THE CLINICAL ROLE OF FIBRINOGEN AND FIBRIN

IN PERIPHERAL ARTERIAL DISEASE

by

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I declare that the work presented in this thesis has been carried out solely by me except where indicated in the text and below.

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DEDICATION

To my wife and family for their patience and support.

SUMMARY

There is a high incidence of peripheral arterial disease in the West of Scotland associated with atherosclerosis. This may manifest itself as intermittent claudication, ischaemic rest pain, gangrene or in the form of abdominal aortic aneurysm.

These patients have a greatly increased risk of thrombotic events not only in the limb arteries but also in the coronary, extracranial and cerebral vessels. Recently there has been increasing interest in the contributions of thrombosis and haemorheology to arterial disease. The aim of this thesis is to investigate the role of fibrinogen, fibrin formation and blood rheology in patients with peripheral arterial disease.

Fibrinogen has roles in thrombosis and haemorheology: the conversion of fibrinogen to fibrin is the central event of coagulation and fibrinogen is a determinant of whole blood viscosity through its effects on plasma viscosity and red cell aggregation.

Fibrin is degraded by plasmin into fibrin degradation products. Fibrin degradation products (FDP's) can now be measured using a specific monoclonal antibody to the D-dimer of cross-linked FDP's. Cross-linked FDP's are therefore markers of in vivo fibrin formation and lysis.

Red cell aggregation is the major determinant of low shear blood viscosity and can now be measured photometrically by a new technique.

Fibrinogen, cross-linked FDP's and red cell aggregation were measured in 115 patients with peripheral arterial disease together with other blood factors of haemostasis and blood rheology. Multivariate analysis showed that the increase in plasma fibrinogen in patients with peripheral arterial disease can be explained by the effect of smoking.

Cross-linked FDP's were also increased in peripheral arterial disease and were associated with the fibrinogen level. This has not previously been reported and suggests that the increased fibrinogen level may promote fibrin formation. Cross-linked FDP's increased with age, but were not related to smoking habit. Cross-linked FDP's correlated with severity of ischaemia and were highest in patients with abdominal aortic aneurysm. Cross-linked FDP's increased following reconstructive vascular surgery suggesting increased fibrin formation, which, if intravascular, could be detrimental to the patency of vascular grafts. Cross-linked FDP's may be prognostic markers for subsequent vascular graft occlusion and this possibility merits further investigation.

Red cell aggregation was also increased in peripheral arterial disease (not previously reported by a photometric method). This may also be related to the increased

fibrinogen level. Red cell aggregation was higher in ischaemic rest pain than in intermittent claudication, and was highest in patients with abdominal aortic aneurysm. High red cell aggregation was found in ex or non smokers compared to current smokers. The reason for this is not clear.

This study also confirmed the increased levels of plasma viscosity, white cell count and triglyceride and decreased HDL cholesterol found in peripheral arterial disease in other studies. Total cholesterol was not increased in this study. There has been recent interest in the rheological properties of white cells. White cell count was related to the severity of ischaemia, being highest in patients with ischaemic rest pain. White cell count and fibrinogen were also increased by cigarette smoking. Fibrinogen and white cell count are two possible mechanisms through which the effects of smoking could lead to progress of peripheral arterial disease.

Fibrinogen is an acute phase reactive protein. The cytokine interleukin - 6 regulates hepatic synthesis of fibrinogen in the acute phase response. In order to assess whether interleukin - 6 regulates the raised fibrinogen level in peripheral arterial disease, interleukin - 6 was measured in 30 patients and found to be significantly increased when compared to controls. This has not been previously reported and is in keeping with an inflammatory process in peripheral arterial disease. Interleukin - 6 was not related

to smoking habit. Some correlation between interleukin - 6 and fibrinogen was found in this study. It is therefore possible that the raised interleukin - 6 level may be one inducer of the raised fibrinogen level in peripheral arterial disease.

Activation of Factor XII initiates the intrinsic system of coagulation when blood comes into contact with a non - endothelial surface. Levels of activated Factor XII were measured in 40 patients with peripheral arterial disease by a new and modified assay in order to assess whether atherosclerosis acts as a "foreign" surface initiating the intrinsic system of coagulation. Levels were not increased in patients compared to controls, suggesting that atherosclerosis does not promote thrombosis through acting as a foreign surface.

Insertion of aortic grafts in 11 patients temporarily increased activation of Factor XII, returning to baseline levels at one day and one week after surgery. It is possible that the graft surface is rapidly coated with a conditioning layer and no longer acts as a foreign surface at one day.

A short term increase in fibrinogen, red cell aggregation and white cell count following reconstructive vascular surgery was probably due to an acute phase response.

In order to assess fibrinolytic activity in patients with peripheral arterial disease plasminogen activator inhibitor was measured in 100 patients with stable intermittent claudication. Levels were increased in the patients, suggesting deficient fibrinolysis in peripheral arterial disease. This has not previously been reported.

Von Willebrand factor is an essential co-factor for platelet adhesion and high shear induced platelet aggregation following vascular injury. Levels of von Willebrand factor were measured and found to be increased in 100 patients with intermittent claudication.

Factor VII is an important risk factor for the development of ischaemic heart disease. It was also increased in 100 patients with intermittent claudication. This finding has not previously been reported, and may also be related to thrombosis in these patients.

Plasminogen activator inhibitor, von Willebrand factor, and factor VII introduce three possible new risk factors for thrombosis in peripheral arterial disease, and provide additional evidence of a hypercoagulable state and deficient fibrinolysis in peripheral arterial disease.

Having established that patients with peripheral arterial disease have a hypercoagulable state and abnormal blood rheology, the next aim of this thesis was to determine if low-dose antiplatelet or anticoagulant therapy would alter

this. A double blind, placebo controlled trial of patients with stable intermittent claudication was performed. 100 patients attending the out patient clinic were randomised to receive minidose warfarin, low-dose aspirin or placebo for three months. However there was no significant alteration in the hypercoagulable state or abnormal blood rheology after three months of treatment.

While this trial does not exclude clinical benefit from long term prevention of thrombotic events, an improved medical management for altering thrombotic factors in peripheral arterial disease is required. Suggestions for this include studies of fish oil extracts which are known to reduce fibrinogen levels, inhibitors of shear induced platelet aggregation and interleukin - 6.

The potential effects of fibrinogen on thrombosis through increased fibrin formation and red cell aggregation suggest the need to lower raised fibrinogen levels in peripheral arterial disease.

CHAPTER 1

General Introduction

Peripheral Vascular Disease

The West of Scotland has a high incidence of cardiovascular disease (Pocock et al., 1980, see Figure 1.1). Peripheral vascular disease may manifest itself as intermittent claudication, ischaemic rest pain, gangrene or in the form of aneurysm. The commonest cause is atherosclerosis. While the vascular surgeon may see most of the effects of atherosclerosis in the limb arteries, it is part of a diffuse cardiovascular disease.This may affect the management of the peripheral arterial disease.

Arteriosclerosis is a generic name introduced by Lobstein (Lobstein, 1829) to describe thickening and induration of the artery. It is not a specific pathological entity but clinically includes atheroma, Monckeberg's sclerosis, and the degenerative changes in arteries associated with age. These often occur together, particularly in the lower limb arteries (McKeown, 1965).

The term atherosclerosis referring to the type of arteriosclerosis affecting the intimal coat of the artery was coined in 1904 by Marchand, a pathologist from Leipzig (Leibowitz, 1970). Improved medical care and standard of living this century have contributed to a longer life expectancy. An increase in the elderly population, together with continued adverse smoking habits and dietary habits have led to an increase in the number of patients

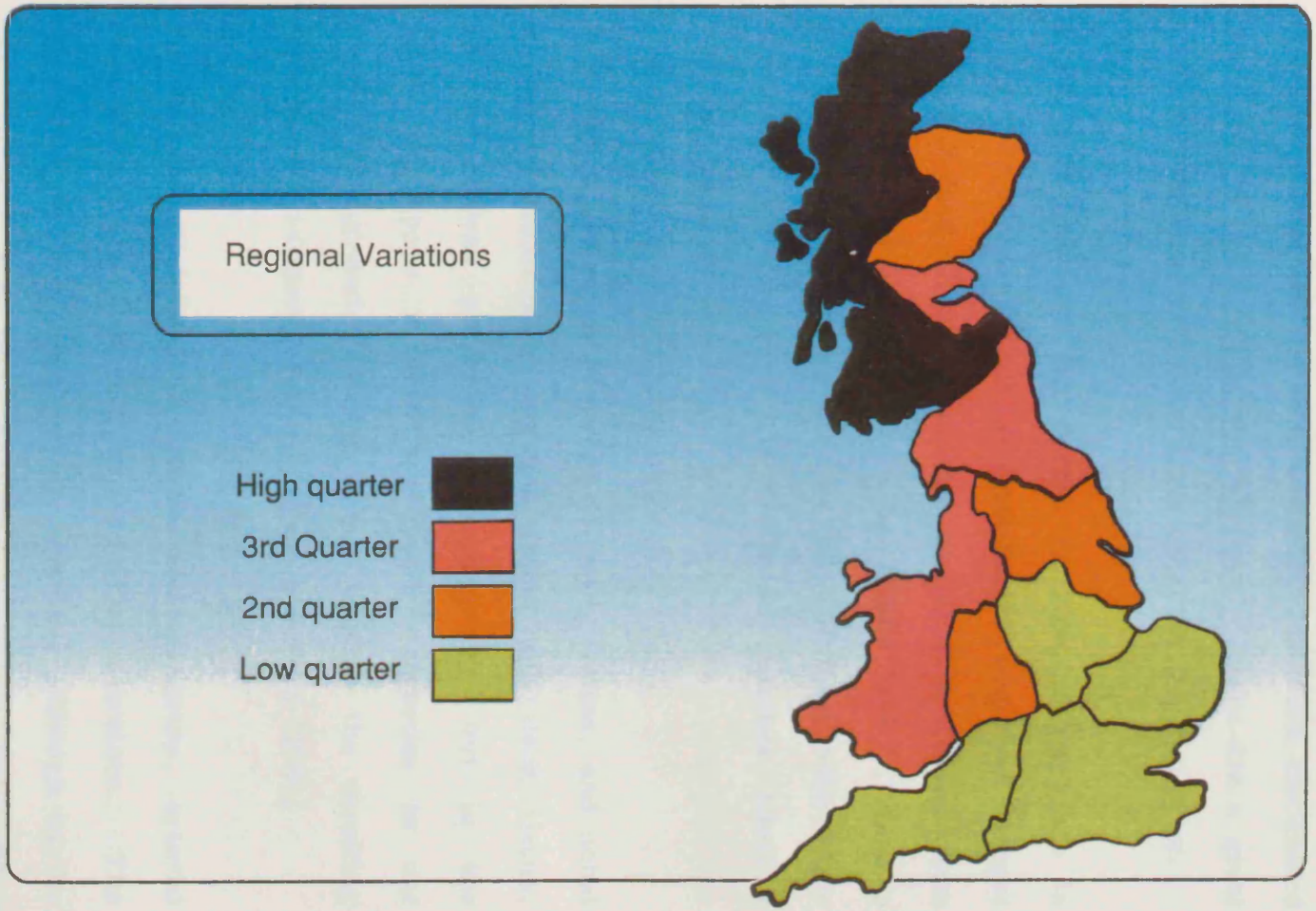

Figure 1.1

Death rates per 100,000 from ischaemic heart disease in regions of Scotland, England and Wales. Shading denotes quartiles. (after Fulton et al., 1978)

presenting with the clinical manifestations of atherosclerotic peripheral arterial disease.

Peripheral vascular disease, however, has been recognised as a clinical entity since ancient times. There are a great many descriptions and recordings found in the literature.

While it is probable that many instances of gangrene in ancient times were due to trauma or ergotism, (ergot poisoning from infected rye; this appeared in epidemic form in Europe from Roman times to the Middle Ages), Leonardo da Vinci illustrated the arteries of a subject with senile arteriosclerosis in one of his anatomical sketches (Reid & Pollock, 1978).

Hippocrates described gangrene following trauma, and noted a line of demarcation between living and dead tissue. Amputation was performed for gangrene, but in the Hippocratic code it was considered dangerous to cut through living and sensitive tissue because the resulting pain caused collapse and death (Reid & Pollock 1978).

As well as peripheral ischaemia and gangrene, arterial aneurysms have featured in historical literature. The commonest cause of aneurysm is atheroma, although in the past syphilis and false aneurysm formation were more common than today. Antyllus described aneurysm in the second century A.D. during the rule of Emperor Julian, when the Roman Empire was at its height (Eastcott, 1982).

Galen, another Roman, having completed his medical training in Alexandria took up his post as surgeon to the school of gladiators in his native town, Pergamos in Asia Minor. He recognised one of the most characteristic features of aneurysm - pulsation (Major, 1954). His aptitude for clinical observation interested him more than surgical treatment.

Aetuis of Amida was an early Byzantine and one of the earliest head and neck surgeons. He also treated aneurysm of the brachial artery, gaining proximal control and then incising the sac and ligating the aneurysm (Ericksen, 1844). Iatrogenic brachial artery aneurysms were not uncommon in those times as the practice of bloodletting was widely carried out.

More recently in 1761, William Hunter, a Scot and elder brother of John Hunter, gave the name to and described four instances of arteriovenous aneurysm caused by attempted phlebotomy (Hunter, 1761).

Definitions:

Intermittent claudication is pain in the muscles of the lower limb on walking relieved by rest. Exercise is always required before it appears. Intermittent claudication is caused by impaired flow of arterial blood to the muscles. The pain classically occurs in the calf muscles; it may extend to the thigh or buttock when vascular lesions involve the aorto-iliac segment.

Ischaemic rest pain is pain in the distal part of the extremity due to severe ischaemia where the blood supply and oxygen delivery are not sufficient to meet the needs of the tissues at rest. It is present at rest and is partly relieved by dependency. (Dependency however leads to oedema which may increase devitalisation of the skin resulting in tissue necrosis, ulceration and gangrene).

Gangrene is necrosis with putrefaction - digestion of dead tissue by saprophytic organisms.

An **aneurysm** is a localised dilatation of the artery. The wall of the true aneurysm is composed of components of the arterial wall. The natural history is characterised by expansion. Aneurysms differ from ectasia which is generalised dilatation of the vessel.

Evolution of the Current Status of Surgery for Peripheral Arterial Disease.

While in the past there was no effective medical or surgical treatment, many ischaemic limbs can now be salvaged by reconstructive vascular surgery (giving much satisfaction to the patient and surgeon) with restoration of function, relief of ischaemic rest pain and restored viability. Currently reconstructive vascular surgery is performed for three groups of patients: patients with intermittent claudication, patients with severe ischaemia requiring limb salvage and patients with aneurysm.

In the past amputation was performed for gangrene: in the 5th Century B.C. amputation was recommended to be performed through devitalised tissue where vessels were thrombosed and would not bleed (Rang et al., 1981).
By the 1st Century A.D. Aulus Cornelius performed amputations at the line of demarcation between healthy and gangrenous tissue. He stated "some of the sound part be cut away than any of the diseased part be left behind" (Harvey, 1929).

By 100 A.D. Archigenes advocated primary ligation of vessels proximal to the level of gangrene (Beasley, 1982), making preparatory ligature of vessels before amputation of the distal limb.

Ambroise Paré also used ligation of vessels for amputation in the sixteenth century. Paré was a military surgeon and recognised the importance of the level of amputation with regard to successful prosthetic limb fitting. He described the first revisional amputation of an officer's leg that had been shot off by a musket ball because the healed stump was too long for useful function with an artificial limb. Even after Paré, ligation of vessels for amputation was not generally accepted because of delayed haemorrhage. This was presumably due to infection or because ligatures were not properly applied (Reid & Pollock, 1978).

The first descriptions in English of the use of ligatures in amputation surgery were by William Clowes and Peter Lowe in 1596 (Clowes, 1596; Lowe, 1596). Lowe founded the Faculty of Physicians and Surgeons of Glasgow and was in Paris when Paré was an old man. He most probably knew him.

John Hunter ligated the femoral artery in the treatment of a popliteal artery aneurysm in 1785 (Eastcott, 1966; Rains, 1966). Animal studies had led him to place the ligature above the anastomotica magna artery, to aid in the thrombosis of the aneurysm, and to confine it to the sac.

Reconstructive vascular surgery is a very recent advance. The experimental work of Carrel in the early years of this century did not lead to immediate clinical success but technical observations were recorded which are still pertinent today (Carrel, 1908). Murphy (1897) sutured an artery together by end to end anastomosis in 1897. Goyanes in Madrid inserted popliteal vein to bridge the defect after excising a popliteal aneurysm (Goyanes, 1906), and in 1912 Lexer repaired an axillary artery aneurysm with vein graft (Lexer, 1913). J Hogarth Pringle successfully treated a brachial artery occlusion and popliteal artery aneurysm using vein graft in Glasgow Royal Infirmary in 1912 (Pringle, 1913).

Reconstructive vascular surgery was greatly aided by arteriography, introduced by R dos Santos in 1929 (dos Santos et al., 1929). His son Cid dos Santos introduced thromboendarterectomy in 1947 (dos Santos, 1947).

Another advance was the introduction of bypass grafts. Kunlin introduced the bypass vein graft (Kunlin, 1951). Eastcott and Hufnagel after World War II found undenatured homografts successful in human aorta. Freeze dried homografts were used to replace occluded segments in large arteries (Eastcott, 1953; Hufnagel et al., 1953). Dubost and his colleagues in Paris successfully inserted a homograft for an abdominal aortic aneurysm in 1952 (Dubost et al., 1952).

Cooley and DeBakey, and Gerbode used homograft for ruptured abdominal aortic aneurysm (DeBakey & Cooley, 1953; Gerbode, 1954). However homografts had disappointing long term results due to dilatation.

The most recent major advance in reconstructive vascular surgery has been the introduction of prosthetic material for arterial grafts. Voorhees introduced Vinyl N Cloth in 1952 (Voorhees et al., 1952). This was followed by Nylon, Teflon and Dacron. Dacron is now a material of choice in the wide bore, high-flow situation (Cooley, 1985). Many modifications in graft technology have been applied to improve long term patency. These include close knitted Dacron, a low internal velour and high external velour, a

strengthened skeleton and gelatin sealant (Wu et al., 1985; Guidoin et al., 1986; Reid & Pollock, 1991;). There has been a progressive reduction in operative mortality in reconstructive vascular surgery and this will remain the mainstay of surgical treatment. In selected cases, percutaneous intra-arterial angioplasty, and more recently laser assisted angioplasty and intra-arterial thrombolytic therapy offer the patient an improved arterial circulation and carry a lower mortality than conventional surgery (Ferguson et al., 1986).

PATHOLOGY OF ATHEROSCLEROSIS

Definition

Atheroma is derived from the Greek "athere" which means porridge. The lesions of atheroma consist of plaques of intimal thickening of the wall of arteries due mainly to accumulation of lipids, proliferation of smooth muscle cells and formation of fibrous tissue.

Pathology

The fatty streak is the earliest lesion, commonly found in children. It is a patch of intimal thickening, a lipid rich lesion consisting of macrophages and smooth muscle cells (McGill, 1968). Fatty streaks are found in early life at similar anatomical sites as atheromatous plaques in older ages (Leary, 1941; Robertson et al., 1963). The role of the fatty streak in atherosclerosis is uncertain because

population studies have shown some to be reversible (Berry, 1989).

