means. Error bars show standard errors. *=ANOVA comparing all three groups.

Neutrophil activation.

(Figure 7.5). At baseline, there was no difference in the expression of CD11b by granulocytes between the three groups. Following exercise, all groups showed an increase in CD11b expression, which reached the highest level in the smoking controls. By 60 minutes post- exercise, CD11b expression had returned to baseline values in all groups.

Figure 7.5 CD11b expression in granulocytes before and after exercise.



Points represent median values. Error bars show interquartile ranges. *=Kruskal-Wallis comparing all three groups.

Discussion

Platelet activation

Previous studies have shown that patients with peripheral arterial disease have increased activation, and reactivity of platelets, although the evidence regarding the response to exercise is equivocal. Our study, using a robust measure of platelet activation, platelet P-selectin expression measured by flow cytometry, has shown that our patients with claudication had less platelet activation than age and sex-matched controls. This discrepancy may be due to the fact that all of the subjects with claudication were taking aspirin, compared to three of the 11 control subjects. Previous studies have not attempted to control for antiplatelet agent usage, and it would have been unethical to discontinue it in the claudicants, or to start it in the control subjects without a suitable indication. In the study by Koksch et al which found an increase in platelet activation in PAD, measured by a similar method to ours, only 40% of the PAD subjects were taking aspirin.[202] From Figure 7.3, it appears that exercise may have some effect in activating platelets, particularly at five minutes. However, in no group was the 5 minute P-selectin expression significantly greater than baseline levels.

Although the use of monocyte-platelet complexes as a marker of platelet activation is thought to be robust and useful, it did not point to any clear effect in this study.

In summary, the high prevalence of aspirin use in our PAD patients seems to have reduced the amount of platelet activation in our patients, both at rest, and after exercise. Although this may have prevented us from making a meaningful comparison with our control groups, it is perhaps reassuring that the high platelet activity noted by other studies in this population, is reduced by aspirin.

Neutrophil activation

Our data have shown that although claudicants have an increase in neutrophil activation following exercise, this is less marked than the increase in controls matched for age, sex and smoking status. These findings are contrary to previous studies which have found higher increases in neutrophil activation in claudicants compared to controls, whether assessed by CD11b expression,[78; 83] or more indirect measures.[205] There is a marked effect of smoking as illustrated by comparing the two control groups, which leads to an enhanced response to exercise in the smoking controls. It is known that activation of neutrophils leads to them becoming stiffer, increasing the time it takes them to pass through the microcirculation. It may be that in patients with peripheral arterial disease, these activated neutrophils are becoming adherent to a damaged endothelium, and becoming trapped – in a similar manner to the white-cell trapping seen in chronic venous disease.[207] However, if neutrophils were trapped in the microcirculation, we might expect to see a late 'wash-out' of activated neutrophils – which we do not.

Previous investigation of the effect of smoking on neutrophil activation has yielded seemingly conflicting results.[208] Although soluble markers of neutrophil activation were higher in smokers compared to non-smokers, the number of activated neutrophils in systemic blood was not higher in the smokers. Incubation of unactivated neutrophils with smoker's plasma led to neutrophil activation, which was not seen with the non-smokers plasma. The conclusion from this study is that smoking leads to neutrophil activation, and sequestration in the microcirculation, with 'leakage' of neutrophil secretion products into the systemic circulation.

The reason for the lower number of activated neutrophils in our claudicants may be explained by the combination of:

1. sequestration of neutrophils, activated by smoking and possibly claudication.
2. a microcirculation lined by endothelial cells affected by atherosclerosis +/- ischaemia-reperfusion.

If neutrophils are becoming trapped in the microcirculation, what implications does this have? We can speculate that this would lead to further localised endothelial damage, and possible the progression of atherosclerosis. Endothelial damage is likely to lead to prothrombotic effects, and further white cell activation (Figure 7.2). Ultimately, these may lead to progression of disease, both locally and systemically. It must be borne in mind, that there is unequivocal evidence of benefit of exercise therapy in claudication. However, if ways can be developed of reducing the potentially deleterious effects, such as neutrophil sequestration, this may enhance the benefit achieved.

Conclusions

The high prevalence of antiplatelet agent use in our claudicant population has prevented us making definite conclusions about the effect of exercise on platelet activation in this study. However, it would appear that the use of flow-cytometry to detect platelet P-selectin expression is a useful technique, and could be used for further work in this area. Following exercise, claudicants have an attenuated increase in systemic neutrophil activation compared to matched controls, possibly reflecting sequestration of activated neutrophils in the microcirculation. Further work is required to determine whether this is the case.

Chapter 8

The effect of walking distance on the fibrinolytic response

Introduction

A possible explanation for the lack of an enhanced fibrinolytic response in patients with claudication may be related to the amount of time spent walking. If the fibrinolytic response depends on the duration of exercise, the response in claudicants would be expected to be reduced as they spent less time, on average, walking than the controls. This hypothesis was tested using the data available from the study in Chapter 6.

Hypothesis.

The lack of an enhanced fibrinolytic response in claudicants is due to the reduced walking time during their exercise period.

Aim.

To examine the influence of walking distance on the fibrinolytic response in claudicants.

Methods.

The data from the study in Chapter 6 were analysed. The preferable method of studying the influence of walking time on the fibrinolytic response would be to construct a logistic regression model. However, as only a relatively small number of subjects with complete data sets were available, this was not possible. It was therefore decided to divide the claudicants into two groups: those walking less than three minutes at the

assessment visit, and those walking more than three minutes. Three minutes was chosen as the cut-off, as this was the duration of exercise in the control groups. This allowed comparison between the two groups of claudicants, and also between the claudicants able to walk for more than three minutes, and the control groups. The biomarkers studied were APC-PCI, tPA activity, tPA antigen, and PAP complexes. Graphs similar to those in Chapters 5 and 6 were plotted, and the area under the curve calculated for each group pre and post-exercise. Data for the non-smoking control group are not plotted for clarity.

Results

Subjects

8/19 subjects walked for longer than 3 minutes at their assessment visit (Table 8.1). It can be seen that there is a considerable 'learning' effect with treadmill walking for some individuals. The group that walked longer than three minutes at the assessment visit, walked a median of 82m at the screening visit, which increased to 174m at the assessment visit. Other than the differences in walking distance, the groups are similar.

Table 8.1 Subject details.

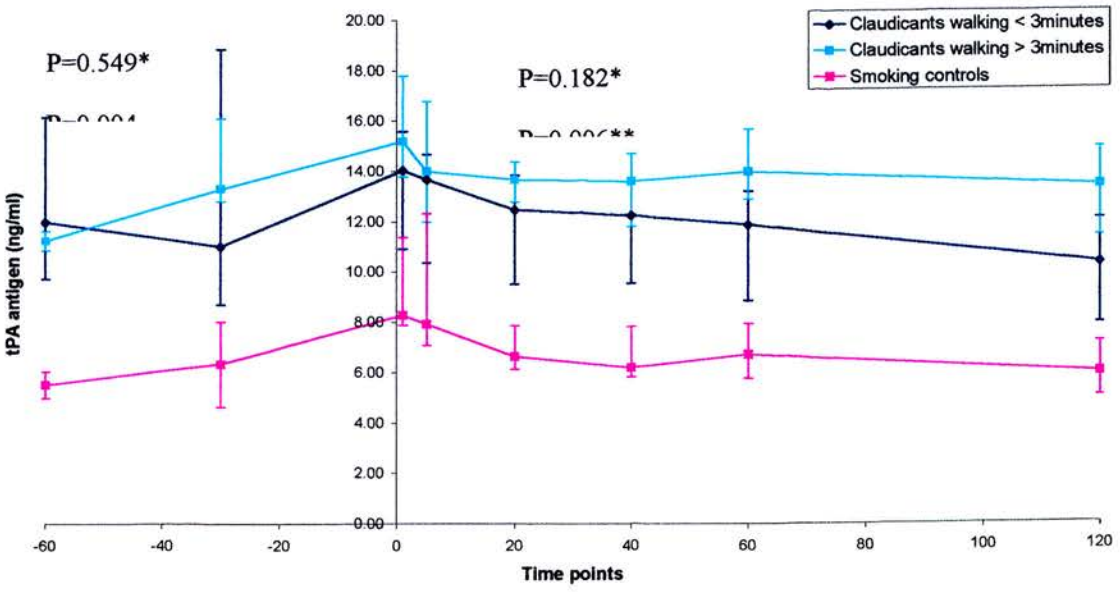
	Claudicants		
	Claudication time < 3minutes n=11	**Claudication time ≥ 3minutes n=8**	**P=**
Age, years (IQR)	61 (58-68)	62 (56-68)	0.968*
Body mass index, kg/m^2	23	24	0.600*
(IQR)	(22-26)	(23-27)	
Smoking			
pack years	47	47	<0.904*
Medical history			
CAD	1	2	0.603†
HT	1	3	0.603†
COAD	2	4	1.0†
Medication			
Aspirin	6	7	1.0†
PAD			
Lowest ABPI	0.59	0.51	0.116*
Walking distance, m,	93	82	0.322*
(IQR) [screening]	(66-116)	(64-118)	
Walking distance, m,	66	174	<0.001*
(IQR) [assessment]	(60-92)	(172-184)	
Cholesterol, mmol/l (IQR)	5.3 (4.6-6.1)	4.8 (4.1-5.21)	0.186*

IQR = interquartile range; CAD = coronary artery disease (angina, myocardial infarction or coronary bypass surgery); CVD = cerebrovascular disease (transient ischaemic attack or stroke); HT = hypertension; COAD = chronic obstructive airway disease; Ca blocker = calcium channel blocker; ACEI = ACE inhibitor. *=Mann-Whitney U Test. †=Fisher's Exact Test

tPA antigen

(Figure 8.1) From Chapter 6, tPA antigen levels were higher both pre and post exercise in claudicants than in controls. There seems to be no difference in tPA antigen levels when the claudicant group are categorised according to walking distance.

Figure 8.1 tPA antigen for claudicants categorised by walking ability, and smoking controls.

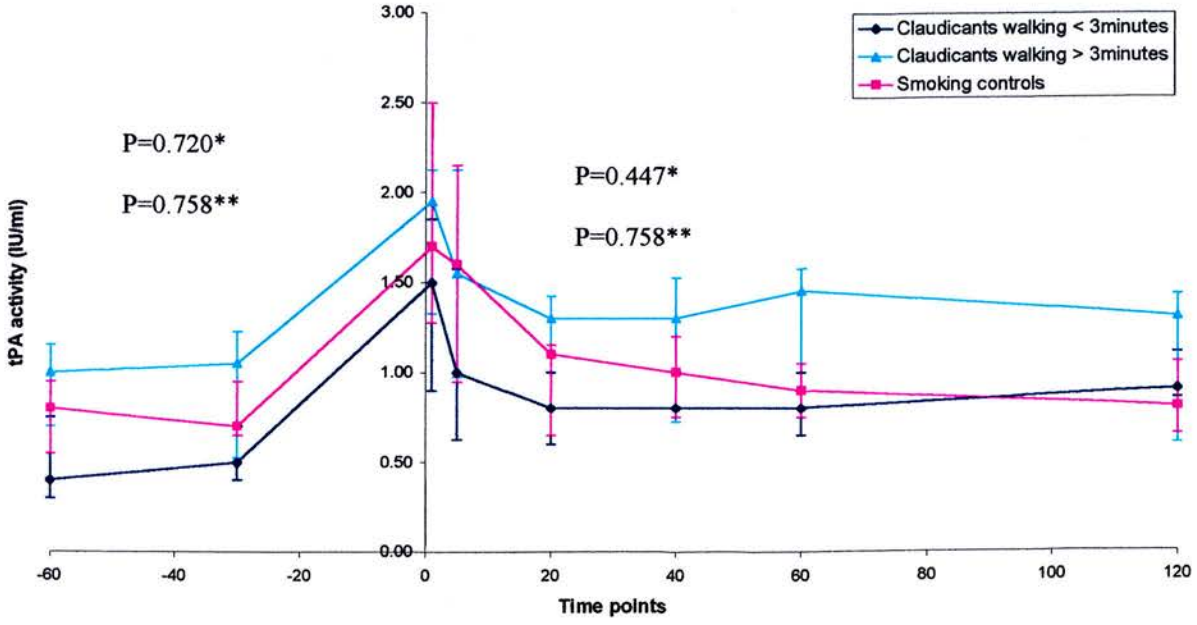


Data presented as medians and interquartile ranges. *=Claudicants walking <3 minutes vs claudicants walking > 3minutes. **=Claudicants walking > 3 minutes vs Smoking controls.

tPA activity.

(Figure 8.2) There was no statistically significant difference between the claudicant group as a whole and the control groups with respect to tPA activity. However, when the claudicants who walked further than three minutes are taken as a separate group, they are seen to have higher tPA activity pre and post exercise when compared to the smoking controls, although not reaching statistical significance.

Figure 8.2 . tPA activity for claudicants categorised by walking ability, and smoking controls.

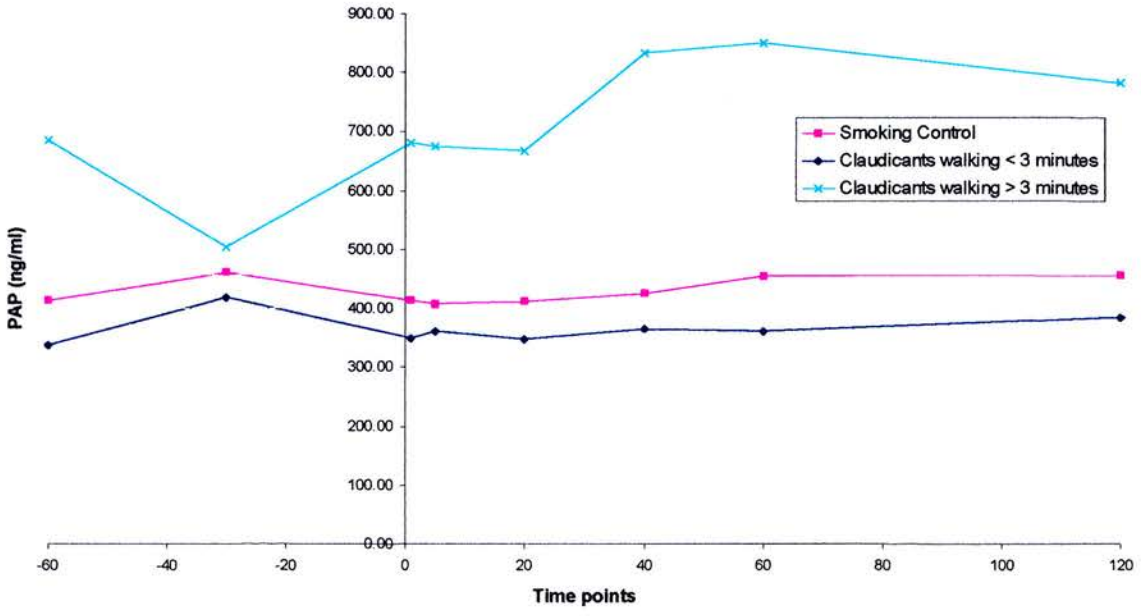


Data presented as medians and interquartile ranges. *=Claudicants walking <3 minutes vs claudicants walking > 3minutes. **=Claudicants walking > 3 minutes vs Smoking controls.

PAP.

(Figure 8.3) Complete data is only available for two claudicants who walked for longer than three minutes and therefore no error bars are shown, or formal statistics calculated. With the obvious cautions about interpreting these data, it appears that the subjects with longer walking distance have a considerable increase in PAP when compared to the other claudicant group, and the smoking controls.

Figure 8.3 PAP for claudicants categorised by walking ability, and smoking controls.

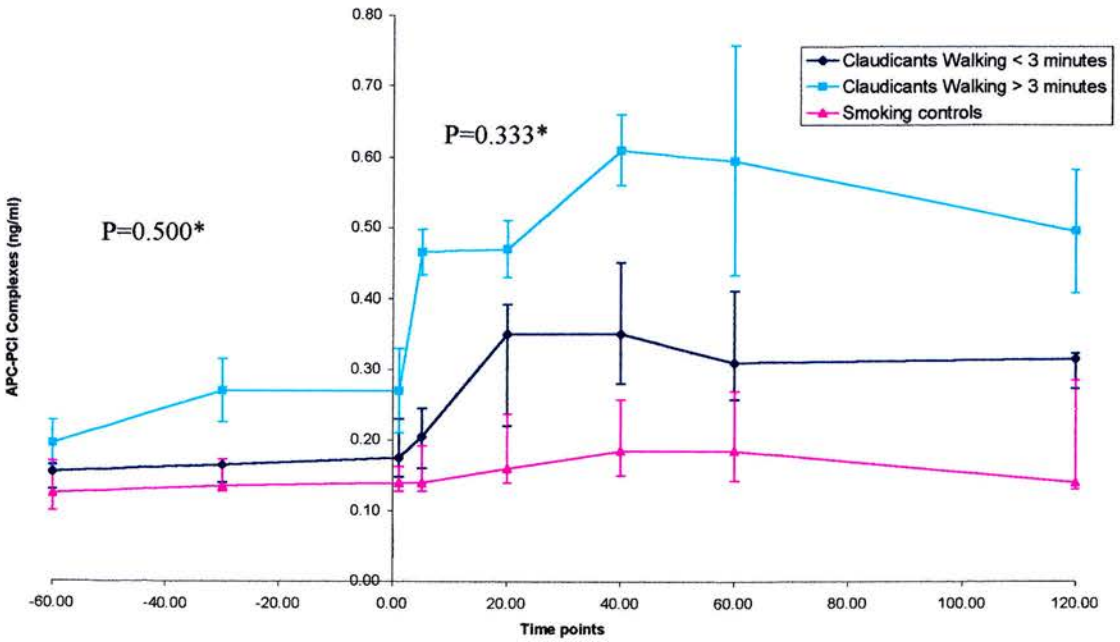


Data presented as medians. Due to the small numbers in the groups, no error bars or statistics are shown.

APC-PCI

(Figure 8.4) Levels of APC-PCI were similar at baseline, but the claudicants who walked for longer than 3 minutes had a larger rise than the other groups, indicating greater thrombin generation.

Figure 8.4 APC-PCI for claudicants categorised by walking ability, and smoking controls.



Data presented as medians and interquartile ranges. *=Claudicants walking <3 minutes vs claudicants walking > 3minutes. **=Claudicants walking > 3 minutes vs Smoking controls.

Discussion

The data from the previous chapters appeared to show that claudication was associated with increased thrombin generation, without a corresponding increase in fibrinolytic response. However one of the main differences between the groups was the distance they walked at the assessment visit (claudicants 82m, controls 174m). It could therefore be argued that the lack of an enhanced fibrinolytic response in the claudicants is due to the shortened duration of exercise, and not to the claudication itself. Our data would seem to confirm this hypothesis. When compared to the smoking controls, and the claudicants walking less than three minutes, the longer walking claudicants had higher levels of tPA activity at rest and post exercise, and possibly higher levels of tPA antigen, and larger increase in PAP – indicating total fibrinolysis. The improved response of the longer walking claudicants, however does not seem to simply be a function of their increased walking distance, as they also have increased levels at rest. This may reflect that they have less damaged endothelium – capable of releasing more tPA, although it is not clear why tPA activity levels are higher than in the smoking controls. It is tempting to speculate this is a physiological attempt to counteract the increased thrombin generation, but the mechanism responsible for this is obscure.

Do these findings alter the significance of the data from the previous chapters? It is difficult to quantify the net effect of increased thrombin generation against the enhanced fibrinolytic response seen in the claudicants. Although claudicants who have a longer walking distance seem to have improved fibrinolysis, they also have the most marked increase in thrombin generation.

Caution needs to be exercised when interpreting the data from this chapter. It is likely that walking distance in the claudicants is not a completely independent variable, and it may be representing the effect of another variable, for example disease severity. In addition, once the claudicant group was divided on the basis of walking ability, the small numbers have hindered formal statistical analysis. However, the graphs presented show consistent trends which we feel merit cautious acceptance.

Conclusion.

The previous finding that increased thrombin generation in claudicants is left unchecked by increased fibrinolysis is over simplistic. Claudicants who walk further, generate more thrombin, and have a more marked fibrinolytic response than other claudicants, and matched controls.

Chapter 9

Normal Variability of Thrombin Generation and Fibrinolysis

Introduction

The previous chapters have suggested that exercise in patients with intermittent claudication (IC) leads to thrombin generation and relative hypofibrinolysis. However, the reproducibility of the measurement of, and the longitudinal variation in, markers of thrombin generation and fibrinolysis in resting and exercising claudicants has yet to be established. The aim of this study, therefore, was to examine the reproducibility of coagulation markers measured before and after exercise, and on two separate occasions two weeks apart, in individuals with IC and age matched controls.

Patients and Methods

22 subjects from the study in Chapters 5 and 6 returned for a second assessment visit, which was conducted in the same manner as assessment visit 1. The number of subjects chosen to assess reproducibility was arbitrary, and was dictated by financial constraints. Blood samples were collected at the same time points, for a selected number of biomarkers. (Table 9.1) AUC's were calculated for pre and post-exercise periods in the same manner as previously. AUC's were then converted into time-controlled values by dividing the pre-exercise values by 30, and the post-exercise values by 119 to produce clinically relevant results. (Figure 9.1)

In order to test the reproducibility of baseline biomarker levels, pre-exercise AUC's were compared between visits. Calculating the difference between time-controlled pre and post-exercise AUC's, and comparing this value for visits 1 and 2 tested the reproducibility of the effect of exercise. The method of Bland and Altman,[209] which

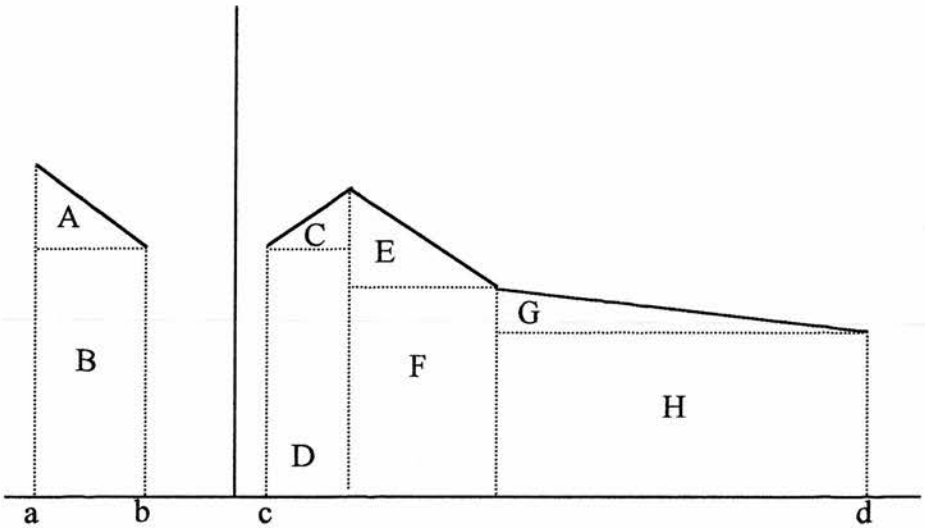
plots the mean of the results from visits one and two on the x-axis, against the difference between the results from the two visits on the y-axis, was used to compare data from the two visits.

Table 9.1 Markers measured at each time point. ; PT = prothrombin time; APTT = activated partial thromboplastin time; tPA = tissue plasminogen activator; PAI-1 = plasminogen activator inhibitor 1; APC = Activated Protein C; PCI = Protein C Inhibitor.

*=commercially available assay kit.

Marker	Method
PT	Bayer, Rapidpoint coag,
APTT	UK*
Thrombin-antithrombin	
complexes	Dade-Behring, IL, USA*
tPA activity	Biopool, CA,USA*
PAI-1 activity	Biopool, CA,USA*
APC-PCI complexes	Stenflo.[173]

Figure 9.1. Calculation of time controlled AUC



Pre-exercise time-controlled AUC = A+B / (b-a)

Post-exercise AUC = C+D+E+F+G+H / (d-c)

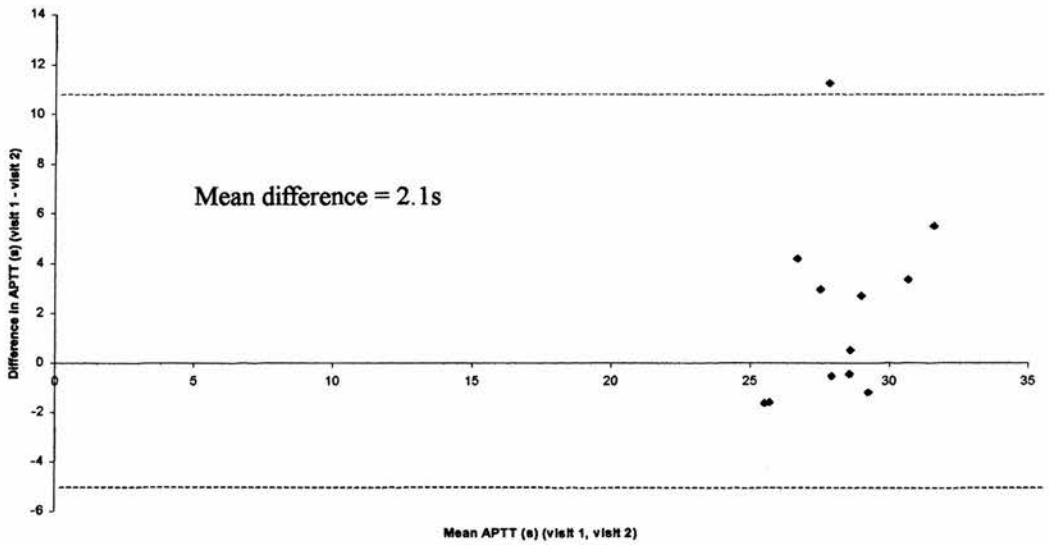
Results

The details of the subjects studied are presented in Table 9.2. Bland-Altman plots for each of the measured parameters are presented in Figures 9.2 to 9.13. Complete data were not available for all subjects, as sufficient sample volume was not obtained at every time point for every parameter at both visits on all subjects. Subjects with incomplete data sets are not plotted. Coefficients of variation for the assays at baseline have also been calculated (Table 9.3), which demonstrates relatively low variability for APTT, PT, APC-PCI complexes, tPA activity and PAI activity, with higher variability for TAT complexes.

Table 9.2 Patient details. IQR = interquartile range; IHD = ischaemic heart disease (angina, myocardial infarction or coronary bypass surgery); CVD = cerebrovascular disease (transient ischaemic attack or stroke); HT = hypertension; COAD = chronic obstructive airway disease; Ca blocker = calcium channel blocker; *=Kruskal-Wallis. †= Fishers Exact Test

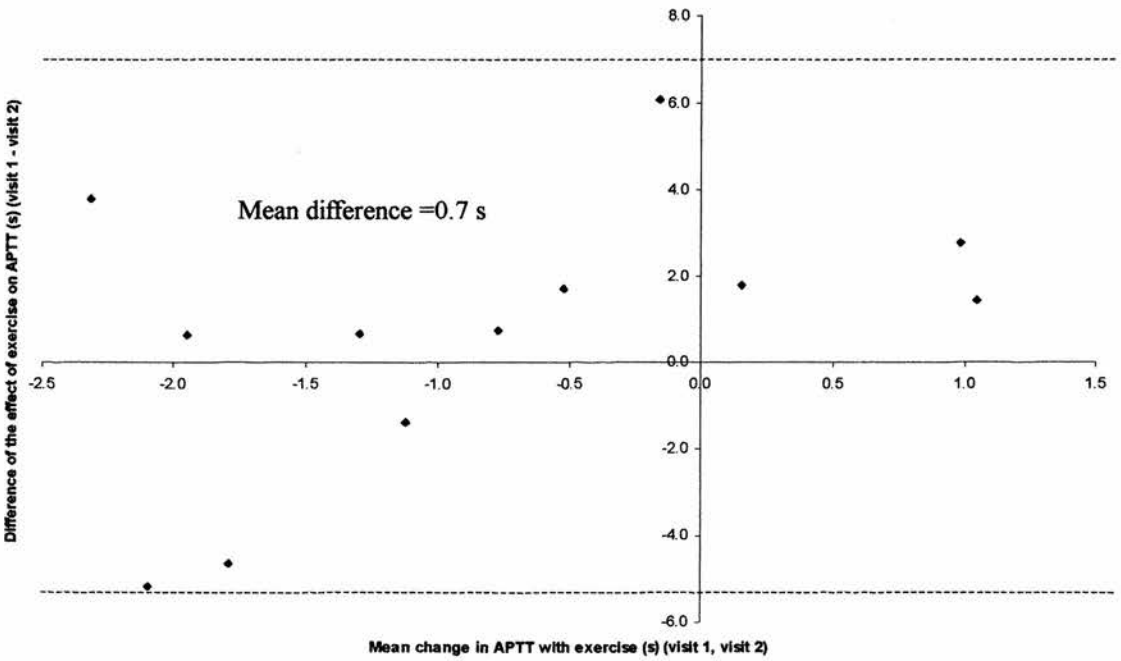
		Controls		
	Claudicants n=10	**Smokers n=6**	**Non-smokers n=6**	**P=**
Age, years (IQR)	59 (58-67)	69 (60-70)	69 (58-70)	0.378*
Body mass index, kg/m^2	24	23	27	0.760*
(IQR)	(22-25.0)	(23-25)	(24-32)	
Smoking				
pack years	44	45	0	0.005*
Medical history				
CAD	2	0	1	0.320†
CVD	0	0	0	
HT	2	0	0	0.325†
COAD	2	0	0	0.325†
Medication				
Aspirin	8	1	1	0.017†
ß-blocker	0	0	1	0.252†
Nitrate	1	0	0	0.615†
PAD				
Lowest ABPI	0.56	1.11	1.12	<0.001*
(IQR)	(0.51-0.62)	(1.06-1.13)	(1.09-1.16)	
Walking distance, m,	108	174	174	<0.001*
(IQR)	(64-174)	(170-174)	(174-174)	
Cholesterol, mmol/l (IQR)	4.9 (4.3-6.1)	4.9 (4.8-6.6)	4.6 (4.3-5.1)	0.794*

Figure 9.2 Reproducibility of baseline APTT measurements



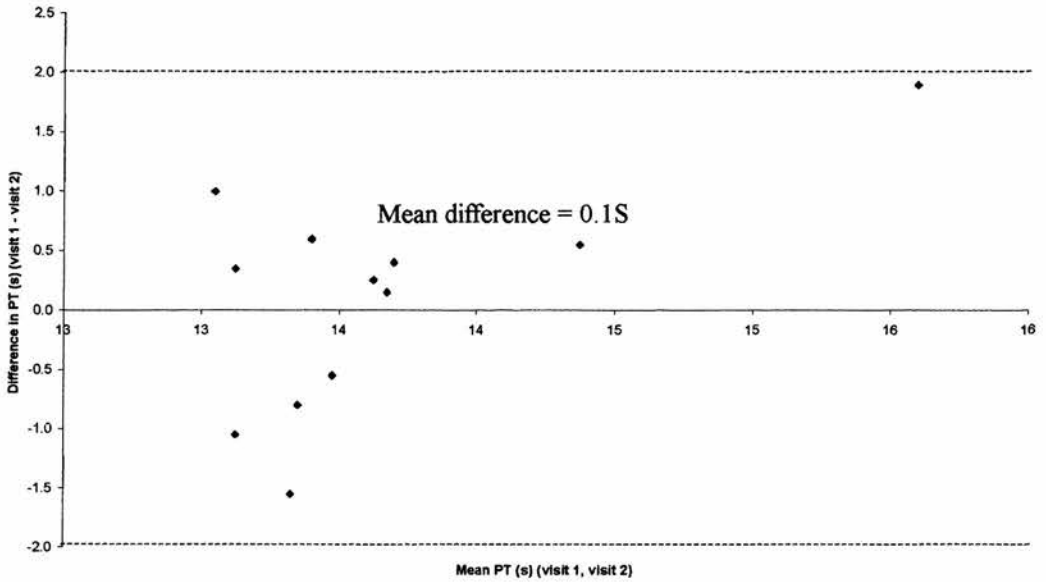
.....= 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Figure 9.3 Reproducibility of the effect of exercise on APTT measurements.



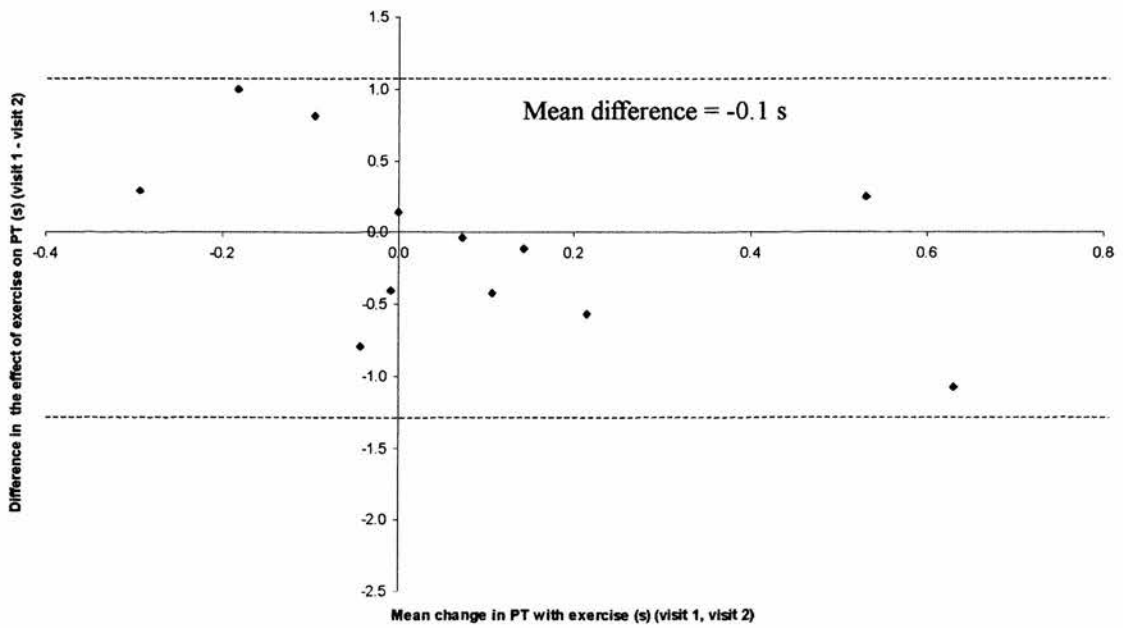
.....= 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Figure 9.4 Reproducibility of baseline PT measurements



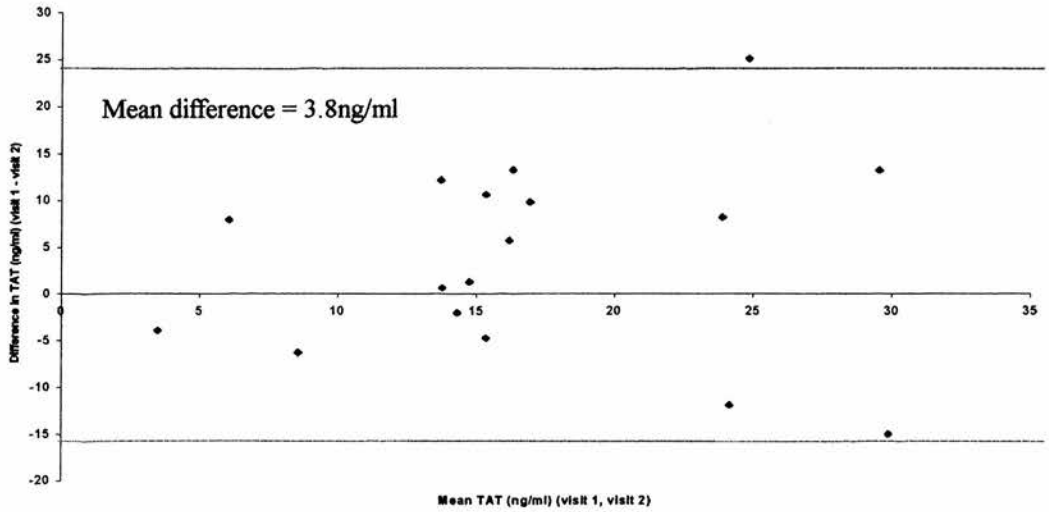
.....= 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Figure 9.5 Reproducibility of the effect of exercise on PT measurements.



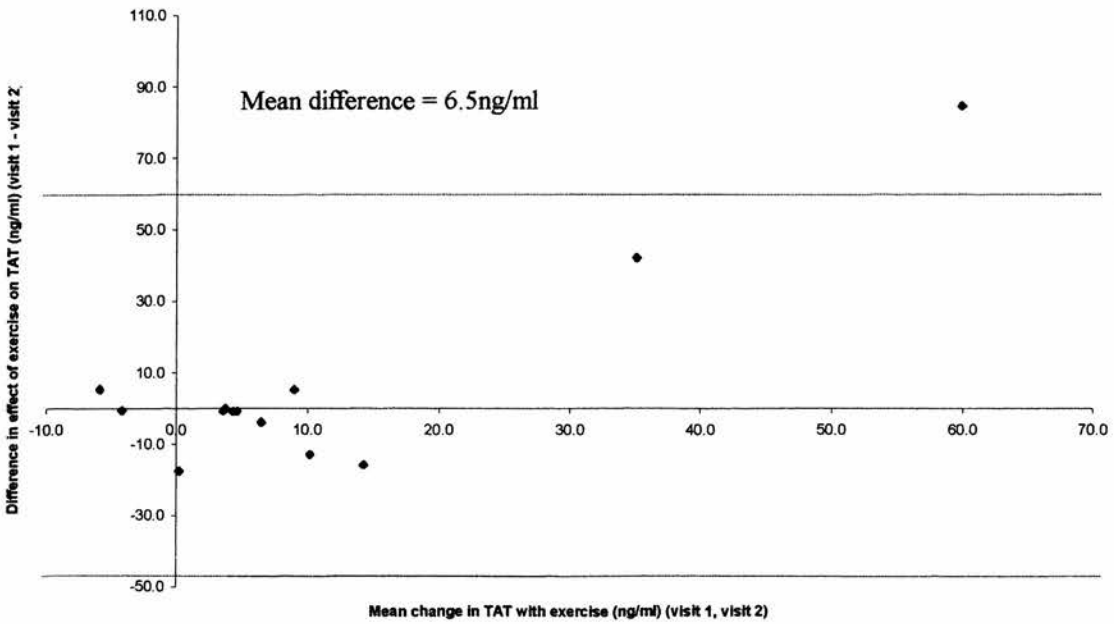
= 95% prediction intervals of difference between measurements at visit 1 and visit 2

Figure 9.6 Reproducibility of baseline TAT measurements.



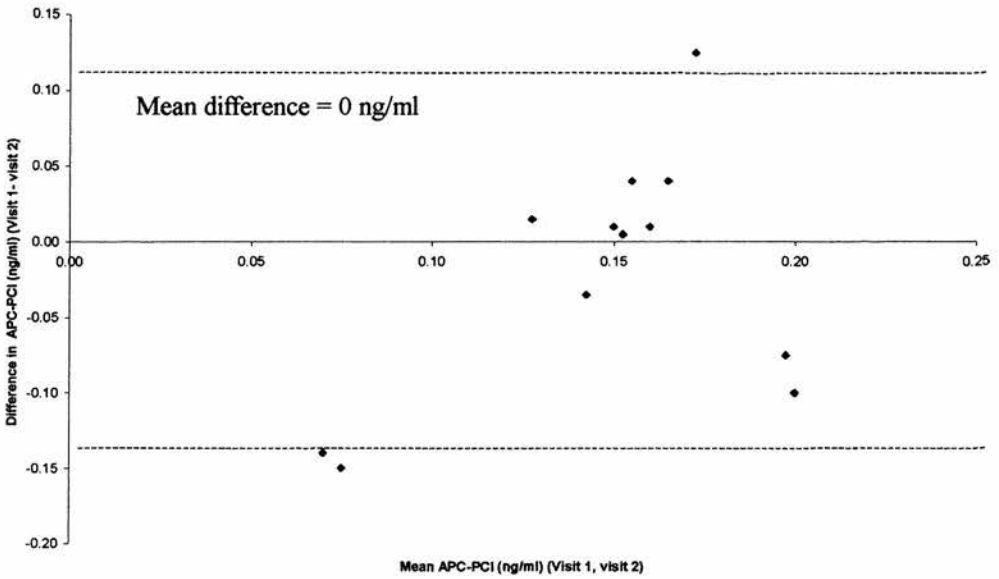
.....= 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Figure 9.7 Reproducibility of the effect of exercise on TAT measurements.



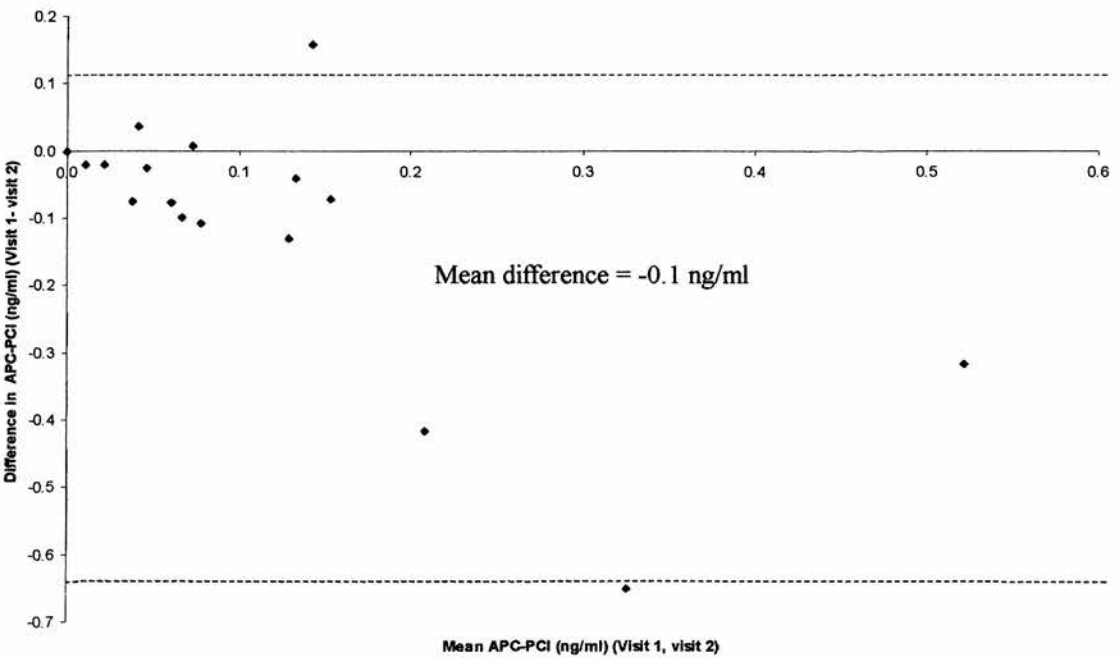
..... = 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Figure 9.8 Reproducibility of baseline APC-PCI measurements.



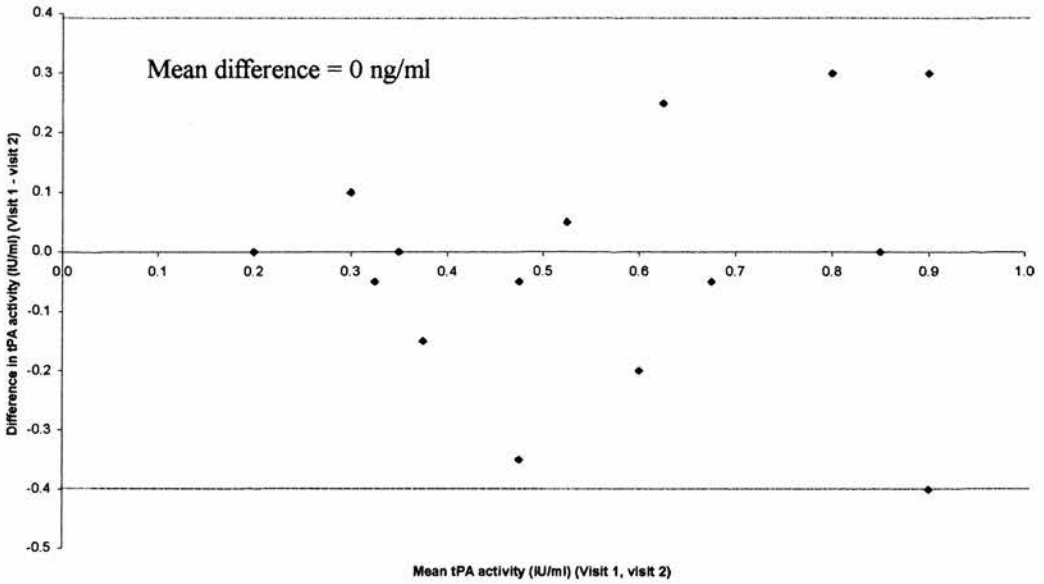
.....= 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Figure 9.9 Reproducibility of the effect of exercise on APC-PCI measurements



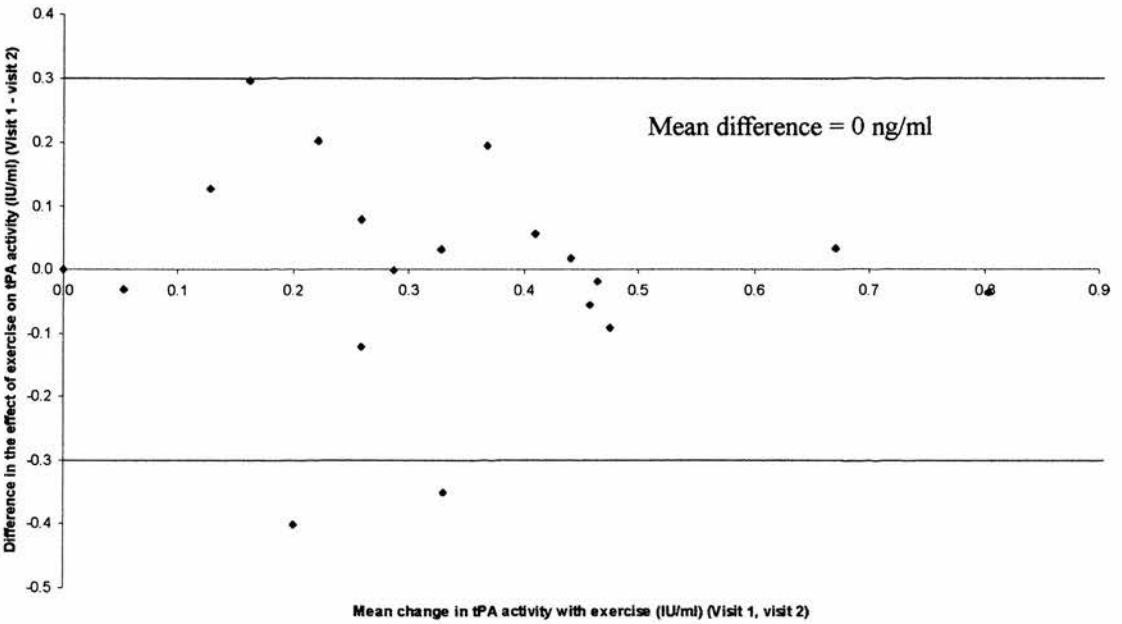
.....= 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Figure 9.10 Reproducibility of baseline tPA activity measurements.



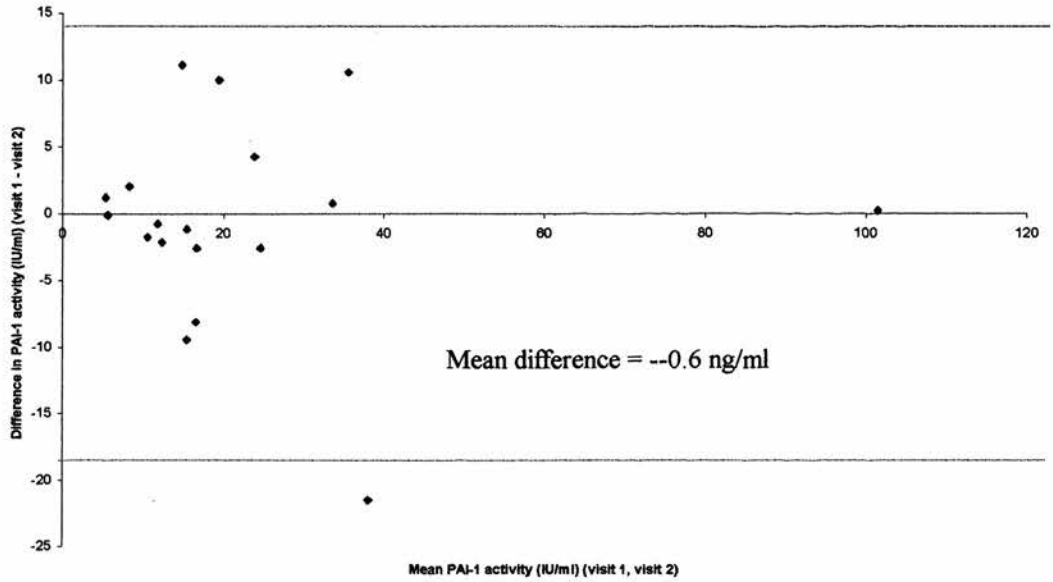
.....= 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Figure 9.11 Reproducibility of the effect of exercise on tPA activity measurements.



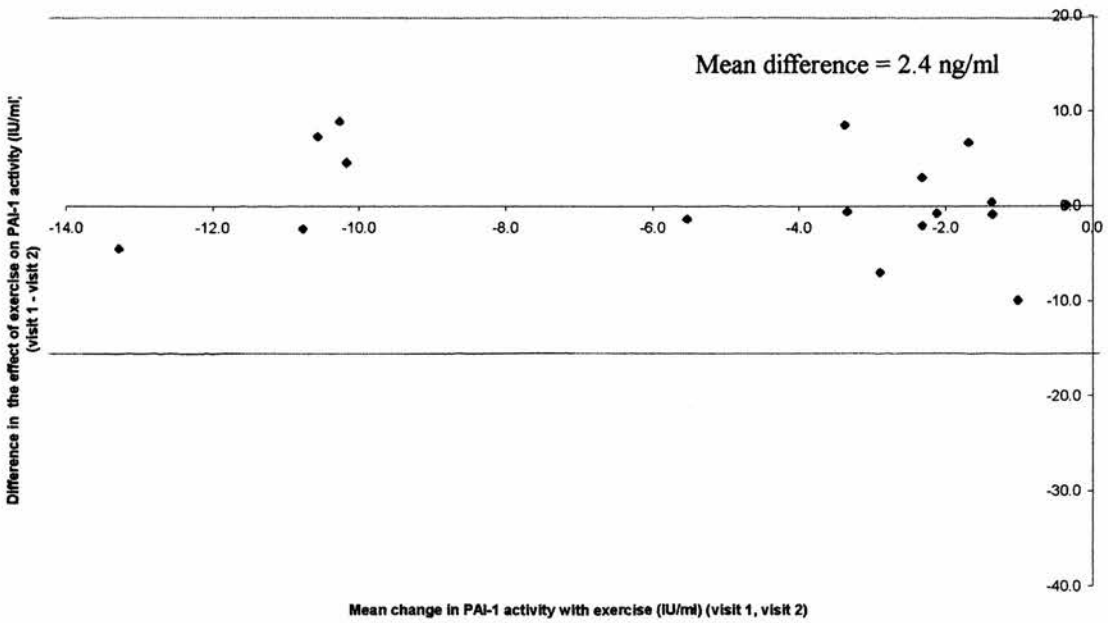
.....= 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Figure 9.12 Reproducibility of baseline PAI-1 activity measurements.



..... = 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Figure 9.13 Reproducibility of the effect of exercise on PAI-1 activity measurements.



..... = 95% prediction intervals of difference between measurements at visit 1 and visit 2.

Table 9.3. Co-efficient of variation for pre-execise AUC values.

Parameter	Coefficient of variation
APTT	7.5%
PT	3.9%
TAT	40.3%
APC-PCI	17.2%
tPA activity	18.5%
PAI-1 activity	18.3%

Discussion

This study has shown that the normal variability of coagulation parameters can be significantly high.

Bland-Altman graphs can be interpreted quantitatively or qualitatively. If a variable's clinically relevant (or normal) range is known, then one can determine whether the 95% prediction limits for estimation of the difference lie within this range. If that range is not known and it is not, therefore, possible to define exactly what level of variation between measurements is clinically acceptable or meaningful, then it is necessary to simply assess the graphs pragmatically on a variability spectrum. When the difference between the two measurements is small in comparison to the mean of the two measurements then variability is low and/or reproducibility is high. If the difference approaches the mean then the variability is high and reproducibility is low. It is

important to point out that although the mean of the differences often approaches zero, this is to be expected statistically, and does not indicate that the variability is low.

Baseline

At baseline, all the markers except PT and APTT, exhibited differences between the two visits approached the mean of the measurements between the two visits in a large number of subjects, indicating significant variability over time with the same subject and/or low assay reproducibility.

Effect of exercise.

These data are calculated by subtracting pre-exercise, from post-exercise AUCs and some of the points have negative x-values, representing a fall in the variable with exercise. Again, APTT, and PT, show reasonable consistency between visits, but most of the other markers vary widely, suggesting that the same individual responds to the similar levels of exercise in different ways on different days and/or the assay lacks reproducibility. There are a number of possible reasons fore this inter-visit variation:

- *The method of blood collection varied from visit to visit.* This is not a likely source of visit to visit variation, as the method, timing and processing of all samples was standardised before the study started. All samples were taken from a pre-placed venous cannula, without a tourniquet, and placed immediately on ice. No changes were made during the study to the tubes the blood was collected in, or the method of processing.
- *The assays themselves lack reproducibility.* Again, this seems unlikely, as assays were performed to strict protocols, in accredited laboratories (except the PT and APTT) according to Good Laboratory Practice. This ensures adequate quality control checks are performed prior to and at the end of each assay run.

In the case of PT and APTT, quality control specimens were run at the beginning and end of each day to ensure correct calibration of the analyser.

- *There are significant actual variations in the levels of these markers in individuals from visit to visit.* Although visits occurred at the same time of the day, and influences known to affect these biomarkers (exercise, smoking, eating and drinking) were controlled as far as possible, this would seem to be the most likely explanation of the intra-visit variation.

The variation in coagulation parameters over time has been studied previously in various patient groups, although not in those with PAD or IC. A study of 26 healthy volunteers showed even greater variation in coagulation markers that observed in the present study (coefficients of variation [CV's]: tPA antigen 15%, TAT 25%, PAP 20%, PAI activity 30% and PAI antigen 47%). The results from these studies are difficult to compare as a variety of statistical methods have been used – including correlation coefficients,[210] coefficients of variation (CV),[211; 212] and reliability coefficients[213; 214]. The use of these summary variables is attractive, as it gives a single figure that attempts to describe the spread of the data. But, such figures can be highly misleading. For example, the CV's of the some of the markers in this study were low (Table 9.3) despite the Bland-Altman plots showing that levels varied widely between visits.

CV's also allow the construction of a power calculation to give the number of subjects (or repeated measures) likely to be required to achieve a statistically meaningful result in longitudinal studies. For example with a within-subject CV of 30%, six measurements must be made for each sample in order to be reasonably sure (α=0.05, β=0.10) of detecting a true halving, or doubling of the marker[211]. For the markers measured in our study: 2 measurements would be required to detect a doubling, or

halving of APTT, or PT; 3 measurements for APC-PCI, tPA activity, or PAI activity; and the high variability of baseline TAT measurements (CV 40%) means that the marker would have to be measured 12 times to be likely (β=0.10, α=0.05) of detecting a true halving or doubling.

On the basis of present and previous data, one can state with some confidence that coagulation parameters can vary significantly in the same individual (healthy and diseased) over time in response to a range of (often unidentified and unrecognised) stimuli such as intercurrent illness. Those undertaking basic science investigations and clinical trials that use biomarkers must be aware of this variability and the important implications it has for study design and interpretation.

Conclusion

It is likely that longitudinal studies investigating coagulation parameters will be undertaken in patients with claudication. Before groups embark upon such investigations, pilot data needs to be collected to allow the calculation of an appropriate power calculation. It appears from our data that markers show significant intra-individual variation with time, despite careful attempts to minimise this. The implication is that large numbers of measurements need to be made, or methods of reducing this variability found.

Chapter 10

Summary and Conclusions

The overriding conclusion of the work presented in this thesis is that research in this area is fraught with difficulties. The data from Chapter 9 regarding variability; the wide error bars from the other chapters; along with the lack of any firm conclusions which can be drawn about fibrinolysis, neutrophil response and platelet response; all stem from the difficulty in study this subject.

Undoubtedly, some of these difficulties could have been reduced by changes to the study design which are more apparent in retrospect.

- A more homogenous control group, for example entirely smokers would have increased the power of the study, reducing the chance of missing possibly significant differences between the groups (ie reducing Type 2 errors).
- As all of the subjects with claudication were taking aspirin, and most of the controls were not, it was unrealistic to expect to see an increase in platelet activation in the claudicant group. It could be argued that the control group could have been given a course of aspirin, so that at least the two groups were comparable. If this were not considered ethical, then a comparison between the groups is probably meaningless.
- It was not anticipated that patients with abdominal aortic aneurysms (AAA) would have obvious coagulation abnormalities. The very high levels of D-dimer found in one of our patients with a AAA may or may not have been due to his AAA, but we felt it merited excluding patients with aneurysms from the study. Future studies should exclude patients with a AAA.

Despite the above reservations, some tentative conclusions can be drawn.

- Walking in patients with intermittent claudication is associated with an increase in thrombin generation, which could produce a pro-coagulant state if left unchecked by a corresponding enhanced fibrinolytic response.
- There is evidence of a reduction in the number of activated neutrophils in the systemic circulation of patients with intermittent claudication.

What then are the implications of these findings? It is accepted that walking exercise improves walking distance, and it seems that walking to the point of maximal claudication produces larger increases in walking distance than walking for shorter distances.[44; 45] Although it is a widely held belief that exercise is generally beneficial, this is largely based on studies with healthy volunteers, or cardiac rehabilitation programmes.[52] No studies to date have yet examined the effect of exercise therapy on total morbidity or mortality in claudicants. Given the findings of the studies in this thesis, it would be reassuring to know that the advice given to claudicants regarding exercise therapy is not harmful in terms of cardiovascular morbidity and mortality. However, it is known that exercise therapy can reduce some of the systemic consequences of exercise in claudicants,[215] so in the absence of firm data regarding total morbidity and mortality, there is no good reason not to encourage claudicants to exercise as before.

As with most research, the research in this thesis has raised many questions with possible areas for future work.

- Is the prothrombotic state seen in claudicants following exercise due to ischaemia-reperfusion as postulated, or is it the effect of exercise in the presence of widespread atherosclerosis? This question could be answered by studying

claudicants exercising without ischaemia-reperfusion – arm exercises for example.

- What is the effect of treatment of claudication on the coagulation response, by exercise therapy, or percutaneous balloon angioplasty for example? There is evidence that exercise therapy can reduce the systemic inflammatory response in claudicants.[215]
- If, as seems likely, the procoagulant state is mediated by cytokines, can this be ameliorated medically by anti-inflammatory drugs?
- It may be that some of the more subtle effects of exercise in claudicants are not detected because we were sampling from the systemic circulation, and some markers / cells may be too diluted to detect changes, or become trapped in the peripheral circulation, or the pulmonary capillaries. Taking blood samples from the femoral vein would answer these issues.

In conclusion, the work presented in this thesis has shown that research in this area is often not straightforward. Whilst it appears that patients with intermittent claudication may generate more thrombin when compared to a control group, it is not clear whether this is related to ischaemia-reperfusion. Furthermore, the clinical relevance of these findings has not been demonstrated and would require further work. Any additional conclusions based on this work are difficult to draw, largely because of the variability of the parameters, and the relatively small numbers of subjects studied.

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Appendix 1

Consent Form and Information Sheet

Study Title: A pilot study to measure biochemical markers of coagulation and fibrinolysis in blood taken from healthy subjects and subjects with intermittent claudication, pre- and post-standardised exercise

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take as much time to read the following information carefully and discuss it with friends, relatives and your doctor if you wish. Ask us if there is anything that is not clear or if you would like more information.

We appreciate you taking the time to read (or have read to you), inquire about, and understand the following information.

What is the purpose of the study?

Pfizer is doing this research study to look at certain naturally occurring chemicals (biochemical markers) in your blood. This study will measure blood coagulation (clotting) chemicals and blood fibrinolysis (clot-breaking) chemicals. By measuring these chemicals it will help further understand the way that the blood coagulates (clots) and then how these clots are normally broken down within the body.

We will be taking blood samples before and after you have walked on a treadmill in order to examine the changes in these chemicals on exercise.

We will be studying: (a) 20 subjects who smoke and are diagnosed with intermittent claudication (pain on walking), (b) 10 healthy volunteers who smoke and (c) 10 healthy volunteers who are lifelong non-smokers.

Why have I been chosen?

You are being asked as you have intermittent claudication or are a healthy male who can be matched to a patient with intermittent claudication.

We require smokers who have a chronic smoking habit (□ 20 cigs/day for □1 year): we require healthy volunteers who are lifelong non-smokers.

40 subjects at this centre will be involved in the study and your participation will last for a period of about 3 weeks.

Do I have to take part?

It is up to you to decide whether or not to take part. You will be given this information sheet to keep. If you do decide to take part you will be asked to sign a consent form, and you will receive a copy of your signed form. If you decide not to take part this will not affect your medical care. You are free to withdraw from the study at any time without giving a reason. This will not affect your medical care.

What will happen to me if I take part?

During the study you will be seen in the outpatient department, for 3 visits. Your travelling expenses between the Birmingham Heartlands Hospital and home will be paid. On completion of the study, you will receive £125 to compensate you for your time and inconvenience.

At the start of the study (Screening Visit) you will have a vascular examination and medical. Your blood pressure and pulse rate will be measured and a blood and urine sample (for laboratory safety tests) will be taken. A small finger-prick blood sample will also be taken to measure your blood glucose and cholesterol. If you are a patient with intermittent claudication, you will undergo an exercise test (walking on a treadmill) if no treadmill assessment has been performed in the last 2 months. You will be asked to walk until the pain in your leg makes you want to stop. A blood sample will be taken at this visit for genetic analysis: we wish to check the variation in your genes for some of the blood chemicals being measured in the study. No other type of genes will be tested for.

If you are suitable for the study, you will be asked to return for Study Visit 1 about 7-14 days after the Screening Visit. Following Study Visit 1, you will be asked to return 7-14 days later for Study Visit 2. (Study Visit 2 is the last visit.)

Transport via taxi will be arranged for you at each study visit. On arrival, you will be asked to sit down in a wheelchair as we aim to minimise body movement as much as possible. You will be asked questions about your health and a small finger-prick blood sample will be taken to measure your blood glucose and cholesterol.

A cannula (a small plastic tube used for blood sampling) will be put into your arm for standard blood sampling. Blood samples will be taken to measure the levels of blood clotting chemicals in your blood, both before and after an exercise test. The exercise test is performed on a treadmill. If you have claudication you will be asked to walk on the treadmill until the pain in your leg makes you want to stop. If you are a normal volunteer you will be asked to walk on the treadmill until you want to stop because of tiredness, or for 3 minutes, whichever is the shorter. Once you have completed the exercise, you will again be asked to sit. The times at which samples will be taken are: 60 minutes before exercise, 30 minutes before exercise, and then 1 minute, 5 minutes, 20 minutes, 40 minutes, 60 minutes and 120 minutes after the exercise test.

Once the final blood sample has been taken, at 120 minutes after exercise, the doctor will check you are fine before you leave.

What do I have to do?

If you are suitable for the study and agree to take part, there are certain restrictions on what you can and cannot do:

- Smokers must not smoke for 1 hour before arriving for each study visit.
- Please eat any main meal, e.g. breakfast or lunch, over an hour before arriving.
- For 48 hours before each study visit you should not make any changes to your normal lifestyle e.g. unaccustomed exercise.
- Drugs which are used to treat intermittent claudication e.g. Prazilene®, Trental® and Hexopal® should not be taken for 48 hours before the study visits.
- You should continue taking all other medications during the whole time you are taking part in the study, but should ensure that you tell the doctor in charge of the study what drugs you are taking.

If you feel unwell during the study you should inform the study doctor, as he/she will need to record details of this as part of the study.

What are the possible risks of taking part?

The treadmill exercise and the blood sampling procedure may cause some minor discomfort. For example, there may be light bruising around the needle-puncture site of the cannula for blood sampling. Participants with claudication will develop their normal leg pain when they are asked to walk on the treadmill.