The fibrous plaque of advanced atherosclerosis is made up of increased intimal smooth muscle cells surrounded by connective tissue matrix and containing variable amounts of intracellular and extracellular lipid (Ross, 1986). At the lumen of the artery this plaque is generally covered by a dense fibrous cap of smooth muscle and connective tissue. Beneath the fibrous cap are smooth muscle cells and macrophages which contain lipid droplets, surrounded by connective tissue (Ross, 1986). When lipid is abundant and the fibrous layer is thin, ulceration may occur (Lindop, 1985). Mural thrombus is then likely to be deposited on the surface of the ulcer. It is common to get deposition of calcium salts in the plaque (Ross, 1986); occasionally bone formation is seen (Reid & Pollock, 1978). Thinning of the media and in some instances extension of plaques into the media can weaken the arterial wall: this may lead to aneurysm formation (Lindop, 1985).

Microscopy

In the early stages of atheroma there is accumulation of lipid in proliferated spindle cells. These spindle cells are shown by electron microscopy to be smooth muscle cells lying in the intima. They are derived by proliferation and migration of smooth muscle cells from the media (Lindop, 1985). Laminae of connective tissue appear between lipid rich smooth muscle cells in the subendothelial intima and

form the fibrous part of the lesion. Then areas of necrosis in the deeper part of the lesion occur resulting in a structureless accumulation of lipids, tissue debris, altered blood and necrosis which extends into overlying fibrous tissue. Calcium deposition may be seen. There is an infiltration of neutrophil leucocytes and other inflammatory cells. Lipid-laden macrophages (foam cells) may appear around lipid deposits and usually contain crystals of cholesterol. The internal elastic lamina is disrupted resulting in lipid deposition, necrosis and fibrosis extending into the adjacent media. The media deep to the plaque is thin and atrophic. Small blood vessels grow into the plaque from the vasa vasorum in the media and sometimes from the intimal surface (Lindop, 1985).

Atheroma often develops first around the origins of the intercostal and lumbar branches in the aorta. In the legs atheroma is often severe resulting in a progressive diminution of the blood supply and peripheral ischaemia.

When collateral blood supply becomes inadequate the ischaemia may be sufficiently severe to cause gangrene (see Plate 1). When the arteries of legs amputated for gangrene are examined they usually show narrowing or obliteration and calcification of the major arteries.

The arteries supplying the upper limbs may also be affected by atheroma and occlusion of vessels in the arms may be

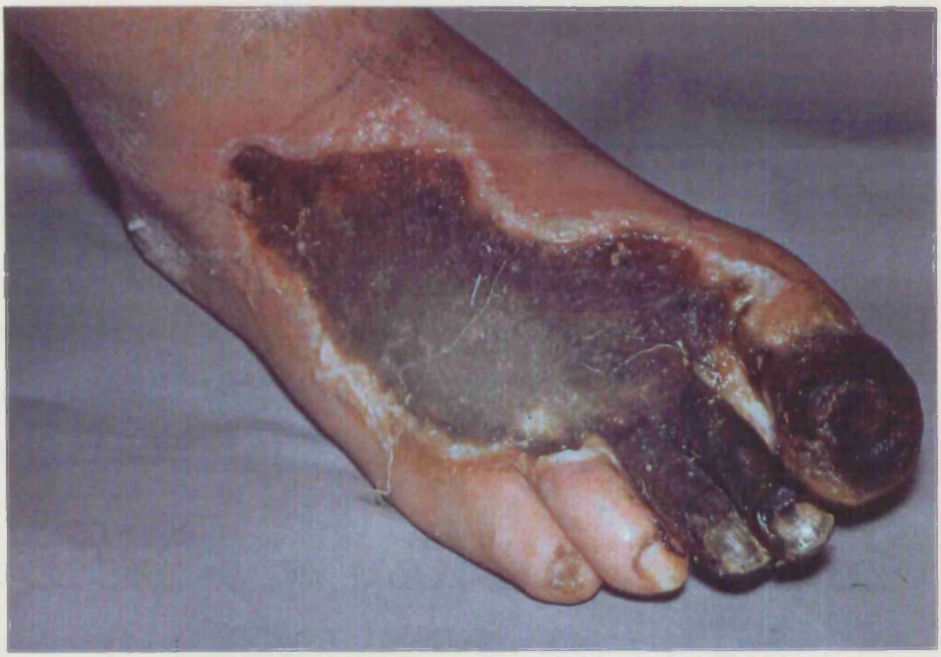

Plate 1 Critical leg ischaemia with gangrene.

detected on routine clinical examination in some patients presenting with other symptoms of peripheral vascular disease. The upper limb has an excellent collateral blood supply and is not exerted in the same way as the lower limb; this may explain why few patients with peripheral vascular disease present with upper limb symptoms.

Complications

Complications of atheroma are occlusive thrombosis, haemorrhage, ulceration or rupture, aneurysm and embolism.

Occlusive thrombosis is the commonest complication. This may be preceded by haemorrhage into a plaque, which increases the amount of luminal narrowing; plaque ulceration, or plaque rupture (Davies & Thomas, 1985). In the aorta complications of atheroma are aneurysm formation as well as thrombosis and embolism.

AETIOLOGY OF ATHEROSCLEROSIS

Although the pathogenesis of atherosclerosis remains unclear many recent observations have indicated new areas for research. These have centred around lipids, platelets, smooth muscle cells, monocytes, fibrinogen and blood rheology.

Lipids and Atherosclerosis

Chronically elevated levels of cholesterol, especially that carried by low density lipoprotein (LDL) and very low density lipoprotein (VLDL), have been associated with increased atherosclerosis in epidemiological studies (Inkeles & Eisenberg, 1981). In contrast, high density lipoprotein (HDL) is considered to be a protective factor in atherosclerosis (Inkeles & Eisenberg, 1981). Clinical trials have suggested that it is beneficial to lower plasma LDL levels since a decrease in cholesterol correlates with a reduction in the clinical sequelae of atherosclerosis (Thompson, 1987).

More than 93% of body cholesterol is located in the cells, while about 7% of body cholesterol is in plasma (Brown & Goldstein, 1981). All the cholesterol in plasma is contained within lipoprotein particles. Brown and Goldstein observed that cells contain specific surface receptors for LDL. When cells require cholesterol for the synthesis of new membranes, bile acids or steroid hormones, plasma LDL binds to receptors. It is taken into the cell and is degraded, providing cholesterol cell metabolism.

Brown and Goldstein in their study of homozygous familial hypercholesterolaemia, proposed that the number of LDL receptors displayed by a cell varies with the need of a cell for cholesterol. This protects the cell against excess cholesterol. When the number of LDL receptors is decreased there is a reduction in the removal of LDL from

the circulation and a consequent increase in the plasma LDL. They have shown in familial hypercholesterolaemia that there is a deficiency of LDL receptors with a consequent rise in plasma LDL levels. However this does not explain how hypercholesterolaemia leads to atherosclerosis (Brown & Goldstein, 1986).

It is possible that hypercholesterolaemia may induce a subtle form of endothelial cell injury. Hypercholesterolaemia alters the cell membrane cholesterol: phospholipid ratio. This might decrease the malleability of the endothelial cells at the branches and bifurcations where they may be damaged by changes in blood flow (Jackson & Gotto, 1976). Hypercholesterolaemia also results in increased monocyte adherence to the endothelial cells (Faggiotto et al., 1984), possibly followed by release of interleukins and growth factors.

Platelets and Atherosclerosis

Endothelial cells normally prevent platelet adherence by their electro-negative charge and by their capacity to form antithrombotic substances such as prostacyclin (Ross, 1986). Platelets contain at least two mitogenic substances - epidermal growth factor and platelet derived growth factor (PDGF) (Oka & Orth, 1983; Ross et al., 1974).

PDGF is produced by megakaryocytes and is stored in platelets (Ross, 1986; Kaplan et al., 1979). Growth factors are also produced by monocytes, macrophages, endothelial

cells and smooth muscle cells (Ross, 1986). PDGF is released during platelet aggregation following the adhesion of platelets to subendothelium and collagen. It has a high affinity for smooth muscle cells and fibroblasts and causes proliferation of these cells. It does not have a high affinity for endothelium (Bowen-Pope & Ross, 1982). PDGF is mitogenic and chemotactic (Grotendorst et al., 1982; Senior et al., 1985). This could permit PDGF to bind to connective tissue at the site of endothelial cell damage and attract smooth muscle cells to migrate from the media into the intima and proliferate (Grotendorst et al., 1982).

Smooth Muscle Cells and Atherosclerosis

It is generally accepted that the migration of smooth muscle cells from the arterial media into the intima is responsible for the development of atherosclerosis. Smooth muscle cells have receptors for LDL and PDGF (Witte & Cornicelli, 1980; Bowen-Pope et al., 1985). They can respond to a number of chemotactic factors which probably explains their accumulation in the atherosclerotic lesion. Smooth muscle cells can also accumulate lipid and can take on the appearance of foam cells (Ross, 1986).

Monocytes, Macrophages and Atherosclerosis

Subendothelial migration and localisation of monocytes are the earliest events in fatty streak formation and thus in atherogenesis (Mazzone et al., 1983). One of the earliest cellular interactions in hypercholesterolaemia is the attachment of monocytes to endothelial cells (Faggioto et

al., 1984). The monocyte is known to be the source of the macrophage (Van Furth, 1982). In the intima, macrophages which possess a high affinity receptor for acetylated LDL actively phagocytose these molecules and this results in intracellular accumulation of significant amounts of cholesterol esters (Goldstein et al., 1979). Lipid droplets appear in the cytoplasm of macrophages and change their morphology. These modified macrophages are the major source of foam cells (Schaffner et al., 1980). Morphological distinction between monocyte and smooth muscle cell derived foam cells is difficult.

Macrophages also secrete a mitogen that is similar if not identical to PDGF which stimulates smooth muscle cell proliferation (Shimokado et al., 1985).

Fibrinogen and Fibrin and Atherosclerosis

The role of fibrin in the aetiology and development of atherosclerosis was first proposed by Rokitansky. Carl Rokitansky was born in Konegsgratz in 1804. He studied in Prague and Vienna and became Professor of Pathological Anatomy in 1844 in Vienna. In 1852 he proposed that deposition of fibrin in the arterial wall initiated the atheromatous process. He stated that " the deposit is an indigenous product derived from the blood and for the most part from the fibrin" (Rokitanky, 1852).

In the 1940s Duguid demonstrated the presence of fibrin on and in plaques, and postulated that it was a major factor in plaque growth (Duguid, 1946; Duguid, 1948). More recently fibrinogen and fibrin have been shown to be present in all fibrous atheromatous plaques (Smith et al., 1978). Smith et al. (1990a) reported that the character of fibrin found in plaques suggests that fibrin is involved in the development of atherosclerotic lesions. They measured fibrinogen and a range of fragments mainly derived from cross-linked fibrin in atherosclerotic plaques. The degree of cross-linkage of fibrin and the total quantity of fibrin degradation products released from histologically early atherosclerotic lesions were higher than in late lesions or in normal intima. They suggested that the fragments may be atherogenic (Smith et al., 1990a). Fibrinogen may play a further role in atherosclerosis by its rheological effect, increasing plasma viscosity and red cell aggregation.

Blood Rheology and Atherosclerosis

The observations that atherosclerotic plaques tend to occur in relation to the origins of branches (e.g. intercostals), bifurcations (e.g. aorta, carotid artery) and bends of major arteries (such as the adductor opening of the superficial femoral artery) have led to the hypothesis that alterations in flow at these sites potentiate the development of atherosclerosis (Glagov et al., 1989). Haemodynamic factors which may be responsible for this localisation include low flow velocity and reduced wall shear stress, increased flow velocity and increased wall shear stress, and flow

separation (turbulence). (Cornhill & Roach, 1976; Fry, 1973; Caro et al., 1971; Wesolowski et al., 1965).

Atherosclerotic plaques are found localised to low flow, low shear areas, which can promote increased red cell aggregation and blood viscosity, particularly when increased levels of fibrinogen and haematocrit exist (Lowe, 1987a). Blood cells and cellular aggregates accumulate in these areas of low shear, re-circulating flow (Goldsmith & Karino, 1982). The long dwell times of activated platelets and white cells should promote their interaction with endothelium (Lowe, 1987a). Increased levels of fibrinogen and haematocrit promote platelet aggregation in whole blood (Lowe, 1987a). The selective deposition of fibrinogen and fibrin in developing arterial lesions (Smith et al., 1978) may be another mechanism by which increased fibrinogen levels could promote arterial disease.

The rheological properties of the monocytes may also be relevant to their proposed involvement in atherogenesis: monocytes are the most adhesive and least deformable type of circulating white cell (Lowe, 1987a).

Hence, fibringoen and rheological abnormalities of the blood may also have a significant role in the development of atherosclerosis.

EPIDEMIOLOGY OF PERIPHERAL ARTERIAL DISEASE

Epidemiology studies the extent, distribution and causes of disease in populations. The epidemiology of atherosclerosis has dealt mainly with ischaemic heart disease and stroke, however there is increasing interest in the epidemiology of peripheral vascular disease (Fowkes, 1988; Dormandy et al., 1989).

Patients with intermittent claudication are up to four times more likely to have coronary artery disease, (Reid et al., 1966; Hughson et al., 1978) and about one half will die from a heart attack (Widmer et al., 1981). Likewise patients with coronary artery disease have at least a five times increase in risk of developing intermittent claudication (Reunanen et al., 1982; Dawber, 1980). These findings reflect the diffuse nature of atherosclerosis.

Epidemiological research into peripheral arterial disease has concentrated on symptomatic patients with intermittent claudication, however studies in the USA and Europe suggest that severe asymptomatic disease is common and that at least 10% of the older population has a major disruption to peripheral blood flow by the age of 60 years (Criqui et al., 1985; Schroll & Munck, 1981; Widmer et al., 1964; Fowkes, personal communication).

The term risk factor expresses the observed relationship with disease. Risk factors are associations, and cannot necessarily be identified as causal factors; they remain a target for modification in intervention studies. In the pathogenesis of atherosclerosis, injury to the endothelium, fibrinogen, lipids, blood platelets, smooth muscle cells and monocyte macrophages have been shown to be potential mechanisms. Risk factors may affect some of these mechanisms.

Fowkes in an epidemiological review of the aetiology of peripheral atherosclerosis stated that cigarette smoking is an independent factor greater than any other risk factor, and it is a more important aetiological variable in peripheral arterial disease than in ischaemic heart disease (Fowkes, 1988). Smoking is associated with increased circulating fibrinogen concentration, blood viscosity, white blood cell count and haematocrit. Fowkes also reported that serum triglyceride level and diabetes mellitus are important risk factors for peripheral arterial disease, but that hypertension and serum cholesterol level (important in cerebrovascular and ischaemic heart disease) have a less important role in peripheral arterial disease.

Conventional Risk Factors

Conventional risk factors are smoking, hypertension, dietary fat, diabetes mellitus, age, sex, lack of physical activity and blood group.

Smoking

Vascular surgeons have long been aware of the adverse effects of cigarette smoking. More than 90% of patients attending hospital with intermittent claudication have a history of smoking (Lord, 1965). In the Framingham study (a prospective epidemiological study carried out in Framingham, Massachusetts, USA) cigarette smoking and diabetes mellitus were the most important risk factors for developing intermittent claudication (Gordon & Kannel, 1972). Smoking was found to be a more important aetiological variable in peripheral arterial disease than in ischaemic heart disease (Dawber, 1980).

Hypertension

Hypertension is known to be a major risk factor for cerebrovascular disease (Gordon & Kannel, 1972). While most epidemiological studies have shown that raised blood pressure is a possible risk factor for peripheral arterial disease a causal association has not been confirmed

(Fowkes, 1989). The common occurrence of severe atherosclerosis in normotensive individuals indicates that hypertension may potentiate or enhance atherosclerosis rather than be essential for its occurrence.

Lipids and Dietary Fat

Ingestion of a diet rich in animal fat leads to an alteration in the serum lipids. In populations who eat a lot of animal fat there is a high incidence of myocardial infarction (Keys, 1980). A reduction in serum cholesterol level in hypercholesterolaemic subjects leads to reduction in the incidence of subsequent myocardial infarction and the benefit is proportional to the decrease in serum cholesterol (Thompson, 1987).

At least 20 studies have examined the relationship between blood cholesterol concentration and peripheral arterial disease: a consistent picture has not emerged (Fowkes, 1988). Serum triglyceride levels have been found to be higher in peripheral arterial disease than in controls but an independent effect of triglyceride has not been shown since triglyceride concentrations are inversely related to high density lipoprotein (HDL) cholesterol. It would be necessary to show that triglyceride acts independently of HDL and other factors (Fowkes, 1989; Hulley et al., 1980).

Diabetes and Insulin

Ischaemic gangrene may be the complaint which reveals the presence of diabetes mellitus. In an autopsy study, Bell found that arteriosclerotic gangrene was 40 times more frequent in diabetics than in non diabetics and more common in female than in male diabetics (Bell, 1950).

A raised casual blood glucose concentration in the Framingham study was more closely related to developing intermittent claudication than heart disease and had an independent effect when other risk factors were taken into account (Kannel & McGhee, 1984). However, Fowkes concludes that more evidence is required to determine whether an impaired glucose tolerance is a risk factor in the general population (Fowkes, 1988).

Insulin could have an aetiological role in atherogenesis. Insulin stimulates the migration (Nakao et al., 1985) and proliferation (Pfeifle et al., 1980; Pfeifle & Ditschuneit, 1981) of arterial smooth muscle cells in vitro by interaction with membrane receptors (Pfeifle & Ditschuneit, 1983).

Three epidemiological studies have reported a correlation, apparently independent of other risk factors, between plasma insulin concentration and the occurrence of coronary artery disease (Ducimetiere et al., 1980; Pyorala, 1979; Welborn & Wearne, 1979).

Fibrinogen and factor VII coagulant activity levels have been shown to be significantly higher in diabetics than controls (Meade, 1987a). In addition, both were higher in diabetics with evidence of microangiopathy (Meade, 1987a). The raised plasma fibrinogen concentration found in diabetics may partly explain their increases in red cell aggregation and plasma viscosity (MacRury & Lowe, 1990)

Age and Sex

Increasing age is probably the most powerful predictor of the risk of ischaemic heart disease (Meade, 1987a). Likewise, symptomatic peripheral arterial disease is commonest in the seventh and eighth decades. Fibrinogen, factor VII coagulant activity and factor VIIIc increase with age in both men and women (Meade & North, 1977), and there is a decrease in the fibrinolytic activity with age which is more marked in men than in women (Meade et al., 1979).

Physical Activity

Morris reported a lower incidence of ischaemic heart disease in people who are physically active (Morris et al., 1980). He found a higher incidence of ischaemic heart disease in London bus drivers compared to bus conductors (Morris et al., 1966). Recently Rosengren et al. (1990) reported that men with a low score for physical activity had a significantly higher plasma fibrinogen level than men with a high score.

Blood Group

In a prospective study of 7662 men (Whincup et al., 1990) the incidence of ischaemic heart disease was higher in those with blood group A than in those with other blood groups (relative risk 1.21, 95% confidence limit 1.01 to 1.46).

Plasma concentration of factor VIIIc is also significantly affected by blood group. Subjects with blood group A or B have on average 8% more factor VIIIc than subjects with blood group O (Preston & Barr, 1964). The higher plasma factor VIII and serum cholesterol concentrations in people with blood group A than in those with blood group O could summate, and contribute to a greater atherogenic potential (Boulton, 1990).

Fibrinogen as a Risk Factor for Arterial Disease

As previously noted, epidemiological research has concentrated on coronary artery disease and stroke. Most studies on peripheral arterial disease have been an adjunct to studies on coronary artery disease. Furthermore, epidemiological studies in peripheral arterial disease are difficult due to the diffuse nature of the disease: some patients with severe atherosclerosis may be asymptomatic and others may have less disease, localised at an important site (Dormandy et al., 1989).

Plasma fibrinogen has emerged as a major risk factor for coronary artery disease. Four prospective studies have reported an association between high levels of plasma fibrinogen and an increased risk of ischaemic heart disease: these are the Northwick Park Heart Study (Meade et al., 1980, 1986), the Goteborg Study (Wilhelmsen et al., 1984), the Leigh Study (Stone & Thorp, 1985) and the Framingham Study (Kannel et al., 1987). All four prospective studies have found a significant relationship between high fibrinogen levels and ischaemic heart disease. This association is a strong one (Meade, 1987b). In the Goteborg study, fibrinogen levels were also predictive of stroke (Wilhelmensen et al., 1984).

Multiple regression analysis from the data of two of the four prospective studies strongly suggests that a substantial part of the effect of smoking and ischaemic heart disease is mediated through fibrinogen (Meade, 1987b). There is also epidemiological evidence that a raised fibrinogen level is associated with peripheral arterial disease. Dormandy et al. (1973a) measured plasma fibrinogen in 126 patients with intermittent claudication. 60% of patients had a plasma fibrinogen concentration above the upper limit of the normal range. Dormandy et al. (1973b) also reported the prognostic significance of the initial fibrinogen level. They prospectively studied 62 patients with untreated intermittent claudication. There was a significant correlation between the initial fibrinogen level and subsequent progress. In patients whose claudication improved the mean initial plasma

fibrinogen was similar to controls, whereas in patients who deteriorated the mean initial plasma fibrinogen was almost double the mean normal.

Studies of intermittent claudication in the community support this. Powell et al used a standard exercise test to detect peripheral arterial disease in asymptomatic people. They demonstrated that fibrinogen was significantly raised in the subjects who had asymptomatic peripheral arterial disease and asymptomatic ischaemic heart disease, compared to disease free subjects (Powell et al., 1987). Further evidence for a role in peripheral arterial disease has come from the general epidemiology of fibrinogen: four risk factors for peripheral arterial disease have increased fibrinogen levels. Levels are higher in smokers than non-smokers while values for ex-smokers are intermediate (Meade et al., 1986; Korsan-Bengsten et al., 1972; Balleisen et al., 1985; Yarnell et al., 1983). Fibrinogen levels are significantly higher in diabetics than non-diabetics (Meade, 1987a), and fibrinogen level increases with age (Meade, 1987a). Hypertension was significantly related to fibrinogen level in the Framingham study (Kannel et al., 1987).

Alcohol consumption appears to result in decreased fibrinogen level (see Table 1.1). It is interesting that both low employment grade and increasing levels of job

Table 1.1
Effects of smoking and alcohol consumption on plasma fibrinogen (g/l) in men. (After Meade et at 1979)

Test	Drinkers	Non-drinkers	Total
Smokers	2.86	3.03	2.92
Non-smokers	2.63	2.77	2.69
Total	2.75	2.87	-

dissatisfaction are associated with increasing levels of plasma fibrinogen (Markowe, 1985), so too is poor physical activity (Rosengren et al., 1990).

The progression of symptoms in patients with peripheral arterial disease is usually associated with atherosclerosis and/or thrombosis. Increased fibrinogen levels could play an important role in these processes: by arterial wall deposition of wall fibrin(ogen) (Smith et al., 1990a), due to its influence in the coagulation system, and also as a determinant of plasma and whole blood viscosity.

THE ROLE OF BLOOD RHEOLOGY AND FIBRINOGEN IN PERIPHERAL ARTERIAL DISEASE.

Introduction

There is still a lack of knowledge about the aetiology and development of peripheral atherosclerosis. Many investigators have examined the role of cholesterol and other lipids, and also cellular aspects focusing on endothelium, arterial smooth muscle cells, macrophages and platelets. More recently the importance of the physical properties of the blood in contributing to peripheral arterial disease has been realised (Dormandy et al., 1973a, 1973b). Dormandy demonstrated that patients with intermittent claudication have increased blood viscosity and raised fibrinogen levels and that the increased blood viscosity was

an important factor in reducing blood flow and worsening of the prognosis in these patients (Dormandy et al., 1973a, 1973b).

Graft occlusion may follow progression of disease of arterial walls proximal or distal to the graft, however recent evidence suggests that plasma fibringoen is also an important factor in graft occlusion (Hamer et al., 1973; Harris et al., 1978; Wiseman et al., 1989).

There is therefore increasing evidence that increased plasma fibrinogen and its consequences have an important part to play in peripheral arterial disease, and it is now possible to investigate some of these possible consequences of fibrinogen elevation using precise laboratory measurements.

This thesis is concerned with investigation of the roles of fibrinogen, fibrin formation and blood rheology in peripheral vascular disease. Before formulating the plan of these investigations, the definitions and concepts of haemorheology are reviewed.

Definitions

Rheology is the study of flow and deformation of matter. The flow and deformation of blood in blood vessels is termed **haemorheology**. (Copley & Seaman, 1981; Copley, 1987)

Viscosity is the intrinsic resistance to flow of a bulk liquid. The intrinsic resistance to flow is caused by the internal friction between the molecular and particular components of the liquid resisting movement against each other. **Fluidity** is the reciprocal of viscosity.

When a liquid travels along a cylindrical pipe it is arranged in theoretical concentric cylindrical layers. The layer nearest the pipe's wall is stationary while the innermost, axial layer travels at the maximum velocity. The concentric layers slide over each other and this process of sliding is called shearing. The **shear rate** is the difference in velocity between two adjacent layers. This is greatest at the outermost layers of the fluid and least at the innermost layers. The resistance to shearing is caused by internal friction forces between adjacent layers. It is the frictional flow resistance that is the liquid's viscosity. The **shear stress** is the force per unit area applied to a fluid layer which causes it to move relative to its adjacent layer.

The Systeme Internationale (SI) units of shear rate are seconds^{-1} (s^{-1}). The SI units of shear stress are pascals (Pa). A pascal equals one newton per square metre (N/m^2).

Viscosity (or the dynamic viscosity of a fluid) is the ratio of shear stress to shear rate.

$$\text{Viscosity (mPa.s)} = \frac{\text{Shear stress (mPa)}}{\text{shear rate } (s^{-1})}$$

In rheology, units are expressed in millipascals (mPa) because of the small forces involved.

Sir Isaac Newton hypothesised in his Principia of 1686 that the shear rate was directly proportional to the shear stress, which makes the viscosity constant. Water is an example of a Newtonian fluid. Blood, however, acts in a different manner. At low shear rates, due mainly to red cell aggregation, the viscosity of blood increases. Therefore the viscosity alters at different shear rates for blood and it is termed a Non-Newtonian fluid.
At high shear rates (200 mPa.s and above) blood viscosity reaches a constant minimum value, about two and a half times the viscosity of plasma and about five times the viscosity of water. At high shear rates red cell aggregates are dispersed and red cells deform their shape into ellipsoids, parallel to the flow. At high shear, blood becomes a Newtonian fluid of minimal viscosity. This phenomenon occurs in many parts of the normal circulation (Lowe, 1987b).

Viscosity varies with temperature. As temperature increases there is a decrease in molecular interactions and hence decreased viscosity. This phenomenon occurs in plasma, serum and whole blood. (Harkness, 1971; Goyle & Dormandy, 1976).

The volume of fluid flowing in a tube depends on the driving pressure along the tube, the length and radius of the tube and the fluid's viscosity; this may be formulated:-

$$Q = \frac{\Delta P . \pi r^4}{8L} \frac{1}{n}$$

where Q = the flow rate

Δ P = the driving pressure

r = radius

L = length

n = viscosity

This is the Hagen-Poiseulle equation.

The **differential viscosity** of a fluid corresponds to the gradient at any given point on the shear stress versus shear rate curve. As this curve tends to be non linear for fluid suspensions the **apparent viscosity** is more commonly used and is defined as the gradient of the line between any point on the curve and the origin. Effectively this is a ratio of the overall shear stress to the overall shear rate, calculated as if both shear stress and shear rate were distributed normally (Chien, 1975).

For the purpose of this thesis the coefficient of the apparent dynamic viscosity is used exclusively, and is referred to simply as "viscosity". Units are expressed in

mPa.s. The normal range for plasma viscosity in healthy subjects from three years to middle age is 1.1-1.5 mPa.s at 37°C (Lowe, 1987b; International Committee for Standardisation in Haematology, 1984).

Blood flows under the driving pressure of the heart and the consequent pressure gradient between arteries and veins. Blood flow is inversely related to the vascular resistance, which is a function of the vessel's geometry (particularly the radius), and the viscosity of the blood.

Increased blood viscosity causes an increased flow resistance through an organ or limb and results in a reduction in blood flow unless compensated by increase in vessel diameter.

Blood Viscosity

The major determinants of blood viscosity are plasma viscosity, haematocrit, red cell microrheology (aggregation and deformability) and shear conditions. Temperature also affects blood viscosity .

Plasma Viscosity

Whole blood is a suspension of dispersed cells in plasma. Therefore the plasma viscosity affects whole blood viscosity. Plasma is composed of plasma proteins. The effect of these plasma proteins on viscosity varies with the concentration of protein (Harkness, 1971). Individual plasma proteins influence the plasma viscosity and hence

whole blood viscosity to different degrees, not only according to their molecular weight but also according to their shape. Fibrinogen is one of the most important plasma proteins in determining plasma viscosity. It is a large asymmetrical protein with a length to diameter ratio of about 18:1, which can rotate during flow and disturb the streamlines of flow and plasma (Harkness, 1971; Chien, 1972). Other plasma proteins with marked effects on plasma viscosity include the immunoglobulins, alpha-2-macroglobulin, and low density and very low density lipoproteins (Harkness, 1981).

Plasma exhibits Newtonian behaviour (Dintenfass, 1971; Harkness, 1971). It can therefore be measured irrespective of shear rate. At normal or increased haematocrits, increases in plasma viscosity result in increased whole blood viscosity (Mayer, 1966; Rand et al., 1970). This effect of plasma viscosity increases with increasing haematocrit (Mayer, 1966).

Haematocrit

Haematocrit is the volume fraction of cells in whole blood, whereas packed cell volume is measured by centrifugation and is 2-5% higher than the actual volume occupied by the cells because of the trapping of a small volume of plasma between the cells (Wintrobe et al., 1981). In clinical practice these terms are synonymous. With a higher haematocrit, i.e. with more concentration of red cells, it is understandable that the viscosity of blood increases. Over

the haematocrit range 0.2-0.6 (found in pathological conditions as well as in health) there is a linear increase in the logarithm of blood viscosity (Begg & Hearns, 1966; Chien et al., 1966; Dormandy, 1970). However over the range of haematocrit found in most people (0.35-0.55) the relationship between haematocrit and whole blood viscosity is essentially a linear one. The effect of haematocrit on blood viscosity is greater at low shear rates (Lowe & Barbenel, 1988), where red cell aggregation occurs increasing the blood viscosity. At high shear rates aggregates are disrupted and red blood cell deformation occurs.

Normal ranges for haematocrit are 0.41-0.54 in males and 0.37-0.47 in females (Wintrobe et al., 1981). Smokers tend to have a higher haematocrit (Isbister, 1987). This is probably due to increased carboxyhaemoglobin concentration which leads to an increase in red cell mass stimulated by tissue hypoxia. Lowe and Barbenel (1988) noted that elevated haematocrit in smokers reflects a larger mean cell volume rather than an increased red blood cell count.

Red Blood Cell Aggregation

Red blood cells form linear aggregates termed "rouleaux" under low flow conditions. This can be observed microscopically (Chien & Jan, 1973). The rouleaux formed also undergo a secondary aggregation into networks of red blood cells.

Rouleaux formation has been of interest to clinicians for a considerable time as it plays a major role in the erythrocyte sedimentation rate (ESR) test. The ESR test indicates illness and conditions where plasma acute phase reactive protein concentrations are raised.

Aggregates in static blood form an elastic network. A finite shear stress (termed the **yield stress**) is needed to disrupt these aggregates. Merrill et al. (1965) showed that when blood in a narrow capillary tube stops flowing, a driving pressure is required to recommence flow. The static aggregates require this yield stress to disrupt them. This may be of some importance in the microcirculation in pathological conditions where the driving pressure is low.

Mechanisms of Red Cell Aggregation

When blood is static only translational Brownian motion will bring red cells into collision with each other. At low shear rates of less than 50 sec^{-1}, collisions lead to the formation of red cell aggregates. Hence low shear forces promote red cell aggregation more than stasis. At increasing shear rate the aggregates disrupt and become completely dispersed at 50 s^{-1} in normal blood (shear stress 0.2 Pa). (Schmid-Schonbein et al., 1968; Chien, 1975).

Under normal circumstances red blood cells repel each other because of an overall negative charge on the cell membrane, primarily due to sialic acid residues (Cook et al., 1961).

Erythrocytes suspended in physiological buffered saline show no sign of aggregation (Lerche, 1983). However in plasma, red blood cells aggregate and this is due to the plasma proteins. The plasma proteins responsible for red cell aggregation are fibrinogen, alpha-2-macroglobulin, IgM and IgG (Schmid-Schonbein et al., 1973; Rovel et al., 1979). Fibrinogen is the most active of the physiological aggregating agents in the blood (Replogle et al., 1967; Chien et al., 1970).

The aggregating plasma proteins are thought to have a bridging effect between two red cells. They are able to link the naturally repellent red cells together, being long molecules. Fibrinogen is a large molecule (MW 340,000) that is elongated and flexible. Its length is about 650-700A (Harkness, 1971; Chien, 1972). This is sufficiently long to act as a bridge between cells at a distance where repulsive forces are small.

Fibrinogen is dimeric, consisting of a central E region and two terminal D regions at opposite ends of the molecule (Figure 1.2).

Rampling demonstrated that the amount of fibrinogen bound to the red blood cell membrane was directly proportional to the concentration of fibrinogen in the suspending medium up to a concentration of 10 g/l with no sign of saturation (Rampling, 1981). He calculated that about 2% of the circulating fibrinogen in vivo is bound to the red blood cell

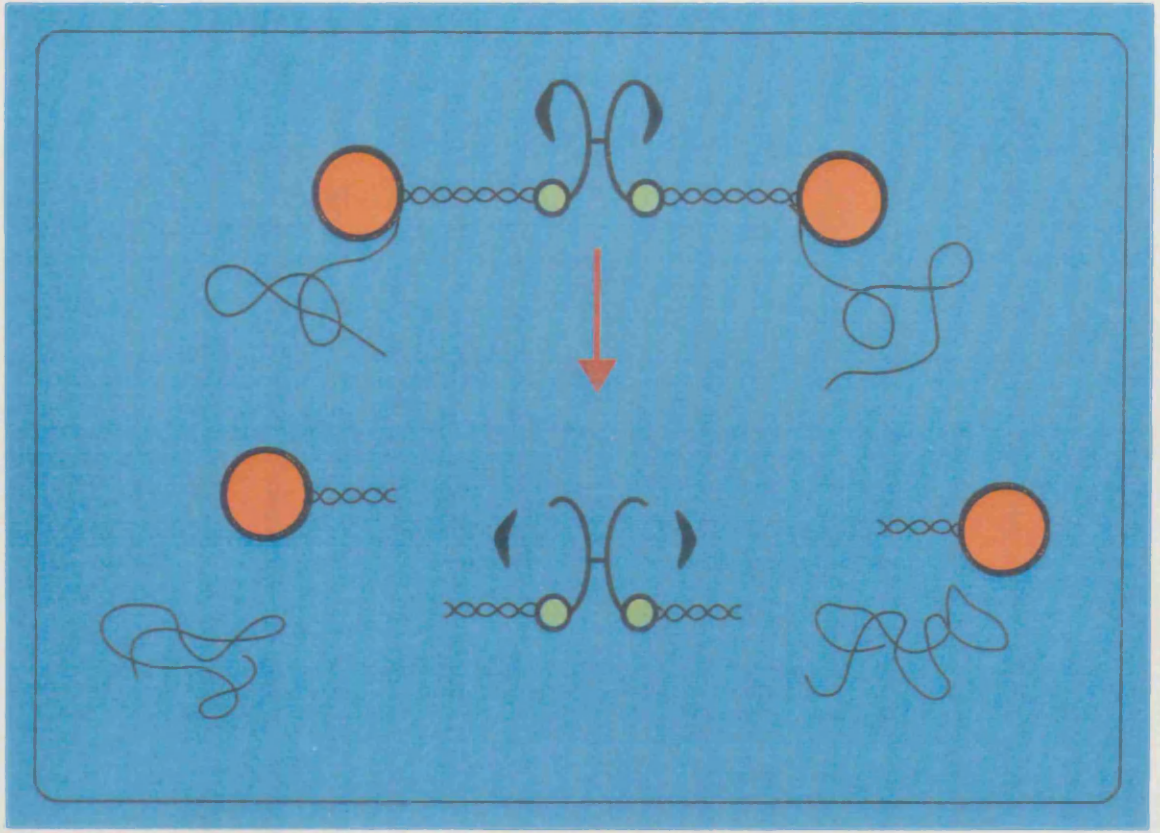

Figure 1.2

Diagram illustrating the central E region and two terminal D regions of fibrinogen, after breakdown by plasmin.

membrane. He also noted that plasmin derived fibrinogen degradation products also adsorb onto red blood cells. The amount bound varied to the type of degradation product. Rampling concluded that this was compatible with adsorption sites on both of the terminal D regions of the fibrinogen molecule. Fibrinogen degradation fragments D and E did not induce aggregation on their own, but synergistically enhanced the effect of fibrinogen (Rampling, 1981). The effect of fibrinogen on aggregation in plasma is more immediate than the effect of fibrinogen on aggregation in a purified system (Chien et al., 1970; Merril et al., 1966). This is because the other plasma proteins play a synergistic role with fibrinogen in red cell aggregation (Rampling, 1988)

Other plasma proteins (alpha-2-macroglobulin, IgM and IgG) also aggregate red cells, forming clump aggregates of different configuration to rouleaux (Schmid-Schonbein et al., 1973; Rovel et al., 1979). These proteins are also large; it is estimated that molecules require a molecular weight of 150,000 or more to cause aggregation (Rampling, 1988).

Red blood cells must be in close proximity to each other to allow aggregation to occur. At low haematocrit the rate of red cell aggregation is low, aggregates are small and separate. Increases in haematocrit within the normal range greatly increase red cell aggregation. At abnormally high haematocrits the intense cell - cell interactions and the

close proximity of red cells reduces the effects of aggregation (Chien, 1988). Hence red cell aggregation exerts its maximal effects on blood viscosity at normal or slightly elevated haematocrits (Chien, 1972).

There is evidence that red cells themselves may have different aggregabilities. This may be due to different cell deformability, surface charge or affinity for bridging proteins (Chien, 1975). Impairment of dextran induced aggregation has been shown for cells rigidified by formaldehyde (Knox et al., 1977). Heat treatment is known to increase membrane viscosity, presumably by protein denaturation, reducing cell membrane deformability (Rakow et al., 1981; Nash & Meiselman, 1985). Lerche and Baumler (1984) heated red blood cells to either 48.4, 48.8 or 49.5°C. They found a progressive reduction in red cell aggregation as cells became more rigid. Nash et al. (1987) separated red blood cells by their density which they proposed related to the cell age. The oldest, most dense cells had a substantial increase in potential for aggregability in dextran. Rampling and Whittingstall (1986a) have produced similar results for aggregability in fibrinogen.

Increases in plasma viscosity caused by increased concentrations of fibrinogen, alpha-2-macroglobulin and immunoglobulins IgM and IgG are accompanied by increased

red cell aggregation. However at very high plasma viscosity, red cell aggregation is attenuated because of the hyperviscous medium that the red blood cells must move in to aggregate.