What are the possible benefits of taking part?

There is no intended clinical benefit from taking part in this study.
All study tests, examinations and medical care required as part of this study are provided free.

What are my rights if something goes wrong?

Whilst Pfizer does not expect you to suffer any health problems by taking part in this study, Pfizer may compensate anyone whose health suffers as a result of participation in this study. You do not have to prove it was anyone's fault; if the health problem arose because of your participation in the study you will be compensated.

Pfizer accepts that this study is being conducted subject to the Association of the British Pharmaceutical Industry (ABPI) guidelines entitled "Clinical Trial Compensation Guidelines", relating to compensation for injury arising in the course of clinical trials, under which any compensation will be considered: a copy of the guidelines are available on request.

Will my taking part in this study be kept confidential?

Your family doctor (General Practitioner) will usually be told that you have decided to take part in this study.

Your records obtained while you are in this study as well as related health records will remain strictly confidential at all times. However, these will need to be made available to others working on Pfizer's behalf and possibly the independent ethics committee members.

By signing the consent form you agree to this access for the current study and any further research that may be conducted in relation to it (even if you withdraw). The information disclosed will remain confidential.

What will happen to the results of the research study?

The results of this study are likely to be published in a medical journal.
You will not be identified in any report/publication.

Who has reviewed the study?

The East Birmingham Ethics Committee has reviewed the study.

If you have any problems with the conduct of the study, you can telephone the secretary of the Ethics Committee on 0121 424 0594, who will arrange for your worries to be investigated.

Data Protection: What use will be made of data collected from this study?

Personal data, which may be sensitive, (e.g. date of birth) will be collected and processed but only for research purposes in connection with this study.

The study data will be sent around the world but you will not be referred to by name or identified in any report or publication nor could the data be traced back to you.
By taking part in this study you agree not to restrict the use of any data even if you withdraw.

Pfizer Limited (who will control the use of the data) will take steps to ensure your personal data is protected.

By agreeing to take part in this study you agree to the transfer of your personal data to other Pfizer companies and to medicines regulatory authorities both within and outside Europe where some data protection laws may not be as good as in Europe.

Contact for further information

Thank you for taking the time to read (or have read to you) the information about this study. If you have any questions or concerns now or at any time about the study, your safety or your rights, please ask your doctor, his/her study staff or the contact person(s) indicated below.

If you enter this study, you can contact the following people listed below if you have any questions or worries.

Contact Details

- Study co-ordinator: Paul Burns
  - Tel: 0121 424 2115 - during office hours
  - Tel: 0121 424 2000 (switchboard) - outside office hours
- The East Birmingham Ethics Committee
  - Tel: 0121 424 0594

CONSENT FORM

AGREEMENT TO PARTICIPATE IN A CLINICAL STUDY

Subject Initials: _____ Subject Name: _____

Date of Birth: _____ *Study doctor's name: _____

		Please initial box (subject)
1	I confirm that I have read and understand the information sheet dated 29 June 2001 for the above study and have had the opportunity to ask questions.	[ ]
2	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	[ ]
3	I understand that others working on Pfizer's behalf and the independent ethics committee will need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw. I agree to this access.	[ ]
4	I consent to the collection, processing, reporting and transfer within and outside Europe of my personal and sensitive data for healthcare and/or medical research purposes.	[ ]
5	I agree not to restrict the use of any data or results, which arise from this study.	[ ]
6	I consent to the collection of a blood sample for targeted genetic analysis (TAFI and related genes).	[ ]
7	I agree to take part in the above study.	[ ]

Signed by subject: _____ **Date**: _____/_____/_____

Subject Name:

_____

[Print please] **please date your own signature at time of signing****

Signed by doctor: _____ **Date**: _____/_____/_____

Study Doctor's Name: _____
[Print please]

**Study doctor should ensure the subject dates his/her own signature at the time of signing.

Appendix 2

Raw Data

Assay: Flow cytometry – Platelet MESF (CD62)

Visit	Time point	Claudicants									
1	-60	122	112	92	113	119	127	101	129	206	150
1	-30	125	107	98	115	126	107	97	119	134	179
1	1	139	111	97	123	138	123	104	138	178	137
1	5	140	733	101	122	129	100	116	134	176	160
1	20	115	96	104	111	**NA**	107	99	122	159	214
1	40	154	110	94	112	131	152	100	141	149	150
1	60	119	116	97	115	138	164	117	180	151	162
1	120	119	167	100	112	140	148	143	140	211	181
2	-60	95	111	121	166	125	108	126	117	NA	NA
2	-30	113	111	119	118	121	128	143	**NA**	NA	NA
2	1	100	117	125	140	136	120	151	132	NA	NA
2	5	121	110	116	129	159	150	144	148	NA	NA
2	20	98	135	101	133	130	144	137	**NA**	NA	NA
2	40	128	119	129	155	123	150	127	124	NA	NA
2	60	109	113	114	130	132	149	135	138	NA	NA
2	120	113	151	146	158	156	244	127	127	NA	NA

NA=no sample received / unsuitable for analysis

Assay: Flow cytometry – Platelet MESF (CD62)

Visit	Time point	Smoking Controls						Non-smoking Controls				
1	-60	144	130	120	126	134	117	152	166	146	125	176
1	-30	165	159	117	138	134	121	143	148	159	139	164
1	1	151	135	123	135	143	123	146	162	151	125	135
1	5	186	129	133	129	156	120	144	207	162	148	167
1	20	142	141	131	141	163	123	138	193	140	136	142
1	40	144	146	126	146	157	131	139	178	157	136	166
1	60	161	148	120	137	170	120	133	161	135	128	163
1	120	170	113	115	133	184	115	133	176	179	159	353
2	-60	136	134	138	137	159	120	144	123	132	131	111
2	-30	135	123	149	138	177	132	146	116	130	171	137
2	1	139	136	155	134	141	124	166	122	126	115	120
2	5	143	143	161	292	157	127	167	127	131	120	130
2	20	138	149	123	130	142	123	175	129	126	136	115
2	40	147	151	150	132	152	133	171	118	190	146	122
2	60	148	146	133	132	128	135	NA	126	144	123	123
2	120	149	127	165	156	141	139	NA	120	175	139	120

NA=no sample received / unsuitable for analysis

Assay: Flow cytometry – % monocyte-platelet complexes

Visit	Time point	Claudicants									
1	-60	3	1	3	3	0	0	0	-1	-3	0
1	-30	3	2	2	1	0	1	1	-2	-1	0
1	1	8	1	2	1	1	1	-2	-1	2	1
1	5	1	6	2	0	0	1	-2	0	0	0
1	20	9	-2	1	4	**NA**	3	0	-1	2	1
1	40	#	3	2	3	1	2	3	-1	0	1
1	60	0	1	2	2	0	2	-1	0	-2	1
1	120	5	2	3	6	0	3	-2	-1	-2	1
2	-60	1	1	0	0	0	-1	-2	6	NA	NA
2	-30	5	2	2	-2	3	1	0	**NA**	NA	NA
2	1	-2	0	1	1	2	0	1	8	NA	NA
2	5	0	0	1	2	-3	1	-1	5	NA	NA
2	20	-3	2	0	0	1	0	0	**NA**	NA	NA
2	40	-2	1	0	-1	-1	0	1	-1	NA	NA
2	60	3	5	0	1	-3	1	0	5	NA	NA
2	120	-7	2	-3	-2	1	1	0	4	NA	NA

NA=no sample received / unsuitable for analysis

Assay: Flow cytometry – % monocyte-platelet complexes

Visit	Time point	Smoking Controls						Non-smoking Controls				
1	-60	-3	0	2	0	0	2	0	-1	-2	-1	0
1	-30	-5	1	0	0	0	0	1	-2	2	2	2
1	1	-3	0	1	0	1	2	1	0	0	2	1
1	5	-2	0	1	1	0	1	0	2	3	1	-1
1	20	-6	3	1	0	0	1	0	0	1	-2	1
1	40	-2	0	1	-1	0	3	-1	-2	-1	1	-1
1	60	-5	1	0	2	0	1	-8	-5	3	3	2
1	120	-2	-1	2	0	1	1	-1	-1	3	-1	-2
2	-60	1	1	0	-1	-1	1	6	-4	0	0	-1
2	-30	-1	0	0	2	-4	0	1	0	2	0	0
2	1	0	1	2	1	3	4	1	0	0	1	1
2	5	1	1	-1	3	0	0	0	0	1	0	0
2	20	0	-1	-2	0	-1	0	0	0	0	4	0
2	40	1	1	-1	1	-1	0	0	0	2	2	0
2	60	1	1	3	0	1	0	NA	0	0	0	2
2	120	-2	2	1	2	0	1	NA	2	0	1	2

NA=no sample received / unsuitable for analysis

Assay: Flow cytometry – CD11b expression on lymphocytes

Visit	Time point	Claudicants									
1	**-60**	2434	1670	2283	1914	2194	3059	2243	2702	3285	2369
1	**-30**	2354	1641	2198	1956	2043	3115	2414	2576	2928	2361
1	**1**	2554	2148	2654	2173	2971	3673	2553	2621	3116	3014
1	**5**	2551	1933	2402	2115	2796	3224	2419	2543	2807	2743
1	**20**	2453	1722	2210	1978	2530	3339	2306	2469	3014	2727
1	**40**	2406	1671	2241	1939	2389	2778	2285	2579	2886	2494
1	**60**	2346	1666	2000	1941	2437	2768	2276	2597	2972	2542
1	**120**	2351	1566	1485	1880	2178	2574	1988	2386	2614	2477
2	**-60**	2797	1599	2163	1960	2604	2620	3445	3684	8455	NA
2	**-30**	2913	1714	2006	2046	2343	2714	3235	3373	8059	NA
2	**1**	3673	2312	2059	2349	4169	3174	3384	3579	9001	NA
2	**5**	3266	2167	1988	2226	3999	3125	3048	3646	9287	NA
2	**20**	3228	2037	2022	2073	3064	3052	3077	3528	8963	NA
2	**40**	3098	1926	1870	2044	2559	2688	2940	3304	7993	NA
2	**60**	2946	1846	1933	2139	2339	2837	2642	3237	7075	NA
2	**120**	3051	1905	1869	1946	2020	2715	2922	3271	8152	NA

NA=no sample received / unsuitable for analysis

Assay: Flow cytometry – CD11b expression on lymphocytes

Visit	Time point	Smoking Controls						Non-smoking Controls				
1	**-60**	2899	2335	2907	3165	3334	2900	NA	2053	2423	3203	4051
1	**-30**	2800	2192	3048	3157	3035	2720	NA	2119	2761	3447	3595
1	**1**	3121	2520	3586	5348	8478	3810	NA	2644	5119	5486	6272
1	**5**	2718	2286	3371	3993	6829	3186	NA	2538	4448	4221	5144
1	**20**	2629	2318	3150	4547	4875	3104	NA	2506	3133	4434	4445
1	**40**	2751	2375	2793	3715	3714	3294	NA	2343	3642	3082	4116
1	**60**	2657	2143	2688	3452	3442	2856	NA	2300	2830	3837	4396
1	**120**	2569	2164	2651	3123	3109	2651	NA	2394	2784	4457	4006
2	**-60**	2667	2491	2629	2685	2956	3054	2586	2567	2801	3112	4385
2	**-30**	2621	2442	2973	2862	2884	3230	2975	2544	2511	2746	4727
2	**1**	3047	2621	3397	4510	8534	3637	5484	3131	4948	4625	6876
2	**5**	3069	2488	3058	3680	6432	3147	4584	2791	4117	3987	6079
2	**20**	2746	2371	2943	3527	4519	3135	3656	2679	3412	3789	5144
2	**40**	2568	2398	2716	3248	3282	3310	3153	2524	2908	4203	4383
2	**60**	2640	2322	2671	3137	NA	3077	2976	2458	2638	3355	4573
2	**120**	2410	2253	2439	2706	NA	3190	3145	2570	2616	3070	4736

NA=no sample received / unsuitable for analysis

Assay: Prothrombin time

Units: seconds

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	14.1	13.7	13.1	18.9	13.2	13.9	14.4	12.5	14	15										
1	-30	13	13.8	13.5	14.2	13.2	13.9	14.4	12.7	13.4	15.4										
1	1	12.9	15.5	13.2	14.9	13.8	13.3	14.7	13.3	13.1	15.5										
1	5	13	NA	15.4	14.5	13.8	14.4	13.9	13.2	13.4	15.6										
1	20	13.2	14.1	15.6	19.6	13.8	14.2	14.3	13	13.2	15.2										
1	40	12.6	13.9	16.2	15	14.9	14.6	14.5	12.9	13.2	14.9										
1	60	12.8	14.3	13.6	19	13.4	13.7	14.8	12.6	12.7	15.1										
1	120	12.9	14	13.6	14.7	13.2	14.2	14.1	12.7	13.8	14.8										
2	-60	12.8	13.7	13	14.8	13.7	13.4	13.8	13.6	13.1	NA										
2	-30	12.3	13.5	12.9	14.5	13.8	13.6	16.1	13.7	NA	NA										
2	1	12.4	13.9	13	15.3	13.1	13.9	14.3	13.4	13.8	NA										
2	5	12.4	13.8	12.9	14.5	13.8	13.3	14.3	13.4	13	NA										
2	20	12.9	13.4	13	14.1	13.7	13.4	16.4	13.5	13.2	NA										
2	40	12.6	13.8	13.5	14.4	13.6	13.4	13.7	13.7	13.4	NA										
2	60	12.5	14.8	12.9	14.2	13.7	13.8	12.8	13.4	13.6	NA										
2	120	13.7	14.5	13	14	13.7	13.6	13.9	13.3	13.4	NA										

NA=no sample received / unsuitable for analysis

Assay: Prothrombin time

Units: seconds

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	14.3	14.7	13.7	14.2	13.6	NA	NA	NA	NA	NA	14	13.2	13.6	NA	13.7	12.8	NA	NA	NA	NA
1	-30	13.9	14.3	13.8	14	13.4	NA	NA	NA	NA	NA	13.4	13.6	14.3	NA	13.4	12.7	NA	NA	NA	NA
1	1	14.5	15.7	13.9	14.3	13.7	NA	NA	NA	NA	NA	13.1	13.2	15	NA	13.2	13	NA	NA	NA	NA
1	5	14.1	15.7	14	14.1	14.2	NA	NA	NA	NA	NA	13.4	13.5	14.9	NA	13.3	13.1	NA	NA	NA	NA
1	20	13.8	15.5	14	14.1	13	NA	NA	NA	NA	NA	13.2	13.4	14.6	NA	13.2	12.8	NA	NA	NA	NA
1	40	14.7	16.6	13.6	13.6	13.7	NA	NA	NA	NA	NA	13.2	13.1	14.1	NA	13.1	12.7	NA	NA	NA	NA
1	60	13.6	15.4	13.7	13.6	13.8	NA	NA	NA	NA	NA	12.7	13.4	14.3	NA	13.2	12.8	NA	NA	NA	NA
1	120	14.1	15.6	13.9	14.5	13.3	NA	NA	NA	NA	NA	NA	13.4	14.3	NA	12.9	13.3	NA	NA	NA	NA
2	-60	13.8	NA	12.9	13.8	13	13	NA	NA	NA	NA	13.2	13.6	14.4	NA	13	13.4	NA	NA	NA	NA
2	-30	14.3	NA	14.2	13.9	13.2	12.8	NA	NA	NA	NA	12.7	13.5	13.7	NA	12.8	12.6	NA	NA	NA	NA
2	1	14	NA	14.1	13.2	14.1	13.2	NA	NA	NA	NA	13.6	13	14.3	NA	12.9	13	NA	NA	NA	NA
2	5	15.2	NA	14.1	12.9	14.2	13.4	NA	NA	NA	NA	13.4	15	14.5	NA	13.1	13.1	NA	NA	NA	NA
2	20	14.2	NA	13.8	13.3	15.7	13.4	NA	NA	NA	NA	12.9	13	14.2	NA	12.9	12.5	NA	NA	NA	NA
2	40	13.8	NA	13.4	13.3	14	13	NA	NA	NA	NA	13.4	13.4	14.4	NA	13.2	12.7	NA	NA	NA	NA
2	60	14.6	NA	16.4	13.4	13.2	13.9	NA	NA	NA	NA	12.7	12.9	14.5	NA	12.9	13	NA	NA	NA	NA
2	120	14.1	NA	13.6	13.6	13.8	13	NA	NA	NA	NA	13.4	13.4	13.8	NA	13.3	12.6	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: Activated partial thromboplastin time

Units: Seconds

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	14.1	13.7	13.1	18.9	13.2	13.9	14.4	12.5	14	15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	-30	13	13.8	13.5	14.2	13.2	13.9	14.4	12.7	13.4	15.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	1	12.9	15.5	13.2	14.9	13.8	13.3	14.7	13.3	13.1	15.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	13	NA	15.4	14.5	13.8	14.4	13.9	13.2	13.4	15.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	13.2	14.1	15.6	19.6	13.8	14.2	14.3	13	13.2	15.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	12.6	13.9	16.2	15	14.9	14.6	14.5	12.9	13.2	14.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	12.8	14.3	13.6	19	13.4	13.7	14.8	12.6	12.7	15.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	120	12.9	14	13.6	14.7	13.2	14.2	14.1	12.7	13.8	14.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-60	12.8	13.7	13	14.8	13.7	13.4	13.8	13.6	13.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	12.3	13.5	12.9	14.5	13.8	13.6	16.1	13.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	12.4	13.9	13	15.3	13.1	13.9	14.3	13.4	13.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	12.4	13.8	12.9	14.5	13.8	13.3	14.3	13.4	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	12.9	13.4	13	14.1	13.7	13.4	16.4	13.5	13.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	12.6	13.8	13.5	14.4	13.6	13.4	13.7	13.7	13.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	12.5	14.8	12.9	14.2	13.7	13.8	12.8	13.4	13.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	13.7	14.5	13	14	13.7	13.6	13.9	13.3	13.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: Activated partial thromboplastin time

Units: Seconds

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	14.3	14.7	13.7	14.2	13.6	NA	NA	NA	NA	NA	14	13.2	13.6	NA	13.7	12.8	NA	NA	NA	NA
1	-30	13.9	14.3	13.8	14	13.4	NA	NA	NA	NA	NA	13.4	13.6	14.3	NA	13.4	12.7	NA	NA	NA	NA
1	1	14.5	15.7	13.9	14.3	13.7	NA	NA	NA	NA	NA	13.1	13.2	15	NA	13.2	13	NA	NA	NA	NA
1	5	14.1	15.7	14	14.1	14.2	NA	NA	NA	NA	NA	13.4	13.5	14.9	NA	13.3	13.1	NA	NA	NA	NA
1	20	13.8	15.5	14	14.1	13	NA	NA	NA	NA	NA	13.2	13.4	14.6	NA	13.2	12.8	NA	NA	NA	NA
1	40	14.7	16.6	13.6	13.6	13.7	NA	NA	NA	NA	NA	13.2	13.1	14.1	NA	13.1	12.7	NA	NA	NA	NA
1	60	13.6	15.4	13.7	13.6	13.8	NA	NA	NA	NA	NA	12.7	13.4	14.3	NA	13.2	12.8	NA	NA	NA	NA
1	120	14.1	15.6	13.9	14.5	13.3	NA	NA	NA	NA	NA	NA	13.4	14.3	NA	12.9	13.3	NA	NA	NA	NA
2	-60	13.8	NA	12.9	13.8	13	13	NA	NA	NA	NA	13.2	13.6	14.4	NA	13	13.4	NA	NA	NA	NA
2	-30	14.3	NA	14.2	13.9	13.2	12.8	NA	NA	NA	NA	12.7	13.5	13.7	NA	12.8	12.6	NA	NA	NA	NA
2	1	14	NA	14.1	13.2	14.1	13.2	NA	NA	NA	NA	13.6	13	14.3	NA	12.9	13	NA	NA	NA	NA
2	5	15.2	NA	14.1	12.9	14.2	13.4	NA	NA	NA	NA	13.4	15	14.5	NA	13.1	13.1	NA	NA	NA	NA
2	20	14.2	NA	13.8	13.3	15.7	13.4	NA	NA	NA	NA	12.9	13	14.2	NA	12.9	12.5	NA	NA	NA	NA
2	40	13.8	NA	13.4	13.3	14	13	NA	NA	NA	NA	13.4	13.4	14.4	NA	13.2	12.7	NA	NA	NA	NA
2	60	14.6	NA	16.4	13.4	13.2	13.9	NA	NA	NA	NA	12.7	12.9	14.5	NA	12.9	13	NA	NA	NA	NA
2	120	14.1	NA	13.6	13.6	13.8	13	NA	NA	NA	NA	13.4	13.4	13.8	NA	13.3	12.6	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: ESR

Units: mm/hr

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	7	6	18	13	14	4	10	25	8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	-30	9	4	18	13	14	4	10	25	10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	1	9	4	18	13	16	4	10	27	12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	9	6	21	13	16	4	10	23	10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	11	6	18	13	16	4	10	24	12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	11	6	18	15	16	4	10	27	12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	11	6	18	13	16	4	10	19	12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	120	11	6	21	15	16	4	12	27	12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-60	8	4	27	16	35	17	12	23	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	10	4	29	16	33	17	12	22	11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	10	6	29	16	34	17	12	25	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	10	6	29	16	34	17	14	23	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	10	6	29	14	33	19	14	23	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	10	6	29	16	34	17	14	22	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	10	6	29	16	37	17	16	23	11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	8	6	29	16	37	20	14	20	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: ESR

Units: mm/hr

Visit	Time point	Smoking Controls											Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	4	NA	16	4	NA	NA	NA	NA	NA	NA	8	4	9	NA	4	NA	NA	NA	NA	NA
1	-30	4	NA	16	4	NA	NA	NA	NA	NA	NA	6	7	9	NA	4	13	NA	NA	NA	NA
1	1	4	NA	16	4	NA	NA	NA	NA	NA	NA	8	7	9	NA	4	11	NA	NA	NA	NA
1	5	6	6	16	4	NA	NA	NA	NA	NA	NA	6	7	9	NA	4	11	NA	NA	NA	NA
1	20	6	4	16	4	NA	NA	NA	NA	NA	NA	8	7	11	NA	10	13	NA	NA	NA	NA
1	40	4	4	16	6	NA	NA	NA	NA	NA	NA	8	7	9	NA	6	13	NA	NA	NA	NA
1	60	4	4	20	4	NA	NA	NA	NA	NA	NA	8	7	11	NA	6	13	NA	NA	NA	NA
1	120	NA	2	18	4	NA	NA	NA	NA	NA	NA	8	7	NA	NA	4	13	NA	NA	NA	NA
2	-60	6	NA	11	4	11	NA	NA	NA	NA	NA	NA	NA	2	NA	4	10	NA	NA	NA	NA
2	-30	6	NA	11	4	11	NA	NA	NA	NA	NA	NA	NA	8	NA	7	4	NA	NA	NA	NA
2	1	4	NA	6	4	10	NA	NA	NA	NA	NA	NA	NA	6	NA	4	12	NA	NA	NA	NA
2	5	6	NA	10	4	11	NA	NA	NA	NA	NA	NA	NA	2	NA	4	12	NA	NA	NA	NA
2	20	4	NA	11	6	11	NA	NA	NA	NA	NA	NA	NA	6	NA	2	10	NA	NA	NA	NA
2	40	6	NA	12	4	11	NA	NA	NA	NA	NA	NA	NA	6	NA	4	12	NA	NA	NA	NA
2	60	6	NA	12	4	11	NA	NA	NA	NA	NA	NA	NA	8	NA	7	10	NA	NA	NA	NA
2	120	6	NA	14	6	11	NA	NA	NA	NA	NA	NA	NA	8	NA	4	6	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: Fibrinogen

Units: mg/dl

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	397	333	488	630	390	318	471	566	383	393	399	498	324	415	345	346	398	460	319	499
1	-30	407	332	482	637	366	331	462	567	375	382	405	480	317	408	350	350	412	631	334	496
1	1	636	338	480	428	388	348	478	571	383	412	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	416	304	514	484	381	390	522	523	383	419	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	418	351	474	444	372	368	499	537	353	403	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	414	307	492	441	355	346	494	574	364	401	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	646	298	511	406	381	388	450	593	356	393	381	501	309	421	347	359	401	463	309	495
1	12	419	363	471	630	350	371	456	593	372	387	413	418	294	406	344	349	382	458	303	462
2	-60	400	319	583	441	659	727	598	602	360	369	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	393	326	582	560	699	605	542	539	325	365	345	503	329	630	283	381	637	506	332	447
2	1	402	345	532	492	529	627	547	556	377	361	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	400	317	525	529	572	640	592	526	387	381	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	511	322	522	489	548	698	530	533	368	377	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	412	322	542	404	561	654	539	549	362	390	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	387	331	546	491	545	665	527	536	358	383	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	12	399	274	529	424	524	667	504	536	353	337	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: Fibrinogen

Units: mg/dl

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	342	416	381	356	488	492	446	544	246	647	364	359	324	221	291	317	251	252	340	245
1	-30	333	420	380	344	495	480	338	721	269	528	480	293	328	191	296	322	297	264	324	250
1	1	362	429	421	367	460	480	NA	NA	NA	NA	365	375	340	258	309	327	NA	NA	NA	NA
1	5	341	404	411	364	464	509	NA	NA	NA	NA	522	365	344	231	315	328	NA	NA	NA	NA
1	20	329	409	371	351	474	520	NA	NA	NA	NA	631	360	312	224	308	316	NA	NA	NA	NA
1	40	335	406	380	336	424	525	NA	NA	NA	NA	409	352	334	235	296	305	NA	NA	NA	NA
1	60	326	414	384	355	408	520	338	555	309	560	397	362	326	204	297	309	248	242	290	269
1	120	346	390	369	357	412	417	361	627	298	561	389	370	328	216	318	300	241	253	275	268
2	-60	342	381	378	354	338	466	NA	NA	NA	NA	462	368	324	250	279	NS	NA	NA	NA	NA
2	-30	324	379	377	282	369	458	662	461	321	496	418	353	334	252	283	277	267	408	350	252
2	1	344	422	383	511	393	467	NA	NA	NA	NA	487	383	346	280	267	NS	NA	NA	NA	NA
2	5	336	417	371	360	408	638	NA	NA	NA	NA	450	379	348	263	282	NS	NA	NA	NA	NA
2	20	332	391	368	401	402	452	NA	NA	NA	NA	429	378	332	258	259	NS	NA	NA	NA	NA
2	40	335	365	368	457	358	440	NA	NA	NA	NA	424	381	331	264	278	NS	NA	NA	NA	NA
2	60	324	398	351	397	362	449	NA	NA	NA	NA	382	371	328	260	276	NS	NA	NA	NA	NA
2	120	337	386	358	386	347	635	NA	NA	NA	NA	376	372	326	260	275	NS	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: TAT

Units: ng/ml

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	2.4	11.3	5.6	13.1	1.3	11.4	14.2	16.3	18.7	20.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	-30	38.9	32.4	50.4	17.7	1.8	14.6	17.8	56.0	26.0	18.0	10.9	17.5	20.5	15.3	8.9	7.7	11.4	3.0	35.0	18.9
1	1	78.1	78.4	54.0	52.0	18.0	12.7	31.6	45.4	20.5	42.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	40.2	>60	101	34.8	4.1	11.0	37.9	41.7	14.7	15.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	46.1	43.0	35.6	58.5	5.6	15.7	32.2	56.8	12.1	30.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	266	53.3	35.5	28.1	8.6	52.5	33.5	149	12.2	15.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	34.6	27.3	40.5	48.3	7.0	28.0	39.7	426	29.0	32.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	12											NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		30.2	38.0	12.5	17.4	4.0	21.3	30.3	47.5	13.2	29.9										
2	-60	8.7	3.0	17.0	15.7	7.9	11.6	10.6	7.2	34.9	10.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	11.4	21.0	22.6	12.5	3.0	23.8	25.8	38.7	39.9	16.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	13.4	42.1	12.4	7.6	10.1	30.5	45.9	40.7	24.0	17.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	18.5	50.2	11.4	9.6	3.9	21.1	47.2	22.6	19.0	22.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	13.1	96.2	9.6	25.8	8.6	40.0	44.8	15.0	37.9	22.3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	11.9	56.9	10.8	22.6	7.7	229	47.0	18.2	30.8	19.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	18.2	178	15.1	23.3	11.8	42.1	37.6	46.1	34.5	17.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	12											NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		8.0	103	7.2	16.4	9.3	52.4	33.2	15.7	34.6	12.8										

NA=no sample received / unsuitable for analysis

Assay: TAT

Units: ng/ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	2.1	20.6	6.0	12.2	5.7	6.8	NA	NA	NA	NA	1.9	1.4	8.7	4.6	1.7	14.6	NA	NA	NA	NA
1	-30	2.1	33.2	9.3	14.7	17.7	3.0	30.1	9.7	12.3	15.3	6.8	26.9	18.4	7.8	3.8	7.4	7.3	5.4	7.6	17.5
1	1	5.0	16.0	11.1	42.7	12.6	42.2	NA	NA	NA	NA	8.1	4.3	25.2	29.9	3.4	14.2	NA	NA	NA	NA
1	5	3.2	19.2	4.1	18.5	8.1	10.5	NA	NA	NA	NA	8.7	7.7	9.0	23.1	3.9	6.0	NA	NA	NA	NA
1	20	6.4	10.2	4.9	18.9	18.1	8.1	NA	NA	NA	NA	7.5	27.5	15.0	32.5	5.2	7.2	NA	NA	NA	NA
1	40	3.2	8.8	3.2	27.2	15.7	20.7	NA	NA	NA	NA	5.8	27.6	35.7	27.7	6.0	16.9	NA	NA	NA	NA
1	60	7.9	8.3	5.1	29.0	21.3	7.1	NA	NA	NA	NA	11.5	4.6	18.4	18.0	8.5	5.1	NA	NA	NA	NA
1	120	5.5	8.9	3.8	22.9	6.5	22.3	NA	NA	NA	NA	3.3	22.5	4.9	17.2	4.9	26.6	NA	NA	NA	NA
2	-60	2.2	9.5	20.9	206	7.6	50.5	NA	NA	NA	NA	2.2	9.5	20.9	206	7.6	50.5	NA	NA	NA	NA
2	-30	6.5	16.4	12.8	323	5.5	12.7	NA	NA	NA	NA	6.5	16.4	12.8	323	5.5	12.7	NA	NA	NA	NA
2	1	10.0	21.0	9.2	44.3	9.2	17.9	NA	NA	NA	NA	10.0	21.0	9.2	44.3	9.2	17.9	NA	NA	NA	NA
2	5	13.0	18.7	4.2	187	7.4	7.0	NA	NA	NA	NA	13.0	18.7	4.2	187	7.4	7.0	NA	NA	NA	NA
2	20	13.3	17.7	6.0	47.2	8.4	40.0	NA	NA	NA	NA	13.3	17.7	6.0	47.2	8.4	40.0	NA	NA	NA	NA
2	40	13.5	16.0	3.5	32.2	11.2	14.6	NA	NA	NA	NA	13.5	16.0	3.5	32.2	11.2	14.6	NA	NA	NA	NA
2	60	4.9	16.3	55.1	26.5	11.3	8.7	NA	NA	NA	NA	4.9	16.3	55.1	26.5	11.3	8.7	NA	NA	NA	NA
2	120	4.3	15.4	4.2	7.3	5.7	8.4	NA	NA	NA	NA	4.3	15.4	4.2	7.3	5.7	8.4	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: PF1+2