Measurement of Red Cell Aggregation

Direct measurement of red blood cell aggregation is possible using microscopic examination. Indirect measurement involves techniques based on the rheological effect of red blood cell aggregation.

(a) Direct Microscopic Measurement

The microscopic aggregation measurement of Chien and Jan (1973) gives a direct measurement. However it is a tedious and time consuming test. It only investigates cell behaviour in a static context.

(b) Indirect Measurement

Low Shear Blood Viscosity

Low shear blood viscosity predominantly measures red blood cell aggregation (International Committee for Standardisation in Haematology, 1986). However measurement is technically demanding. The major disadvantage of low shear viscometry is the phase separation that occurs with rapid settling of aggregation during measurement. This can lead to an underestimation of red cell aggregation (Lowe & Barbenel, 1988).

ESR

The erythrocyte sedimentation rate test was developed by Fahraeus (1921). The Westergren method is recommended by the International Committee for Standardisation in Haematology (1973). It detects major changes in aggregating proteins (Lowe, 1987b). However the ESR is a poor measure of the influence of red cell aggregation on blood flow because of its dependence on haematocrit (Lowe, 1987b).

Viscoelasticity

The elastic component of blood can be used as an index of red cell aggregation (Thurston, 1979). This type of measurement is sensitive to haematocrit, plasma viscosity and to the the artefacts of cell settling (Lowe, 1987b).

Photometry

The rate and extent of red cell aggregation can be measured photometrically by the changes in light transmission through whole blood (Schmid-Schonbein et al., 1973). The amount of light transmitted through whole blood varies with the shear rate. At low shear rates, cell-free gaps concomitant with red cell aggregation occur, resulting in an increase in light transmission. At high shear rates light transmission is minimal, as aggregates are dispersed. An automated system has recently become available. This is the Myrenne aggregometer (MA1) (Schmid-Schonbein et al., 1982).

These five techniques for measuring red cell aggregation have been compared (Rampling & Whittingstall, 1986b). The correlations between the responses of these techniques were generally satisfactory. Direct microscopy gives unequivocal data. The photometric Myrenne Aggregometer was the simplest and technically easiest method, requiring the smallest amount of whole blood. The aggregometer was the most sensitive test between fibrinogen concentrations of 3g/l - 8g/l. This is ideal for measurement in pathological conditions. The Myrenne aggregometer is used for the measurement of red cell aggregation in this thesis, and is described in detail in chapter 2.

Low shear blood viscosity is increased in patients with peripheral arterial disease (Dormandy, 1973a). Increased low shear blood viscosity could be important in limiting blood flow, distal to a stenosis. Low shear blood viscosity is difficult to measure, requiring experience, a sensitive viscometer and a fresh and well mixed, heated sample (International Committee for Standardisation in Haematology, 1986; Lowe & Barbenel, 1988). The Myrenne photometric aggregometer on the other hand is easy to work and could be used in routine laboratories.

White Blood Cells

There is increasing evidence that white blood cells may contribute to arterial disease through atherogenesis, thrombosis and ischaemia. In this regard the role of the white blood cell has been studied, especially the granulocyte and monocyte, in promoting endothelial injury and microvascular occlusion.

Chien has studied the rheology of the white cell in the microcirculation (Chien, 1988). In normal blood vessels less than 10 microns in diameter, white blood cells make a significant contribution to flow resistance because of the larger cell volume and lack of deformability. The mean cell volume of a white blood cell is 200 micrometres^3. The white blood cell is much less deformable than the red blood cell - it is spherical whereas the red blood cell is discoid and it has a high internal viscosity coefficient, 1000 times that of the red cell (Chien, 1988).

In high flow states white blood cells are dispersed in large vessels. The larger white blood cells tend to occupy the central region of blood flow, increasing white blood cell velocity. In low flow states red cell aggregation occurs and the white blood cells are displaced to the vessel wall by the aggregates. The white blood cell concentration is reduced in the central region of flow. Leucocytes in contact with endothelium may roll along on the endothelium at lower velocity than the main blood stream, and adhere. This has been observed experimentally (Schmid-Schonbein et al.,

1980). Therefore a decrease in blood flow favours white blood cell interactions with the endothelium and adhesions may occur in response to chemotactic factors. White blood cell adhesion also reduces the vessel lumen and flow rate and increases flow resistance.

At capillary bifurcations the entrance of a blood cell into one branch causes an increase of resistance and a decrease of flow in that branch, so the next cell will enter the other branch, giving a balance of resistance and cell distribution. When a white blood cell enters one branch it causes a greater increase in resistance due to its larger cell diameter and lower deformability. Subsequent red blood cells are directed into the other branch until the resistance due to them matches that due to the one white blood cell. Thereafter the entering red blood cells will alternate with the branches. One white blood cell may be equivalent to 20-30 red blood cells in terms of resistance change at the capillary bifurcation (Chien, 1988). Hence the entry of a white blood cell into a capillary network has a large effect on the distribution of red blood cells.

In large vessels the rheological influence of the white blood cell on flow resistance is negligible because of the relatively low concentration in normal blood compared to red blood cells. The rheological influence is increasingly pronounced in vessels with diameters smaller than white blood cells.
The white blood cell count is very labile. Granulocytes are in three pools, the marrow pool, the tissue pool and the

circulating pool. Traffic among these can be acutely altered by physiological influences (corticosteroids and vasoactive amines) and pathological influences (bacterial products and complement activation). The white blood cell count and differential count can vary from day to day (Ernst et al., 1987a). It is important to take this into account when measuring white blood cell count.

In recent years the role of white blood cells in atherogenesis, thrombosis and ischaemia has stimulated many epidemiological surveys, as well as clinical and animal research.

Alderman et al. (1981) reported that patients with intermittent claudication have an increased white cell count compared to controls.

A prospective study of 7,000 males for an average of 6.5 years found that in smokers with a white blood cell count greater than 9000/microlitre, the incidence of myocardial infarction was four times as great as in smokers with a white blood cell count less than 6000/microlitre (Zalokar et al., 1981). The strongest association was with neutrophils. Lymphocyte count was not predictive.

In a study of 2026 patients the white blood cell count taken after recovery from a first myocardial infarction correlated strongly with the risk of re-infarction (Lowe et al., 1985). The white blood cell count co-varied with tobacco use, but

its association with re-infarction was still significant when tobacco and other co-variants were taken into account.
In the Multiple Risk Factor Interventional Trial a decrement of 1,000 white blood cells/ml was associated with a decrease of 14% in the risk of cardiac death. This was independent of tobacco smoking (Grimm et al., 1985).

There are two possible explanations for these epidemiological findings: the white blood cell count could be a marker for one or more disease processes (recognised or unrecognised) that lead to vascular injury; alternatively the white blood cell could play a pathogenic role in vascular injury.
In animal studies, one of the earliest cellular interactions that occurs in hypercholesterolaemia is the attachment of monocytes to the endothelial cells (Ross, 1986). Monocytes are known to be able to transform into macrophages and secrete growth factors as well as interleukin-1 which induces the adherence of further neutrophils and monocytes to endothelial cells (Lowe, 1990). Subendothelial migration and localisation of these monocytes are the earliest events in fatty streak formation (Ross, 1986).

Nash et al. (1988) have shown that in critical limb ischaemia there is a leukocyte activation (activation being determined by impaired filterability), and this returns to normal after amputation of the ischaemic limb. Using microfiltration techniques they found that white blood cells taken from blood draining critically ischaemic legs had an impaired ability to flow through five and eight micron filters. They

also found that white blood cells in blood draining the ischaemic leg had worse filterability than white cells from blood draining from the arm. After amputation of the ischaemic limb, the flow properties of the white blood cells were no different from the controls. They concluded that this impaired filterability was due to activation of the white blood cells caused by factors released in the ischaemic tissue (Nash et al., 1988).

More recently Hickey et al. (1990) reported that there was no significant difference in white cell filterability between resting claudicants and controls. However filterability was significantly reduced after exercise in claudicants. There was no change in filterability with exercise in controls or in the claudicants when the tests were repeated after bypass surgery.

Evidence for ischaemia as an activator of white cells is the observation that after removal of hindlimb tourniquets in animals, there is pulmonary entrappment of white cells with subsequent lung injury (Anner et al., 1987; Klausner et al., 1988a). Activated leucocytes promote tissue damage: Klausner et al. (1988b) demonstrated that leucocyte depletion in animals is an effective protection to tissue injury following hindlimb ischaemia and reperfusion. Activation of leukocytes may cause reperfusion injury by

increased microvascular permeability, production of oxygen free radicals, leukotrienes, lysosomal enzymes, and platelet aggregation (Klausner et al., 1988b; Paterson et al., 1989; Korthius & Granger, 1986; Mehta et al., 1986; Lowe, 1990).

Paterson et al. (1989) showed that thromboxane mediates the production of oxygen free radicals by activated leucocytes and that inhibition of thromboxane synthesis or thromboxane receptor blockade prevents tissue injury after ischaemia and reperfusion. Actual cytotoxic injury to endothelium appears to result largely from oxidative assault (Ernst, 1987a).

In summary, white blood cells are independent risk factors for coronary heart disease and may contribute to the progression of peripheral arterial disease. White blood cell count is increased in peripheral arterial disease and in smokers. White blood cells could have significant rheological effects in the microcirculation, and may have a causal role in peripheral arterial disease.

Fibrinogen

Although the importance of fibrinogen in blood coagulation has been known for many years it is only recently that fibrinogen has been linked to cardiovascular disease, (Meade et al., 1980). In the Framingham Study the project had progressed for 20 years before fibrinogen estimation was introduced to the risk factors for cardiovascular disease (Kannel et al., 1987), and epidemiological studies

have tended to concentrate on ischaemic heart disease and stroke (Lee et al., 1990). There is a need for investigation into the role of fibrinogen in peripheral arterial disease.

Fibrinogen is a plasma protein synthesised by hepatic parenchymal cells. It is essential for wound healing and platelet function. The central event in the coagulation of blood is the thrombin catalysed conversion of fibrinogen into fibrin. Fibrin constitutes the physical basis for all blood clots and provides a framework for the permanent haemostatic plug. Fibrin is degraded by plasmin in a proteolytic process of fibrinolysis into fibrin degradation products.

Before considering the role of fibrinogen in peripheral arterial disease it is necessary to consider its genetics, biochemistry, haemostatic and rheological effects.

Genetics

The fibrinogen gene is on chromosome 4 (Kant et al., 1985). There are separate genes for the three chains: gamma, alpha and beta - one gene for each chain (Crabtree & Kant, 1981). Humphries et al. (1987) have shown that individuals have different genotypes for fibrinogen and that a "high fibrinogen" genotype exists. The same group showed a strong association between certain genotypes coding for the fibrinogen protein and plasma

fibrinogen concentrations. This means that some people may be genetically predisposed to high fibrinogen levels and their consequences.

Biochemistry

The molecular structure of fibrinogen has been determined (See Figure 1.3) Fibrinogen is a complex molecule with a molecular weight of 340,000 and 2,964 amino acids (Doolittle, 1987). X-ray diffraction techniques identified supercoiled alpha-helices along a long axis (Bailey et al., 1943). The molecule is arranged in a tier of three globules. This was observed by electron microscopy, (Hall & Slayter, 1959) with a central "domain" globule and two terminal "domain" globules. The amino acid sequence of human fibrinogen was determined in the 1970`s (Henschen & Lottspeich, 1977; Doolittle et al., 1979). It is composed of two alpha-chains of 51 residues, two beta-chains of 118 residues and two gamma-chains of 78 residues. The amino acid sequence and 29 disulphide bonds between 58 cysteine residues allow the molecule to have six cysteine residues interconnected spatially in four rings. These "disulphide rings" hold all the chains together with alpha-helices interconnecting the three domains. Calcium binding sites have also been identified and quantified on the terminal domains (Marguerie et al., 1977; Nieuwenhuizen & Haverkate, 1983). Calcium ions have been known for a long time to influence the fibrinogen-fibrin system.

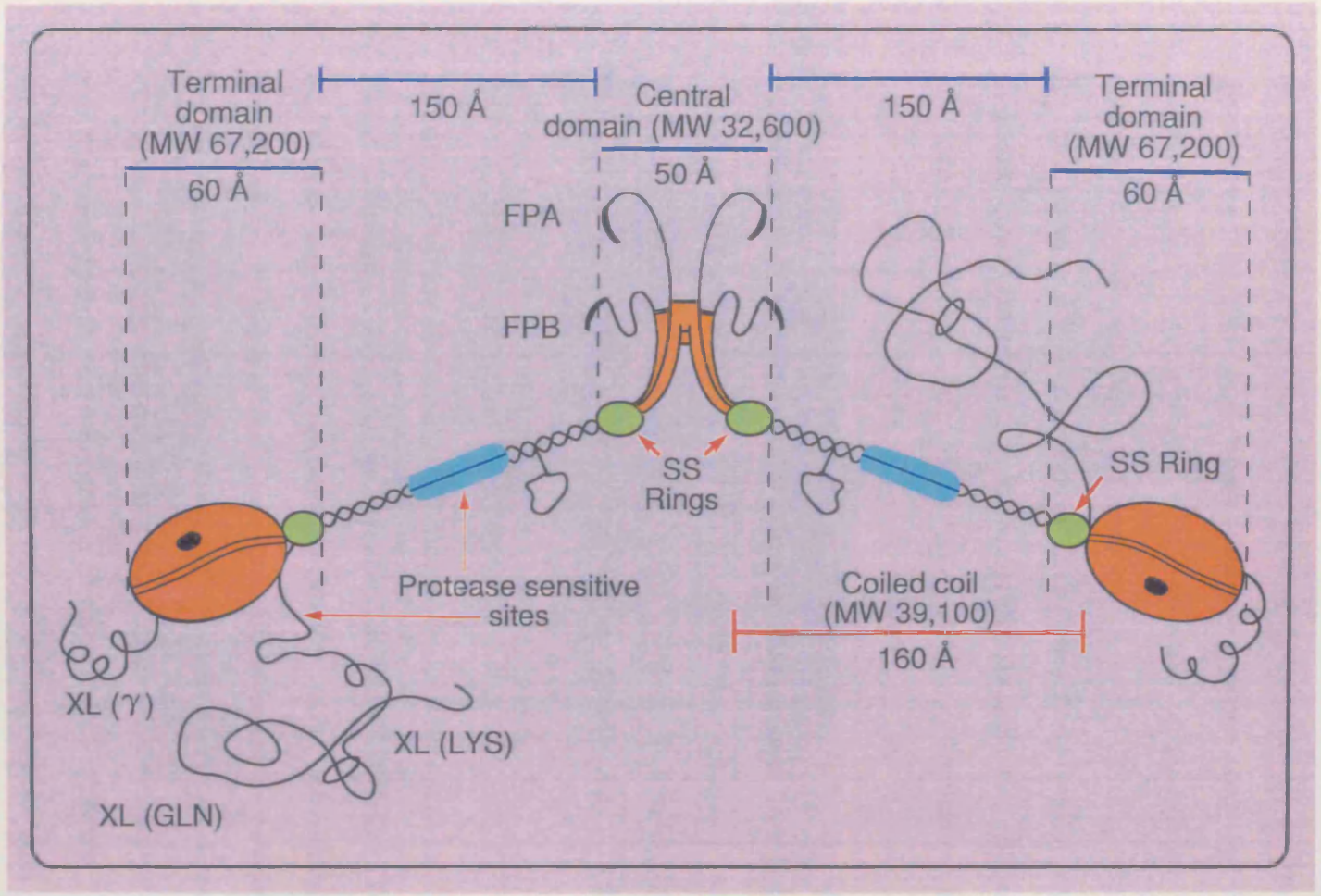

Figure 1.3

The molecular structure of fibrinogen. (after Marder et al., 1982)
(SS = disulphide rings, FPA = Fibrinopeptide A, FPB = Fibrinopeptide B)

Role of Fibrinogen in Haemostasis

The triggering event for the transformation of fibrinogen into fibrin is the thrombin catalysed release of the fibrinopeptides A & B. The fibrinopeptides A & B were discovered in 1951 and their characterisation led to the realisation that only about 3 % of the mass of fibrinogen is removed by the reaction of thrombin. (Bailey et al., 1951; Lorand, 1951). The removal of these relatively small polar peptides from the central domain allows the mutual approach of two molecules of fibrinogen which spontaneously polymerise into the fibrin network (Doolittle, 1987).

This process initially creates a dimer stage between two molecules. A second set of interactions comes into play when a third molecule is added at which time gamma-chains in neighbouring molecules come into juxtaposition, favourable for covalent cross-linking by factor XIII. (See Figure 1.4)

Stabilisation (cross-linking) of the polymer strengthens the fibrin network. The plasma enzyme, activated factor XIII, stabilises the fibrin network. Factor XIII is calcium dependent. It allows the formation of isopeptide covalent bonds between suitably disposed lysine and glutamine residues of gamma-chains (Doolittle, 1987). This results in a glutamyl-lysine cross-link. Cross-linked fibrin is mechanically stronger than non cross-linked fibrin and is more resistant to fibrinolysis (Gerth et al., 1974). (See Figure 1.4).

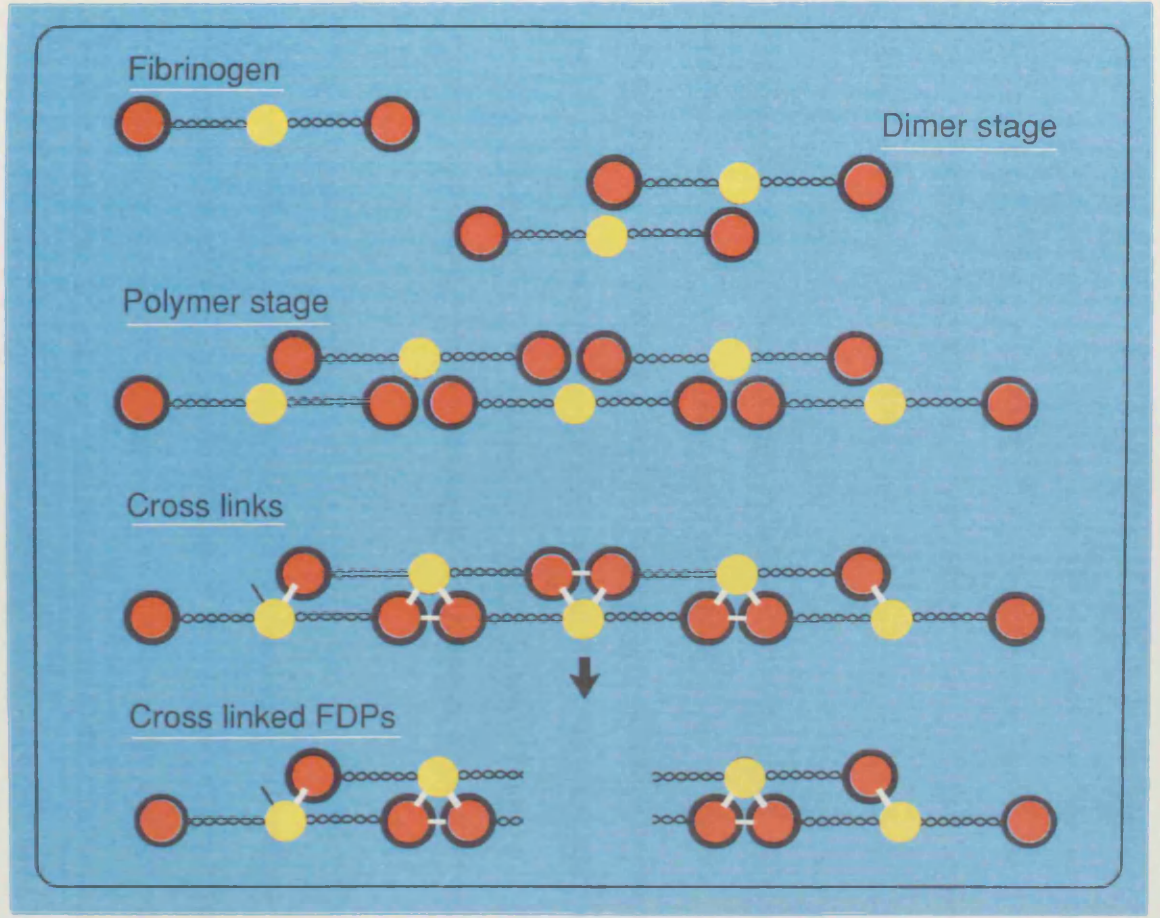

Figure 1.4

Fibrin formation, stabilisation (cross linked) and fibrinolysis creating cross linked fibrin degradation products.

Fibrin clots are not designed to be permanent. Under the usual circumstances of wound healing, fibroblasts move through the fibrin meshwork and lay down a more permanent framework composed of collagen fibres. The superfluous fibrin network is then proteolytically dismantled. As a reaction to fibrin formation the fibrinolytic system is activated giving rise to plasminogen activation. The resulting plasmin degrades insoluble fibrin to soluble degradation products and this process disintegrates a blood clot.

In haemostatic balance, the relative rate at which fibrin is formed at least equals the rate at which it is degraded. A coagulation system relatively more active than fibrinolysis may cause thrombosis, while overactive fibrinolysis may cause bleeding. In cases of overt fibrinolysis not only fibrin but fibrinogen is attacked by plasmin (Niewenhuizen, 1987a).

Plasmin cleaves the slender interdomainal connections at protease sensitive sites, resulting in cross-linked fibrin degradation products, composed of a double terminal domain complexed with a central domain of another unit to which it was polymerised prior to fibrinolysis. The complex is called a D_2E fragment; a D fragment being equivalent to the terminal domain and the E fragment equivalent to the central domain (Doolittle, 1987). (See Figure 1.3).

Measurement of Fibrin Degradation Products

In the past, measurement of fibrin degradation products (FDPs) was performed on serum, to remove fibrinogen, as the polyclonal antibodies, used previously, cross reacted with fibrinogen. Newer monoclonal antibodies are specific for FDPs and do not cross react with fibrinogen. Measurement is now therefore performed on plasma.

At least six monoclonal antibody bound plasma assays are available for the measurement of the breakdown of fibrin (Niewenhuizen, 1987b). One of these monoclonal antibody assays is specific for the D-dimer fragment, measuring cross-linked FDPs (Rylatt et al., 1983). Elevated levels of cross-linked fibrin degradation products represent a secondary fibrinolytic response, therefore providing an indirect but reliable index of underlying fibrin formation (Whittaker et al., 1987).

Measurement of cross-linked FDPs has recently been applied to certain clinical conditions. Increased cross-linked FDPs are found in patients with deep venous thrombosis, pulmonary embolism, myocardial infarction, patients on thrombolytic therapy, renal transplantation and rheumatoid arthritis (Whittaker et al., 1987).

Hence cross-linked FDPs can be measured accurately, and are elevated in thrombotic and fibrinolytic conditions. They are markers for in vivo fibrin formation. In patients with peripheral arterial disease, elevated levels could indicate

either thrombosis in the vessel lumen or excess chronic deposition of fibrin on the vessel wall (Smith et al., 1990a).

Effect of Haemorheology on Blood Flow in Peripheral Arterial Disease

The Hagen-Poiseuille equation can be used to relate blood flow and the circulation in vivo.

Flow is proportional to :-

$$\frac{\text{Perfusion pressure (P artery-P vein)}}{\text{Vascular resistance X Blood viscosity}}$$

Blood flow depends on the perfusion pressure produced by the heart, on the resistance to flow of the vessels (where diameter is particularly important). and on blood viscosity.

Blood viscosity is dynamic: it changes in different parts of the circulation and this mostly relates to the different shear rates and vessel diameter encountered at different sites. In health, the circulation minimises blood viscosity in parts of the microcirculation where most flow resistance occurs, i.e. arterioles and capillaries. Blood viscosity is kept at a minimum because the high shear conditions in the healthy circulation ensure maximum red blood cell deformation, and minimal red cell aggregation. Red cells travel in axial streamlines in rapid flow. They travel faster than the plasma, which is displaced to the slower outer flowing

streamlines at the vessel wall. This results in a consequent fall in the dynamic haematocrit (The Fahraeus effect: Fahraeus, 1929). Hence at high shear the healthy circulation maintains a minimum blood viscosity due to a low dynamic haematocrit.

However blood viscosity in the microcirculation alters in disease. In overt ischaemia with decreased perfusion pressure and maximal vasodilation there is a reduction in shear rate. This favours the formation of red cell aggregates, which may further increase blood viscosity and reduce blood flow, initiating a vicious cycle (Dintenfass, 1971; Chien, 1972). This form of positive feedback could be enhanced by elevated levels of haematocrit and aggregating proteins, especially fibrinogen. Rampling and Challoner (1983) demonstrated that increased rouleaux formation limits flow in medium to large vessels particularly at low driving pressures.

Low shear conditions are necessary for red cell aggregation. Peripheral arterial disease favours low shear conditions which may exist at areas of flow separation such as arterial bifurcations, and distal to arterial stenoses, as well as the microcirculation (Lowe, 1987b).

Increased blood viscosity has been shown to be an important factor in reducing blood flow in patients with intermittent claudication. Dormandy et al. (1973a) found that 126 patients with intermittent claudication had

significantly higher blood viscosity than controls, when measured at high ($230s^{-1}$) and low ($23s^{-1}$) shear rates. They noted that patients with the same clinical and arteriographic lesion (a localised superficial femoral artery occlusion) had widely differing claudication distances. The patients with a shorter claudication distance had a higher blood viscosity than patients with a longer claudication distance. They suggested that in a proportion of cases the increased blood viscosity was the critical factor responsible for the symptoms.

Dormandy et al. (1973b) then went on to assess the prognostic significance of the initial blood viscosity in 62 untreated patients with intermittent claudication over a three year period. They found that there was a significant correlation between blood viscosity and subsequent deterioration in claudication distance. Viscosity was most significant as a prognostic marker when measured at a high shear rate. High levels of haematocrit, fibrinogen and whole blood viscosity and decreased red blood cell filterability have all been shown to have a poor prognostic significance in terms of clinical outcome - increasingly severe claudication, development of ischaemic rest pain and gangrene, and the need for surgery (Dormandy, 1988; Dormandy et al 1973b; Reid et al., 1976).

Primary or secondary polycythaemia, myeloma or other paraproteinaemias, and leukaemia may all present clinically with leg ischaemia in the absence of any significant vessel disease. These cases are rare, but specific treatment of the underlying cause is often successful (Dormandy, 1988).

Since abnormally high levels of fibrinogen, haematocrit, whole blood viscosity and red blood cell deformability are significant risk factors for progression of peripheral arterial disease, therapy to reduce these levels would be expected to improve clinical outcome. Reduction in haematocrit by venesection, normovolaemic haemodilution, pharmacological defibrinogenation, pharmacological improvement of blood cell rheology and plasma exchange have all been studied (Ford et al., 1978; Yates et al., 1979; Lowe et al., 1982; Lowe et al., 1984; Walker et al., 1983; Ernst et al., 1987b). At present however, there is no medical treatment proven to reduce the risk of arterial events.

Reconstructive Vascular Surgery

Surgical bypass involves inserting either autogenous vein or prosthetic graft material. Prosthetic graft materials are more thrombogenic than autogenous vein graft (Bergan et al., 1982; Michaels, 1989). Bypass of an occluded distal artery may prevent an amputation, yet even when autogenous saphenous vein is available, reconstructive surgery is not always successful. Early graft occlusion is usually attributed to a technical problem or an insufficient in-flow or out-flow. Late graft occlusion is more common

and is attributed to anastomotic intimal hyperplasia, graft stenosis or thrombosis or due to progression of distal or proximal arterial disease (Naylor et al., 1989). Plasma fibrinogen concentration was found to be the most important variable predicting vascular graft occlusion in a recent prospective study of 157 patients undergoing saphenous vein femoropopliteal bypass grafts (Wiseman et al., 1989). Median plasma fibrinogen was higher in patients with occluded grafts than in those with patent grafts one year after operation. The next most important variables predicting graft occlusion were smoking markers - carboxyhaemoglobin concentration and serum thiocyanate concentrations. Median concentrations of carboxyhaemoglobin and thiocyanate were significantly higher in patients with grafts that failed. These smoking markers also indicated that one quarter of the patients were untruthful about their smoking habit. Surprisingly, increased serum cholesterol and increased plasma LDL cholesterol levels were associated with improved graft patency. Increased blood viscosity and a tendency to thrombosis are two possible mechanisms whereby high plasma fibrinogen levels could influence occlusion of bypass grafts. The role of fibrinogen in thrombosis and blood rheology is therefore of interest to the vascular surgeon.

Summary of Chapter 1

There is a high incidence of peripheral arterial disease in the West of Scotland associated with atherosclerosis. The earliest descriptions and treatments of atherosclerosis, gangrene and aneurysm are described. The severity and type of peripheral arterial disease are defined, intermittent claudication, ischaemic rest pain, gangrene and aneurysm. Also the evolution of surgical treatment and reconstructive vascular surgery is described. The pathology, complications and aetiology of atherosclerosis are reviewed in relation to lipids and cell apoprotein receptors, platelets, smooth muscle cells and monocytes.

Recent research on the significance of white cell count and the monocyte in atherogenesis, rheology, and tissue ischaemia is reviewed.

There is lack of knowledge of the aetiology and development of atherosclerosis. Epidemiological studies of fibrinogen have dealt mainly with ischaemic heart disease, but there are clinical studies of symptomatic peripheral arterial disease and these are reviewed.

The role of fibrinogen in the development of the atheromatous plaque is described as is its haemostatic and haemorheological importance in peripheral arterial disease and vascular graft occlusion.

Fibrinogen is also a determinant of whole blood viscosity, by its effects on plasma viscosity and red cell aggregation. It is prognostic for progress and deterioration of peripheral arterial disease. It is also implicated in the aetiology of atherosclerosis.

The transformation of fibrinogen to fibrin is described and, also the cross-linked stabilisation of the fibrin network by activated factor XIII. The subsequent fibrinolytic action of plasmin degrades insoluble fibrin to soluble degradation products, and their measurement by monoclonal antibody techniques provides a reliable, albeit indirect, index of in vivo fibrin formation.

Precise laboratory methods are now available to measure red cell aggregation, and also cross-linked fibrin degradation products as a measure of in vivo fibrin formation. The relation of these variables to peripheral arterial disease and plasma fibrinogen level is the main topic of this thesis.

Introduction to Aims

Increased fibrinogen levels are present in patients with peripheral arterial disease (Hamer et al., 1973; Dormandy et al., 1973a), and may have roles in thrombosis and haemorheology.

Increased fibrinogen levels might cause increased red cell aggregation and could promote increased fibrin formation in these patients. These possibilities are explored in chapter

3 as red cell aggregation and cross-linked fibrin degradation products can now be measured by two new methods.

Fibrinogen is an acute phase reactive protein (Castell et al., 1988a). Interleukin - 6 (IL-6) is a cytokine which regulates the hepatic synthesis of fibrinogen (and other acute phase reactive proteins) in the acute phase response (Castell et al., 1988a). Interleukin - 6 is produced by lymphoid cells and by endothelial cells, fibroblasts and macrophages and is the most potent inducer of acute phase protein synthesis in human hepatocytes (Hirano & Kishimoto, 1990; Heinrich et al., 1990). Since fibrinogen is increased in peripheral arterial disease, this raises the possibility that it may be increased because of an inflammatory process in atherosclerosis, regulated by interleukin - 6. This possibility is explored in chapter 4 as interleukin - 6 can now be measured by a recently introduced assay.

When blood comes into contact with a non-endothelial surface e.g. collagen, factor XII is activated initiating the intrinsic system of coagulation (Forbes & Courtney, 1987). This results in the conversion of fibrinogen to fibrin. Fibrinogen is preferentially adsorbed on to foreign surfaces (Vroman et al., 1980; Forbes & Courtney, 1987) and

thrombus formation often occurs on atherosclerotic plaques (Davis & Thomas, 1985). Atherosclerotic plaques might act as a foreign surface activating factor XII and initiating the intrinsic system of coagulation.

Since it is now possible to measure levels of activated factor XII with a recently introduced and modified assay, this is explored in chapter 5.

Reconstructive vascular surgery often introduces prosthetic arterial grafts which are foreign surfaces that come into direct contact with the blood. Activation of factor XII in relation to reconstructive vascular surgery is also explored in chapter 5 and its association with fibrinogen and cross-linked FDPs.

At present there is no proven medical treatment to reduce the risk of arterial events in peripheral arterial disease. However an improved management might be obtained by preventing thrombosis. It is therefore reasonable to evaluate the effects of anticoagulant and antiplatelet therapy on the hypercoagulable state and abnormal blood rheology found in patients with peripheral arterial disease. Mini dose warfarin has been shown to be effective in the prevention of venous thrombosis (Poller et al., 1990) and low dose aspirin remains pharmacologically active inhibiting

thromboxane A2 without inhibiting prostacyclin (Hanley et al., 1981). Such low doses have minimal side effects. A randomised double blind placebo controlled trial of low-dose aspirin and minidose warfarin is reported in chapter 6.

In health, fibrin formation and fibrinolysis are maintained in physiological balance (Nolf, 1908), however patients with peripheral arterial disease have an increased tendency to thrombosis. It is possible that inhibition of the fibrinolytic system might lead to increased fibrin formation and thrombosis in these patients. This is also explored in chapter 6 as a rapid inhibitor of plasminogen activator (plasminogen activator inhibitor - PAI) can now be measured. There is at present no published report on PAI levels in peripheral arterial disease, or on the effects of antithrombotics on PAI levels.

Increased factor VII levels have been shown to be an important risk factor for ischaemic heart disease (Meade et al., 1980, 1986) but its levels have not previously been studied in peripheral arterial disease. Warfarin is known to reduce factor VII levels (Meade, 1990).

Von Willebrand factor is essential for platelet aggregation and adhesion. It has been previously reported to be increased in peripheral arterial disease and may indicate endothelial cell damage due to atherosclerosis (Christe et al., 1984).

The effects of minidose warfarin and low dose aspirin on these factors are also examined in chapter 6.

AIMS

The aim of this thesis is to investigate the role of fibrinogen, fibrin formation and red cell aggregation in patients with peripheral arterial disease.

Aims (Chapter 3)

(1) To measure plasma cross-linked fibrin degradation products and red cell aggregation in patients with peripheral arterial disease and controls. This is to determine if cross-linked fibrin degradation products and red cell aggregation are increased in peripheral arterial disease, and their association with plasma fibrinogen level and white cell count.

(2) To determine if the severity of ischaemia or type of atherosclerotic disease (abdominal aortic aneurysm) correlates with the levels of fibrinogen, cross-linked fibrin degradation products or red cell aggregation.

(3) To evaluate the effects of age, sex, cigarette smoking, and reconstructive vascular surgery on fibrinogen, cross-linked FDP's and red cell aggregation. Patients were followed up serially for 3-12 months (mean 7 months) in order to evaluate the effects of changes in smoking and reconstructive vascular surgery.

Aims (Chapter 4)

(1) To determine if interleukin-6 levels are increased in patients with peripheral arterial disease.

(2) To determine if interleukin-6 levels correlate with the increased fibrinogen level and raised white blood cell count found in peripheral arterial disease.

(3) To determine any correlation of interleukin-6 with cross-linked fibrin degradation products and red cell aggregation.

(4) To determine if interleukin-6 is increased by cigarette smoking in peripheral arterial disease.

Aims (Chapter 5)

(1) To determine if there is an increase in the level of activated Factor XII in patients with peripheral arterial disease compared to controls.

(2) To determine if activated Factor XII is increased by insertion of prosthetic vascular grafts.

(3) To find out if activated Factor XII correlates with fibrinogen level or cross-linked fibrin degradation products in patients with peripheral arterial disease.

Aims (Chapter 6)

(1) To determine if low dose warfarin or low dose aspirin alter the hypercoagulable state and the haemorheological disturbance in patients with peripheral arterial disease (100 patients with intermittent claudication) compared to placebo. This has been carried out in a prospective randomised double blind placebo controlled trial.

(2) To determine what, if any side effects occur with these drugs in patients with peripheral arterial disease.

(3) To determine the levels of plasminogen activator ihibitor, von Willebrand factor antigen, and factor VII activity in patients with peripheral arterial disease.

CHAPTER 2

Subjects, Materials and Methods

Introduction

In this Chapter, the subjects, materials and methods used and the experimental procedures employed are outlined.

They have been presented here in a separate chapter to prevent, where possible, repetition: many of the tests were performed in each of the investigative chapters.

In order to overcome the diversity of rheological methods, the International Committee for Standards in Haematology have published guidelines (ICSH, 1973, 1984, 1986) for blood sampling, subsequent sample handling, and measurement of plasma and red blood cell rheology. These guidelines provide a basis for standardisation and improve comparability between rheological work performed in different laboratories. The experimental work described in this thesis was performed in accordance with these guidelines.

Control Selection and Assessment

Control subjects were not on medication known to be rheologically active. They had no other serious illnesses known to increase fibrinogen concentration, fibrin degradation or red cell aggregation (e.g., rheumatoid arthritis, malignancy, infection) nor evidence of peripheral arterial, cerebrovascular or myocardial disease.

122 control subjects selected from the general population, hospital staff and from hospital patients without arterial disease or inflammatory conditions or malignancy had measurement of red cell aggregation, fibrinogen, plasma viscosity, haematocrit, full blood count, lipids, height and weight. Smoking, body mass index, age and sex were also recorded. A further 231 control subjects in a random sample of the general population (Scottish Heart Health Study) had plasma measurements of cross-linked fibrin degradation products. These 231 controls were aged between 25-64 years and were used to establish a more confident population range because of the highly skewed distribution of cross-linked fibrin degradation products. They were all asymptomatic for arterial disease.

Patient Selection and Assessment

All patients studied were either in-patients or out-patients attending the Unit for Peripheral Vascular Surgery, Glasgow Royal Infirmary. The Unit is referred patients from the Eastern district of Greater Glasgow Health Board and also from other parts of the West of Scotland, being a specialised vascular unit.

A full clinical history and complete physical examination was performed on every patient, with documentation of peripheral pulses. The presence, type and severity of disease were recorded and divided into groups of intermittent claudication (confirmed by ankle brachial pressure index (ABPI) less than or equal to 0.9), ischaemic

rest pain (confirmed by ABPI less than or equal to 0.4), gangrene, and abdominal aortic aneurysm. This is in accordance with the standards suggested in reports dealing with lower extremity ischaemia prepared by the Ad Hoc Committee on Reporting Standards (1986), Society for Vascular Surgery/North American Chapter, International Society for Cardiovascular Surgery.

Patients were diagnosed to have peripheral vascular disease only when there were clinical symptoms as well as an ankle brachial pressure index of less than, or equal to 0.9. Radiographic evidence of vascular disease was obtained using aortography in 90% of the in-patients. This was because most in-patients were admitted with a view to reconstructive vascular surgery.

Abdominal ultrasound was used to detect the presence of abdominal aortic aneurysm. During this present study ultrasound was performed to determine the incidence of abdominal aortic aneurysm in 100 patients with intermittent claudication examined with a 3.5 MHz ultrasound probe.

Sex, age, height, weight and blood pressure were also recorded. Body mass index was defined as weight/height^2 (kg/m^2).