Units: ng/ml

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	0.80	0.70	0.70	0.70	0.70	1.10	1.60	1.00	0.90	0.90	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	-30	1.00	0.80	1.20	0.70	0.70	1.50	2.50	1.50	1.00	1.00	1.10	0.90	0.50	0.90	0.80	0.60	0.60	0.60	0.90	0.50
1	1	1.40	1.40	1.20	1.20	0.60	1.00	2.30	1.80	0.90	1.30	1.00	0.50	0.60	0.90	0.60	0.80	0.80	0.60	0.70	0.50
1	5	1.30	0.90	1.10	0.80	0.70	1.30	2.40	3.10	0.90	0.90	0.90	0.60	0.40	0.70	0.50	0.90	0.70	0.80	0.70	0.50
1	20	1.30	1.10	1.00	1.00	0.80	1.40	2.30	2.20	0.80	1.10	0.90	0.40	0.70	1.10	0.50	0.70	1.10	0.50	0.80	0.60
1	40	3.70	1.20	0.80	0.80	0.90	1.60	2.50	3.30	0.80	0.80	0.90	0.50	0.70	1.10	0.40	1.00	0.50	0.70	1.80	0.60
1	60	1.10	0.70	1.00	1.00	0.90	1.80	2.60	2.80	0.90	1.00	1.00	0.50	0.40	0.90	0.40	0.80	1.10	0.40	0.70	0.60
1	120	1.10	0.90	0.70	0.60	0.70	1.40	1.90	1.60	0.80	1.00	1.10	0.50	0.40	0.90	0.30	1.00	0.70	0.40	0.60	0.70
2	-60	0.90	0.70	0.70	0.60	0.80	1.10	1.30	1.00	1.50	0.50	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	0.90	0.70	1.00	0.80	0.90	1.40	2.00	1.40	1.60	0.60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	0.60	1.00	0.60	0.60	0.70	1.10	2.40	2.30	1.30	0.70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	0.90	2.10	0.80	0.90	1.00	1.50	2.60	1.40	1.60	0.80	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	0.70	1.40	0.70	1.00	1.10	2.00	2.00	1.30	1.40	0.90	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	0.70	1.30	0.50	1.00	1.20	2.80	2.60	1.30	1.00	0.60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	0.80	2.20	0.60	1.10	1.00	2.10	2.20	1.80	1.20	0.70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	0.70	1.40	0.60	0.90	1.10	2.20	2.00	1.10	1.10	0.60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: PF1+2

Units: ng/ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	0.80	0.80	0.70	1.00	0.90	0.70	NA	NA	NA	NA	0.40	0.60	0.90	0.50	0.80	1.40	NA	NA	NA	NA
1	-30	0.80	1.20	0.70	1.00	1.00	0.60	0.60	0.70	0.90	1.00	0.50	0.80	1.20	0.60	0.90	1.30	0.40	0.60	0.40	1.20
1	1	0.90	0.90	0.80	1.30	0.80	1.00	0.90	0.60	1.10	0.90	0.50	0.50	1.50	0.80	0.80	1.30	0.40	0.50	0.50	0.70
1	5	0.80	0.90	0.70	1.00	0.80	0.80	0.70	0.40	0.90	0.60	0.60	0.70	0.70	0.70	0.90	1.10	0.50	0.60	0.60	0.80
1	20	0.80	0.70	0.80	1.00	0.90	0.80	1.00	0.60	1.40	1.00	0.60	1.00	0.80	0.80	1.10	1.00	0.50	0.50	0.60	0.90
1	40	0.60	0.60	0.80	1.10	0.80	0.90	0.60	0.40	1.30	0.90	0.60	1.10	1.50	0.90	1.00	1.50	0.40	0.60	0.90	0.70
1	60	0.80	0.60	0.90	1.20	0.80	0.80	0.50	0.40	3.70	0.50	0.70	0.80	1.10	0.80	1.10	1.20	0.50	0.50	1.20	0.70
1	120	0.70	0.70	0.70	1.10	0.70	0.90	0.80	0.50	1.40	0.50	0.50	1.00	0.80	0.60	1.10	1.60	0.30	0.50	0.50	0.90
2	-60	1.20	1.10	0.90	3.00	0.50	1.40	NA	NA	NA	NA	0.50	0.60	1.20	0.70	0.60	NS	NA	NA	NA	NA
2	-30	1.40	0.70	0.70	3.40	0.50	0.70	NA	NA	NA	NA	0.60	0.70	1.10	0.70	0.60	NS	NA	NA	NA	NA
2	1	1.30	1.20	0.60	1.80	0.60	0.80	NA	NA	NA	NA	0.50	0.70	1.50	0.90	1.20	NS	NA	NA	NA	NA
2	5	1.30	0.60	0.60	2.80	0.60	0.70	NA	NA	NA	NA	0.60	0.70	1.50	0.60	0.50	NS	NA	NA	NA	NA
2	20	1.00	1.00	0.70	1.60	0.60	1.30	NA	NA	NA	NA	0.70	0.70	1.10	0.70	0.60	NS	NA	NA	NA	NA
2	40	1.00	0.60	0.70	1.70	0.50	1.00	NA	NA	NA	NA	0.80	0.70	1.20	0.60	0.50	NS	NA	NA	NA	NA
2	60	0.90	0.70	1.50	1.50	0.40	0.90	NA	NA	NA	NA	0.70	0.60	0.90	0.90	0.70	NS	NA	NA	NA	NA
2	120	1.10	0.60	0.60	1.30	0.40	0.90	NA	NA	NA	NA	0.70	0.50	0.80	0.60	0.50	NS	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: APC-PCI

Units: ng/ml

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	0.19	0.11	0.16	0.13	0.15	0.16	3.80	0.13	0.18	0.26	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	-30	0.13	0.14	0.17	0.18	0.16	0.21	3.90	0.14	0.17	0.36	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	1	0.23	0.09	0.18	0.15	0.17	0.23	4.30	0.14	0.23	0.39	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	0.23	0.11	0.24	0.40	0.18	0.26	4.25	0.13	0.17	0.53	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	0.37	0.76	0.17	0.39	0.22	0.33	4.25	0.40	0.22	0.55	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	0.31	1.33	0.35	0.51	0.25	0.39	4.20	NA	0.24	0.71	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	0.41	1.13	0.12	0.27	0.26	0.41	4.10	0.35	0.25	0.92	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	120	0.33	0.32	0.31	0.32	0.43	0.25	4.00	0.18	0.28	0.67	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-60	0.25	0.16	0.15	0.11	0.13	0.12	2.55	0.10	0.12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	0.22	0.16	0.16	0.19	0.17	0.17	2.55	0.14	0.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	0.33	0.25	0.16	0.17	0.13	0.17	3.20	0.15	0.23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	0.33	0.40	0.18	0.16	NA	0.21	3.05	0.17	0.19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	0.33	0.66	0.19	0.20	0.15	0.21	3.15	0.20	0.19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	0.36	0.65	0.16	0.15	0.16	0.21	3.25	0.13	0.23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	0.38	0.50	0.21	0.27	0.23	0.27	3.05	0.13	0.24	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	0.33	0.45	0.18	0.21	0.18	0.32	3.45	0.12	0.18	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: APC-PCI

Units: ng/ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	0.10	0.21	0.15	0.23	0.10	0.14	NA	NA	NA	NA	0.14	NA	NS	0.16	NA	0.22	NA	NA	NA	NA
1	-30	0.12	0.25	0.14	0.27	0.13	0.13	NA	NA	NA	NA	0.14	NA	0.15	0.37	NA	0.28	NA	NA	NA	NA
1	1	0.13	0.32	0.15	0.20	0.12	0.21	NA	NA	NA	NA	0.14	NA	0.18	0.40	NA	0.31	NA	NA	NA	NA
1	5	0.13	0.35	0.15	0.32	0.12	0.24	NA	NA	NA	NA	0.15	NA	0.19	0.38	NA	0.33	NA	NA	NA	NA
1	20	0.14	0.33	0.18	0.41	0.14	0.29	NA	NA	NA	NA	0.17	NA	0.22	0.56	NA	0.37	NA	NA	NA	NA
1	40	0.12	0.31	0.16	0.40	0.21	0.29	NA	NA	NA	NA	0.16	NA	0.17	0.56	NA	0.35	NA	NA	NA	NA
1	60	0.12	0.43	0.15	0.42	0.22	0.28	NA	NA	NA	NA	0.16	NA	0.13	0.47	NA	0.37	NA	NA	NA	NA
1	120	0.13	0.63	0.15	0.69	0.13	0.37	NA	NA	NA	NA	0.13	NA	0.14	0.36	NA	0.46	NA	NA	NA	NA
2	-60	0.14	NA	NS	0.16	NA	0.22	NA	NA	NA	NA	0.14	0.16	0.08	NA	NA	NA	NA	NA	NA	NA
2	-30	0.14	NA	0.15	0.37	NA	0.28	NA	NA	NA	NA	0.15	0.14	0.13	NA	NA	NA	NA	NA	NA	NA
2	1	0.14	NA	0.18	0.40	NA	0.31	NA	NA	NA	NA	0.14	0.15	0.09	NA	NA	NA	NA	NA	NA	NA
2	5	0.15	NA	0.19	0.38	NA	0.33	NA	NA	NA	NA	0.16	0.14	0.12	NA	NA	NA	NA	NA	NA	NA
2	20	0.17	NA	0.22	0.56	NA	0.37	NA	NA	NA	NA	0.17	0.19	0.14	NA	NA	NA	NA	NA	NA	NA
2	40	0.16	NA	0.17	0.56	NA	0.35	NA	NA	NA	NA	0.17	0.18	0.18	NA	NA	NA	NA	NA	NA	NA
2	60	0.16	NA	0.13	0.47	NA	0.37	NA	NA	NA	NA	0.21	0.20	0.17	NA	NA	NA	NA	NA	NA	NA
2	120	0.13	NA	0.14	0.36	NA	0.46	NA	NA	NA	NA	0.18	0.19	0.19	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: D-dimer

Units: ng/ml

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	26	28	33	85	48	45	544	65	248	282	63	16	17	102	5	5	19	19	9	9
1	-30	46	30	39	39	51	45	629	63	244	246	84	21	3	124	0	18	15	26	11	5
1	1	109	47	37	64	56	47	468	71	150	232	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	60	14	35	42	29	47	555	54	245	182	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	57	42	21	28	59	36	398	45	214	302	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	48	57	32	62	32	37	659	76	248	338	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	35	18	33	24	40	63	585	52	232	320	76	23	10	105	1	15	25	20	5	4
1	120	57	46	53	35	63	45	607	69	250	345	91	26	6	132	0	9	24	23	15	10
2	-60	38	28	37	26	84	58	679	50	323	177	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	44	19	32	28	84	64	666	41	285	132	52	24	5	90	2	20	19	8	42	20
2	1	49	18	46	32	81	61	798	39	338	163	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	56	22	35	26	84	62	497	63	321	131	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	35	22	49	28	64	56	776	45	304	144	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	46	31	33	30	64	62	863	52	321	148	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	39	18	21	12	70	61	686	58	298	172	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	52	72	42	39	72	62	639	97	311	150	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: D-dimer

Units: ng/ml

Visit	Time point	NA										NA									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	37	164	25	135	36	80	9	5	4	16	22	44	44	6	12	72	0	27	0	0
1	-30	45	162	41	123	24	76	2	10	0	26	9	20	21	5	39	56	0	27	7	24
1	1	37	142	29	128	30	71	NA	NA	NA	NA	12	20	13	14	11	66	NA	NA	NA	NA
1	5	45	114	29	125	65	90	NA	NA	NA	NA	15	15	21	12	72	88	NA	NA	NA	NA
1	20	0	108	27	118	15	80	NA	NA	NA	NA	8	13	19	9	12	34	NA	NA	NA	NA
1	40	47	128	33	116	66	92	NA	NA	NA	NA	11	19	26	8	27	82	NA	NA	NA	NA
1	60	29	154	23	95	23	56	0	2	2	22	6	15	37	0	36	65	0	23	3	23
1	120	45	172	41	121	11	92	17	10	6	35	12	26	37	27	40	55	0	64	16	18
2	-60	35	93	49	161	9	59	NA	NA	NA	NA	2	20	34	5	NA	NA	NA	NA	NA	NA
2	-30	37	88	55	114	1	76	2	4	4	29	27	20	24	24	19	33	0	59	18	6
2	1	47	103	53	238	9	64	NA	NA	NA	NA	16	23	45	8	6	NA	NA	NA	NA	NA
2	5	39	100	37	87	9	70	NA	NA	NA	NA	16	16	34	9	31	NA	NA	NA	NA	NA
2	20	33	83	35	142	19	70	NA	NA	NA	NA	9	22	26	6	21	NA	NA	NA	NA	NA
2	40	39	110	96	169	11	58	NA	NA	NA	NA	20	32	21	23	7	NA	NA	NA	NA	NA
2	60	37	102	29	161	14	63	NA	NA	NA	NA	11	19	30	2	11	NA	NA	NA	NA	NA
2	120	58	119	61	135	19	70	NA	NA	NA	NA	29	36	29	114	7	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: FbDP

Units: ng fibrinogen equivalents /ml

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	364	235	374	339	247	342	2570	462	827	1130	1025	501	441	1680	294	222	401	367	266	269
1	-30	308	269	367	354	254	340	2655	475	836	1303	815	544	374	1728	284	217	259	355	257	224
1	1	359	276	360	150	214	346	2542	461	800	1323	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	399	ns	357	342	257	361	2695	450	807	1263	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	332	288	385	354	138	390	2851	486	801	1165	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	358	327	369	364	262	354	2659	497	894	1132	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	347	294	360	334	276	349	2683	488	875	1268	809	582	396	2034	298	232	261	366	262	224
1	120	348	335	395	350	267	363	2728	4104	903	1359	864	651	378	19113	313	266	294	384	277	249
2	-60	336	239	357	363	496	455	21034	337	1059	1297	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	324	237	378	357	519	442	2566	357	1019	1295	651	538	492	1003	263	220	261	295	363	3113
2	1	347	259	144	156	521	479	2483	359	977	1178	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	358	276	346	367	545	469	2641	369	976	1213	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	374	290	372	364	589	485	2844	300	1056	1087	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	325	276	331	360	549	471	2452	358	1110	1119	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	323	306	141	357	478	503	2459	367	1015	1134	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	305	303	337	351	447	465	2635	370	1029	1301	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: FbDP

Units: ng fibrinogen equivalents /ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	377	847	204	476	309	301	154	231	305	448	150	181	236	256	186	348	241	366	166	233
1	-30	416	640	232	4114	340	288	176	282	205	446	157	1104	252	237	203	362	276	442	158	291
1	1	402	812	215	4104	352	307	NA	NA	NA	NA	158	1104	237	274	198	NA	NA	NA	NA	NA
1	5	388	617	222	484	363	298	NA	NA	NA	NA	161	175	248	262	211	NA	NA	NA	NA	NA
1	20	356	891	243	519	374	281	NA	NA	NA	NA	157	172	253	285	190	NA	NA	NA	NA	NA
1	40	3103	704	211	505	344	299	NA	NA	NA	NA	151	180	1113	271	220	NA	NA	NA	NA	NA
1	60	336	953	210	463	351	315	162	222	280	449	158	178	166	263	196	349	238	437	207	288
1	120	356	943	220	479	320	330	179	207	267	377	150	190	1104	244	196	336	294	391	148	292
2	-60	356	991	236	568	346	2103	NA	NA	NA	NA	180	186	129	277	216	NA	NA	NA	NA	NA
2	-30	366	1056	231	607	316	2103	170	237	300	258	189	198	148	278	214	361	201	536	214	221
2	1	352	9113	279	604	329	260	NA	NA	NA	NA	184	194	177	303	219	NA	NA	NA	NA	NA
2	5	344	1012	255	633	342	279	NA	NA	NA	NA	184	202	173	305	225	NA	NA	NA	NA	NA
2	20	333	869	258	684	323	2104	NA	NA	NA	NA	173	188	230	289	214	NA	NA	NA	NA	NA
2	40	326	888	238	664	339	268	NA	NA	NA	NA	1104	182	166	300	221	NA	NA	NA	NA	NA
2	60	335	982	242	615	337	265	NA	NA	NA	NA	180	178	180	284	215	NA	NA	NA	NA	NA
2	120	344	1088	233	613	300	281	NA	NA	NA	NA	177	175	218	251	187	NA	NA	NA	NA	NA

Assay: tPA antigen

Units: ng/ml

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	13.8	9.6	12.0	12.0	8.3	18.5	8.5	9.9	38.1	10.5										
1	-30	20.2	6.6	11.4	13.3	7.5	20.7	9.1	10.8	27.4	10.2	11.0	12.8	14.7	12.9	18.4	16.1	16.2	14.8	8.0	9.9
1	1	11.9	15.7	10.6	13.9	8.2	20.8	10.60	13.3	34.2	11.6	14.8	13.8	17.8	15.2	18.7	18.7	17.2	15.3	10.4	11.7
1	5	13.6	12.7	10.0	12.0	7.2	20.2	8.6	10.2	29.9	11.4	13.7	13.7	17.0	14.0	19.3	16.8	14.7	15.0	10.9	10.6
1	20	12.1	9.9	10.6	13.7	5.7	20.4	7.8	9.4	29.2	10.4	12.5	12.8	14.4	13.7	13.5	16.3	15.6	14.3	9.0	9.6
1	40	9.7	9.5	10.5	13.6	4.7	18.3	7.5	9.8	30.9	8.5	12.3	11.8	14.2	13.6	16.9	14.8	14.7	12.2	8.8	9.8
1	60	13.6	9.1	10.3	15.0	4.5	15.5	8.7	8.8	27.2	10.2	11.9	12.9	14.0	13.3	18.1	15.7	16.1	12.1	8.1	9.8
1	120	12.1	6.7	9.4	13.6	6.9	14.9	7.9	11.9	24.8	9.0	11.8	12.7	13.4	11.4	15.0	14.9	14.9	8.8	7.6	9.1
2	-60	17.0	9.2	10.8	14.1	8.1	13.3	7.9	9.1	25.4	11.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	16.0	9.5	10.9	12.0	7.2	13.2	6.5	10.5	21.1	12.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	13.3	12.0	10.4	10.6	7.7	15.4	7.1	14.2	25.5	15.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	14.0	9.8	9.7	13.1	7.8	13.2	6.2	14.7	25.1	13.3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	17.8	8.1	9.7	10.5	6.0	11.9	6.0	13.3	24.8	13.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	13.7	7.2	9.3	11.0	4.7	12.1	6.9	12.8	22.2	13.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	10.7	6.9	9.0	14.1	6.7	14.6	6.2	11.4	22.4	13.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	12.5	6.8	9.1	12.1	5.3	11.0	7.3	11.8	21.6	12.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: tPA antigen

Units: ng/ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	5.1	8.9	6.2	6.0	4.7	10.3					11.0	8.7	9.7	6.8	5.8	6.5				
1	-30	4.5	8.6	7.0	5.9	4.7	10.7	4.0	6.8	11.1	13.4	11.0	8.6	9.7	6.1	5.4	8.0	8.2	10.5	7.7	15.3
1	1	6.2	9.7	7.9	8.2	11.4	10.9	7.9	8.4	11.4	18.9	12.8	8.9	12.0	NA	7.3	10.3	9.4	13.0	9.9	14.3
1	5	3.1	9.2	7.3	8.5	13.4	10.6	6.5	7.4	12.0	18.7	12.2	9.4	12.6	11.3	7.5	9.2	8.8	11.4	9.9	13.9
1	20	4.4	8.2	6.9	6.8	6.4	10.1	5.4	6.5	10.8	13.9	12.3	7.8	8.8	7.9	7.1	7.8	9.0	7.8	8.4	14.0
1	40	5.0	7.7	6.8	6.1	5.9	9.6	5.6	6.3	10.9	14.7	8.4	7.5	7.6	7.5	6.5	7.7	8.8	12.2	8.3	12.3
1	60	4.6	10.3	6.7	6.2	6.9	8.8	4.3	6.8	11.2	14.2	10.2	8.9	5.5	7.3	6.6	7.8	8.5	10.5	8.3	11.5
1	120	5.9	7.0	5.0	6.0	4.9	9.3	5.0	6.2	10.0	13.0	10.6	8.7	6.6	7.4	6.5	6.4	7.2	9.7	8.2	10.8
2	-60	7.6	12.5	7.6	4.5	9.4	11.4	NA	NA	NA	NA	11.2	13.6	7.2	8.5	9.4	NA	NA	NA	NA	NA
2	-30	8.9	10.3	6.7	3.6	8.5	10.3	NA	NA	NA	NA	10.9	8.2	8.5	8.2	6.6	NA	NA	NA	NA	NA
2	1	9.1	13.5	8.7	6.2	18.0	12.3	NA	NA	NA	NA	12.7	8.9	13.4	14.0	8.2	NA	NA	NA	NA	NA
2	5	10.0	12.9	7.2	4.1	15.4	11.6	NA	NA	NA	NA	9.3	7.8	7.5	11.8	7.6	NA	NA	NA	NA	NA
2	20	8.9	10.1	6.7	6.1	6.4	11.3	NA	NA	NA	NA	10.5	6.8	5.6	9.1	7.1	NA	NA	NA	NA	NA
2	40	7.8	9.9	6.7	6.8	8.3	11.2	NA	NA	NA	NA	10.3	6.6	4.2	8.3	6.8	NA	NA	NA	NA	NA
2	60	8.9	10.0	6.5	5.4	7.2	10.3	NA	NA	NA	NA	11.0	7.4	5.2	7.5	6.1	NA	NA	NA	NA	NA
2	120	8.1	9.0	5.4	4.9	7.6	10.5	NA	NA	NA	NA	10.7	5.5	4.5	8.1	4.7	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: tPA activity

Units: IU/ml

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	0.80	0.40	0.30	0.30	0.70	0.30	0.70	0.30	0.30	1.00	0.60	BLQ	0.70	1.10	1.50	0.30	0.70	1.20	1.20	1.20
1	-30	0.70	0.50	0.40	0.30	0.70	0.40	0.90	0.30	0.40	1.10	0.60	0.20	0.60	1.20	1.60	0.30	1.00	1.30	1.40	1.30
1	1	1.40	3.00	0.70	0.70	1.50	1.50	1.10	0.80	1.00	2.50	1.70	0.70	1.40	1.90	3.00	1.10	2.00	2.50	2.00	2.00
1	5	NS	0.90	0.60	0.70	1.50	1.10	1.00	0.40	0.50	3.00	1.60	0.60	1.10	1.60	2.50	0.60	2.00	1.80	2.00	1.50
1	20	0.80	0.60	0.30	0.60	1.00	0.70	0.90	0.60	0.80	1.50	1.00	0.30	0.70	1.30	1.90	0.50	1.40	1.40	1.60	1.30
1	40	0.80	0.80	0.50	0.80	1.00	0.70	1.10	0.40	1.00	1.60	1.00	0.40	0.80	1.30	1.90	0.50	1.30	1.30	1.50	1.50
1	60	0.80	0.70	0.30	0.80	1.10	0.60	1.10	0.50	0.90	1.80	0.90	0.40	0.80	1.50	3.00	0.60	1.40	1.30	1.60	1.50
1	120	1.00	1.00	0.50	0.90	1.20	0.50	1.30	0.80	0.90	1.30	0.90	0.40	0.60	1.30	2.50	0.60	1.40	1.20	1.50	1.50
2	-60	0.50	0.50	BLQ	0.60	1.10	0.20	0.70	0.30	0.30	0.70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	0.50	0.50	BLQ	0.70	1.10	0.30	0.70	0.40	0.40	0.80	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	1.00	3.00	0.20	2.00	2.50	1.30	0.90	1.10	1.10	3.50	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	0.80	1.30	BLQ	1.40	2.50	0.90	0.50	0.70	0.80	3.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	0.70	1.10	BLQ	1.10	1.30	0.60	0.60	0.70	0.80	1.40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	0.70	0.80	BLQ	0.90	1.20	0.50	0.90	0.70	0.80	1.30	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	0.80	0.70	0.20	1.10	1.90	0.60	0.90	0.80	0.80	1.30	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	1.00	1.00	0.40	1.00	1.10	0.50	1.00	1.00	0.70	1.10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis. BLQ=Below limit of quantification

Assay: tPA activity

Units: IU/ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	0.8	0.5	0.4	0.3	1.0	0.2	1.0	0.9	BLQ	0.7	BLQ	0.2	0.6	0.8	0.5	0.3	0.2	0.4	0.3	0.9
1	-30	0.7	0.6	0.6	0.3	0.9	0.3	1.1	1.0	BLQ	0.7	BLQ	0.2	0.7	0.9	0.6	0.3	0.3	0.5	0.3	0.8
1	1	1.6	1.8	1.3	1.2	10.0	1.3	2.5	1.8	0.5	2.5	BLQ	1.0	5.0	6.0	1.6	1.1	0.8	1.5	0.9	1.6
1	5	1.0	1.0	0.9	0.8	9.0	0.8	1.8	1.6	BLQ	2.5	BLQ	0.5	3.0	3.5	1.2	0.7	0.6	1.1	0.7	1.3
1	20	0.7	0.8	0.6	0.5	1.5	0.5	1.1	1.1	BLQ	1.2	BLQ	0.3	1.1	1.6	0.8	0.4	0.5	0.7	0.4	1.1
1	40	0.7	0.8	0.8	0.6	1.1	0.5	1.3	1.3	BLQ	1.0	BLQ	0.3	0.8	1.1	0.7	0.4	0.6	0.8	0.4	1.1
1	60	0.7	1.5	0.8	0.7	0.9	0.6	1.2	1.3	BLQ	0.9	BLQ	0.2	0.9	0.9	0.7	0.4	0.5	0.9	0.4	1.2
1	120	0.8	0.9	0.8	0.5	0.7	0.6	1.2	1.4	0.2	1.0	BLQ	0.2	0.8	1.0	0.7	0.4	0.5	0.9	0.5	1.1
2	-60	0.5	0.4	0.6	0.4	0.6	BLQ	NA	NA	NA	NA	BLQ	0.2	0.6	0.8	0.5	NA	NA	NA	NA	NA
2	-30	0.5	0.7	0.8	0.5	0.7	BLQ	NA	NA	NA	NA	BLQ	0.2	0.8	0.9	0.5	NA	NA	NA	NA	NA
2	1	1.3	1.8	1.6	1.3	8.5	0.5	NA	NA	NA	NA	BLQ	1.0	4.5	NS	1.6	NA	NA	NA	NA	NA
2	5	0.9	1.5	1.2	0.9	6.0	0.3	NA	NA	NA	NA	BLQ	0.5	3.0	NS	1.1	NA	NA	NA	NA	NA
2	20	0.7	0.9	0.9	0.8	1.3	0.2	NA	NA	NA	NA	BLQ	0.2	1.2	1.1	0.7	NA	NA	NA	NA	NA
2	40	0.7	1.0	0.9	0.6	0.9	0.3	NA	NA	NA	NA	BLQ	0.2	0.9	1.0	0.8	NA	NA	NA	NA	NA
2	60	0.8	1.0	0.9	0.7	0.8	0.3	NA	NA	NA	NA	BLQ	0.2	1.0	0.9	0.8	NA	NA	NA	NA	NA
2	120	0.9	1.0	1.1	0.4	0.8	0.3	NA	NA	NA	NA	BLQ	0.2	0.7	1.2	0.7	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis. BLQ=Below limit of quantification

Assay: PAI-1antigen

Units: ng/ml

Visit	Time point	Claudicants																			
		001	**002**	**003**	**004**	**005**	**006**	**007**	**008**	**009**	**010**	**011**	**012**	**013**	**014**	**015**	**016**	**017**	**018**	**019**	**020**
1	-60	115	43.0	169	171	67.5	46.1	92.4	145.3	216.8	66.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	-30	35.0	75.0	195	119	71.5	48.0	39.9	167.5	147.1	54.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	1	290	134.1	189	63.6	58.2	27.8	63.4	169.2	130.5	84.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	233	63.4	57.6	125	115.4	30.7	79.7	54.5	61.5	67.3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	212	48.7	29.3	88.8	43.9	19.4	66.4	109.2	145.3	67.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	195	78.5	63.8	111	27.9	50.7	60.2	27.1	226.5	49.3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	310	49.4	117	55.1	14.6	52.9	32.8	150.9	213.5	73.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	120	105	36.3	135	85.9	109.1	35.3	47.7	115.0	199.1	73.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-60	132	117.2	69.3	107	121.0	43.4	119.8	139.6	211.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	116	168.5	76.2	134	148.2	48.9	60.2	159.1	194.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	145	29.3	54.7	56.4	132.7	33.3	62.0	28.6	139.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	109	229.2	68.1	130	225.8	29.3	11.5	97.3	233.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	50	49.2	138	114	78.2	27.2	16.7	109.6	135.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	119	73.4	106	63.5	92.6	27.5	34.5	115.6	122.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	229	67.3	61.8	73.0	175.4	30.6	29.2	111.9	175.3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	157	25.8	48.1	83.7	126.9	26.9	29.0	123.8	139.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: PAI-1 antigen

Units: ng/ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	65.9	74.1	77.4	57.3	63.3	45.4	NA	NA	NA	NA	106	103	14.7	NA	51.9	71.4	NA	NA	NA	NA
1	-30	49.8	107	63.2	66.2	43.7	42.9	NA	NA	NA	NA	111	90.9	14.6	NA	40.9	73.8	NA	NA	NA	NA
1	1	70.1	59.4	57.8	288	38.3	38.0	NA	NA	NA	NA	119	102	10.6	NA	46.5	73.8	NA	NA	NA	NA
1	5	52.4	75.3	54.8	69.1	41.8	47.6	NA	NA	NA	NA	127	71.4	12.1	NA	40.7	76.2	NA	NA	NA	NA
1	20	43.2	37.4	50.8	41.4	18.2	57.9	NA	NA	NA	NA	133	35.7	9.8	NA	54.4	66.0	NA	NA	NA	NA
1	40	177	64.6	42.9	58.4	34.4	66.3	NA	NA	NA	NA	118	52.4	10.4	NA	49.7	70.8	NA	NA	NA	NA
1	60	41.5	56.8	60.4	41.1	34.0	33.7	NA	NA	NA	NA	61.5	48.5	6.0	NA	60.7	43.9	NA	NA	NA	NA
1	120	62.1	256	46.9	54.0	30.7	72.5	NA	NA	NA	NA	82.5	61.7	8.4	NA	41.3	74.9	NA	NA	NA	NA
2	-60	153	NA	33.0	43.4	NA	60.3	NA	NA	NA	NA	139	70.1	9.5	NA	NA	NA	NA	NA	NA	NA
2	-30	146	NA	26.6	67.2	NA	48.0	NA	NA	NA	NA	137	108	11.5	NA	NA	NA	NA	NA	NA	NA
2	1	146	NA	71.9	63.2	NA	61.7	NA	NA	NA	NA	159	116	7.4	NA	NA	NA	NA	NA	NA	NA
2	5	127	NA	66.0	311	NA	59.9	NA	NA	NA	NA	167	141	8.6	NA	NA	NA	NA	NA	NA	NA
2	20	71.4	NA	63.7	37.9	NA	44.7	NA	NA	NA	NA	140	101	8.1	NA	NA	NA	NA	NA	NA	NA
2	40	89.3	NA	57.3	70.7	NA	60.3	NA	NA	NA	NA	136	74.8	8.0	NA	NA	NA	NA	NA	NA	NA
2	60	111	NA	46.8	79.9	NA	76.6	NA	NA	NA	NA	131	78.7	7.0	NA	NA	NA	NA	NA	NA	NA
2	120	108	NA	46.5	47.5	NA	74.6	NA	NA	NA	NA	110	109	7.2	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: PAI-1 activity

Units: IU/ml

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	12.6	15.0	32.7	25.8	13.3	25.0	7.3	27.3	39.9	12.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	-30	8.9	14.8	22.0	23.1	9.6	21.7	6.9	24.7	41.8	10.2	14.0	36.3	31.9	6.3	8.8	34.7	11.2	36.6	18.1	4.7
1	1	12.1	15.6	20.0	NA	9.0	13.7	5.4	16.9	35.6	11.7	10.4	31.1	34.9	4.6	9.1	30.8	8.9	25.6	19.8	6.0
1	5	18.4	8.3	17.7	15.5	6.5	14.4	8.3	18.7	30.2	14.4	10.2	30.5	28.4	4.3	6.4	29.1	8.6	29.4	24.1	6.1
1	20	14.5	9.6	14.5	13.2	8.8	12.3	6.9	17.2	26.0	11.3	9.6	29.0	25.0	4.6	7.9	22.8	7.9	32.1	17.8	5.7
1	40	16.3	10.1	21.1	12.0	7.6	11.5	5.9	11.5	30.3	11.3	8.7	26.9	23.5	4.0	8.3	24.5	6.6	28.1	12.4	6.8
1	60	15.0	8.4	13.2	7.2	7.6	13.2	6.8	12.2	22.6	10.0	10.0	26.9	15.1	3.6	5.0	24.1	6.8	15.6	14.4	5.0
1	120	12.3	12.6	17.6	7.3	7.2	16.7	6.1	12.6	28.1	8.3	12.2	27.9	25.1	4.1	6.9	18.8	9.5	32.2	9.3	5.7
2	-60	22.3	16.1	48.9	13.6	11.7	26.2	6.1	22.4	31.3	14.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	18.2	16.0	48.8	15.3	12.7	25.7	4.4	21.1	29.3	12.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	17.2	13.8	44.6	8.5	14.4	14.2	3.4	18.2	27.2	10.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	21.3	13.3	45.2	9.7	8.9	16.5	3.4	18.6	27.3	10.3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	17.8	11.5	44.1	8.6	10.6	13.4	3.8	18.3	23.8	13.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	12.5	11.7	35.6	6.5	9.5	11.5	5.1	15.6	27.2	12.3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	12.9	7.0	33.7	8.1	12.9	12.2	3.4	12.4	20.0	11.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	12.8	10.1	20.6	10.5	11.7	17.6	4.8	10.4	23.7	10.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: PAI-1 activity