Smoking Habit

Current smoking habit was monitored in every patient (cigarettes, cigars, pipe tobacco). If a patient had stopped smoking for more than one month he was considered to be an ex-smoker, otherwise he was considered a current smoker. The quantity of cigarettes or tobacco smoked per day was recorded (less than 20 cigarettes per day or 20 cigarettes or more per day). Carboxyhaemoglobin levels were measured in all patients to establish whether or not they were truthful about their smoking habit every time that blood was sampled. A carboxyhaemoglobin level of less than 2.5% was used to confirm that the patients were ex-smokers or non-smokers. A carboxyhaemoglobin level of 2.5% or greater indicated that the patients were current smokers (Russell, 1982).

Blood Sampling

A plastic syringe and 19 gauge needle were used to collect blood. A rubber tourniquet was applied around the upper arm to generate a full vein in the antecubital fossa which was then punctured. The tourniquet was then released and the blood collected (the use of a tourniquet has been shown to induce stasis which leads to haemoconcentration (Lewis, 1982) and to release of endothelial tissue plasminogen activator (Levin et al., 1984).

Sample Handling

For the laboratory measurements of haemorheology, full blood count, platelet count and carboxyhaemoglobin, 4 mls of blood were added to tubes containing 0.1 ml of liquid dipotassium edetate (EDTA, Steyne Laboratories, Ltd) giving a final concentration of 1.5 mg/ml blood. EDTA prevents the thrombin mediated conversion of fibrinogen to fibrin by sequestering free calcium ions. This concentration of EDTA was chosen because of its minimal effect on red cell morphology and plasma and whole blood viscosity (Harkness, 1971; Wintrobe et al., 1981). EDTA is recommended for measurement of blood viscosity and red cell aggregation (ICSH, 1986; Myrenne Aggregometer Manual). The EDTA blood was stored at laboratory temperature (20-25°C) until measurement of whole blood viscosity, red cell aggregation and haematocrit were performed. The samples were mixed on a rotating and gently oscillating oblique wheel mixer to prevent phase separation.

Haemorheological measurements of whole blood viscosity, red cell aggregation, and hematocrit were performed within six hours of sample collection - usually within two hours. Plasma viscosity was performed within 48 hours - usually within six hours.

EDTA-anticoagulated blood for plasma viscosity was centrifuged at laboratory temperature (20-25°C) at 2,500 g for 10 minutes. Approximately 2 mls of plasma was

aspirated with a plastic Pasteur pipette and stored in a small stoppered plastic tube at laboratory temperature. As recommended by Harkness, all sample tubes were stoppered throughout sampling to prevent evaporation which increases plasma viscosity (Harkness, 1971).

One tube of EDTA blood was sent to the Department of Haematology, Glasgow Royal Infirmary for a full blood count and platelet count (Coulter S Counter).

For the laboratory measurements of coagulation (fibrinogen, crosslinked fibrin degradation products, factor XII, activated partial thromboplastin time, prothrombin time, plasminogen activator inhibitor, von Willebrand factor, and factor VII) 9 mls of blood were added to a polystyrene tube containing 1 ml of trisodium citrate (0.109 M). The citrate tubes were freshly prepared and stored at 4 °C. During sampling they were stored in a bucket of melting ice (4 °C). Each tube was checked before use to make sure that no leakage or evaporation of anticoagulant had occurred. After venesection the sample tubes were sealed and gently mixed. The citrated blood was centrifuged at 4 °C at 2,500g for 10 minutes and the plasma separated for measurement.

Plasma separated for fibrinogen, crosslinked-fibrin degradation products, factor XII, PAI and von Willebrand factor were stored in small stoppered plastic tubes at -20°C. Measurements were then done in batches at a later

stage. Measurements of activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured immediately. Factor VII was measured within 7 days.

Blood obtained for the measurement of Interleukin-6 (IL-6) and C-Reactive protein (CRP) and alpha-2-macroglobulin was added to a 10 ml glass tube that contained no anticoagulant, and was centrifuged at 2,500 g for 10 minutes and the serum stored in a small stoppered plastic tube at -20 °C.

Blood for lipid measurement (total cholesterol, high density lipoprotein cholesterol and total triglyceride) was added to a 10 ml glass tube that contained no anticoagulant and sent to the University Department of Pathological Biochemistry, Glasgow Royal Infirmary.

LABORATORY METHODS

(1) Coagulation

Blood coagulation measurements and times were performed using the University Department of Medicine's Coagulation Laboratory standard methods. During the period of study the laboratory's performance of these tests was satisfactory according to the National External Quality Control Assurance Scheme (NEQAS).

Measurement of Fibrinogen, Activated Partial Thromboplastin Time, Prothrombin Time, and Factor VII

Fibrinogen, activated partial thromboplastin time, prothrombin time and factor VII were measured with a Coag-A-Mate X2 automated coagulometer (General Diagnostics, Warner-Lambert Company, Morris Plains, New Jersey, USA).

Plasma fibrinogen concentration was measured by the standard Clauss assay with reagents and standards from Organon Teknika. The APTT was measured using reagents from Organon Teknika. The prothrombin time was measured using the Manchester reagent. Factor VII was measured using the technique described by Poller et al. (1987).

Measurement of cross-linked FDPs

Measurement of cross-linked fibrin degradation products was made using the "Dimer Test", Enzyme Linked Immunoassay (ELISA; Agen USA Inc, California).

Principle

A D-dimer specific capture monoclonal antibody (DD-36) which reacts only with D-dimer and related high molecular weight derivatives, was used to measure D-dimer concentration in plasma.

The D-dimer specific capture monoclonal antibody is bonded to a polystyrene stripwell plate and reacts with plasma D-dimer. A Tag monoclonal antibody (DD-4D2) conjugated to horseradish peroxidase (HRP0) then binds to the D-dimer, and is coloured when 2,2 azinobis (3-ethyl benzthiazolinesulfonic acid) and hydrogen peroxide are added. The colour developed is read by absorbance spectrometry.

Procedure

Buffer solution (5 mls, Tween 20, 40% v/v and Phosphate Buffer Salts in 1 litre of distilled water) and standard dilutions of D-dimer were prepared immediately before each assay.

25 microlitres of plasma from samples, and 25 microlitres of standards were incubated for one hour in stripwells coated with the D-dimer specific capture monoclonal antibody and 100 microlitres of buffer solution. The wells were then thoroughly washed with buffer for three minutes and emptied. 50 microlitres of reconstituted Tag antibody was added to each well and incubated for one hour. The wells were washed again with buffer solution for three minutes and emptied. 100 microlitres of ABTS substrate activated with hydrogen peroxide was added to each well for 15 minutes to allow colour development. 50 microlitres of stopping reagent was then added to stop the reaction (see Plate 2).

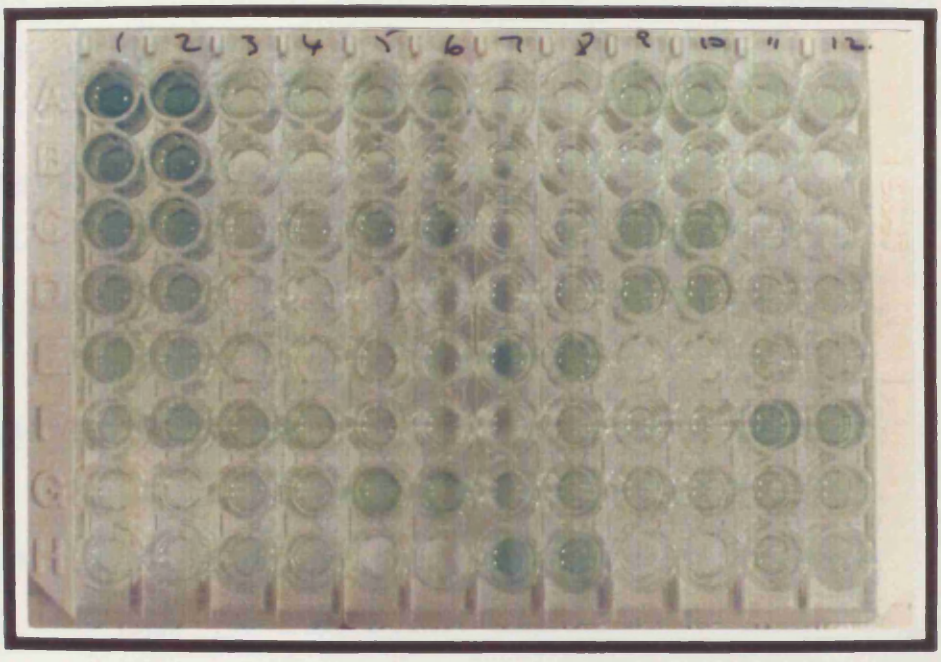

Plate 2

Stripwell plate for measurement of cross linked fibrin degradation products.

The colour developed was read by absorbance spectrometry at 405 nm. The optical density is related to the concentration of D-dimer (cross-linked FDP's). This was calculated from the standard curve for each sample (see Figure 2.1). Two measurements were made of each sample and an average value obtained.

Plasminogen Activator Inhibitor Activity (PAI)

PAI activity was measured using a chromogenic substrate assay with a kit from Kabi Diagnostica, Epsom, Surrey.

Von Willebrand Factor

Von Willebrand Factor was measured by an ELISA technique (Daco Ltd, High Wycombe, Bucks, UK).

(2) **Rheology**

Red cell aggregation

Red cell aggregation was measured photometrically using an automated Myrenne MA1 Aggregometer (Myrenne GMBH, Roetgen, West Germany. Schmid-Schonbein et al., 1982). Measurement is based on the light transmitting properties of aggregating suspensions which were described as early as 1935 and have since been confirmed by a number of groups (reviewed by Klose et al., 1972).

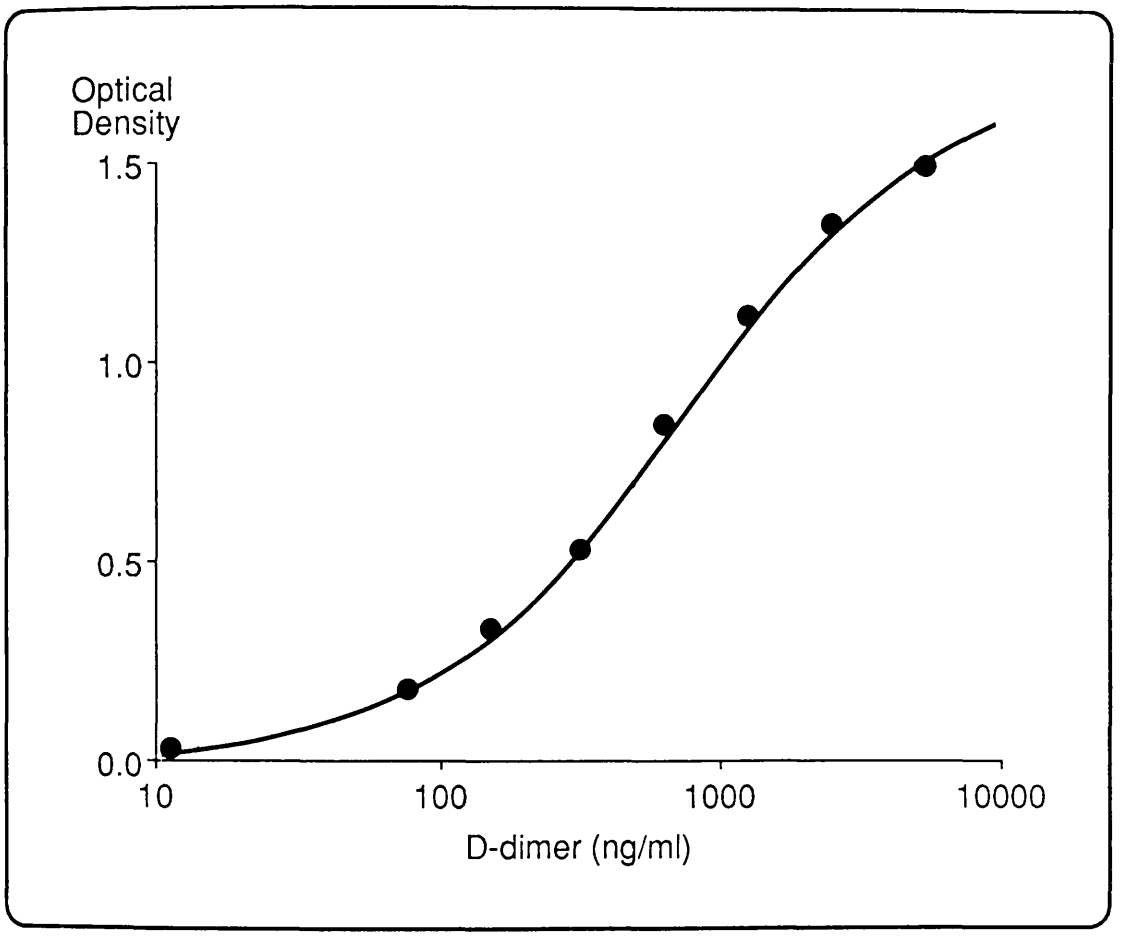

Figure 2.1

Typical standard curve in the measurement of cross linked fibrin degradation products. The optical density is related to the concentration of D-dimer (cross linked fibrin degradation products)

The Myrenne aggregometer consists of a transparent perspex cone and plastic plate rotational viscometer. The plate is fixed in position. Shearing of the sample is achieved by rotating the cone. Infra-red light transmitted through the sample is measured by a photometer which produces photovoltages processed in the microprocessor unit within the machine. The cone part of the viscometer rotates, achieving high shear rates of about 600 s^{-1} for 10 seconds and then stops. This disrupts any aggregates. During the phase of high shear the red cells are deformed and align with flow streamlines. During the five seconds of the static phase there is an increase in light transmission through the sample as red cell aggregation occurs at stasis. This increase in light transmission is measured photometrically (see Plate 3).

Procedure

Prior to introducing the sample the intensity of the light source transmitted through the cleaned cone and plate were logged with the chamber closed, and stored by pressing the 'A' (Adjust) key. Once this process was completed 25 microlitres of the blood sample to be tested were pipetted onto the centre of the cone. The plate was then lowered into position by closing the cover. The 'M' mode key was pressed. This resulted in a shear rate of 600 s^{-1} for 10 seconds, dispersing any red blood cell aggregates. This meant that all

Plate 3

Myrenne Photometric Aggregometer for measurement of red cell aggregation.

measurements were performed from a zero aggregation baseline. The increase in light transmission was then measured over a five second period of stasis. The increase in light transmission was integrated by the microprocessor unit and displayed digitally as a dimensionless aggregation index. The aggregation was measured two times for each sample; an average was taken of the duplicate readings. If these did not agree to within 0.5 units the sample was removed and the measurement repeated until two measurements agreed within 0.5 units. The value of the aggregation index was termed the mean extent of aggregation (MEA). The MEA was found to be consistent for up to eight sequential readings on the same sample. This meant that measurements could be made without having to replace the test sample.

The measuring system was cleaned with distilled water and dried with tissue before another sample could be measured. Red cell aggregation was measured at native haematocrit since MEA is relatively constant over the haematocrit range of 0.3-0.5 except at very high fibrinogen concentrations (Myrenne Aggregometer Manual). Previous studies of red cell aggregation in normals and diabetics at both native haematocrit, and at a standard haematocrit of 0.4 showed no significant difference in clinical situations (MacRury, 1990).

Plasma Viscosity and Whole Blood Viscosity

Plasma viscosity and whole blood viscosity were measured at 37 °C and at high shear rates with a Coulter-Harkness semi-automatic capillary viscometer (Coulter Electronics Limited, Harpenden, Hertfordshire). This instrument is preferred because of its constant pressure head, accurate measurement (experimental error less than 1% - Harkness, 1971), reproducibility (CV less than 1%) and speed of measurement (Harkness, 1981; Lowe, 1987b). (See Plate 4).

The plasma or whole blood sample was warmed to 37 °C then pipetted into a small cup. The sample was then drawn through a capillary tube using a constant head of pressure (mercury head). The time taken for the plasma or blood to flow through the tube was recorded electronically as the meniscus moves between two electrodes. The movement activates an electronic timer. The velocity of flow was determined by measuring the time for the mercury meniscus moving in a capillary parallel with the sample to pass between two electrodes. This activates an electronic timer.
The viscometer was calibrated daily with standard viscosity solutions and the temperature of measurement checked.

Corrected viscosity is whole blood viscosity corrected to a standard haematocrit of 0.45. This was calculated

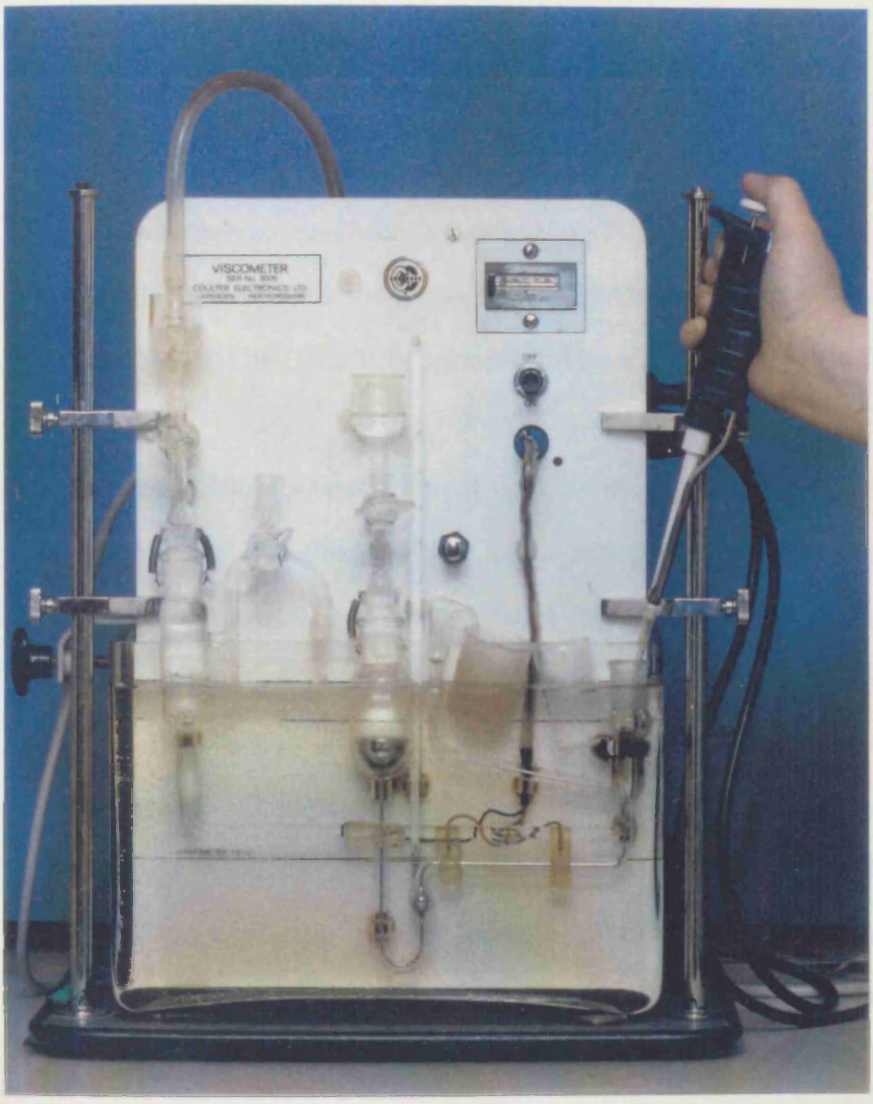

Plate 4 Coulter Viscometer.

using the formula of Matrai et al. (1987). Corrected viscosity assesses the contributions of factors other than haematocrit to blood viscosity.

Haematocrit

Haematocrit was measured by the Hawksley microhaematocrit method (Hawksley & Sons, Lancing, Sussex). This is widely used in clinical practice and accords with the ICSH recommendations (ICSH, 1986).

Well mixed blood was drawn up from a 4 mls EDTA tube into two duplicate 1 mm diameter glass capillaries. One end of each capillary tube was sealed over a Bunsen flame. The capillary tubes were then placed in the Hawksley Microcentrifuge and centrifuged at 13,000 g for five minutes. The haematocrit was then read as a percentage ratio of the height of the red cell pack to that of the whole sample. No correction was made for plasma trapping. Duplicate readings agreed to within 1%. An average value was taken to the nearest percentage; measurement was made at laboratory temperature (20-25 °C).

(3) **Full Blood Count and Platelet Count**

Full blood count and platelet count were measured in the Department of Haematology, Glasgow Royal Infirmary (Coulter S Counter).

(4) **Lipids**

Serum lipids were measured in the University Department of Pathological Biochemistry, Glasgow Royal Infirmary. Total cholesterol and triglyceride were measured on a Hitachi 717 automatic analyser using Boehringer Mannheim (UK Ltd) enzymatic reagents. HDL cholesterol was measured by precipitation of the apolipoprotein B containing lipoprotein, using heparin manganese. HDL cholesterol in the remaining supernatant was then measured on the Hitachi 717 automatic analyser with Boehringer Mannheim (UK Ltd) reagents.

(5) **Carboxyhaemoglobin Measurement**

Carboxyhaemoglobin was measured on EDTA anticoagulated blood in a CO-Oximeter 282 (Instrumentation Laboratory, Lexington, USA) in the University Department of Pathological Biochemistry, Glasgow Royal Infirmary.

The methods for the measurement of interleukin-6, C-reactive protein, alpha-2-macroglobulin, and Factor XII are discussed separately in Chapters 4 and 5.

Ankle Brachial Pressure Index

The ankle brachial pressure index (ABPI) correlates with the severity of ischaemia (Yao et al., 1969; Yao, 1973). Subjects were rested for at least five minutes. The brachial, anterior tibial and posterior tibial artery

systolic blood pressures were measured with a standard sphygmomanometer and a Sonicaid Blood Velocimeter (Bognor Regis, UK) using an 8 MHz doppler probe. The ABPI was calculated in both lower limbs using the highest tibial artery pressure divided by the brachial artery pressure. The limb with the lowest ABPI was used for assessment of severity of ischaemia in any individual patient.

Statistics

Statistical analyses were performed by myself on an Amstrad PC 2086 using a Statgraphics programme (Plus Ware STSC Inc, USA) and in collaboration with G D Murray, Senior Lecturer in Medical Statistics, University of Glasgow, Department of Surgery, Glasgow Royal Infirmary. Statistical analyses in Chapter 6 were performed in collaboration with The Department of Statistics and Community Medicine, University of Edinburgh.

Unless otherwise stated all grouped data in this thesis are expressed as the mean and standard deviation as most variables appeared normally distributed. In Chapters 3, 4 and 5 exceptions were cross-linked fibrin degradation products, red cell aggregation, interleukin-6, and Factor XII which were not normally distributed. In Chapter 6, plasminogen activator inhibitor, von Willebrand factor, factor VII, prothrombin time, fibrinogen, cross linked FDPs, red cell aggregation, platelet count and carboxyhaemoglobin

were not normally distributed. When there was a statistically significant difference between groups the p-value is given. Significance in the test is displayed as a probability value ($p<0.05$, 0.01 or 0.001) i.e. the level at which the null hypothesis can be rejected. Conventionally, probability values greater than 0.05 are considered statistically non-significant. Since the groups appeared to be normally distributed for most variables the Student's 't'-test was usually employed for the comparison of two groups where indicated. The 95% confidence intervals for differences are given where appropriate. Where variables were not normally distributed, logarithmic transformation or non-parametric methods were used.

The Spearman's rank correlation was used to show associations between two variables. This correlation is less sensitive to outliers than the Pearson correlation method and is appropriate for non-parametric data. The Spearman's rank correlation ranges from 1 to -1. A zero correlation indicates that no association exists (null hypothesis). A negative correlation indicates an inverse correlation.

CHAPTER 3

Fibrin Degradation Products, Red Cell Aggregation and Fibrinogen in Peripheral Arterial Disease.

Thrombosis is an important feature of peripheral arterial disease. Recent work has suggested the importance of blood rheology in peripheral arterial disease. It has been shown that patients with peripheral arterial disease have increased fibrinogen, increased blood viscosity, increased white cell count and a higher haematocrit compared to the normal population (Dormandy et al., 1973a; Alderman et al., 1981).

At low shear rates red cell aggregation disrupts flow streamlines and greatly increases whole blood viscosity (Fahraeus, 1958). Fibrinogen is the precursor of fibrin, and also the most important red cell aggregating plasma protein. The measurement of cross-linked fibrin degradation products and red cell aggregation in peripheral arterial disease has not been previously reported. It is now possible to measure the plasma levels of cross-linked FDP's with a specific monoclonal antibody test. Cross-linked FDP's are markers of in-vivo fibrin formation. It is also now possible to measure red cell aggregation using a new method with a Myrenne photometric aggregometer (See Chapter 2).

This chapter describes measurements of cross-linked fibrin degradation products and red cell aggregation which were performed in 118 patients with peripheral arterial disease,

compared to a control population. This was to determine if their levels are increased in peripheral arterial disease, and their association with plasma fibrinogen. Possible causes for the increased plasma fibrinogen level found in patients with peripheral arterial disease were also examined.

100 of these patients were serially followed up for 3-12 months, to determine the effects of changes in cigarette smoking and reconstructive vascular surgery on fibrinogen, cross-linked fibrin degradation products, red cell aggregation and other blood factors of coagulation and rheology.

Aims

1 To measure by two new techniques, cross-linked fibrin degradation products and red cell aggregation in patients with peripheral arterial disease and controls. This was to determine if cross-linked FDP`s and red cell aggregation are increased in peripheral arterial disease.

2 To determine if cross-linked FDP`s and red cell aggregation were associated with the increased fibrinogen level found in peripheral arterial disease.

3 To relate fibrinogen, cross-linked fibrin degradation products, and red cell aggregation to other measured haemorheological factors: plasma viscosity, haematocrit, white blood cell count, full blood count, platelets and lipids.

4 To determine if the severity of ischaemia, or the type of arterial disease (abdominal aortic aneurysm) correlated with the levels of fibrinogen, red cell aggregation or cross-linked FDP's.

5 To examine the effect of cigarette smoking, age and sex on fibrinogen, cross-linked FDPs and red cell aggregation in patients with peripheral arterial disease and controls.

6 To examine the effects of reconstructive vascular surgery on fibrinogen, cross-linked FDP's and red cell aggregation in patients with peripheral arterial disease.

METHODS

Selection and assessment of patients and controls are described in Chapter 2.

118 consecutive patients had measurement of fibrinogen, cross-linked fibrin degradation products and red cell aggregation. Plasma viscosity, microhaematocrit, full blood

count, and lipids were also measured. 80 patients had intermittent claudication, 20 patients had abdominal aortic aneurysms, and 15 patients had ischaemic rest pain without tissue necrosis. 3 patients had gangrene or ulceration. These 3 patients have been excluded from further study because infection is known to increase plasma fibrinogen level and white cell count, red cell aggregation and blood visocity (Lowe, 1987a; Gaillard et al., 1985; Friederichs et al., 1984). This left 115 patients for study. The mean age of the patients was 61 years (range 39-86). Medication was also recorded, specifically use of antiplatelet agents or anticoagulants, and the presence of diabetes mellitus was also noted. 46 patients underwent reconstructive vascular surgery.

The patients were compared with 122 controls without evidence of arterial disease for all blood measurements except cross-linked fibrin degradation products. A further 231 controls from the general population were used to compare cross-linked fibrin degradation products. This was from a population study of the level of cross-linked fibrin degradation products being carried out in the University Department of Medicine Laboratory, Glasgow Royal Infirmary, at the same time as this study (Scottish Heart Health Study), and as previously discussed gave a large sample to define the population range of this skewed variable.

100 of the 115 patients with peripheral arterial disease were serially followed up for 3-12 months (mean 7 months), and their blood measurements repeated, together with a full clinical examination, questioning about smoking habit, medication and symptoms. Ankle brachial pressure indices were repeated. Of the 15 patients who were unable to be followed up, 6 had died in the intervening period, and 9 were lost to follow up (5 due to considerable distances involved). At the follow up examination, lipid measurement was not performed as it was impractical to get sufficient patients fasting at the out-patient clinic.

Statistical Analysis

With the exception of cross-linked FDP's and red cell aggregation the distribution of all of the variables under study were well approximated by normal distribution, and so in general, normal-based techniques of t-tests and one-way analysis of variance were used for the univariate analyses. The corresponding non-parametric procedures (the Mann-Whitney U test and the Kruskal Wallis test) were used for cross-linked FDP's and red cell aggregation.

Further multivariate analyses (multiway analysis of covariance, including tests for interaction) were used to compare patients and controls after adjusting for imbalances in smoking habit, age and sex distribution.

Since there do not exist corresponding non-parametric procedures, prior to the multivariate analysis, cross-linked FDP's and red cell aggregation were transformed to approximate normality by using a logarithmic transformation.

To examine the effects following reconstructive vascular surgery, the mean change in patients undergoing surgery was compared to the mean change in patients not getting surgery by the Student's t- test. This was performed on a logarithmic scale for cross-linked FDP's and red cell aggregation which were highly skewed. Baseline values of patients undergoing surgery were not statistically different from patients not getting surgery.

RESULTS

Table 3.1 compares patients and controls for age, sex and smoking habit.

Cross-linked Fibrin Degradation Products

The levels of cross-linked FDP's were significantly increased in patients with peripheral arterial disease compared to the controls. The median value for cross-linked FDP's was 117 ng/ml in the patients compared to 86 ng/ml in the controls ($p<0.001$). (See Table 3.2 and Figure 3.1).

Table 3.1
Comparison of controls and patients for sex, age and smoking habits

	FDP Controls	Other Controls	Patients
n	231	122	115
Sex			
Male	127 (55%)	50 (41%)	77 (67%)
Female	104 (45%)	72 (59%)	38 (33%)
Age (years)			
Mean	50	52	61
S.D.	6.1	17.5	10.6
Minimum	25	21	39
Maximum	64	88	86
Smoking Habit			
Current	85 (37%)	34 (28%)	63 (55%)
Ex-smokers	146 (63%)	88 (72%)	48 (42%)
Non-smokers			4 (3%)

Table 3.2
Comparison of blood tests in controls and patients with peripheral arterial disease

Test	Controls	Patients	p value
Cross linked FDPs (ng/ml)			
Median	86	117	p<0.001
IQR	49	77	
n	231	102	
Red cell aggregation (units)			
Median	3.30	4.45	p<0.001
IQR	1.8	1.75	
n	114	112	
Fibrinogen (g/L)			
Mean	2.97	3.47	p<0.001
S.D.	0.84	0.99	
n	87	111	
Plasma viscosity (mPa.s)			
Mean	1.285	1.329	p<0.01
S.D.	0.078	0.104	
n	71	111	
White cell count ($x10^9$/L)			
Mean	6.5	8.1	p<0.001
S.D.	2.1	2.5	
n	59	115	
Triglyceride (mmol/L)			
Mean	1.13	1.89	p<0.001
S.D.	0.41	0.99	
n	47	98	
HDL cholesterol (mmol/L)			
Mean	1.66	1.28	p<0.001
S.D.	0.42	0.28	
n	45	83	
Total cholesterol (mmol/L)			
Mean	5.98	6.06	N.S.
S.D.	1.36	1.32	
n	47	97	
Haematocrit			
Mean	0.43	0.45	N.S.
S.D.	0.04	0.05	
n	66	111	

Table 3.2. continued

Test	Control	Patients	p value
Haemoglobin (g/dL)			
Mean	13.7	14.2	N.S.
S.D.	1.6	1.5	
n	58	114	
Red blood count ($x10^{12}$/L)			
Mean	4.47	4.57	N.S.
S.D.	0.46	0.57	
n	58	111	
Mean cell volume (fl)			
Mean	90.4	91.6	N.S.
S.D.	5.4	5.5	
n	59	114	
Platelet count ($x10^{9}$/L)			
Mean	281	296	N.S.
S.D.	68	90	
n	55	114	

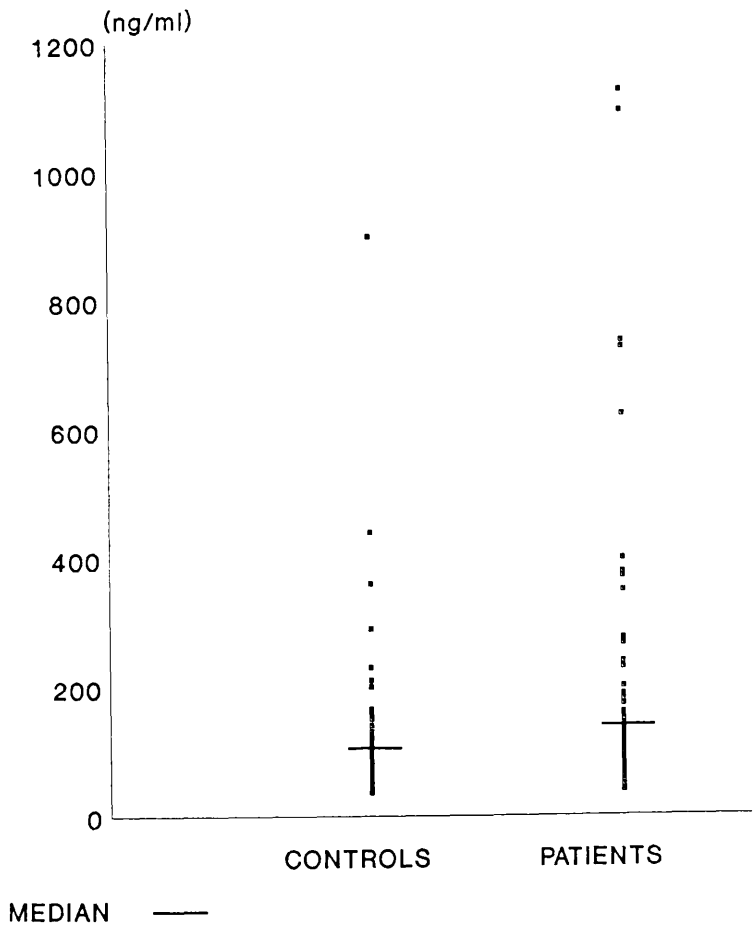

Figure 3.1

A comparison of levels of cross linked fibrin degradation products (FDPs) in controls and patients with peripheral arterial disease.

There was a significant correlation between cross-linked FDP's and plasma fibrinogen in patients with peripheral arterial disease ($r=0.26$, $p<0.01$).

Cross-linked FDP`s also correlated significantly in the patients with :

Age	r= 0.56	p<0.001
ABPI	r=-0.35(inversely)	p<0.001
Haematocrit	r=-0.31(inversely)	p<0.001
Plasma viscosity	r= 0.27	p<0.01
Haemoglobin	r=-0.26(inversely)	p<0.01

(See Table 3.3a).

The significant correlation coefficients for all variables in the controls are shown in Table 3.3b.

Cross-linked FDP`s were significantly increased in patients with intermittent claudication (median 95 ng/ml), ischaemic rest pain (median 128 ng/ml) and in abdominal aortic aneurysm (median 148 ng/ml) compared to controls (median 86 ng/ml). (See Figure 3.2). One way analysis of variance (Kruskal-Wallis) showed that cross-linked FDP`s were significantly different between these three patient groups ($p<0.01$). The level of cross-linked FDP`s therefore was related to the severity of ischaemia and to the type of disease (Table 3.4). There was a significant inverse correlation between cross-linked FDP's and the ankle brachial pressure index ($r=-0.35$, $p<0.001$).

Table 3.3a
Spearman's Rank Correlation Matrix in Patients

	Cross linked FDPs	Red cell aggregation	Fibrinogen	Plasma Viscosity	White cell count	Triglyceride	HDL cholesterol	Total cholesterol	Haematocrit	Haemoglobin	Red blood count	Mean cell volume	Platelet count	Age	Body mass index	Carboxy-haemoglobin	ABPI
Cross linked FDPs																	
Red cell aggregation	-																
Fibrinogen	•• 0.26	-															
Plasma Viscosity	•• 0.27	-	••• 0.58														
White cell count	-	•• -0.27	•• 0.29	•• 0.25													
Triglyceride	-	-	-	-	-												
HDL cholesterol	-	-	-	-	-	••• -0.41											
Total cholesterol	-	-	-	-	-	••• 0.52	-										
Haematocrit	••• -0.31	••• -0.38	-	-	••• 0.37	-	-	•• 0.31									
Haemoglobin	•• -0.26	-	-	-	••• 0.37	•• 0.27	-	• 0.24	••• 0.83								
Red blood count	-	-	-	• 0.24	••• 0.39	-	-	• 0.23	••• 0.79	••• 0.83							
Mean cell volume	-	-	-	-	-	-	-	-	-	-	••• -0.37						-
Platelet count	-	-	••• 0.44	••• 0.43	••• 0.36	-	-	-	-	-	-	-					
Age	••• 0.56	-	-	• 0.20	-	-	-	-	•• -0.31	••• -0.35	•• -0.29	-	-				
Body mass index	-	• 0.20	-	-	-	•• 0.28	• -0.27	• 0.24	-	• 0.22	-	-	-	-			
Carboxy-haemoglobin	-	-	-	-	• 0.21	• 0.26	-	-	-	•• 0.30	• 0.23	-	-	• -0.21	-		
ABPI	••• -0.35	-	-	• -0.20	• -0.22	-	-	-	-	-	-	-	• -0.20	•• -0.30	•• 0.27	-	

• $p<0.05$, •• $p<0.01$, ••• $p<0.001$

Table 3.3b
Spearman's Rank Correlation Matrix in Controls

	Cross linked FDPs	Red cell aggregation	Fibrinogen	Plasma Viscosity	White cell count	Triglyceride	HDL cholesterol	Total cholesterol	Haematocrit	Haemoglobin	Red blood count	Mean cell volume	Platelet count	Age	Body mass index
Cross linked FDPs															
Red cell aggregation	-														
Fibrinogen	-	-													
Plasma Viscosity	-	-	••• 0.41												
White cell count	-	-	-	• 0.31											
Triglyceride	-	• 0.21	• 0.29	-	-										
HDL cholesterol	-	-	-	-	-	• -0.27									
Total cholesterol	-	-	-	-	-	•• 0.30	• 0.22								
Haematocrit	-	-	-	-	-	-	-	-							
Haemoglobin	-	-	-	-	-	-	-	-	••• 0.96						
Red blood count	-	-	-	-	-	-	-	-	••• 0.86	••• 0.78					
Mean cell volume	• 0.62	-	-	-	-	-	-	-	• 0.34	•• 0.42	-				
Platelet count	-	-	-	-	-	-	-	-	-	-	-	-			
Age	••• 0.29	-	-	-	• 0.29	-	-	-	-	-	-	-	-		
Body mass index	-	-	-	-	-	-	-	• 0.24	-	• 0.35	-	-	-	-	

• $p<0.05$ •• $p<0.01$ ••• $p<0.001$

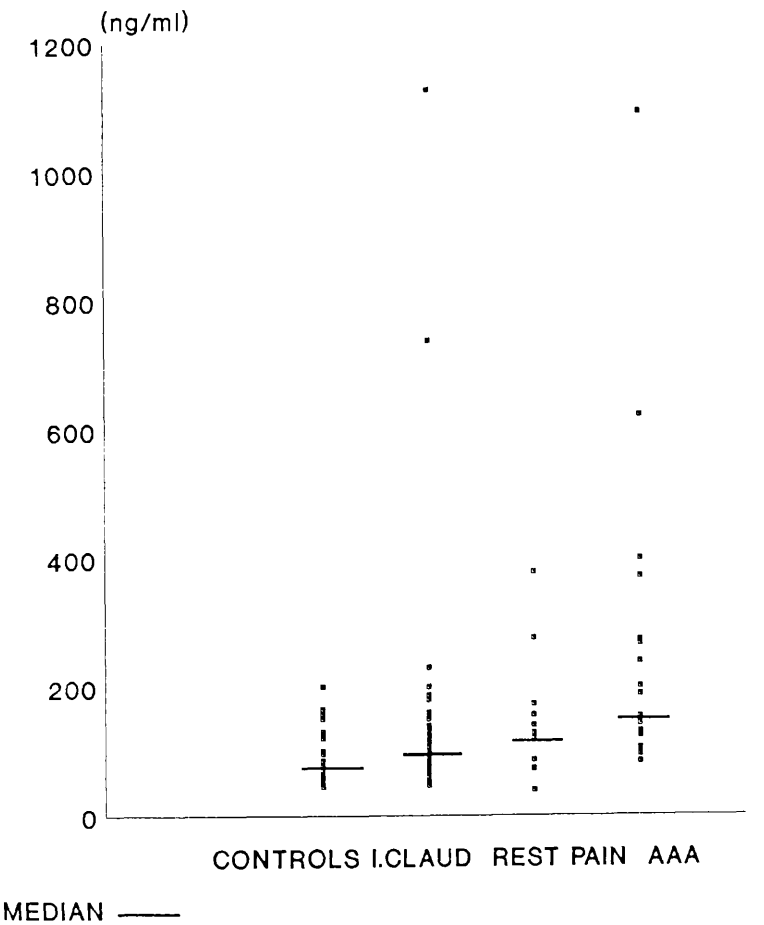

Figure 3.2

A comparison of levels of cross linked fibrin degradation products (FDPs) in controls and patients with intermittent claudication (I.CLAUD), ischaemic rest pain and abdominal aortic aneurysm (AAA).