Units: IU/ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	37	164	25	135	36	80	9	5	4	16	22	44	44	6	12	72	0	27	0	0
1	-30	45	162	41	123	24	76	2	10	0	26	9	20	21	5	39	56	0	27	7	24
1	1	37	142	29	128	30	71	NA	NA	NA	NA	12	20	13	14	11	66	NA	NA	NA	NA
1	5	45	114	29	125	65	90	NA	NA	NA	NA	15	15	21	12	72	88	NA	NA	NA	NA
1	20	0	108	27	118	15	80	NA	NA	NA	NA	8	13	19	9	12	34	NA	NA	NA	NA
1	40	47	128	33	116	66	92	NA	NA	NA	NA	11	19	26	8	27	82	NA	NA	NA	NA
1	60	29	154	23	95	23	56	0	2	2	22	6	15	37	0	36	65	0	23	3	23
1	120	45	172	41	121	11	92	17	10	6	35	12	26	37	27	40	55	0	64	16	18
2	-60	35	93	49	161	9	59	NA	NA	NA	NA	2	20	34	5	0	NA	NA	NA	NA	NA
2	-30	37	88	55	114	1	76	2	4	4	29	27	20	24	24	19	33	0	59	18	6
2	1	47	103	53	238	9	64	NA	NA	NA	NA	16	23	45	8	6	NA	NA	NA	NA	NA
2	5	39	100	37	87	9	70	NA	NA	NA	NA	16	16	34	9	31	NA	NA	NA	NA	NA
2	20	33	83	35	142	19	70	NA	NA	NA	NA	9	22	26	6	21	NA	NA	NA	NA	NA
2	40	39	110	96	169	11	58	NA	NA	NA	NA	20	32	21	23	7	NA	NA	NA	NA	NA
2	60	37	102	29	161	14	63	NA	NA	NA	NA	11	19	30	2	11	NA	NA	NA	NA	NA
2	120	58	119	61	135	19	70	NA	NA	NA	NA	29	36	29	114	7	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: tPA-PAI-1

Units: ng/ml

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	8.10	3.90	7.80	7.40	5.50	11.50	7.90	6.00	18.40	7.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	-30	8.10	4.90	7.20	9.10	6.00	8.20	8.40	8.00	16.00	7.10	9.7	14.5	7.1	6.1	9.8	15.4	10.1	7.5	3.7	2.9
1	1	7.90	5.20	5.70	4.00	3.30	8.40	6.50	7.10	16.60	7.60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	10.40	2.30	7.30	5.20	3.20	8.50	8.80	5.60	17.00	8.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	7.60	4.20	8.00	2.70	3.40	8.50	7.40	7.20	21.20	4.40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	7.70	4.20	7.20	5.50	2.80	4.30	7.80	5.40	18.60	4.90	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	7.70	3.60	2.20	4.40	2.60	5.10	3.80	3.90	16.80	5.30	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	120	10.80	3.40	5.00	2.20	2.80	4.60	4.20	6.00	19.60	6.10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-60	8.40	4.30	7.80	2.80	6.80	11.60	8.10	5.90	17.20	12.20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	9.30	5.20	7.10	5.90	7.00	7.20	4.20	4.50	17.10	11.90	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	10.20	3.60	7.00	3.10	6.30	8.50	5.50	4.70	17.00	10.50	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	9.60	3.20	7.60	3.30	4.40	7.60	7.50	5.40	16.00	11.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	8.50	5.20	13.30	4.40	4.10	6.60	6.10	3.90	19.40	9.50	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	8.70	4.10	10.30	2.10	6.20	7.00	7.30	4.80	14.20	9.70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	10.00	3.60	6.20	5.30	5.60	5.00	7.10	4.20	17.80	8.20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	7.80	4.20	7.70	4.90	4.00	7.70	6.40	4.50	14.80	8.80	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: tPA-PAI-1

Units: ng/ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	6.40	5.00	10.2	6.90	4.00	7.60	NA	NA	NA	NA	18.9	7.10	5.70	4.20	4.40	9.00	NA	NA	NA	NA
1	-30	4.40	7.00	8.70	6.80	3.70	9.40	2.20	4.30	11.0	9.40	18.0	7.50	4.50	5.00	4.20	11.8	8.00	5.00	7.90	10.2
1	1	3.30	6.90	13.0	6.40	4.30	4.80	NA	NA	NA	NA	25.2	6.60	5.30	5.70	4.10	9.50	NA	NA	NA	NA
1	5	4.70	6.40	9.40	5.70	4.20	7.70	NA	NA	NA	NA	33.9	5.70	4.90	5.90	4.10	10.6	NA	NA	NA	NA
1	20	2.70	6.30	7.40	5.40	3.90	6.10	NA	NA	NA	NA	31.8	5.60	5.00	4.80	4.20	9.00	NA	NA	NA	NA
1	40	5.00	3.00	9.30	4.60	3.60	8.00	NA	NA	NA	NA	25.5	6.60	5.00	4.50	4.30	10.4	NA	NA	NA	NA
1	60	3.90	3.80	8.10	2.70	3.00	5.60	NA	NA	NA	NA	21.3	6.60	5.20	4.00	4.10	7.80	NA	NA	NA	NA
1	120	3.30	5.70	8.00	3.30	3.90	7.40	NA	NA	NA	NA	19.8	6.40	3.80	4.20	4.00	6.60	NA	NA	NA	NA
2	-60	6.50	7.40	9.20	3.10	5.90	9.60	NA	NA	NA	NA	37.2	6.40	4.70	5.40	6.10	NA	NA	NA	NA	NA
2	-30	8.20	6.30	8.90	2.0	5.70	10.1	NA	NA	NA	NA	31.8	6.50	7.40	6.10	6.70	NA	NA	NA	NA	NA
2	1	6.80	7.10	8.80	3.00	5.10	7.80	NA	NA	NA	NA	36.3	8.00	3.70	6.40	6.00	NA	NA	NA	NA	NA
2	5	6.80	6.70	8.40	3.30	5.10	7.10	NA	NA	NA	NA	33.6	6.30	5.20	6.40	5.30	NA	NA	NA	NA	NA
2	20	6.50	6.00	8.40	2.90	5.60	6.90	NA	NA	NA	NA	30.3	7.10	4.70	5.40	6.30	NA	NA	NA	NA	NA
2	40	3.60	4.80	9.30	4.20	4.20	7.30	NA	NA	NA	NA	35.7	6.50	4.70	5.30	5.60	NA	NA	NA	NA	NA
2	60	3.60	4.70	6.90	3.10	4.90	7.20	NA	NA	NA	NA	25.2	6.50	4.50	5.00	5.50	NA	NA	NA	NA	NA
2	120	5.30	5.00	8.00	3.10	4.70	6.60	NA	NA	NA	NA	24.3	8.10	2.80	4.80	4.80	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: PAP

Units: ng/ml

Visit	Time point	Claudicants																			
		001	**002**	**003**	**004**	**005**	**006**	**007**	**008**	**009**	**010**	**011**	**012**	**013**	**014**	**015**	**016**	**017**	**018**	**019**	**020**
1	-60	409	338	253	433	344	311	925	498	465	686	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	-30	425	329	268	417	359	308	906	509	465	660	482	285	335	818	495	320	514	775	385	677
1	1	451	363	272	ns	378	336	914	509	491	682	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	447	ns	281	433	384	339	920	519	482	675	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	470	348	281	432	211	355	907	510	487	668	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	479	366	294	486	412	362	927	516	495	833	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	520	355	317	425	410	361	977	545	509	851	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	120	537	369	309	440	425	384	907	551	532	782	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-60	407	317	217	447	602	419	809	397	511	589	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	401	315	210	447	632	433	812	409	499	560	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	413	344	139	239	662	474	815	449	525	585	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	425	333	221	464	659	465	828	434	525	583	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	419	339	222	472	670	460	861	453	517	583	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	433	327	225	477	689	468	855	452	518	614	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	465	319	126	477	678	487	855	466	515	670	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	460	342	251	476	688	501	825	504	525	652	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: PAP

Units: ng/ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	474	378	251	450	548	334	NA	NA	NA	NA	197	329	364	264	515	NA	NA	NA	NA	NA
1	-30	476	364	261	445	489	326	476	843	399	534	186	312	357	260	511	391	243	288	255	301
1	1	500	371	279	458	508	370	NA	NA	NA	NA	197	337	385	286	543	NA	NA	NA	NA	NA
1	5	477	365	280	448	519	368	NA	NA	NA	NA	202	329	396	302	532	NA	NA	NA	NA	NA
1	20	473	373	282	450	523	355	NA	NA	NA	NA	195	328	414	297	508	NA	NA	NA	NA	NA
1	40	477	386	287	464	532	361	NA	NA	NA	NA	187	343	426	290	509	NA	NA	NA	NA	NA
1	60	507	461	278	447	676	366	NA	NA	NA	NA	180	334	425	299	643	NA	NA	NA	NA	NA
1	120	485	473	293	440	575	377	NA	NA	NA	NA	173	343	446	285	663	NA	NA	NA	NA	NA
2	-60	338	459	304	482	429	283	NA	NA	NA	NA	215	307	345	270	508	NA	NA	NA	NA	NA
2	-30	345	466	331	497	435	292	NA	NA	NA	NA	203	303	339	276	523	NA	NA	NA	NA	NA
2	1	382	507	344	512	466	296	NA	NA	NA	NA	208	320	400	295	553	NA	NA	NA	NA	NA
2	5	362	489	345	509	469	300	NA	NA	NA	NA	215	329	400	305	526	NA	NA	NA	NA	NA
2	20	361	473	341	507	495	291	NA	NA	NA	NA	204	316	412	298	540	NA	NA	NA	NA	NA
2	40	365	497	353	512	507	303	NA	NA	NA	NA	203	318	448	309	547	NA	NA	NA	NA	NA
2	60	355	536	376	531	546	294	NA	NA	NA	NA	210	309	421	311	568	NA	NA	NA	NA	NA
2	120	361	544	391	520	522	309	NA	NA	NA	NA	192	310	455	292	550	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: TAFI antigen

Units: ng/ml

Visit	Time point	Claudicants																			
		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020
1	-60	5.9	3.6	5.4	3.0	3.0	4.6	4.0	4.3	2.1	2.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	-30	6.4	3.2	5.3	3.6	4.2	4.6	3.7	3.9	2.0	2.9	4.2	3.3	5.7	4.4	5.7	4.2	3.7	5.2	3.0	4.2
1	1	6.7	3.5	4.7	3.7	2.6	4.9	3.8	4.2	2.9	3.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	5	7.2	1.5	5.1	4.8	3.4	4.6	3.7	4.5	2.9	3.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	20	6.4	3.3	5.4	3.2	3.4	4.4	3.6	4.4	2.9	3.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	40	6.1	3.4	4.9	2.3	2.5	5.0	3.8	4.3	2.7	2.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	60	8.1	3.7	4.7	2.4	2.6	5.0	4.2	4.1	2.5	2.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	120	6.7	3.3	4.4	3.0	2.8	5.2	4.1	4.3	2.3	3.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-60	5.9	3.1	4.6	2.1	3.0	4.9	3.5	3.6	2.3	3.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	-30	6.7	3.1	4.7	3.3	2.7	4.3	3.2	3.3	2.0	4.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	1	6.1	3.5	5.3	3.7	3.4	4.9	3.5	2.9	2.3	4.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	5	5.9	3.1	5.5	4.5	3.6	5.0	3.1	4.1	2.9	3.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	20	6.2	3.0	5.3	3.7	3.1	4.6	3.3	3.5	2.5	3.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	40	5.9	3.1	4.1	3.4	2.8	4.4	3.4	3.4	2.1	2.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	60	7.8	3.6	4.6	3.7	3.0	5.0	4.1	2.7	2.0	3.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	120	6.1	3.0	4.8	3.0	3.7	5.2	3.9	2.8	1.9	3.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Assay: TAFI antigen

Units: ng/ml

Visit	Time point	Smoking Controls										Non-smoking Controls									
		101	103	105	107	109	111	113	115	117	119	201	203	205	207	209	211	213	215	217	219
1	-60	7.3	6.1	5.0	3.0	4.2	3.4	NA	NA	NA	NA	3.2	5.2	4.9	3.4	2.6	3.6	NA	NA	NA	NA
1	-30	5.4	5.5	5.6	4.0	3.9	2.7	4.6	5.8	6.4	5.2	2.7	5.1	4.2	2.9	2.7	4.5	2.3	4.9	4.6	5.4
1	1	5.8	4.9	6.5	3.9	3.4	3.0	NA	NA	NA	NA	2.8	4.8	5.5	3.6	2.9	3.9	NA	NA	NA	NA
1	5	4.6	5.2	5.1	3.1	4.7	3.8	NA	NA	NA	NA	3.4	4.9	5.5	4.2	2.6	4.6	NA	NA	NA	NA
1	20	7.8	5.2	5.7	3.6	4.4	3.7	NA	NA	NA	NA	3.4	5.2	6.0	3.6	3.1	5.5	NA	NA	NA	NA
1	40	7.0	5.1	4.4	3.3	4.4	4.7	NA	NA	NA	NA	2.8	5.2	5.2	3.3	2.9	4.0	NA	NA	NA	NA
1	60	6.9	7.2	5.8	2.5	3.3	3.4	NA	NA	NA	NA	3.8	5.5	4.8	3.2	2.5	4.6	NA	NA	NA	NA
1	120	6.3	5.4	6.4	2.8	2.6	3.1	NA	NA	NA	NA	2.9	6.1	4.1	3.3	2.0	4.2	NA	NA	NA	NA
2	-60	6.7	4.2	6.0	2.9	3.6	3.5	NA	NA	NA	NA	3.1	4.9	4.9	2.9	2.8	NA	NA	NA	NA	NA
2	-30	6.2	4.0	5.1	2.8	4.1	3.9	NA	NA	NA	NA	2.7	6.3	5.3	2.3	3.0	NA	NA	NA	NA	NA
2	1	6.8	5.1	5.3	2.9	5.3	3.2	NA	NA	NA	NA	3.1	4.2	4.5	3.4	3.5	NA	NA	NA	NA	NA
2	5	5.5	4.7	6.3	4.0	4.3	3.9	NA	NA	NA	NA	2.6	5.1	5.7	4.1	2.8	NA	NA	NA	NA	NA
2	20	5.2	4.2	4.4	3.3	4.1	4.7	NA	NA	NA	NA	2.9	5.3	4.9	3.5	2.8	NA	NA	NA	NA	NA
2	40	4.9	4.0	5.5	2.9	4.6	3.3	NA	NA	NA	NA	3.1	6.7	5.8	3.5	3.1	NA	NA	NA	NA	NA
2	60	6.5	4.1	5.6	3.0	4.2	3.5	NA	NA	NA	NA	3.7	4.8	4.3	3.6	3.0	NA	NA	NA	NA	NA
2	120	6.4	4.2	4.1	2.4	4.3	3.5	NA	NA	NA	NA	3.0	4.7	4.1	3.2	3.1	NA	NA	NA	NA	NA

NA=no sample received / unsuitable for analysis

Appendix 3

Published Articles

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Exercise in Claudicants is Accompanied by Excessive Thrombin Generation

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Background: *exercise in IC leads to ischaemia-reperfusion injury of leg muscles and a systemic inflammatory response, but the effect of on coagulation is unknown.*
Objective: *to compare the effect of exercise on thrombin formation and fibrin turnover in patients with IC (n = 10), and age and sex matched smokers ([S] n = 5) and non-smokers ([NS] n = 5) without peripheral vascular disease.*
Methods: *blood was taken from subjects 60 and 30 min before, and 1, 5, 20, 40, 60 and 120 min after, treadmill exercise. Markers of thrombin generation (thrombin-antithrombin complexes [TAT] and prothrombin fragments 1 + 2 [PF1 + 2]) and fibrin turnover (D-dimer and fibrin degradation products [FbDP]) were assayed at each time point.*
Results: *following exercise, thrombin generation was significantly greater in the claudicant group compared to the control groups (Area Under Curve [AUC] post exercise IC vs S vs NS; TAT 3960 vs 1623 vs 1476 vs = 0.007 Kruskal–Wallis [KW]; PF1 + 2 163 vs 107 vs 123 p = 0.024 KW). Pre and post-exercise, fibrin turnover in claudicants was similar to smoking controls, but higher than non-smoking controls. (AUC post exercise IC vs NS; D-dimer 6340 vs 2754 p = 0.055 Mann–Whitney U[MW]; FbDP 45113 vs 21511 p = 0.009 MW).*
Conclusion: *when compared to non-claudicants, exercise in IC is associated with excessive production of thrombin. Despite this, claudicants have a similar level of fibrin turnover suggesting a possible defect in fibrinolysis. This prothrombotic state may contribute to the excess thrombotic morbidity and mortality suffered by claudicants.*

Key Words: Coagulation; Claudication; Ischaemia-reperfusion.

Introduction

Intermittent claudication affects 5% of the middle-aged (55–75 years) European population. While only 1–2% claudicants progress to critical limb ischaemia each year, they suffer an annual cardiovascular mortality that is 3–4 times greater than that observed in an age and sex matched non-claudicant population.[1] Although this may simply reflect the burden of multi-system atherosclerosis, even when the extent of concomitant coronary and extracranial vascular disease is taken into account, claudicants still appear to suffer an excess of thrombotic vascular events.[2] The reasons for this remain ill-defined. However, several groups have shown that when claudicants walk and then rest they suffer repeated episodes of leg muscle ischaemia- and reperfusion injury (IRI), which in turn leads to the development of a systemic inflammatory response (SIR). It has been suggested that this SIR injures the endothelium, accelerates atherosclerosis and may increase thrombotic risk.[3] However, to date, the effect of exercise in claudicants on haemostasis has not been adequately defined. The aim of the present study, therefore, was to compare the effect of treadmill exercise on thrombin production and fibrin turnover in claudicants and controls without peripheral arterial disease (PAD).

Methods

Ten smoking male claudicants and 10 aged-matched (±3 years) male controls (five smoking, five non-smoking) without PAD were recruited (see Table 1 for inclusion and exclusion criteria and Table 2 for baseline patient and control characteristics). To avoid walking patients were transported to hospital by taxi, and then to a quiet, temperature-controlled (25–28 °C) room within the Vascular Studies Unit (VSU) by wheelchair. Subjects were asked to abstain from smoking, or eating a heavy meal for 1 h prior to arrival, and avoid unaccustomed exercise in the

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Table 1. Inclusion and exclusion criteria.

Inclusion criteria		Exclusion criteria
Claudicants	Controls	
Male		Diabetes mellitus
Walking unrestricted by co-morbidity		
Claudicant, as defined by Edinburgh Claudication Questionnaire, for 6 months	No history of peripheral arterial disease	Haemoglobin concentration <11 g/dt Current viral infection Current therapy with warfarin
Resting ankle-brachial pressure index <0.8	Palpable pedal pulses, and resting ankle-brachial pressure index 1.0	
Absolute claudication distance 25–300 m		
Smoker of equivalent of at least 20 cigarettes per day, for >10 years	*Smoker of equivalent of at least 20 cigarettes per day, for >10 years †Non-smoker for >15 years	

*Smoking controls only.
†Non-smoking controls only.

Table 2. Patient details.

	Claudicants $n=10$	Controls		
		Smokers $n=5$	Non-smokers $n=5$	p
Age, years (IQR)	61 (58–70)	69 (60–70)	69 (60–72)	0.57*
Body mass index, kg/m^2 (IQR)	23 (21–25)	25 (23–29)	27 (23–34)	0.21*
Smoking				
Pack years	42	50	0	0.77*
Medical history				
IHD	0	0	1	
CVD	0	0	0	
HT	0	0	0	
COAD	6	0	0	0.01†
Medication				
Aspirin	9	2	1	0.02†
β-blocker	0	0	1	
ACEI	0	0	1	
PAD				
Lowest ABPI (median)	0.56	1.07	1.13	<0.001*
Walking distance (median), m	112	174	174	0.005*
Cholesterol (mmol/l)	187	197	168	0.33*

IQR = interquartile range; IHD = ischaemic heart disease (angina, myocardial infarction or coronary bypass surgery); CVD = cerebrovascular disease (transient ischaemic attack or stroke); HT = hypertension; COAD = chronic obstructive airway disease; Ca blocker = calcium channel blocker; ACEI = ACE inhibitor.
*Kruskal–Wallis.
†Fishers Exact Test.

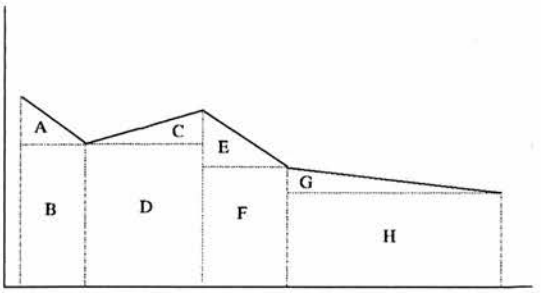

Fig. 1. Calculation of the area under the curve (AUC). Total AUC = area of A + B + C + D + E + F + G + H.

preceding 24 h. While in the VSU the only exercise or movement allowed was on the treadmill; for example, if patients required the toilet they were taken by wheelchair. All the studies commenced between 08.30 and 09.00 am. An 18 gauge cannula inserted into a right antecubital fossa vein, which was kept patent with boluses of 0.9% saline. After 60 and 90 min of sitting, blood was drawn from the cannula. After sitting for a total of 120 min, subjects underwent a treadmill exercise test (3.5 km/h at a gradient of 12) until the absolute claudication distance was reached (claudicants), or for 3 min (controls). At the end of the exercise blood was collected at 1, 5, 20, 40, 60 and 120 min. Fibrinogen, thrombin-antithrombin (TAT) complexes and prothrombin fragments (PF) 1 + 2 (all Dade-Behring, IL, USA); D-dimer (American Diagnostica, CT, U.S.A.); fibrin-degradation products FbDPs) (Organon Teknika, Turnhout, Belgium) were assayed at each time point using commercial assays. Reference ranges for these assays are appended.

SPSS version 10.0.5 package (Statistical Package for Social Sciences Inc, Chicago, IL, U.S.A.) was used for all statistical analysis. Pre-exercise values were calculated by taking the average of the results of the two pre-exercise samples. For samples taken post-exercise, the area under the curve (AUC) was calculated for each individual, at each visit (Fig. 1). Subjects mean

pre-exercise, and post-exercise values were calculated from the two visits, so that each individual had one pre-exercise, and one post-exercise value for statistical analysis. Groups were compared pre- and post-exercise with the Kruskal–Wallis test. Where this returned a value of $p < 0.05$, the Mann–Whitney *U*-test was used to identify the groups that had significantly different values. A value of $p < 0.05$ was taken to be statistically significant.

Results

The groups were well controlled for age and body mass index (Table 2). Significantly more claudicants than controls were taking aspirin, which was to be expected. There were also significantly more claudicants with chronic obstructive airways disease than in either control group, even though the smoking controls had a similar smoking history. At baseline, fibrinogen concentrations were significantly higher in claudicants than the non-smoking controls and trended higher than in smoking controls. These differences persisted after exercise. All groups showed a small rise in fibrinogen concentrations with exercise (Fig. 2). At baseline, claudicants and the smoking controls had similar levels of TAT and PF1 + 2. However, treadmill exercise lead to significantly higher levels of TAT and PF1 + 2 in the claudicant group than the controls (Figs 3 and 4). At baseline and post-exercise,

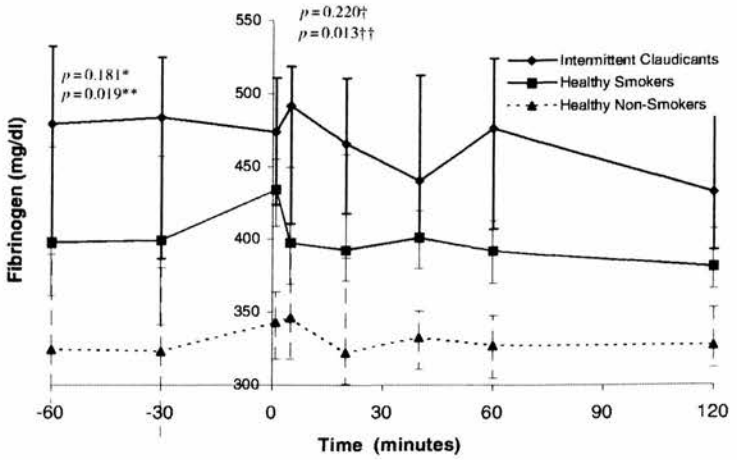

Fig. 2. Plot of fibrinogen with time, for the three subject groups. *Average pre-exercise, claudicants vs smoking controls (Mann–Whitney *U*). **Average pre-exercise, claudicants vs non-smoking controls (Mann–Whitney *U*). †AUC post-exercise, claudicants vs smoking controls (Mann–Whitney *U*). ††AUC post-exercise, claudicants vs non-smoking controls (Mann–Whitney *U*).

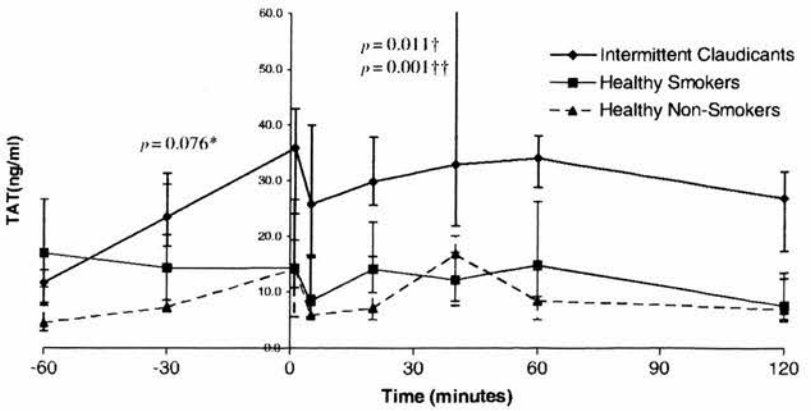

Fig. 3. Plot of thrombin-antithrombin complexes with time, for the three subject groups. *Average pre-exercise, between three groups (Kruskal–Wallis). †AUC post-exercise, claudicants vs smoking controls (Mann–Whitney *U*). ††AUC post-exercise, claudicants vs non-smoking controls (Mann–Whitney *U*).

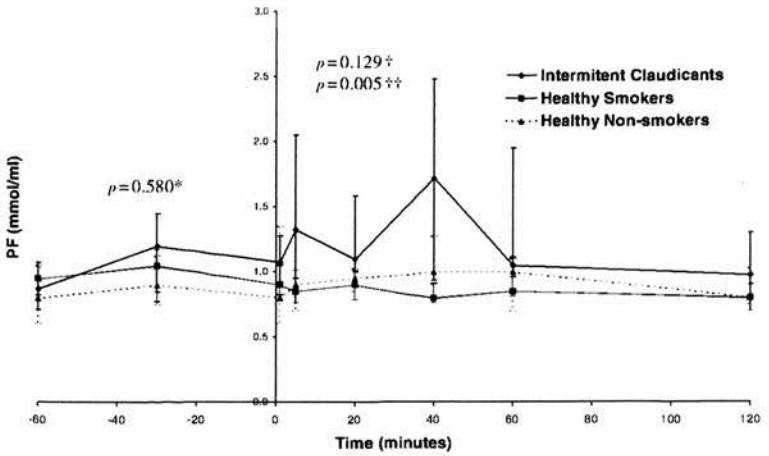

Fig. 4. Plot of prothrombin fragments 1 + 2 with time, for the three subject groups. *Average pre-exercise, between three groups (Kruskal–Wallis). †AUC post-exercise, claudicants vs smoking controls (Mann–Whitney *U*). ††AUC post-exercise, claudicants vs non-smoking controls (Mann–Whitney *U*).

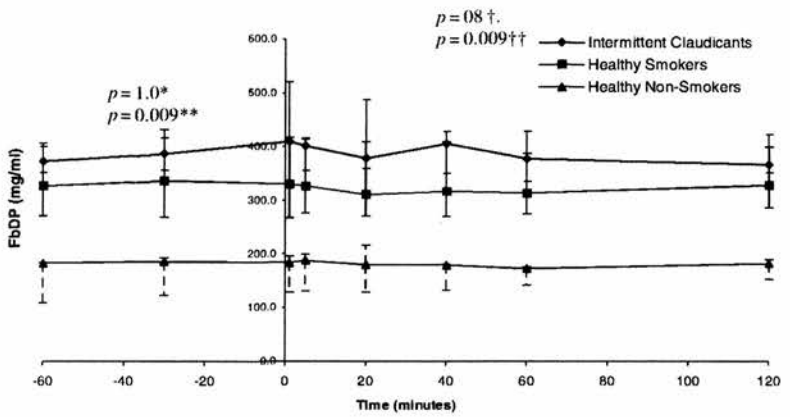

Fig. 5. Plot of fibrin-degradation products with time, for the three subject groups. *Average pre-exercise, claudicants vs smoking controls (Mann–Whitney *U*). **AUC pre-exercise, claudicants vs non-smoking controls (Mann–Whitney *U*). †AUC post-exercise, claudicants vs smoking controls (Mann–Whitney *U*). ††AUC post-exercise, claudicants vs non-smoking controls (Mann–Whitney *U*).

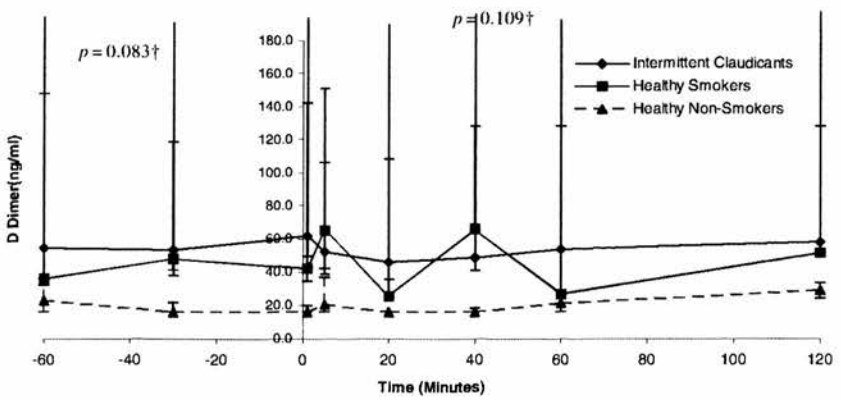

Fig. 6. Plot of d-dimer with time, for the three subject groups. *Average pre-exercise, between three groups (Kruskal–Wallis). †AUC post-exercise, between three groups (Kruskal–Wallis).

FDP and D-dimer levels were similar in the claudicant group, and the smoking controls. Claudicants had consistently higher levels than the non-smoking controls (Figs 5 and 6).

Discussion

The coagulation markers measured, and their role in the haemostatic system, are shown in Fig. 7. The main findings of the present study are that treadmill exercise in claudicants is associated with excessive thrombin generation and that this is not accompanied by an increase in fibrin turnover, which suggests a defect in fibrinolysis. This pro-thrombotic response to exercise was not seen in a carefully matched control group.

The effect of exercise on thrombin generation in claudicants and controls has been examined in three previous studies (Table 3). Two studies observed high baseline TAT and PF1 + 2 but showed no effect of exercise.[4,5] However, the patients in these studies were incompletely described or controlled for factors such diabetics, smoking or hypertension that are known to influence thrombin generation. The third study, which did include a well-matched control group, showed similar levels of thrombin generation at baseline, with a significant increase post-exercise in the claudicant group.[6]

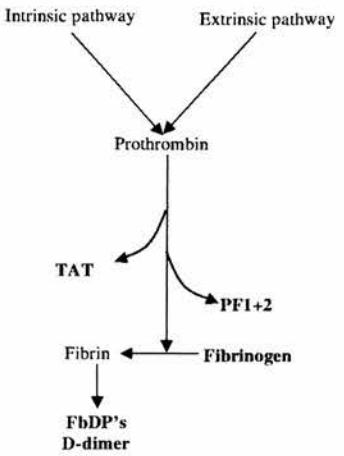

Fig. 7. Simplified depiction of the coagulation cascade, with the measured markers highlighted. TAT = thrombin-antithrombin complexes. PF1 + 2 = prothrombin fragments 1 + 2. FbDP's = fibrin degradation products.

Similar studies of patient with coronary artery disease (CAD) have shown no increase in thrombin generation with exercise, suggesting that the changes observed in the present study may be due to leg muscle IRI rather than simply the presence of generalised atherosclerosis.[7,8] However, further detailed work looking at coagulation, fibrinolysis and endothelial cell function to elucidate the exact mechanisms underlying the current observations is required and currently underway in this department.

One potential criticism of this study is the poor matching of the groups with respect to medical and drug history. However, a previous study of patients with CAD found that aspirin had no effect on thrombin generation.[7] Additionally, although the claudicants had a higher prevalence of chronic obstructive airways disease, this was never a restricting factor in the treadmill exercise.

What is the clinical importance of these findings? Firstly, the discovery of this pro-thrombotic diathesis in patients with claudication re-emphasises the need to institute best medical therapy (BMT) in all PAD patients.[9,10] Secondly, while it has been shown that long-term compliance with an exercise programme can improve walking distance[11] and ameliorate the SIR[12] in highly selected claudicants, present data suggest that in certain circumstances lower limb exercise

Table 3. Summary of previous studies examining thrombin generation in claudicants.

Author and year	Subjects	Sampling time points	Baseline differences	Effect of exercise	Notes
De Buyzere, 1993[4]	34 claudicants 30 age, sex-matched controls	30 min rest before sampling Sampled "before and after".	TAT, PF higher in claudicants	No change in either group with exercise	No mention made of number of diabetics, smokers, or hypertensives
Herren, 1994[5]	22 claudicants 13 age, sex-matched controls	3 min before and after exercise. No mention of rest prior to sampling	TAT, PF higher in claudicants	No change in either group with exercise	Groups poorly matched for smoking, hypertension, hyperlipidaemia.
Mustonen, 1997[6]	15 claudicants 15 age, sex matched controls	15 min rest. Sampling before, and immediately after exercise	TAT levels equal in claudicants and controls	Increase in TAT in claudicants, no change in controls	Matched for smoking, hypertension, hyperlipidaemia and diabetes mellitus

could be harmful. Worryingly, while exercise therapy has been shown to reduce mortality in patients with coronary artery disease, no such benefit has yet been demonstrated in patients with PAD.[13] At present the effect of revascularisation, either by means of angioplasty or bypass, on the prothrombotic diathesis observed in the present study is unknown. However, one could hypothesise that by restoring normal arterial flow and abolishing calf muscle IRI, such procedures might ameliorate SIRS and the haemostatic changes seen with exercise seen in patients treated medically. We are currently examining these issues within the confines of the U.K.-based, multi-centre, Exercise versus Angioplasty in Claudication Trial (EXACT) where claudicants are being randomised to BMT, BMT plus angioplasty and BMT plus supervised exercise. By combining data from these basic science and clinical studies it should be possible to tailor therapy to the individual patient in order to maximise safety, clinical and cost-effectiveness.

In conclusion, exercise in claudicants is associated with excessive thrombin generation, which is not seen in non-claudicants. Further work is required to determine whether this has long-term clinical consequences or should influence management.

Acknowledgements

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Clinical review

Management of peripheral arterial disease in primary care

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Best medical treatment for peripheral arterial disease, including managing hypertension and diabetes, reduces morbidity and mortality and can obviate the need for invasive intervention

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One in five of the middle aged (65-75 years) population of the United Kingdom have evidence of peripheral arterial disease on clinical examination, although only a quarter of them have symptoms. The most common symptom is muscle pain in the lower limbs on exercise—intermittent claudication.[1] Invasive interventions (angioplasty, stenting, surgery) undoubtedly have a role in the management of peripheral arterial disease. However, in common with coronary artery disease, the morbidity and mortality associated with peripheral arterial disease can be greatly reduced, and the results of intervention significantly improved, by the institution of so called "best medical treatment," much of which can be implemented in primary care.

Summary points

- Diagnosis of peripheral arterial disease is based mainly on the history, with examination and ankle brachial pressure index being used to confirm and localise the disease
- Peripheral arterial disease is a marker for systemic atherosclerosis; the risk to the limb in claudication is low, but the risk to life is high
- Patients with intermittent claudication should initially be treated with "best medical treatment"; some patients may be candidates for percutaneous angioplasty, but this treatment is not based on evidence
- Patients should be referred to a vascular surgeon if there is doubt about the diagnosis or evidence of aortoiliac disease or if the patient has not responded to best medical treatment or has severe disease

Sources and selection criteria

We used Medline to identify recent reviews and articles on the epidemiology, assessment, and treatment of peripheral arterial disease and intermittent claudication, by using the terms "intermittent claudication," "peripheral arterial disease," and "peripheral vascular disease." We also consulted standard textbooks, national and local guidelines, and service frameworks.

Diagnosis and assessment

A diagnosis of intermittent claudication can usually be made on the basis of the history—the Edinburgh claudication questionnaire is highly specific (91%) and sensitive (99%) for the condition (table A on bmj.com).[2] The differential diagnosis includes both venous and neurogenic claudication (table 1). Examination usually reveals weak or absent pulses, and further investigations (duplex ultrasonography, angiography) are usually reserved for the small minority of patients in whom invasive intervention is being considered (fig 1).

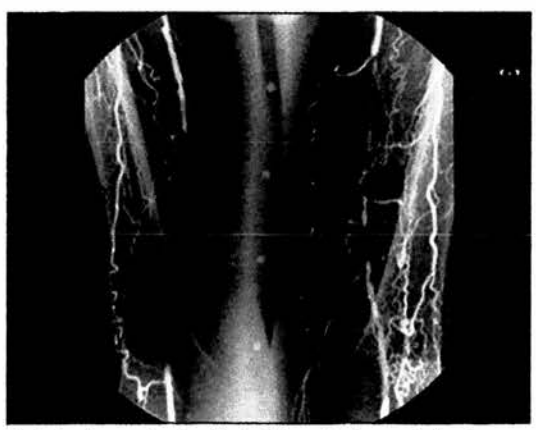

Fig 1 Angiogram showing bilateral femoral artery occlusions in a patient with claudication

The rationale for best medical treatment

Contrary to popular belief, the risk of a person with claudication progressing to critical limb ischaemia and needing amputation is low (< 1% a year). However, the risk of death, mainly from coronary and cerebrovascular events, is high (5-10% a year), some three to four times greater than that of an age and sex matched population without claudication (fig 2 and fig A on bmj.com[15]). Initial management should consist of modification of vascular risk factors and implementation of best medical treatment in the expectation that this will extend life, reduce still further the risk of critical limb ischaemia, and

Extra references, tables, figure, and information are on bmj.com

improve the patient's functional status. Only when best medical treatment has been instituted and given sufficient time to take effect should endovascular or surgical intervention be considered, as most patients' symptoms improve with best medical treatment to a point where invasive intervention is no longer needed.[3] Best medical treatment is beneficial even in patients who eventually need invasive treatment, as the safety, immediate success, and durability of intervention is greatly improved in patients who adhere to best medical treatment.[4,5]

Components of best medical treatment

Table 2 summarises the components of best medical treatment and their effects on peripheral arterial disease, vascular events, and mortality.

Smoking cessation

Complete and permanent cessation of smoking is by far the single most important factor determining the outcome of patients with intermittent claudication.[w6] Unfortunately, rates of cessation after simple oral or written advice from a doctor are as low as 13% at two years.[6] Randomised controlled trials have shown that nicotine replacement treatment approximately doubles the cessation rate in unselected smokers.[w2] Bupropion has a similar benefit when used with intensive support.[w3] Both treatments are now available on prescription, and every patient with claudication should be offered nicotine replacement treatment in the first instance. Not all nicotine replacement preparations (patches, gum, sprays) are the same, and if one preparation is unsuccessful then other preparations, or combinations with different delivery profiles, should be tried. The Cochrane group found smoking classes but not alternative therapies (hypnotherapy, acupuncture, or "aversive smoking") to be beneficial.[7-9,w4]

Antiplatelet agents

The Antiplatelet Trialists' Collaboration showed that prescription of an antiplatelet agent, usually aspirin, reduced vascular death in patients with any manifestation of atherosclerotic disease by about 25% and that antiplatelet agents were equally effective in patients who present with coronary artery disease and with peripheral arterial disease.[10,w8] Some indirect evidence shows that some antiplatelet agents may also improve walking distance in people with claudication.[w10] Clopidogrel is at least as effective as, and possibly more effective than, aspirin in patients with peripheral arterial disease and has a better side effect profile.[w9] However, it is much more expensive and is generally reserved for the sizeable minority of patients with peripheral arterial disease who cannot take aspirin or who continue to have events on aspirin. No data exist to support the routine use of combination treatment (aspirin and clopidogrel) in patients with peripheral arterial disease, but trials are under way.

Management of diabetes mellitus

Diagnosis of type 2 diabetes, or its exclusion, is important in patients with peripheral arterial disease (box), but this is not straightforward.[11] A threshold of fasting glucose >7.0 mmol/l, as recommended by Diabetes UK, should be supported by symptoms of diabetes and may miss a large number of asymptomatic patients (20-30%). The oral glucose tolerance test is the "gold standard" but is logistically difficult. In practice, random blood glucose may be the easiest measure to obtain; a random blood glucose >11.1 mmol/l (plasma glucose performed in an accredited laboratory not finger prick, capillary glucose) is diagnostic of type 2 diabetes, and a random blood glucose of 7.0-11.1 mmol/l should followed with an oral glucose tolerance test.

Table 1 Differential diagnosis of intermittent claudication

Characteristic	Intermittent claudication	Venous claudication	Nerve root pain
Quality of pain	Cramping	"Bursting"	Electric shock-like
Onset	Gradual, consistent	Gradual, can be immediate	Can be immediate, inconsistent
Relieved by	Standing still	Elevation of leg	Sitting down, bending forward
Location	Muscle groups (buttock, thigh, calf)	Whole leg	Poorly localised, can affect whole leg
Legs affected	Usually one	Usually one	Often both

Hypertension

The benefit of treating hypertension in terms of reducing stroke and coronary events is well accepted; data indicate a target of less than 140/85 mm Hg for non-diabetic patients and 140/80 mm Hg for patients with type 2 diabetes.[w13] However, in the short term a reduction in blood pressure may worsen intermittent claudication. This is true of whatever drug treatment has been used, and no evidence exists that β blockers are particularly culpable.[12] The heart outcomes prevention evaluation study has shown that ramipril, an angiotensin converting enzyme inhibitor, reduces cardiovascular morbidity and mortality in patients with peripheral arterial disease by around 25%.[w15,w16] Patients did not have to be hypertensive to be included in the study, and the observed risk reduction could not be accounted for by the relatively modest reduction in blood pressure. The implication of the heart outcomes prevention evaluation study is that most patients with peripheral arterial disease would benefit from an angiotensin converting enzyme inhibitor, provided that treatment is not associated with a deterioration of renal function due to occult renal artery stenosis.

Exercise

A recent Cochrane review has shown that exercise treatment can produce a significant and clinically meaningful increase in walking distance (150%) in most people with claudication who adhere to it.[w20] Although the exact

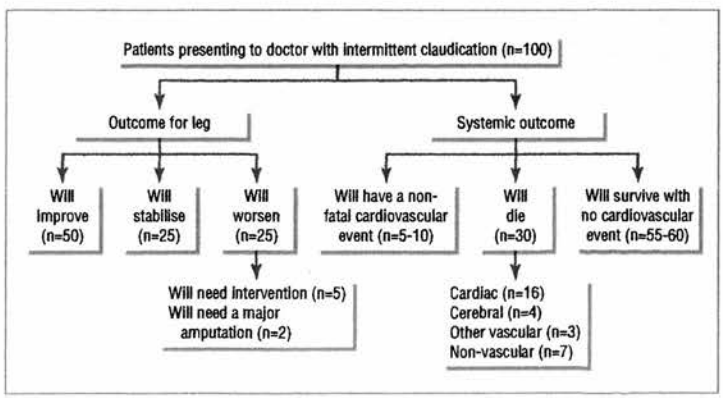

Fig 2 Outcome for patients with intermittent claudication over five years[14]

Table 2 Components of best medical treatment in peripheral arterial disease

Component	Recommendation	Effect on mortality or vascular events	Effect on peripheral arterial disease
Smoking cessation	Repeated advice[w1*] Nicotine replacement therapy or bupropion[w2,w3*] Behavioural therapy (smoking cessation classes)[w4*]	Cessation leads to a reduction in 10 year mortality from 54% to 18%[w5]	Rest pain in 0% of quitters compared with 16% of continued smokers at seven years[w5]
Reduction in cholesterol	All patients to be on a statin to achieve a 25% reduction in cholesterol[w6] Additional treatment may be needed if HDL is low or triglycerides are high (referral to lipid clinic)	RR=0.81 (0.72 to 0.87) for major vascular event (myocardial infarction, stroke, or revascularisation)[w6]	No evidence of clinical benefit[w7]
Antiplatelet agent	Aspirin 75 mg daily[w8] Clopidogrel 75 daily if intolerant of aspirin[w9]	22% reduction in vascular events[w8]	Possible improvement in walking distance[w10]
Treatment of diabetes mellitus	Screen for type 2 diabetes[w11*,w12*]	Intensive control with insulin or sulphonylurea leads to RR=0.94 (0.8 to 1.1) for total mortality[w11] Intensive control with metformin in overweight patients leads to RR=0.64 (0.45 to 0.91) for total mortality[w12]	RR=0.51 (0.01 to 19.64) for risk of lower limb amputation[w11*]
Blood pressure	Reduce blood pressure to <140/85 mm Hg[w13*]	RR=0.87 (0.81 to 0.94) for total mortality[w14*]	Not known
ACE inhibitors	Should be considered in all patients, even if normotensive[w15*,w18*]	RR=0.73 (0.61 to 0.86) for composite endpoint of myocardial infarction, stroke, and cardiovascular death (in diabetes RR=0.75 (0.64 to 0.88))[w15*,w16*]	Not known
Exercise	Patients with lower limb disease should be given written advice about exercise and enrolled in a supervised programme if available[w17,w18]	24% reduction (primary and secondary prevention) in cardiovascular mortality[w19*]	Improvement in walking distance of 150%[w20]
Cilostazol	Useful addition in patients who have unacceptable symptoms despite adherence to best medical therapy[w21-w23]	Unknown	Significant increase in walking distance[w21-w23]

ACE=angiotensin converting enzyme; HDL=high density lipoprotein cholesterol; RR=relative risk (95% confidence interval).
*Evidence from patients without peripheral arterial disease.

mechanisms by which exercise leads to clinical improvement have not been precisely defined, several factors that help to maximise benefit from exercise treatment have been identified (table B on bmj.com). The clinical effectiveness and cost effectiveness of best medical treatment, best medical treatment plus supervised exercise, and best medical treatment plus angioplasty are currently being evaluated in the exercise versus angioplasty in claudication trial funded by Health Technology Assessment.

Reduction in cholesterol

The heart protection study has shown that lowering total cholesterol and low density lipoprotein cholesterol by 25% with a statin reduces cardiovascular mortality and morbidity in patients with peripheral arterial disease by around a quarter, irrespective of age, sex, or baseline cholesterol concentration.[w6] The implication is that every patient with peripheral arterial disease should be treated with a statin. The lipid profile should be measured before and six weeks after starting treatment, to ensure that a 25% reduction in cholesterol is being achieved and to identify those few patients with very high cholesterol concentrations or hypertriglyceridaemia who may benefit from referral to a specialist lipid clinic.

Rationale for screening for diabetes mellitus in intermittent claudication

- Up to 20% of patients with intermittent claudication have diabetes; in up to 50% of cases this may be undiagnosed at the time of presentation
- The United Kingdom prospective diabetes study has shown that intensive glycaemic control reduces the microvascular complications of type 2 diabetes and that the use of metformin reduces macrovascular complications in overweight people with diabetes[w8,w9]
- Most studies show that diabetes is a powerful risk factor for progression to critical limb ischaemia[16]
- Patients with diabetes should have tighter limits placed on blood pressure and, possibly, lipid concentrations[17 18]
- Diabetic patients are more likely to have spuriously high ankle pressures[19]
- Diabetic patients respond less well to surgical intervention but gain a greater benefit from medical treatments for cardiovascular disease than do non-diabetic patients[20]
- Many diabetic patients have neuropathy, which, in combination with arterial insufficiency, puts them at increased risk of neuroischaemic tissue loss

Adjuvant treatment

Cilostazol has been shown to significantly increase (35-109%) walking distance in people with claudication in several large double blind placebo controlled randomised trials.[w21-w25] The precise role of cilostazol remains to be defined, but a trial of the drug is probably indicated in patients who have unacceptable symptoms despite three to six months of adherence to best medical treatment. No convincing evidence supports treatment with other drugs or vitamins,[13] but trials evaluating the effect of folate and vitamin B-12 on hyperhomocysteinaemia, a putative vascular risk factor, are near completion.

When should a patient be referred to a vascular surgeon?

Local circumstances vary considerably, but referral is appropriate if

- The primary care team is not confident of making the diagnosis, lacks the resources necessary to institute and monitor best medical treatment, or is concerned that the symptoms may have an unusual cause
- The patient has unacceptable symptoms despite a reasonable trial of, and adherence to, best medical treatment

- The patient has weak or absent femoral pulse(s) (see below).

Patient with critical limb ischaemia (rest pain, gangrene, or ulceration) should be referred urgently (preferably by telephone) to the next vascular surgical clinic. The patient should also be referred urgently if an abdominal aortic aneurysm is suspected on abdominal examination or if the history suggests a carotid territory transient ischaemia attack or amaurosis fugax.

Vascular and endovascular surgery

No convincing evidence supports the use of percutaneous balloon angioplasty or stenting in patients with intermittent claudication.[14] Two randomised controlled trials have shown that although successful percutaneous balloon angioplasty may lead to a short term (six months) improvement in walking distance, in the longer term (two years) best medical treatment is better than percutaneous balloon angioplasty in terms of walking distance and quality of life measures.[4] The exercise versus angioplasty in claudication trial is further evaluating the role of percutaneous balloon angioplasty.[5] In the United Kingdom bypass surgery is performed only infrequently for intermittent claudication because

- The risks of surgery are generally believed to outweigh the benefits in most patients who improve on best medical treatment
- Even though symptoms are frequently unilateral, most people with claudication have bilateral disease; revascularising one leg often simply serves to unmask hitherto asymptomatic contralateral disease.

In general, the threshold for percutaneous balloon angioplasty, stenting, and surgery is lower in patients who have predominantly aortoiliac (suprainguinal) disease because

- In terms of walking distance, such patients seem to benefit less from best medical treatment, although they gain just as much in terms of protecting life and limb; this may be because the body is less able to collateralise around an aortoiliac block
- Percutaneous balloon angioplasty and stenting in the aorta or iliac arteries is more durable than that below the inguinal ligament, presumably because larger calibre, high flow arteries are involved
- Aortoiliac reconstruction deals with both legs at the same time.

This greater readiness to intervene in patients with absent or diminished femoral pulses in no way undermines the key role of best medical treatment. Furthermore, aortoiliac reconstruction in a patient who also has severe infrainguinal disease is unlikely to lead to a clinically significant reduction in symptoms. See bmj.com for more details on endovascular techniques.[4 5 14 21 22]

Ongoing research

Several recent landmark trials have confirmed the clinical effectiveness and cost effectiveness of best medical treatment for peripheral arterial disease, and further trials are under way. The exercise versus angioplasty in claudication trial will help to define the role of adjuvant treatments such as percutaneous balloon angioplasty and supervised exercise (see bmj.com). The main challenge facing people caring for patients with peripheral arterial disease is applying what we know already. Primary care teams are best placed to deliver this highly effective and evidence based care, possibly through the establishment of community based, nurse led, protocol driven vascular clinics to which general practitioners can refer any "vascular" patient who needs best medical treatment. Interested general practitioners or secondary care specialists in vascular medicine or surgery could oversee such clinics, which would have clear and widely agreed policies for further investigations and referral to secondary care. Such clinics would need additional funding in the short term but would be likely to be cost neutral, or even beneficial, in the medium and long term through the prevention of expensive vascular events such as stroke and amputation.

Additional educational resources

ABC of arterial and venous disease. *BMJ* 2000;320. Review articles on

- Non-invasive methods of arterial and venous assessment: p 698-701
- Acute limb ischaemia: p 764-7
- Chronic limb ischaemia: p 854-7
- Secondary prevention of arterial disease: p 1262-5

Cochrane review of exercise therapy in peripheral arterial disease—Leng GC, Fowler B, Ernst E. Exercise for intermittent claudication. *Cochrane Database Syst Rev* 2000;(2):CD000990

Consensus document on peripheral arterial disease—TASC Working Group. Management of peripheral arterial disease: transatlantic intersociety consensus (TASC). *Eur J Vasc Endovasc Surg* 2000;19(suppl A):S1-244. (250 page evidenced based document produced by international expert panel, covering all aspects of peripheral arterial disease (also available at www.tasc-pad.org))

Information for patients

The Vascular Surgical Society of Great Britain and Ireland produces patient information sheets on intermittent claudication, arteriograms, percutaneous balloon angioplasty, and amputations—available from www.vssgbi.org

Competing interests: None declared.

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Interactive case report

A 66 year old woman with a rash: presentation

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Editorial by Richards and Peile

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Ruth is a 66 year old housewife who presented to her general practitioner with a two to three month history of feeling generally "out of sorts" followed by the development of a rash (figures). The rash first appeared on her fingers and spread to the backs of her hands. The most noticeable changes were on the nail folds, which became erythematous and swollen. The rash then spread to the elbows, knees, and V of her neck. Her husband had noticed a purple pigmentation around her eyes, and she reported that sunshine made the rash worse.

Ruth is a fit, active lady who has never smoked and is teetotal. Her weight is steady and her appetite good. She is currently taking no drugs, although she took hormone replacement therapy for 4.5 years after her menopause 14 years ago.

Ten years ago she was investigated for rectal bleeding. A full blood count, liver function tests, and rigid sigmoidoscopy gave normal results. She has had had no further episodes of rectal bleeding, although she gets intermittent bouts of constipation.

Questions

1 Ruth's general practitioner wondered if she could have dermatomyositis. What do you think?

2 What tests would you do next?

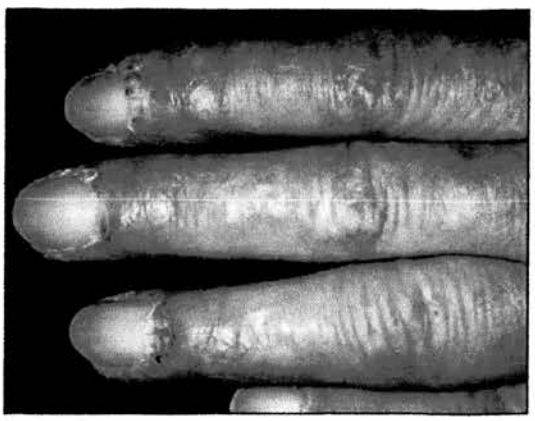
3 What would you tell Ruth at this stage and why?

Please respond through bmj.com

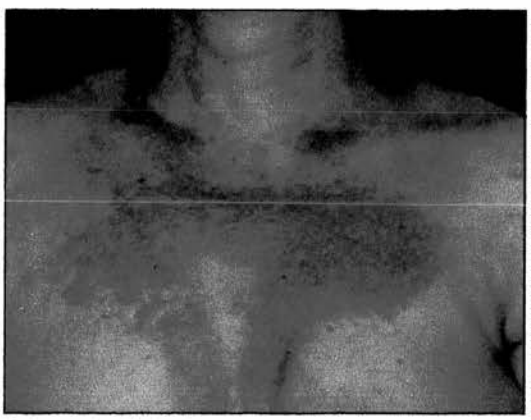
Her mother and grandfather both died of bowel cancer (aged 62 and 48 years). One sister died of breast cancer, but Ruth's mammogram one year before the episode of rectal bleeding showed no abnormality. Recently, her other sister died after a dissection of the ascending aorta.

Competing interests: None declared.

This is the first of a 3 part case report where we invite readers to take part in considering the diagnosis and management of a case using the rapid response feature on bmj.com Next week we will report the case progression and in four weeks' time we will report the outcome and summarise the responses



Rash on fingers



Rash spread to V of neck

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Second Best Medical Therapy

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***Background**: best medical therapy (BMT) provides patients with peripheral arterial disease (PAD) substantial protection against future vascular events.*
***Objective**: to determine the quality of BMT received by PAD patients in this vascular surgery unit.*
***Methods**: retrospective case-note review of 50 consecutive patients in each of the following groups: intermittent claudication (out-patients), symptomatic carotid artery disease (out-patients), lower limb angioplasty, lower limb bypass surgery, carotid endarterectomy.*
***Results**: overall BMT use was poor. Fifteen percent of smokers had assistance with smoking cessation noted. Seventy-eight percent of patients were taking an antiplatelet agent, 38% cholesterol-lowering medication and 51% antihypertensive medication. Fifty-three percent of patients had a cholesterol measurement, 50% of out-patients had a blood pressure measurement and 53% of non-diabetics had a random blood glucose performed. Sixteen of the patients with lower limb disease were given advice about exercise. Patients with a history of coronary artery disease were more likely to be taking cholesterol lowering, or antihypertensive medication.*
***Conclusions**: BMT is poorly used in patients with PAD, which will result in an excess of cardiovascular morbidity and mortality. Strategies need to be developed to increase the use of BMT in our patients.*

Key Words: Best medical therapy; Peripheral arterial disease.

Introduction

Peripheral arterial disease (PAD) affects approximately 20% of the adult population; is the commonest single cause of stroke, major amputation and non-cardiac sudden death; and is also associated with a marked (3–4 fold) increase in cardiac morbidity and mortality due to co-existing coronary artery disease (CAD).[1] Best medical therapy (BMT) (comprising principally of smoking cessation strategies, cholesterol lowering, blood pressure control, diagnosis and control of diabetes mellitus (DM), prescription of an antiplatelet agent and exercise therapy) has long been recognised as the cornerstone of management for patients with CAD because it leads to a very substantial reduction in future vascular events and is highly cost-effective.[2] Although it is overwhelmingly likely that BMT would offer the same degree of protection to patients with PAD,[3] it has been suggested that many such patients go untreated.[4–7] The aim of this study was to determine the quality of BMT provided to patients with PAD presenting to a hospital based vascular surgery unit.

Methods

This was a retrospective case-note review of 50 consecutive patients in each of the following groups:

(1) Patients presenting for the first time to clinic with intermittent claudication (IC), (2) patients presenting for the first time to clinic with symptomatic carotid artery disease, (3) patients undergoing percutaneous balloon angioplasty (PTA) of the lower limb arteries, (4) patients undergoing lower limb bypass surgery, and (5) patients undergoing carotid endarterectomy (CEA).

The institution of each component of BMT (smoking cessation, antiplatelet agent use, cholesterol lowering therapy, blood pressure control, diagnosis of diabetes mellitus and exercise therapy) at the end of each finished hospital episode (out-patient visit or in-patient stay) was recorded. For the purposes of the study hypercholesterolaemia was defined as a total serum cholesterol >5.5 mmol/l, an abnormal random blood glucose was defined as >7 mmol/l, and hypertension as a blood pressure (BP) greater than 160/90 mmHg.

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In the 54 patients identified as having hypercholesterolaemia, either on ($n=32$) or off ($n=22$) treatment, new or altered lipid lowering therapy was instituted in 15. Taken together these data mean that 110 of the 150 patients undergoing intervention (PBA, bypass or CEA) had either not be screened or treated, or were on inadequate treatment for hypercholesterolaemia (cholesterol still >5.5 mmol/l) at the time of their procedure.

Diabetes screening

Sixty-two (25%) patients were known to be diabetic. Of the remainder, 99 (53%) were screened for diabetes, all by random blood glucose, revealing 20 abnormalities (random blood glucose >7 mmol/l). No out-patients ($n=4$) had an abnormality acted upon; 6 of 16 in-patients with a high random blood glucose had the result investigated (test repeated, oral glucose tolerance test, or diabetic referral) (Fig. 4).

Exercise therapy

There is no supervised exercise programme available at our unit. Ten (7%) patients with lower limb disease were noted to have been given general advice on exercise (i.e. "keep walking"), 6 (4%) patients were noted to have been given specific exercise advice ("walk for 30 min, 3 times per week, walking to the point of maximum claudication before stopping").

Blood pressure (BP) control

One hundred and twenty-seven (51%) patients were already receiving anti-hypertensive treatment. All in-patients had their BP measured on admissions (data not shown). Less than 50% of out-patients had their BP checked, and few patients had abnormal blood pressure results acted upon (Fig. 5).

Influence of CAD

Fifty-nine (24%) patients had a history of CAD (angina, myocardial infarction or coronary artery surgery). These patients were more likely to be taking cholesterol lowering, or anti-hypertensive medication at their consultation. However, a history of CAD did not seem to influence vascular surgeons in whether patients were screened for hypercholesterolaemia, hypertension or diabetes (Table 1). Similar proportions from each group were taking an antiplatelet agent when seen.

Discussion

The inescapable conclusion to be drawn from this study is that PAD patients going through this vascular surgery unit have not been receiving BMT, including a significant proportion of patients undergoing surgical or endovascular procedures. This is despite compelling "level 1" evidence indicating that BMT offers

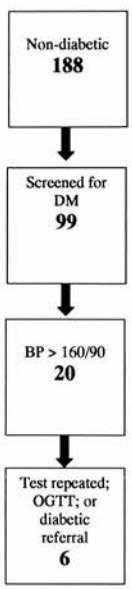

Fig. 4. Number of non-diabetic patients who were screened for DM; found to have an abnormal result, and whether high results were acted upon. OGTT = Oral glucose tolerance test.

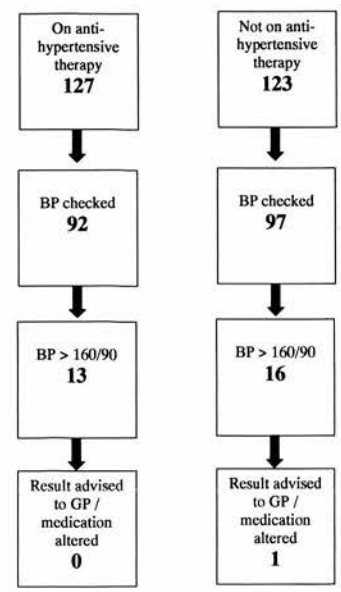

Fig. 5. Flow chart showing number of patients receiving antihypertensive medication, and the number who had their BP checked, found to be high, and acted upon.

in vascular surgery teaching, it will be instituted more completely.

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General Clinical Chemistry

Effects of Temperature on Stability of Blood Homocysteine in Collection Tubes Containing 3-Deazaadenosine

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Background: **The accuracy of homocysteine (Hcy) results is currently compromised by the requirement to separate the plasma within 1 h of sample collection. We studied the effect of temperature on the stability of plasma Hcy over a 72-h time course in blood collected into evacuated tubes containing either EDTA alone or both EDTA and 3-deazaadenosine (3DA).**

Methods: **We recruited 100 volunteers, including both diseased and healthy individuals with a range of baseline plasma Hcy values, from two centers. Blood samples were collected into tubes containing EDTA, and EDTA plus 3DA and stored at ambient temperature (20–25 °C) or refrigerated (2–8 °C). Aliquots of blood were centrifuged at various times up to 72 h, the plasma was removed, and Hcy was measured by HPLC.**

Results: **Plasma Hcy measurement covering the sample collection and storage conditions during the whole time course was possible on samples from 59 of those recruited. One-way ANOVA for repeated measures within subjects revealed that only samples that were collected into tubes containing EDTA plus 3DA and stored refrigerated were stable over 72 h ($P = 0.2761$).**

Conclusions: **A combination of 3DA and storage at 2–8 °C will allow collection of samples for plasma Hcy measurement outside of the hospital setting and wider population screening.**

Homocysteine (Hcy),[4] usually present in small amounts in plasma, is formed in mammals solely from methionine *(1)*. McCully and Ragsdale *(2, 3)* highlighted the importance of this pathway when they found that marked increases in plasma Hcy are a common factor in the presence of vascular lesions, which were brought about either by enzyme deficiencies in methionine metabolism or experimentally in rabbits. Since these initial observations were reported, links between hyperhomocysteinemia and a multitude of disorders have been postulated, including cancer *(4)*, neural tubes defects *(5)*, cognitive decline *(6)*, and dementia *(7)*. However, there is still argument about whether increased Hcy is the cause or result of tissue damage *(8)*. Those studies exhibiting a link between Hcy and vascular disease tend to show that relatively small changes in Hcy concentration lead to large increases in relative risk. In a metaanalysis of 27 such studies *(9)*, a 5 μmol/L Hcy increase led to odds ratios for coronary artery disease of 1.6 (95% confidence interval, 1.4–1.7) in men and 1.8 (95% confidence interval, 1.3–1.9) in women. Dietary fortification with folic acid supplements has been shown to decrease Hcy concentrations *(10–12)*, but the results of prospective studies are needed to show whether lowering Hcy is beneficial in reducing the risk of disease *(13–15)*. If confirmed, then the debate is strengthened in favor of screening programs designed to lower the incidence of heart disease. Such moves have been facilitated by the recent emergence of commercial systems allowing the routine clinical laboratory to perform testing previously performed only in research facilities.

The remaining problem is how to stabilize plasma Hcy concentrations in blood before processing, centrifugation, and storage. Erythrocyte Hcy concentrations are ~10-fold

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[4] Nonstandard abbreviations: Hcy, homocysteine; 3DA, 3-deazaadenosine; and SAHH, *S*-adenosylhomocysteine hydrolase.

lower than in plasma; therefore, any increase in plasma Hcy after sample collection is not attributable to leakage from the cells, but from continued metabolism and excretion into the plasma *(16)*. The liver and pancreas are mainly responsible for Hcy removal in vivo *(1)*. Because this removal pathway is absent in vitro, Hcy has been shown to increase by as much as 10% per hour over the first few hours after sample collection *(16, 17)*. The current recommendation is to place samples on ice and centrifuge within 1 h. Plasma Hcy may then be stable for at least 24 h at room temperature and for several months, if not years, when stored frozen *(17)*.

If a 5 μmol/L increase is associated with an 80% increased risk of vascular disease, artifactual increases in plasma Hcy caused by delays in sample processing could easily lead to false-positive results being reported. Although the treatment for hyperhomocysteinemia is simple and noninvasive, proper risk assessment demands accurate data.

Storage temperature *(18–21)*, acid citrate *(18, 22–24)*, sodium fluoride *(25, 26)*, and erythrocyte lysis *(27–29)* have all been considered in attempts to stabilize plasma Hcy. However, several of these methods cause sample dilution, either through the addition of liquid or, particularly with sodium fluoride, through osmotic effects caused by production of hypertonic plasma *(30)*. To date, the only method that has been shown to stabilize Hcy concentrations in whole blood for any longer than a few hours, without readjustment of reference intervals or immediate centrifugation, is the use of 3-deazaadenosine (3DA) *(31)*. At 100 μmol/L in EDTA whole blood, al Khafaji et al. *(31)* reported that 3DA stabilized plasma Hcy for 72 h at room temperature before centrifugation. 3DA prevents Hcy production through competitive inhibition of the enzyme *S*-adenosylhomocysteine hydrolase (SAHH), the final enzyme in Hcy production from methionine.

In pilot studies *(32)*, we investigated the use of 100 μmol/L 3DA in EDTA whole blood before developing a commercially available evacuated blood collection system. However, because 3DA acts through competitive inhibition, its effectiveness is influenced by temperature. To avoid ambiguity when using phrases such as "ambient" temperature, we controlled storage temperatures between 20 and 25 °C; however, the mean Hcy increased from 8.5 μmol/L to 11.5 μmol/L over 72 h. At 2–8 °C, the mean Hcy decreased by a statistically insignificant 0.5 μmol/L.

In light of the pilot study results, a trial batch of evacuated tubes was produced that contained 3DA spray-dried into tubes containing EDTA. Here we report clinical validation of these blood collection tubes, conducted in such a way as to confirm whether a combined effect of low temperature and 3DA was sufficient to provide long-term stability of plasma Hcy in whole blood.

Most studies on sample stability have been conducted on apparently healthy populations. In the clinical validation of our tubes, we were interested in comparing the stability of samples collected from a more diverse population, including patients with vascular disease and elderly patients, in addition to healthy volunteers.

Materials and Methods

Volunteers were recruited in accordance with the current revision of the Helsinki Declaration of 2000 *(33)*. The only exclusion criterion was individuals known to have recently taken drugs that can interfere with the method used for Hcy analysis (captopril, cysteamine, *N*-acetylcysteine, and *N*-2-mercaptopropionyl glycine).

To ensure a range of starting concentrations, two centers were selected such that blood was collected from 50 individuals, patients, and employees at a hospital in the United Kingdom (Birmingham Heartlands Hospital) and from another 50 volunteers, mainly healthy students and employees, at a university campus in the US (University of Maryland at Baltimore). Local approval was obtained from the appropriate ethics committees, and informed consent was given by all participants.

Blood was collected, by venipuncture, into tripotassium EDTA Vacutainer™ Tubes (Becton Dickinson) and DS30 Hcy Blood Collection Tubes (Drew Scientific Ltd., Barrow in Furness, United Kingdom). The DS30 Hcy blood collection tubes contain dipotassium EDTA as anticoagulant and 100 μmol/L 3DA (final concentration) to inhibit Hcy production. Each tube was evacuated to collect 2.5 mL of whole blood. Once collected, each tube was mixed and aliquoted for storage at ambient temperature (20–25 °C) and under refrigeration at 2–8 °C. Samples were taken from each blood tube for baseline Hcy measurements. At 3, 6, 24, and 72 h after blood collection, an aliquot of blood was removed from each tube at each storage temperature and centrifuged for 10 min at 11 000*g*. The plasma was removed and stored at −80 °C until analysis.

Hcy measurement was performed at two sites using the Drew DS30 Hcy Analyzer (Drew Scientific). The Drew analyzer uses reversed-phase HPLC with fluorescence detection to separate ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F)-derivatized thiols in plasma. Reduction of disulfide bonds by tris(2-carboxyethyl)phosphine allows measurement of all forms of homocyst(e)ine. To avoid between-batch variability, each individual's set of samples was measured within a single run. Within-batch imprecision for this method has been shown to be <2% *(34)*.

Changes in Hcy concentration for each storage condition over time were assessed for statistical significance by one-way ANOVA within subjects with repeated measures.

Results

Of the 100 volunteers recruited, 59 yielded a complete set of results for all time points and storage conditions under investigation. Reasons for lost results included poor

bleeds from elderly volunteers and identifiable mistakes in sample analysis. Incomplete datasets were not included.

The 59 volunteers comprised 38 females and 21 males. The median age was 47 years, with a range of 17–91 years. Initial plasma Hcy values ranged from 3.9 to 28.7 μmol/L.

Shown in Fig. 1 are the changes in plasma Hcy concentration over time. One-way ANOVA within subjects with repeated measures showed that only a combination of the DS30 Hcy blood tube and refrigerated storage provided stability of plasma Hcy concentrations in whole blood at all times up to 72 h (no significant difference, $P = 0.2761$). Hcy changes under the three other storage conditions were all highly significant ($P < 0.0001$).

Using the Tukey ω-procedure *(35)*, we assessed the time taken for Hcy values to change significantly from baseline for each condition. In EDTA alone, Hcy increased significantly before 3 h had elapsed, regardless of whether the tubes were refrigerated or left at ambient temperature. However, in DS30 Hcy blood collection tubes, 3DA used in combination with EDTA provided added stability at ambient temperature (6 h).

DS30 Hcy blood tube samples from 17 individuals yielded enough blood to allow analysis of plasma after 1 week (168 h) at 2–8 °C. However, these volunteers did not produce a full dataset for the other storage conditions. Again, no change in Hcy concentration was observed ($P = 0.4690$). The initial mean Hcy for this group was 14.0 μmol/L. Even after 1 week at 2–8 °C, the concentration had increased by only 0.3 μmol/L.

Previous stability studies have reported changes in Hcy over time as either percentages or absolute changes against initial values. In the present study, in the absence of inhibitor, whole-blood samples stored at 20–25 °C showed an average Hcy increase of 8% per hour in the first few hours after sample collection, but those individuals whose Hcy was at the lower end of the reference interval [5.2–15.1 μmol/L *(36)*] showed increases as large as 20% per hour. Dividing the study population into 5 μmol/L bands (Table 1), according to baseline Hcy, highlighted a significant difference ($P < 0.001$) in the percentage change in plasma Hcy in the whole-blood samples over 72 h across the different bands. When analyzed according to absolute changes in Hcy, this statistical significance disappeared ($P = 0.1460$). This shows that the mass of Hcy produced over time varies little between blood samples taken from a large group of individuals, even over a wide range of initial Hcy concentrations. It therefore follows that the percentage change will be greater in those samples with the lowest starting concentrations.

In light of these findings, we believed that data obtained from individuals with higher starting Hcy might mask any changes over time in those samples with initial Hcy in the lower groups. We therefore subdivided the data into two groups (≤10 μmol/L and >10 μmol/L) containing 43 and 16 individuals, respectively, and analyzed them (Table 2). Irrespective of the baseline Hcy, storage of whole blood at 2–8 °C in EDTA or 20–25 °C in the presence or absence of 3DA led to significant changes in plasma Hcy over 72 h ($P < 0.0001$), whereas samples showed stability over 3 days of storage at 2–8 °C in the DS30 Hcy blood tube independent of whether the initial Hcy was less than or greater than 10 μmol/L ($P = 0.3682$ and 0.3140, respectively).

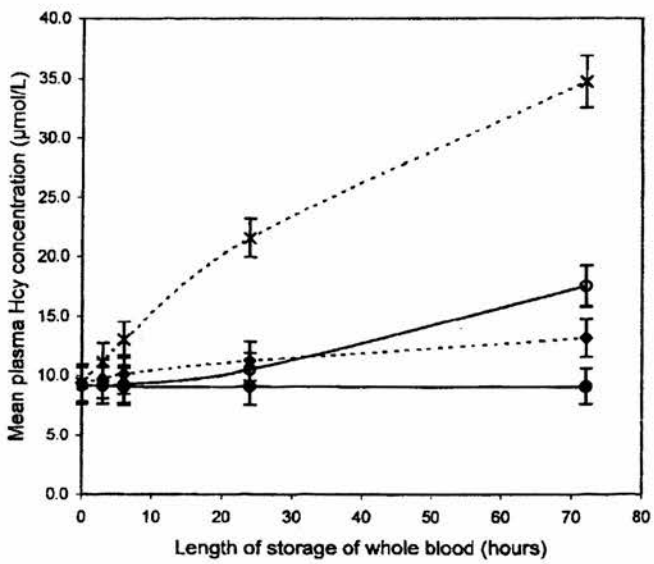

Fig. 1. Effect of whole-blood storage on plasma total Hcy over 72 h in standard EDTA tubes or in DS30 Hcy blood tubes.

Data points are the mean ± 2 SE (*error bars*) for samples from 59 individuals. ●, DS30 Hcy blood tube stored at 2–8 °C; ◆, EDTA tube stored at 2–8 °C; ○, DS30 Hcy blood tube stored at 20–25 °C; ×, EDTA tube stored at 20–25 °C.

Discussion

If blood samples are stored under ambient conditions before centrifugation, Hcy production by erythrocytes leads to an increase in plasma concentrations *(16, 17)*. Previously, 10% per hour has been quoted as the initial rate of Hcy production after venipuncture; a similar average rate was observed in the present study (8% per hour). However, an inverse relationship existed between baseline Hcy and the rate of Hcy production (expressed as a percentage of the initial Hcy). Consequently, because a 5 μmol/L increase in Hcy may be associated with a 80% increased risk of vascular disease *(9)*, strict sampling conditions must be observed to prevent false increases in plasma concentrations.

Storage on ice before centrifugation may stabilize plasma Hcy for up to 6 h *(20)*, although current recommendations suggest that processing should occur within 1 h. As we were interested in providing a solution to sample collection away from a centralized laboratory, we chose to look at refrigerated conditions. However, for samples in which EDTA alone was used, the maximum stability was only 3 h.

If ice is unavailable, acid citrate may also provide

Table 1. Percentage and absolute changes in plasma Hcy concentrations in EDTA whole blood over 72 h of storage at 20–25 °C.

Hcy values, μmol/L	Initial Hcy,[a] μmol/L	Absolute change,[a] μmol/L	Percentage change[a]	n
0 to <5	4.5 (0.1)	23.8 (1.6)	526.9 (36.7)	9
5 to <10	6.8 (0.2)	26.1 (1.2)	397.0 (22.0)	34
10 to <15	12.7 (0.6)	29.8 (4.0)	241.9 (37.2)	7
15 to <20	17.1 (0.8)	20.8 (3.6)	121.2 (20.6)	4
20 to <25	23.0 (0.9)	20.0 (0.9)	87.5 (5.7)	3
25 to <30	27.5 (1.4)	19.8 (2.7)	72.7 (13.6)	2

[a] Results are mean (SE).

stability for up to 6 h at "room temperature". However, at higher temperatures, samples are less stable *(24)*. Ducros et al. *(18)* found that sample stability also depends on the method of analysis. Chromatographic methods showed sample stability for at least 4 h in acid citrate at room temperature, whereas Hcy results increased over the same time course with an immunochemical method (fluorescence polarization immunoassay). Similar findings were reported by Salazar et al. *(23)* and O'Broin et al. *(22)*, who showed a significant increase after 2 h or a 10% increase after 6 h, respectively. Whereas chromatographic methods measure Hcy itself or a Hcy derivative, immunochemical methods are indirect. Hcy is estimated by the amount of its metabolic precursor, *S*-adenosylhomocysteine, formed during the assay by the reverse reaction of the enzyme SAHH. In acid citrate, the low pH may prevent Hcy build up by inhibiting SAHH, but precursor build up is not prevented, thereby giving the impression of sample instability. Where stability is reported, there are conflicting reports about whether acid citrate increases *(22, 24)* or decreases *(23)* baseline Hcy compared with EDTA samples kept on ice. This confusion may be related to corrections for dilution because acid citrate is added as a liquid, requiring hematocrit estimates. Either way, separate reference intervals are required for interpretation.

Similar problems complicate Hcy measurements on capillary whole-blood lysates *(27–29)*. The method appears quite attractive: a simple fingerprick followed by cell lysis, with stability for 2 days at ambient temperature provided by deactivation of the enzymes that produce Hcy. However, even after correction for dilution caused by the lysing agents, results are lower than in plasma because of further dilution by low intracellular Hcy concentrations.

Sodium fluoride samples at 2–3 h after collection have shown Hcy concentrations similar to baseline EDTA values *(25, 26)*. On closer inspection, however, Hcy continued to increase over time. The effect was attributable to an initial concentration drop caused by the formation of hypertonic saline, which led to fluid shifts. This observation was confirmed by Hughes et al. *(30)*, who saw a sodium fluoride concentration-dependent decrease in hematocrit. Fluoride inhibits anaerobic glycolysis and, therefore, ATP production, which is required for methionine conversion to *S*-adenosylmethionine, the first step in Hcy production. However, Hcy production may continue because of cellular reserves of *S*-adenosylmethionine, first postulated by Andersson et al. *(16)*.

Despite these studies, each method has its own weaknesses. After the publication by al Khafaji et al. *(31)*, we investigated the production of evacuated blood tubes containing 3DA. These tubes promised stability for up to 72 h under ambient conditions, without recalculation of Hcy results or changes to currently accepted reference

Table 2. Plasma Hcy concentrations in whole blood over time, subdivided into initial values of ≤10 μmol/L (n = 43) and >10 μmol/L (n = 16), for each storage condition.

Sample	Storage temperature, °C	Initial Hcy, μmol/L	Mean (SE) Hcy concentration, μmol/L, at incubation time of						
			0 h	3 h	6 h	24 h	72 h	P[a]	ω
EDTA	20–25	≤10	6.3 (0.2)	8.0 (0.3)[b]	10.0 (0.3)[b]	18.9 (0.6)[b]	32.0 (1.0)[b]	<0.0001[c]	1.6
		>10	17.6 (1.4)	19.4 (1.5)	21.1 (1.3)	28.6 (1.4)[b]	42.0 (2.0)[b]	<0.0001[c]	3.8
DS30 Hcy blood tubes	20–25	≤10	6.2 (0.3)	6.2 (0.3)	6.2 (0.2)	7.6 (0.3)[b]	14.9 (0.7)[b]	<0.0001[c]	1.1
		>10	17.3 (1.5)	17.1 (1.4)	17.3 (1.5)	17.9 (1.2)	24.2 (1.6)[b]	<0.0001[c]	2.8
EDTA	2–8	≤10	6.4 (0.2)	6.6 (0.3)	6.9 (0.3)[b]	8.0 (0.3)[b]	9.9 (0.3)[b]	<0.0001[c]	0.3
		>10	17.5 (1.5)	18.1 (1.5)	18.7 (1.4)[b]	19.7 (1.5)[b]	21.6 (1.4)[b]	<0.0001[c]	0.9
DS30 Hcy blood tubes	2–8	≤10	6.2 (0.2)	6.1 (0.2)	6.1 (0.3)	6.1 (0.3)	6.1 (0.2)	0.3682[d]	
		>10	17.2 (1.5)	17.4 (1.4)	17.0 (1.5)	17.1 (1.5)	16.8 (1.4)	0.3410[d]	

[a] *P* values calculated using one-way within-subject ANOVA (repeated measures).
[b] Significant differences from 0 h by Tukey's ω-procedure ($\alpha = 0.05$).
[c] Highly significant.
[d] Not significant.

ranges. However, as observed with acid citrate, stability was temperature-dependent *(32)*. In pilot studies at 20–25 °C, plasma Hcy increased from a mean of 8.5 μmol/L to 11.5 μmol/L over 72 h, an increase of 35%, which conflicted with the 10% increase previously reported *(31)*. Even with a 3DA concentration of 200 μmol/L, Hcy production could not be prevented (D.M. Hill and A.C. Kenney, unpublished results). We therefore considered a combination of SAHH inhibition by use of 3DA and a slowing of Hcy precursor production by chilling to 2–8 °C. Controls were used to ensure that pilot study results were confirmed and to verify that chilling alone was not sufficient for sample stabilization.

Because volunteers were recruited to observe the effects of Hcy stabilization at a range of baseline Hcy values, ANOVA was performed within subjects. Only samples that were collected in DS30 Hcy blood collection tubes and stored refrigerated (2–8 °C) showed stability over 72 h.

The mean plasma Hcy for each storage condition over time is shown in Fig. 1. Under ambient conditions (20–25 °C), the rapid increase in Hcy in samples collected into EDTA alone is clearly visible. Even at 72 h, Hcy production is evident. Therefore, if whole blood is left at room temperature for only a few hours without a preservative to stabilize Hcy, false-positive results may be reported. Chilling samples or storage at ambient temperature in the presence of a SAHH inhibitor (3DA) may both stabilize Hcy to some degree. In fact, 3DA at 20–25 °C stabilizes samples for 6 or even 24 h, depending on the initial Hcy concentration. This offers advantages for samples collected near the site of processing, providing some relief in the requirement to have ice on hand and to deliver the sample quickly to the laboratory. However, if there is a longer delay in transport, refrigerated storage in DS30 Hcy blood collection tubes can offer prolonged stability for 72 h and possibly up to 1 week.

Sample cooling may slow the processes involved in Hcy production. This theory appears to be supported by the data shown in Fig. 1, where a slow but steady increase in plasma Hcy occurs over 72 h in EDTA samples stored at 2–8 °C. In contrast, 3DA inhibits Hcy production in the early period under ambient conditions. However, the block is at the final stage in Hcy production. As the precursors to Hcy accumulate, SAHH inhibition by 3DA is finally overcome. A combination approach appears to be more effective, as chilling will prevent the build up of *S*-adenosylhomocysteine.

Despite the wide range of initial Hcy values observed, the absolute increase in Hcy in unpreserved samples showed very little difference after 72 h of storage. Consequentially, we observed significant differences in the percentage change over time that showed an inverse relationship to baseline Hcy. Similar phenomena have been noted previously over 24 h by Fiskerstrand et al. *(19)* and most recently over 4 h by Duarte et al. *(37)*. It follows that the thiol pool or the rate of Hcy production must be very similar within the collected samples. Andersson et al. *(16)* suggested that Hcy in whole-blood samples may be produced from a preformed pool of *S*-adenosylmethionine. If this is the case, it appears that the concentration of this pool is very similar within blood samples, is independent of Hcy concentration, and therefore, is independent of the efficiency of remethylation in vivo.

We observed significant differences in the degree of error in measurements when samples were stored for prolonged periods without a preservative, according to baseline Hcy concentrations (Table 1). Investigators and clinicians must be aware of this effect when claims are made about stability.

This trial was designed to investigate stability in as wide a range of starting Hcy concentrations as was possible to obtain from an unscreened group, such that stability could be compared in a healthy population vs a group that could be defined as having increased Hcy. When the sample population was divided into two groups, those with higher initial Hcy concentrations were stable over longer periods, e.g., up to 24 h compared with up to 6 h, in the Drew DS30 Hcy collection tubes stored at 20–25 °C. Moreover, any concern over whether significant differences in Hcy had been masked in the samples that were stored at 2–8 °C in DS30 Hcy blood tubes because of the presence of individuals with high baseline Hcy concentrations was unfounded.

We report the first commercially available blood collection tube to allow stability of plasma Hcy in whole blood over the course of 3 days without the need for centrifugation. Although we realize that the requirement for refrigeration is not ideal, several samples may be collected and stored in doctors' offices or in mobile clinical trial facilities before they have to be transported to a centralized laboratory for processing, thereby facilitating wider population screening. If refrigerated conditions are not readily available, the use of DS30 Hcy blood collection tubes may offer sample stability over 6 h, within a hospital environment, which may be particularly useful in cardiovascular clinics. These tubes can be used with a range of methods, providing that the method does not rely on the action of SAHH; consequently, tubes containing 3DA should not be used in some of the immunochemical methods currently available *(38, 39)*.

Ultimately, our aim is to produce a method of whole-blood collection that allows transport at a range of ambient temperatures. In the interim, we have developed a method that allows samples to be collected and stored for up to 72 h before laboratory intervention. We have successfully used this procedure to offer a mail-in Hcy test where local testing was not available.

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REVIEW ARTICLE

What Constitutes Best Medical Therapy for Peripheral Arterial Disease?

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Peripheral arterial disease (PAD) is associated with a high morbidity and mortality, largely from coronary and cerebrovascular disease, which often overshadows the PAD itself. Best Medical Therapy (BMT), comprising smoking cessation, antiplatelet agent use, cholesterol reduction, exercise therapy, and the diagnosis and treatment of hypertension and diabetes mellitus; is evidenced based and can result in significant reductions in cardiovascular risk, as well as some improvement in PAD. Previous data have largely been restricted to patients with coronary artery disease, and their relevance to PAD has been extrapolated. However, data are now starting to become available, such as the Heart Protection Study, with data specific to PAD patients. This article reviews the data regarding the use of BMT in patients with PAD, and based on this, makes recommendations for the use of BMT in this group of patients.

Key Words: Best medical therapy; Peripheral arterial disease.

Introduction

Peripheral arterial disease (PAD) is common, with over 20% of the population having asymptomatic disease, and up to 5% having lower limb symptoms; most commonly, intermittent claudication.[1] Although intermittent claudication is relatively benign in terms of limb-loss (1–2% per year), it is associated with a vascular mortality (5–10% per year) 2–4 times greater than that of an age and sex matched non-claudicant population; a risk that is, in fact, greater than that experienced by patients with angina.[2] There are several reasons for this.

- PAD is a marker for severe, multi-system atherosclerosis affecting the cerebral, visceral and coronary arteries.[3]
- In the presence of exercise-limiting intermittent claudication, even severe ischaemic heart disease may be asymptomatic and thus go unrecognised and untreated.
- There is some evidence that repeated ischaemia-reperfusion of leg muscles may lead to a systemic inflammatory response that accelerates atherosclerosis and promotes thrombotic events.[4]
- But most importantly, research into the benefits of risk factor modification and best medical treatment (BMT) in PAD patients has lagged far behind that directed towards symptomatic ischaemic heart disease. This in turn has resulted in:
  - a less compelling evidence base for treatment.
  - a lack of awareness of the vascular risk faced by these patients.
  - a belief that the costs of instituting BMT in patients with PAD could not be justified.

For these reasons, rather than viewing the PAD patient in a holistic way as a vascular "time-bomb", those treating PAD have tended to focus on the arterial lesion and its surgical or endovascular (angioplasty, stenting) treatment. Unfortunately, with the notable exception of carotid intervention for high-grade symptomatic disease,[5–7] there is little or no level 1 evidence to support intervention for PAD that is not immediately life or limb-threatening. What little data are available suggest that invasive intervention for claudication can lead to an early (1 year) improvement in symptoms, but there is no evidence this is sustained.[8–11] Such interventions are expensive,

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potentially hazardous, usually of limited durability and do not impact upon the patients' high underling vascular risk.[10,11] By contrast, there is increasing and compelling evidence that BMT comprising anti-smoking strategies, antiplatelet agents, lipid lowering and exercise programmes dramatically reduce the vascular risk and significant increase functional status.[12,13] BMT is also relatively inexpensive and virtually free from risk. With the release of data from the Heart Protection Study, which included over 6000 patients with PAD and confirmed the benefits of lipid lowering, it is timely to review what BMT should comprise and how it can be instituted universally in patients with PAD.[14]

Anti-smoking strategies

There is overwhelming evidence that smoking is the single most important risk factor for the development and progression of PAD and that it significantly increases the risk, and reduces the success, of peripheral arterial intervention.[12,15–20] Despite the clear benefits of smoking cessation in PAD patients, only a minority (11–48%) of patients manage to quit.[21] Simple oral advice is ineffective,[22] but more intensive counselling has been shown to be effective in unselected smokers, although not in PAD patients.[23–25] Nicotine replacement therapy, whether delivered by patch, gum, intranasal spray, inhaler or sublingual tablet, is safe, and leads to significant improvements in smoking cessation (odds ratio 1.72, 95% confidence interval 1.60–1.84); at least in the short term.[26,27] Bupropion is at least as effective as nicotine replacement therapy, but appears to confer no additional benefit in combination with nicotine replacement therapy.[28] The Cochrane group on tobacco addiction has found alternative therapies such as acupuncture, hypnotherapy, and "aversive smoking", to be ineffective.[29–31]

Hypercholesterolaemia

Hypercholesterolaemia is clearly an independent risk factor for the development and progression of PAD.[2,15,16,32] Cholesterol lowering has been shown to slow the progression of peripheral atherosclerosis in a number of large, including randomised, anatomical and pathological studies,[33,34] although none have shown benefit with respect to PAD symptoms. The recently concluded Heart Protection Study has, for the first time, demonstrated a benefit of statins in PAD patients by reducing coronary events by 20%. Furthermore, this was achieved irrespective of starting total cholesterol[35] (Fig. 1). Detailed, peer-reviewed

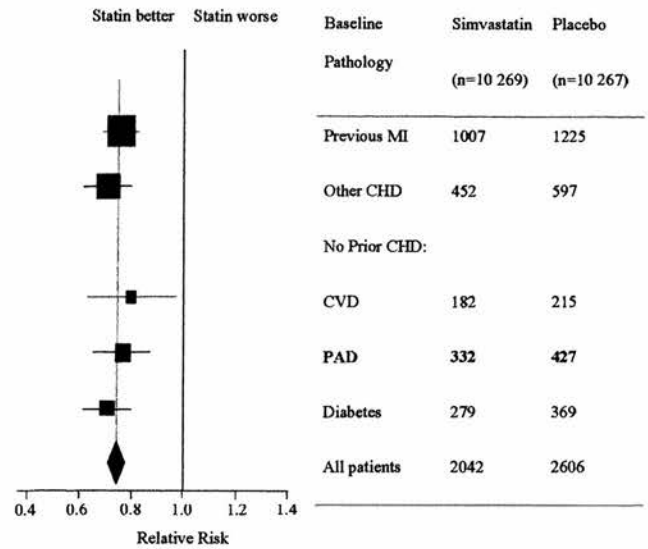

Fig. 1. Benefit of 40 mg simvastatin from The Heart Protection Study. Number of vascular events by prior disease. Data taken from www.hpsinfo.org. MI: myocardial infarction; CHD: coronary heart disease; CVD: cerebrovascular disease.

results are awaited, but this could have significant implications for cholesterol lowering in patients with PAD. Whether there is benefit from raising high density lipoproteins, and reducing triglycerides levels is less clear.[36]

In summary, all patients with PAD should have their cholesterol reduced with aggressive statin therapy, regardless of starting total cholesterol. In the longer term it seems possible that statin therapy will be indicated for any patient with objective evidence of asymptomatic PAD; for example, as demonstrated by a reduced ankle : brachial pressure index. As patents expire, and generic drugs become available, the financial consequences of these massive changes in prescribing practice will ease and, of course, the prevention of large numbers of vascular events will also help to offset the costs.

Antiplatelet Therapy

Early studies suggested antiplatelet agents could produce angiographic improvement,[37] increase walking distance,[38,39] and reduce the requirement for vascular intervention.[40] There is overwhelming evidence from the Antiplatelet Trialists' Collaboration that the prescription of an anti-platelet agent, usually aspirin, reduces vascular death in patients with symptomatic atherosclerotic disease by about 25%.[41] Most of the studies were in patients with ischaemic heart disease and, when taken in isolation, data from the few studies looking specifically at patients with PAD were

not conclusive. However, more recently, a review of 24 trials has shown that, when compared with placebo, APA treatment reduced the risk of death by about a quarter in patients with PAD.[42]

In summary, all patients with PAD should be on an antiplatelet agents because it reduces vascular event and death, improves the patency rates of surgery and endovascular interventions and may improve walking distance. For reasons of cost, non-enteric aspirin (75 mg) is a reasonable first-line choice as there is no clear evidence that a higher dose is more effective (but will cause more adverse events) or that enteric coating is associated with less gastric upset (and is more expensive). Patients who cannot take aspirin should be considered for clopidogrel.

Exercise

There is little doubt that exercise leads to a significant improvement in exercise tolerance (most studies show at least a doubling in walking distance) in patients with PAD.[43–45] It is also likely, though not specifically proved, that exercise will reduce vascular risk. However, clinicians and academics alike have largely neglected this simple, inexpensive and effective therapy; and as such, many important questions remain unanswered.

How does exercise work? Whilst early animal studies suggested that exercise may improve blood flow by the development of collaterals, studies in humans using venous occlusion plethysmography, Xenon-133 clearance and duplex ultrasonography have not confirmed this.[46] Despite this, exercise training can lead to increased clearance of Xenon-133 injected into calf muscles, possibly indicating that blood is being diverted towards more active muscles. Exercise training in claudicants leads to increases in oxidative enzymes, and enhanced utilization of fatty acids in the calf muscles, maximising the use of oxygen delivered to the tissues. Improvements in walking distance may also be due to improvements in walking biomechanics[47] and blood rheology.[48]

What is the best form of exercise? It has generally been thought to be walking but recent data have suggested that arm exercise, may be at least as beneficial, which further questions the mechanism by which exercise achieves its benefit.[49]

Does exercise have beneficial effects on risk factor profile? A small non-randomised controlled trail showed that exercise training for claudicants, can lead to modest reductions in blood pressure, cholesterol and glucose levels.[51] Whether this translates to a significant improvement in cardiovascular risk, has not been specifically determined in claudicants, but data from ischaemic heart disease patients suggests that it may.[50]

Should exercise be supervised and, if so, how and for how long? Supervised exercise programmes would seem intuitively to be better, but there is little evidence to support this. Gardner and Poehlman reviewed 21 studies of exercise therapy in PAD, and found that supervised exercise programmes were no better than unsupervised. A small randomised study of 54 patients, comparing a 12 week supervised exercise programme and unsupervised exercise, did suggest that the supervised programme was superior (improvement in maximum walking distance 207 vs 70% at 6 months). What is unclear, is the durability of any benefit. It might be speculated that any advantage of supervised exercise will diminish with time, although there is no evidence to support this.

Until these issues are addressed one must approach this aspect of care in a pragmatic way based upon local resources. PAD patients should certainly be repeatedly and specifically informed that exercise is beneficial and that it is not (as far as we know) harmful to try to "walk through" their claudication pain. Written advice may be a useful adjunct although this suggestion is not evidence-based. Although supervised programmes may be superior, at least in the U.K., such programmes are not widely available. The ongoing, U.K.-based, Heath Technology Assessment funded Exercise versus angioplasty in claudication trial (EXACT) will provide more information about the relative benefits of exercise and angioplasty when it reports (for more information please contact the senior author).

Diabetes

Diabetics have a 3–5 fold increased risk of PAD, and are at increased risk of progression from intermittent claudication to critical limb ischaemia.[52,53] The U.K. Prospective Diabetes Study has shown that intensive control decreased the risk of microvascular but not macrovascular vascular complications of the disease.[54] However, it is extremely important that the diagnosis of diabetes be specifically confirmed or excluded in patients with PAD because it will affect other areas of their treatment, such as blood pressure and lipid control.[55,56]

Blood Pressure Control

Hypertension, particularly, systolic hypertension, is associated with a three-fold increase in the risk

of developing PAD.[57–59] No study has specifically investigated PAD patients but it is quite clear that in general the treatment of hypertension significantly reduces coronary events and stroke.[60] Traditionally, blood pressure has been treated to a level of 160/90, but more recent data suggest that tighter control (130–140/85 mmHg) might confer additional benefits.[61] It is frequently taught that β blockers are contra-indicated PAD, but there is no evidence to support this.[62]

Other Risk Factors

Hyperhomocysteinaemia is becoming increasingly recognised as an important risk factor for development of atherosclerosis, and cross-sectional studies have linked it specifically to PAD.[63] However, the effect of reducing homocysteine levels has yet to defined, but should be answered by several ongoing trials. Observational studies have suggested that low levels of anti-oxidant vitamins are associated with PAD, although no studies, including the Heart Protection Study, have yet shown any benefit from vitamin supplementation.[14,64,65]

The relationship between alcohol and PAD appears to be J shaped, with minimal risk occurring at around 2 units of alcohol per day.[66] Excess alcohol consumption is clearly associated with an increased vascular risk. Oestrogen has been proposed as being cardio protective, on the basis of reduced cardiovascular morbidity and mortality in women taking hormone replacement therapy, but a recent randomised controlled trials showed no difference in cardiovascular events between groups randomised to oestrogen/progestogen, and placebo.[67] In summary, these factors may represent important risk factors in PAD, but at present there is insufficient evidence to justify targeting them for treatment.

Prevalence of BMT use in PAD

Despite the overwhelming evidence for BMT in patients with PAD, clinical experience and the literature indicated that it has been poorly applied in the past (Table 1). The proportion of patients taking any kind of anti-thrombotic therapy or warfarin ranges from 39% to 66%, and prevalence of cholesterol lowering therapy ranges from 5% to 46%. Patients who also have symptomatic ischaemic heart disease seem more likely to be treated but, in general there seems little or no relationship between the prevalence of treatment, the severity of the underlying disease and thus the potential benefits of BMT.[68–71] In other words, treatment is haphazard rather that the result of evidence.

The current situation is unacceptable, and clearly strategies need to be put in place to ensure that PAD patients do not miss out on evidence-based life saving treatment. The initial step needs to be the education of health professionals working with PAD patients about the increased cardiovascular risk of these patients, and the benefits of BMT. Articles such as this should raise the profile of these important issues. One strategy to increase the institution of BMT is the use of record cards (Fig. 2). These chart the level of individual risk factors over time, and allow easy recognition for healthcare professionals of untreated, or inadequately treated risk factors. These charts could be held in the case notes, or by the patient. Another possibility for increasing BMT use is to have dedicated staff in out-patient clinics. This is an ideal role for clinical nurse specialists, who are increasing in number. Whatever technique is employed, it is important to co-ordinate the patient's care with primary care.

Summary

There is overwhelming evidence for the efficacy of BMT in PAD, in terms of cardiovascular risk reduction, and improvement in PAD symptoms. Recommendations for the use of BMT, based on the best evidence available to date are presented in Table 2. Despite the evidence of benefit, BMT is grossly

Table 1. The use of cholesterol-lowering and anti-thrombotic therapy (antiplatelet agent of warfarin) in patients with PAD.

Study	*n*	Patient population	*n* with IHD (%)	*n* receiving	
				Cholesterol-lowering therapy (%)	Anti-thrombotic therapy (%)
Clark *et al.* (1999)[68]	299	Admitted for angiography	106 (36)	26 (9)	140 (47)
Anand *et al.* (1999)[70]	195	Admitted for peripheral arterial surgery	106 (54)	31 (16)	94 (49)
Bismuth *et al.* (2000)[69]	147	Critical limb ischaemia	66 (45)	8 (5)	58 (39)
McDermott *et al.* (1997)[71]	202	ABPI < 0.9 or abnormal Doppler waveform	103 (51)	93 (46)	133 (66)

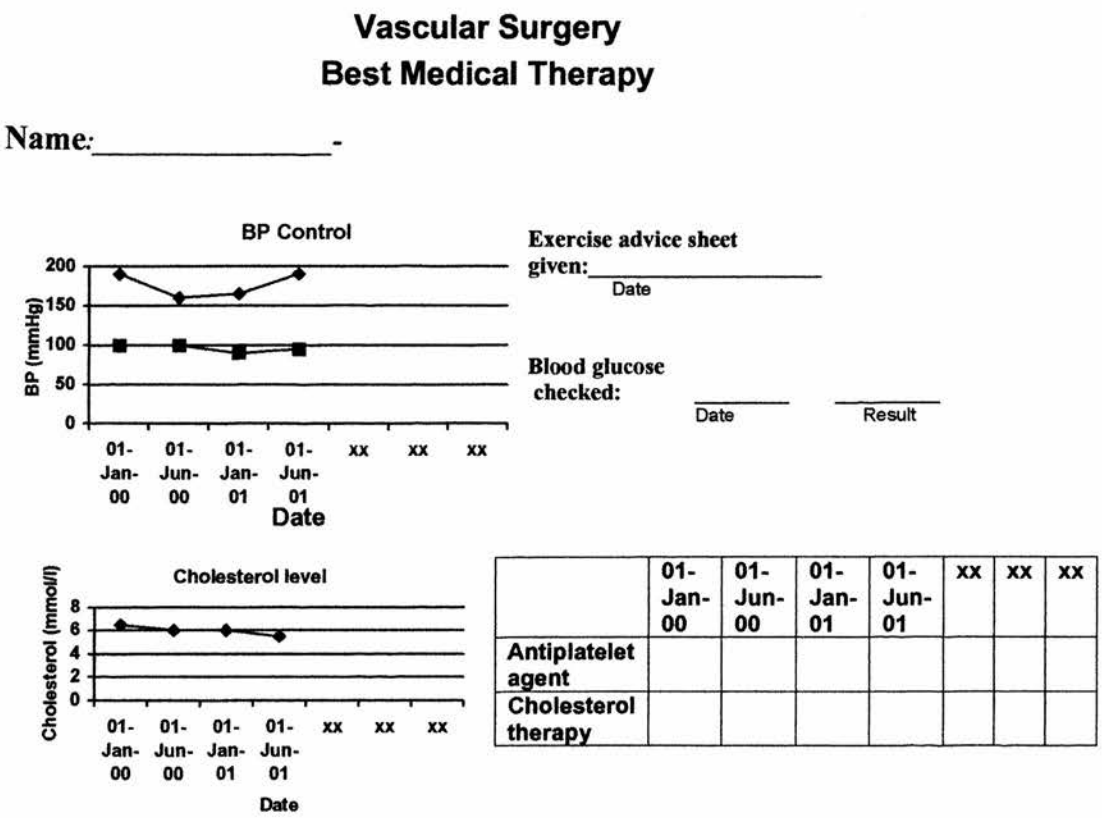

Fig. 2. Best Medical Therapy chart.

Table 2. Recommendations for best medical therapy (BMT) in patients with PAD.

Component of BMT	Recommendation
Smoking cessation	Repeated advice Nicotine replacement therapy Behavioural therapy (Smoking cessation classes)
Cholesterol reduction	Cholesterol checked yearly Statin therapy if total cholesterol >5.0 Additional treatment will be required if HDL low, or triglycerides high (Referral to lipid clinic)
Antiplatelet agent	Aspirin Clopidogrel if aspirin intolerant
Diabetes mellitus	Screen for diabetes mellitus
Blood pressure	Reduce blood pressure to <140/80 mmHg
Exercise	Patients with lower limb disease should be prescribed a supervised exercise programme

underused in PAD patients. If BMT use increases, this will lead to a decrease in cardiovascular morbidity and mortality, a reduction in the requirement for peripheral vascular intervention, and an improvement in outcome for those interventions that are required. It is imperative that those involved in the care of patients with PAD are aware of the benefit of BMT, and develop strategies to help improve its implementation.

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Prevalence and Significance of Thrombophilia in Peripheral Arterial Disease

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Key Words: Thrombophilia; Peripheral arterial disease; Screening.

Introduction

In addition to well-established risk factors such as smoking, diabetes, hypercholesterolaemia and hypertension, an increasing number of novel humoral and endothelial factors have recently been implicated in the aetiology and progression of vascular disease. Thrombophilia may be defined as a propensity to thrombosis secondary to abnormalities in haemostasis.[1] Thrombophilia has long been recognised as contributing to venous thrombosis, but is increasingly associated with arterial disease. It is important because screening may identify patients at high risk of thrombosis who may then be offered prophylaxis. This review will focus on the prevalence and significance of thrombophilic states associated with peripheral arterial occlusive disease (PAOD) and discuss possible strategies for screening and treatment.

Prevalence of Thrombophilia

General coagulation activation

If thrombophilia is important in PAOD then there should be evidence of activation of coagulation in affected patients. Thrombin and fibrinogen, and products of their metabolism, including thrombin-antithrombin (TAT) complexes, prothrombin fragments (PF) 1+2 and fibrin degradation products (FDPs) can be used to measure coagulation activation. Cross-sectional[2–5] and longitudinal[6] epidemiological studies have demonstrated an association between activation of coagulation and PAOD. Furthermore, in 1988, Boneu showed that PAOD was associated with inhibition of fibrinolysis.[7] In young patients (<51 years old) undergoing lower limb revascularisation, as many as 76% may have a hypercoagulable state (increased platelet aggregation or coagulation abnormality).[8]

Homocysteine

A mild elevation of homocysteine levels (hyperhomocysteinaemia) affects 5% or more of the population and is increasingly recognised as an independent risk factor for atherosclerosis and thrombosis.[9] Hyperhomocysteinaemia can cause increased Factor V activity, possibly via a decrease in thrombomodulin cell surface activity and a corresponding decrease in activated protein C (Fig. 1).[10–13] The prevalence of hyperhomocysteinaemia in PAOD may be between 50 and 60%[14–16] and many cross-sectional studies have demonstrated a clear association between plasma homocysteine levels and PAOD.[17]

Antithrombin III

Anti-thrombin III (AT III) is an endogenous anti-coagulant, produced by the liver, which inactivates thrombin and factor Xa. Deficiency of AT III is inherited in an autosomal dominant fashion. In a population-based study of 7983 subjects over 55 years old 3.1% had deficiency of AT III, defined as <75% activity.[18]

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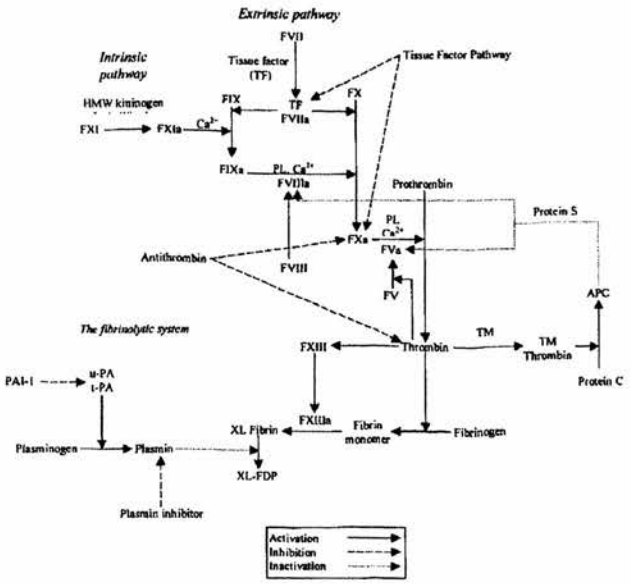

Fig. 1. Overview of coagulation system.

Fibrinogen

Fibrinogen is the substrate on which the end-product of the coagulation cascade, thrombin, acts to produce fibrin, and ultimately, a blood clot. Its effects are diverse and include increases in blood viscosity, red cell aggregation, platelet aggregation and activation.[19] Fibrinogen deposited in the arterial intima may also lead to smooth muscle cell proliferation and leukocyte migration.[20–23] Hyperfibrinogenaemia has long been associated with cardiovascular disease and is present in more than 50% of patients with PAOD.[24–26]

Antiphospholipid antibodies

Antiphospholipid antibodies (aPL) are a group of auto antibodies originally thought to be targeted towards negatively charged phospholipid, although recent work suggests that they are directed against β_2-glycoprotein I.[27] aPLs are of two types, detected by different laboratory methods: the anticardiolipin antibody (aCL) enzyme linked immunoassay and the lupus anticoagulant (LAC) coagulation assay. Although the lupus assay relies on the *in-vitro* effect of aPL to prolong coagulation assays, the *in-vivo* effect is procoagulant, the mechanism for which is uncertain. Antiphospholipid antibodies may inhibit protein C and protein S, and have prothrombotic effects via enhanced platelet reactivity or endothelial cell surface molecules such as heparan sulphate and tissue factor.[27]

Cross-sectional studies have shown that the prevalence of aPL amongst patients with PAOD requiring intervention varies between 26% and 45%.[28–30] This mostly comprises patients with aCL who constitute 84–94% the total. A small proportion of patients with aPL have both LAC and aCL (2–3%).[28,30,31] No studies have yet compared the prevalence of aPL in PAOD with the prevalence in the general population, and no large cross-sectional studies have been performed to give a population prevalence of aPL.

Protein C deficiency

Protein C is a vitamin K dependent protein which, when activated by the thrombin-thrombomodulin complex, inactivates factors Va and VIIIa (Fig. 1). Protein C deficiency is established as a risk factor for venous thrombosis, but its role in arterial pathology is less clear. Few studies have investigated the prevalence of protein C deficiency in PAOD. In a recent study of 116 claudicants, deficiency of protein C was found in 2 (1.7%).[31] Other studies have shown the prevalence of protein C deficiency to be between 2.5 and 15% in PAOD patients, but no comparisons were made with control groups.[8,32–34]

Activated protein C resistance/factor V Leiden

Activated protein C (APC) resistance is the most common inherited risk factor for thrombosis. The prevalence varies in different ethnic populations; in U.K. it is 3.5–4.9%, Africans 0% and in Cyprus 13%.[35,36] The most common cause of APC resistance is a mutation in the factor V gene leading to the replacement of Arginine 506 with Glutamine, (factor V Leiden, fVL) which renders it more resistant to degradation by protein C. This is responsible for 90–95% of APC resistance, the remainder of which is made up of acquired conditions such as aPL, pregnancy and the oral contraceptive pill.[37–40] APC resistance is measured using a plasma assay and exogenous activated protein C, and is indicated by a lowering of the APC ratio (normal range 2.2 to 2.6). This will identify the majority, but not all of patients with fVL. fVL may also be identified directly using genomic analysis, but not all mutations lead to lowering of the APC ratio. fVL is thought to underlie 18–30% of venous thromboses, but its importance in arterial disease is less well defined.

Both APC resistance and fVL have been demonstrated to be more common in patients with PAOD

compared with the general population. Sampram found the prevalence of fVL and APC resistance (defined as ratio <2.6) to be higher (26.4%) in 359 patients with PAOD than in 278 controls (12.2%).[41] A smaller study by Foley in patients who had undergone lower limb arterial bypass surgery reported a 17.8% prevalence of fVL, compared with a local population prevalence of 3.5%.[35] Evans only reported one positive APC resistant patient in 116 claudicants.[31] Variations in these reported figures may be explained by the preferential use of DNA analysis or APC ratio to define fVL; variations in the lower end of the normal range for defining the normal APC ratio and the severity of the presenting PAOD.

Protein S deficiency

Protein S is a vitamin K dependent plasma protein and an essential co-factor for the anticoagulant and profibrinolytic effect of activated protein C.[42] Protein S deficiency has been identified as a cause of venous thrombosis, and more recently has been proposed as a factor in arterial disease. The prevalence of protein S deficiency in the general population is thought to be around 0.7%.[43] There are only a few small studies investigating the prevalence of protein S deficiency in PAOD. Allart in 1990 showed protein S deficiency to be present in 3 out of 45 patients (8%) less than 45 years old who required surgical treatment for PAOD.[42] A study of 33 patients undergoing arterial surgery, and 10 controls found a prevalence of protein S deficiency in PAOD patients of 15%. Although no statistical difference was shown between patients and healthy controls, all five subjects with protein S levels less than normal were PAOD patients.[44]

Prothrombin 20210A

A G to A transition at position 20210A of the prothrombin gene is associated with an increased risk of venous thrombosis, although the underlying mechanism is not clear. The prevalence of this mutation is 1.2% to 4.3% in patients with venous thrombosis, 5.7% in patients with PAOD, and 0.7% in controls.[45,46] However, no specific studies have been performed investigating the association between prothrombin 20210A and arterial disease.

Despite studies screening for different states, using a variety of methods, in patients with a range of disease severity, it is clear that there is an increased prevalence of thrombophilic states in PAOD, perhaps as high as 60%. Although common, the clinical relevance of thrombophilia in PAOD is a more important issue, which will now be discussed.

Significance of Thrombophilia

Studies of general coagulation activation

There is a correlation between the level of coagulation activation and the severity of PAOD as determined by walking distance,[4] ankle–brachial pressure index (ABPI),[47,48] duplex ultrasonography, angiography[49] and clinical symptoms.[25] For example, Ray reported the prevalence of thrombophilia (protein C deficiency, protein S deficiency, antithrombin III deficiency, lupus anticoagulant) to be 11% in controls, 27% in claudicants and 40% in patients who had received a revascularisation.[34]

The importance of a hypercoagulable state in PAOD has also been revealed through the association between coagulation abnormalities and the progression of PAOD. In the Edinburgh Artery Study, whole blood viscosity, plasma viscosity and fibrinogen levels were predictive of the requirement for vascular intervention,[50] or fall in ABPI,[6] over a six-year follow-up period. Furthermore, whole blood viscosity, and fibrinogen levels have been shown to be predictive for the progression of PAOD as determined by walking distance.[51]

Thrombophilic states may also be important causes of failure of arterial interventions. In 1994, Ray studied 124 patients undergoing arterial reconstruction and reported 75 graft occlusions after a mean follow up of 44 months.[34] Almost half (49%) of these were subsequently identified as having a thrombophilia, compared with 27% of patent reconstructions. Abnormalities identified in the graft occlusion group were: protein C deficiency (21% of occlusions), protein S deficiency (17%), lupus anticoagulant (25%) and multiple abnormalities (12%). A subsequent prospective study, investigated the presence of a thrombophilia prior to arterial reconstruction in 60 patients with one-year follow-up.[52] A pre-operative thrombophilia was identified in 65% of patients whose graft subsequently occluded within one year, compared with 20% of those with a patent graft ($p<0.05$). The presence of thrombophilia was particularly significant in early graft failures, where 11 of the 12 occlusions within one month had a pre-operative hypercoagulable abnormality. A prospective study of 137 patients undergoing a mixture of arterial reconstructions identified 14 patients (10%) with a hypercoagulable state.[33] Three of these patients (27%)

suffered a graft thrombosis within 30 days, compared with two of 123 patients with a normal thrombophilia screen (1.6%). Eldrup Jorgensen studied 20 young (<51 years old) patients undergoing aorto-iliac (7) or infra-inguinal (13) vascular surgery.[8] Four patients suffered an early (<30 days) post-operative thrombosis, all of who had thrombophilia identified pre-operatively. Patients with multiple coagulation abnormalities appear to be at special risk. Thus, of 124 patients undergoing revascularisation studied by Ray, 11 had multiple thrombophilias, all of whom had had a previous revascularisation. Nine of these patients had a further occlusion during the follow-up period.[34]

Homocysteine

Hyperhomocysteinaemic patients have an increased rate of vein graft stenosis and increased failure of bypass grafts and angioplasty.[16,53] Patients undergoing peripheral arterial bypass surgery with elevated homocysteine have evidence of pre-existing intimal hyperplasia in saphenous vein biopsies.[15] A prospective study investigating hyperhomocysteinaemia and progression of PAOD, with mean follow-up of 37 months found a trend towards an association, but this was not statistically significant.[54] This may, however, represent a type II error as only a relatively small number of patients (22) were judged to have progression of PAOD during follow-up.

Fibrinogen

Fibrinogen levels correlate with the severity of PAOD, higher levels being associated with more severe disease, as determined by claudication distance,[25] angiography,[55,56] and ABPI.[47,49,57] Hyperfibrinogenaemia has been shown to be predictive for the progression of PAOD, as measured by change in claudication distance,[51] or the requirement for intervention.[50]

Given that hyperfibrinogenaemia is associated with the development and progression of PAOD, it is unsurprising that high levels of fibrinogen are predictive of failure of interventions for PAOD.[58] Prospective studies have shown that hyperfibrinogenaemia is associated with failure of vein and prosthetic femoral popliteal bypass grafts.[59–62] In addition, associations have been demonstrated between raised fibrinogen levels and graft stenosis, implying that it is not simply an increased thrombotic tendency underlying the failure of such interventions.[58,63] Data regarding fibrinogen and patency following percutaneous angioplasty are conflicting. Two prospective studies have shown that hyperfibrinogenaemia is associated with poorer patency rates, while another prospective study showed that high fibrinogen levels measured prior to angioplasty were associated with improved patency rates.[64–66]

At present there are no selective treatments available to lower fibrinogen and consequently no reported trials confirming benefit in treating hyperfibrinogenaemia. While there is a great deal of evidence associating fibrinogen levels and PAOD, it is difficult to conclude that this relationship is causal until such trials are available. Fibrinogen is an acute phase protein, and its increased levels in arterial disease may merely be representative of an underlying low-grade inflammatory process.

Antiphospholipid antibodies

No studies to date have demonstrated an association between the prevalence of aPL and the progression of PAOD. However, aPL and the antiphospholipid syndrome are associated with an increased risk of thrombotic complications of vascular surgery, although the majority of these studies are retrospective.[28,67–69] Ahn retrospectively identified seven patients with aPL who underwent a total of 18 vascular procedures.[70] Three of these patients, none of whom were anticoagulated developed multiple post-operative thrombotic complications and all eventually required amputation. The remaining four vascular patients in this study were taking steroids, anticoagulants, or vitamin K at the time of the initial operation. A similar study found that 16 of 19 patients with aPL undergoing a vascular procedure suffered a thrombosis, 12 of who died.[71] In a retrospective report of 234 patients undergoing vascular surgery, aPLs were associated with a shorter bypass patency period (17 vs 58 weeks) and a risk of occlusion that was 5.6 times greater than patients without aPLs.[28]

The only prospective study to investigate the association between aPL and the outcome of vascular intervention, showed a trend towards the presence of aPL and failure of arterial bypass surgery, but this did not reach statistical significance.[30] This result is unfortunately confounded by the fact that, significantly more of the aPL group were anticoagulated post-operatively thus diminishing any likely difference between the groups.

Activated protein C resistance/factor V Leiden

Ouriel prospectively monitored 76 patients who underwent lower limb revascularisation for a mean of 47 months. 60% of those with APC resistance (defined as APC ratio <2.0) had an occlusion of their graft, while only 24% of those without APC resistance suffered a graft failure ($p<0.02$).[72] A similar finding was seen in Sampram's study in which 32% of those with fVL and 49% of those with APC resistance suffered a graft occlusion (both $p<0.001$).[41] A study from Foley *et al.* reported no association between fVL and graft occlusion, but excluded patients whose graft occluded within six weeks of surgery, a time that others have reported as important in graft occlusion associated with thrombophilia.[35]

Protein S deficiency

Although the prevalence of protein S deficiency is higher in patients with PAOD, its significance is unknown. Allart investigated the families of young (<45 years old) PAOD patients who were found to be heterozygotes for protein S deficiency, but no association was found between likelihood of protein S deficiency, and arterial thrombosis.[42] This finding corroborated a previous study, which showed that relatives of protein S deficient individuals did not have an increased incidence of arterial thrombosis.[73]

Deficiency of protein S was identified in 4 of 20 patients (20%) whose arterial reconstruction failed compared with 6 of 40 (15%) of those with a successful reconstruction at 30 days post surgery, although this difference did not reach statistical significance.[42]

Antithrombin III deficiency

In Van der Bom's population study, examination of 7983 subjects revealed a complex relationship between level of AT III and ABPI.[18] In men, mild PAOD was associated with a high level of ATIII, while severe PAOD was associated with lower levels of ATIII. Whilst in women, there was in an inverse relationship between ABPI and ATIII level. The authors suggest that levels of ATIII rise in the presence of cardiovascular disease as a protective mechanism, but as vascular disease progresses, ATIII becomes consumed, leading to lower levels. The reason for the difference between the sexes is not clear.

The poor results of intervention in patients with thrombophilias, in terms of intervention failure and mortality, reinforce the clinical importance of these states in patients with PAOD. The presence of two or more co-existent thrombophilias, seems to have an additive effect, and be particularly dangerous clinically. However, many thrombophilic states may be asymptomatic for many years and the "two-hit" hypothesis suggests that thrombophilic states only become apparent when a subject is exposed to some other thrombogenic trigger such as surgery, oestrogen-containing medication, dehydration or systemic upset.

Table 1. Patients to be investigated for thrombophilia.

1	Venous thromboembolism before the age of 40–45 years
2	Recurrent venous thrombosis or thrombophlebitis
3	Thrombosis in an unusual site, e.g. mesenteric vein, cerebral vein etc.
4	Unexplained neonatal thrombosis
5	Skin necrosis, particularly if on coumarins
6	Arterial thrombosis before the age 30 years
7	Relatives of patients with thrombophilic abnormality
8	Patients with clear family history of venous thrombosis
9	Unexplained prolonged activated partial thromboplastin time
10	Patients with recurrent foetal loss, idiopathic thrombocytopaenia or SLE

Clinical implications

Screening

The British Committee for Standards in Haematology (BCSH) identified 10 groups of patients who should be screened for thrombophilia (Table 1).[1] The treatment of thrombophilic abnormalities is complex, and the decision for treatment, which may be lifelong anticoagulation, should only be made after careful consideration of the patient, the individual thrombophilia and any triggering factors that may have precipitated a previous thrombosis. It is our recommendation that such patients are referred to a haematologist.

In patients with PAOD who do not fall into one of the groups in Table 1, thrombophilia screening is still likely to reveal an abnormality in approximately 30–60% of patients. In those who are not undergoing a vascular intervention, there is no evidence to suggest that treatment of the thrombophilia will alter the progression of arterial disease. There is evidence however, that patients with a thrombophilia undergoing a vascular intervention have a poor prognosis, with increased risk of graft occlusion, limb loss and death, and this can be partially offset by treatment. It is therefore recommended that all patients undergoing a vascular intervention should be screened for a thrombophilic tendency.

Testing for thrombophilia should depend on the individual abnormality. Antiphospholipid antibodies, activated protein C resistance, and hyperhomocysteinaemia are the commonest abnormalities, and should form the basis of a thrombophilia screen. Screening for protein C and S deficiency, prothrombin 20210A, and antithrombin III deficiency may be useful, but likely to yield less positive results, although no less significant.

Assays for homocysteine have previously been difficult to perform, due to the requirement for immediate cooling of the sample and separation within 1 h. New techniques are being developed to improve the stability of blood samples for homocysteine analysis, increasing the ease by which homocysteine assays can be performed.[74,75]

The cost of thrombophilia screening is used as an argument against its use. However, if screening were targeted to high-risk groups, such as those in Table 1, or those undergoing intervention, the cost of screening may be offset against the reduced risk of failure of vascular intervention. The treatment of intervention failure may include prolonged hospital stay, repeated intervention, or amputation, all with significant costs. A more detailed cost-benefit analysis is beyond the scope of this article, and would be difficult to perform given that the lack of trials in this area means the true benefit of screening and/or treatment cannot be quantified.

Treatment

Although numerous different treatments are available for thrombophilias, they have not been formally studied in patients with PAOD to determine whether improved outcomes can be attained.

Hyperhomocysteinaemia

Patients with hyperhomocysteinaemia, who are undergoing a vascular intervention, should be treated with homocysteine lowering therapy prior to surgery if there is sufficient time. If the surgery is urgent, consideration should be given to formally anticoagulating these patients until the level of homocysteine can be reduced. Hyperhomocysteinaemia may be corrected simply with folic acid, and vitamins B_{12} and/or B_6, although it has yet to be demonstrated whether such treatment will lead to a reduction in cardiovascular risk or improvement in patency rates. Trials are presently being undertaken to determine whether lowering homocysteine levels is beneficial in terms of outcome for vascular patients both in PAOD and in cardiac and cerebrovascular disease. It seems sensible in the absence of current evidence however, to lower homocysteine levels in PAOD patients undergoing intervention.

Anti-coagulation

Anticoagulation in non-thrombophilic patients is of benefit in femoro-popliteal bypass grafts when compared with no treatment, but when compared to aspirin, the data are conflicting.[76] The largest study was a multicentre, randomised controlled trial investigating the effectiveness of oral anticoagulation (to maintain an INR 4.0–4.5) against aspirin (80 mg daily) in 2690 patients undergoing infrainguinal bypass surgery,[77] which showed no overall benefit of either treatment in preventing graft occlusion. Patients with antiphospholipid antibodies who are anticoagulated (with heparin and subsequently warfarin) when they underwent vascular surgery were noticed to suffer fewer complications.[70] No studies to date have prospectively investigated the use of anticoagulation in PAOD patients with a thrombophilia. However, Khamashita *et al.* retrospectively studied the effectiveness of anticoagulation in patients with antiphospholipid syndrome.[78] They showed that anticoagulation with warfarin to an international normalised ratio (INR) of >3 was significantly more effective in preventing recurrent thrombosis than anticoagulating to an INR <3, or aspirin. This study was not confined to patients with PAOD, but is significant in demonstrating a benefit of aggressive anticoagulation in thrombophilia.

Steroids

Whilst the thrombophilias discussed previously are not thought to be associated with a vasculitis, patients with the lupus anticoagulant who are taking steroids seem to have a reduced thrombotic risk.[70] The protective effect of steroids in conjunction with aspirin has been demonstrated previously in obstetric patients, and leads to a decrease in lupus anticoagulant levels.[79] There are no data on the use of steroids for PAOD patients with thrombophilia.

Anti-platelet agents

Aspirin is beneficial in obstetric patients with the lupus anticoagulant.[80] The use of aspirin has not been investigated in PAOD with a thrombophilia, but it is suggested that it be used in patients with aPL, with no history of thrombosis. Patients with aPL undergoing surgery, or with a history of thrombosis should be formally anticoagulated, as these patients are at high risk of thrombosis.

Factor replacement

An alternative treatment for patients with protein C or S deficiency undergoing surgery is the use of perioperative fresh frozen plasma or protein C concentrate. In the case of peripheral vascular surgery, patients will usually require formal anticoagulation to ensure the patency of the graft.

Nucleic acid therapy

Recently, oligonucleotides have been shown to have *in-vitro* anticoagulant effects through specific protein binding.[81] It remains to be seen whether this will translate into improved outcomes in thrombophilias.

Conclusion

The evidence to date supports an association between certain thrombophilias and peripheral vascular disease. Hyperhomocysteinaemia, hyperfibrinogenaemia, APCR and aPL syndrome are more common in PAOD, but there is no clear evidence for the other thrombophilias.

Thrombophilic states in general are associated with an increased failure rate of vascular reconstruction. This is particularly marked when considering patients with multiple thrombophilias, and early intervention failures. No conclusive evidence yet exists to show that treatment of these thrombophilic states can lead to an improvement in the course of PAOD, or the results of intervention. While it may be appropriate to anticoagulate patients identified with a thrombophilia who are undergoing a vascular intervention, it cannot yet be justified to recommend screening of all patients with PAOD for thrombophilia. There is a pressing need for well-designed trials of therapeutic intervention in patients with thrombophilia to determine whether outcomes are genuinely improved.

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