Table 3.4
Comparison of severity of ischaemia and type of disease (abdominal aortic aneurysm)

Test	Intermittent claudication	Ischaemic rest pain	Abdominal aortic aneurysm	p value
Cross linked FDPs (ng/ml)				
Median	95	128	148	p<0.01
IQR	60	93	165	(Kruskal-Wallis)
n	65	12	18	
Red cell aggregation (units)				
Median	4.23	4.30	5.30	p<0.01
IQR	1.66	1.95	1.66	(Kruskal-Wallis)
n	70	15	20	
Fibrinogen (g/L)				
Mean	3.34	3.56	3.55	N.S.
S.D.	0.78	1.11	1.20	(F-test)
n	69	15	20	
Plasma Viscosity (mPa.s)				
Mean	1.313	1.349	1.344	N.S.
S.D.	0.093	0.135	0.073	(F-test)
n	70	15	19	
White cell count ($x10^9$/L)				
Mean	7.7	10.3	7.7	p<0.001
S.D.	1.8	4.4	1.8	(F-test)
n	73	15	20	
Triglyceride (mmol/L)				
Mean	2.02	1.69	1.85	N.S.
S.D.	1.13	0.56	0.76	(F-test)
n	62	11	18	
HDL cholesterol (mmol/L)				
Mean	1.31	1.32	1.14	N.S.
S.D.	0.26	0.42	0.24	(F-test)
n	54	10	13	

Table 3.4 continued

Test	Intermittent claudication	Ischaemic rest pain	Abdominal aortic aneurysm	p value
Total cholesterol (mmol/L)				
Mean	6.18	6.16	5.98	N.S.
S.D.	1.21	1.61	1.50	(F-test)
n	61	11	18	
Haematocrit				
Mean	0.45	0.48	0.43	$p<0.05$
S.D.	0.04	0.07	0.04	(F-test)
n	69	15	20	
Haemoglobin (g/dL)				
Mean	14.3	14.8	13.8	N.S.
S.D	1.3	2.0	1.2	(F-test)
n	72	15	20	
Red blood count ($x10^{12}$/L)				
Mean	4.57	4.90	4.47	N.S.
S.D.	0.44	0.97	0.49	(F-test)
n	72	13	20	
Mean cell volume				
(fl) Mean	92	90	91	N.S.
S.D.	5	7	4	(F-test)
n	72	15	20	
Platelet count ($x10^9$/L)				
Mean	282	328	291	N.S.
S.D.	72	142	79	(F-test)
n	72	15	20	
Ankle brachial pressure index				
Mean	0.79	0.29	0.87	$p<0.001$
S.D.	0.23	0.22	0.25	(F-test)
n	72	14	20	
Age (years)				
Mean	58.4	65.5	68.4	$p<0.001$
S.D.	9.9	13.0	6.3	(F-test)
n	73	15	20	
Body mass index (kg/m^2)				
Mean	25.00	24.37	25.27	N.S
S.D.	3.34	3.49	4.22	(F-test)
n	73	13	19	

There was no significant difference between current and carboxyhaemoglobin - proven ex or non-smokers in either patients (Figure 3.3) or controls (See Table 3.5a, 3.5b). A serial study of the effects of stopping smoking was performed. In the 100 patients followed up for 3-12 months 57 were initial current smokers, 42 who initially claimed to be ex-smokers and only 1 patient who had never smoked before. In this group of 100, 41 of the 57 smokers continued to smoke. 18 claimed to have stopped. After confirming ex-smoker status with carboxyhaemoglobin levels only 14 patients were considered to have stopped smoking. 10 of these patients proceeded to reconstructive vascular surgery, leaving only 4 patients. This was insufficient for meaningful analysis of the effects of stopping smoking, independent from the effects of reconstructive surgery. Cross-linked FDP`s correlated strongly with age ($r=0.56$, $p<0.001$). Multivariate analysis showed evidence of an interaction with age (i.e. the relationship of cross-linked FDP's to age differed between patients and controls). When the effect of age was taken into account, there was still a significant difference between patients and controls ($p<0.05$, Table 3.6). There was no sex difference: the level of cross-linked FDP`s was not different between males and females in patients or controls.

Of the 100 patients who were followed up, 46 patients underwent reconstructive vascular surgery. 54 patients did not undergo surgery. The ankle brachial pressure index was significantly increased in patients following surgery

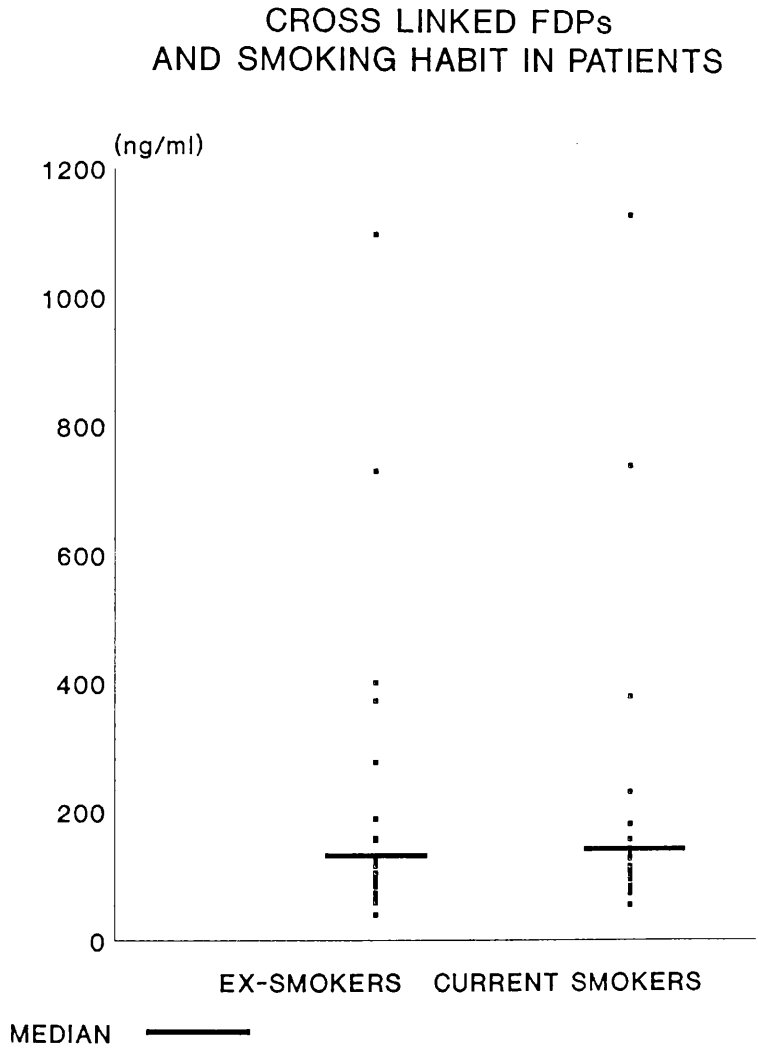

Figure 3.3

A comparison of levels of cross linked fibrin degradation products (FDPs) in ex-smokers and current smokers in patients with peripheral arterial disease.

Table 3.5a
Comparison of ex-smokers and smokers in patients with peripheral arterial disease

Test		Ex-Smokers	Current Smokers	p value
Cross linked FDPs				
(ng/ml)	Median	118	130	N.S.
	IQR	65	75	
	n	32	57	
Red cell aggregation				
(units)	Median	4.65	4.20	p<0.05
	IQR	1.60	1.65	
	n	35	61	
Fibrinogen (g/L)				
	Mean	3.17	3.60	p<0.05
	S.D.	0.81	1.02	
	n	34	60	
Plasma viscosity				
(mPa.s)	Mean	1.314	1.322	N.S.
	S.D.	0.092	0.096	
	n	34	61	
White cell count				
($x10^9$/L)	Mean	7.0	8.4	p<0.001
	S.D.	1.8	2.0	
	n	35	63	
Triglyceride (mmol/L)				
	Mean	1.72	1.86	N.S.
	S.D.	0.71	1.09	
	n	30	55	
HDL Cholesterol				
(mmol/L)	Mean	1.27	1.28	N.S.
	S.D.	0.33	0.25	
	n	26	45	
Total cholesterol				
(mmol/L)	Mean	6.10	5.90	N.S.
	S.D.	1.40	1.29	
	n	30	54	

Table 3.5a continued

Test		Ex-smokers	Current smokers	p value
Haematocrit				
	Mean	0.43	0.45	$p<0.05$
	S.D.	0.05	0.05	
	n	34	60	
Haemoglobin (g/dL)				
	Mean	13.6	14.3	$p<0.05$
	S.D.	1.5	1.5	
	n	35	62	
Red blood count				
($x10^{12}$/L)	Mean	4.37	4.61	N.S.
	S.D.	0.46	0.61	
	n	34	60	
Mean cell volume (fl)				
	Mean	91	92	N.S.
	S.D.	6	6	
	n	35	62	
Platelet count ($x10^9$/L)				
	Mean	268	306	N.S.
	S.D.	83	94	
	n	35	62	

Table 3.5b
Comparison of non-smokers and current smokers in controls

Test		Non smokers	Current smokers	p value
Cross linked FDPs				
(ng/ml)	Median	85	86	N.S.
	IQR	49.5	33	
	n	146	85	
Red cell aggregation				
(units)	Median	3.4	2.45	N.S.
	IQR	1.3	2.65	
	n	73	28	
Fibrogen (g/L)				
	Mean	2.85	3.37	$p<0.05$
	S.D.	0.83	0.90	
	n	56	22	
Plasma viscosity				
(mPa.s)	Mean	1.288	1.325	N.S.
	S.D.	0.076	0.121	
	n	73	28	
White cell count				
($x10^9$/L)	Mean	6.2	7.6	$p<0.05$
	S.D.	1.4	3.2	
	n	38	15	
Triglyceride (mmol/L)				
	Mean	1.16	1.33	N.S.
	S.D.	0.65	0.48	
	n	60	24	
HDL cholesterol				
(mmol/L)	Mean	1.62	1.62	N.S.
	S.D.	0.35	0.50	
	n	58	23	
Total cholesterol				
(mmol/L)	Mean	5.60	5.92	N.S.
	S.D.	1.16	1.30	
	n	60	24	

Table 3.5b continued

Test		Non smokers	Current smokers	p value
Haematocrit				
	Mean	0.43	0.44	P<0.05
	S.D.	0.04	0.04	
	n	74	29	
Haemoglobin (g/dL)				
	Mean	13.6	14.4	N.S.
	S.D.	1.4	1.9	
	n	38	14	
Red blood count				
($x10^{12}$/L)	Mean	4.47	4.64	N.S.
	S.D.	0.44	0.50	
	n	38	14	
Mean cell volume (fl)				
	Mean	90	91	N.S.
	S.D.	5	7	
	n	38	15	
Platelet count ($x10^9$/L)				
	Mean	285	286	N.S.
	S.D.	69	71	
	n	36	15	

Table 3.6
Multivariate analysis, comparing patients and controls corrected for age, sex and smoking habit

1

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Cross linked FDPs (analysed on logarithmic scale)	Constant	2.358	0.584	4.04	0.000
	Sex	0.052	0.052	1.01	0.315
	Age	0.028	0.004	6.27	0.000
	Smoking	-0.044	0.053	-0.84	0.399
	Group	0.772	0.335	2.31	0.022
	† Age by Group	-0.016	0.006	-2.70	0.007

† interaction

2

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Red cell Aggregation	Constant	4.211	0.603	6.98	0.000
	Sex	-0.205	0.180	-1.14	0.256
	Age	0.011	0.006	1.79	0.076
	Smoking	0.496	0.183	2.72	0.007
	Group	-0.995	0.200	-4.96	0.000

(no interactions)

3

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Fibrinogen	Constant	4.010	0.458	8.75	0.000
	Sex	-0.104	0.140	-0.74	0.458
	Age	0.007	0.005	1.46	0.146
	Smoking	-0.466	0.139	-3.34	0.001
	Group	-0.199	0.154	-1.29	0.198

(no interactions)

4

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Plasma Viscosity	Constant	1.316	0.048	27.58	0.000
	Sex	-0.018	0.014	-1.24	0.217
	Age	0.001	0.001	2.27	0.025
	Smoking	-0.017	0.014	-1.19	0.235
	Group	-0.018	0.016	-1.15	0.250

(no interactions)

5

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
White cell count	Constant	10.577	1.051	10.07	0.000
	Sex	-0.365	0.325	-1.12	0.262
	Age	0.007	0.011	0.65	0.518
	Smoking	-1.172	0.318	-3.69	0.000
	Group	-0.888	0.364	-2.44	0.016

(no interactions)

Table 3.6 continued

6

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Triglyceride (analysed on logarithmic scale)	Constant	0.450	0.119	3.79	0.000
	Sex	-0.029	0.034	-0.86	0.390
	Age	-0.001	0.001	0.41	0.680
	Smoking	-0.024	0.031	-0.78	0.436
	Group	-0.180	0.039	-4.68	0.000

(no interactions)

7

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
HDL cholesterol	Constant	0.557	0.215	2.59	0.011
	Sex	0.161	0.064	2.53	0.013
	Age	0.003	0.002	1.44	0.153
	Smoking	-0.034	0.057	-0.60	0.552
	Group	0.361	0.071	5.11	0.000

(no interactions)

8

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Total cholesterol	Constant	5.517	0.832	6.63	0.000
	Sex	0.329	0.237	1.39	0.168
	Age	0.001	0.009	0.03	0.977
	Smoking	0.226	0.217	1.04	0.300
	Group	-0.243	0.270	-0.90	0.371

(no interactions)

9

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Haematocrit	Constant	56.155	2.307	24.34	0.000
	Sex	-3.553	0.698	-5.09	0.000
	Age	-0.069	0.025	-2.77	0.006
	Smoking	-1.614	0.692	-2.33	0.021
	Group	-0.205	0.768	-0.27	0.790

(no interactions)

10

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Haemoglobin	Constant	18.481	0.715	25.86	0.000
	Sex	-1.544	0.219	-7.04	0.000
	Age	-0.027	0.008	-3.41	0.001
	Smoking	-0.480	0.215	-2.23	0.027
	Group	0.060	0.246	0.25	0.806

(no interactions)

Table 3.6 continued

11

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Red blood count	Constant	5.755	0.262	22.01	0.000
	Sex	-0.482	0.079	-6.08	0.000
	Age	-0.007	0.003	-2.55	0.012
	Smoking	-0.123	0.078	-1.58	0.117
	Group	0.085	0.089	0.98	0.343

(no interactions)

12

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Mean Cell Volume	Constant	95.624	2.970	32.19	0.000
	Sex	-0.533	0.917	-0.58	0.562
	Age	-0.006	0.032	-0.20	0.844
	Smoking	-1.313	0.898	-1.46	0.146
	Group	-1.000	1.026	-0.97	0.331

(no interactions)

13

Variable	Predictor	Coefficient	S.D.	t-ratio	p value
Platelet count	Constant	273.220	44.810	6.10	0.000
	Sex	34.720	13.750	2.53	0.013
	Age	0.269	0.486	0.55	0.580
	Smoking	-21.670	13.400	-1.62	0.108
	Group	-11.380	15.510	-0.73	0.464

(no interactions)

compared to those not undergoing surgery ($p<0.001$). Cross-linked FDP's were significantly increased in patients following reconstructive vascular surgery compared to those not undergoing surgery ($p<0.05$). There was no increase in the patients who did not have surgery (Table 3.7).

Red Cell Aggregation

The levels of red cell aggregation were significantly increased in peripheral arterial disease compared to controls. The median value for patients was 4.45 units compared to 3.30 units in controls ($p<0.001$). (See Table 3.2 and Figure 3.4).

The correlation with plasma fibrinogen was not significant $r=0.010$ ($p=0.29$).

Red cell aggregation in the patients correlated significantly with:

Haematocrit	r=-0.38(inversely)	p<0.001
White Cell Count	r=-0.27(inversely)	p<0.01
Body mass index	r= 0.20	p<0.05

Red cell aggregation did not correlate significantly with cross-linked FDP's, $r=0.16$, $p=0.11$. (See Table 3.3a).

Red cell aggregation was significantly increased in intermittent claudication (median 4.23 units), ischaemic rest pain (median 4.30 units) and in abdominal aortic aneurysm

Table 3.7
Comparison of the mean change between patients having surgery and patients not having surgery

Test	No surgery	Surgery	p value 95% C.I.
Cross linked FDPs (ng/ml) (on a log scale)			
Mean change	-0.01	0.15	p<0.05
S.D	0.19	0.39	(0.02, 0.31)
n	42	35	
Red cell aggregation (units)			
Mean change	-0.14	-0.03	N.S.
S.D.	1.22	1.61	(-0.48, 0.69)
n	49	44	
Fibrinogen (g/L)			
Mean change	-0.07	-0.33	N.S.
S.D.	1.01	0.82	(-0.65, 0.14)
n	47	42	
Plasma viscosity (mPa.s)			
Mean change	0.007	0.027	N.S.
S.D.	0.085	0.102	(-0.020, 0.061)
n	45	39	
White cell count ($x10^9$/L)			
Mean change	0.2	0.5	N.S.
S.D.	2.2	2.0	(-0.6, 1.1)
n	52	43	
Haematocrit			
Mean change	0.00	-0.01	N.S.
S.D.	0.03	0.04	(-0.02, 0.01)
n	49	41	
Haemoglobin (g/dL)			
Mean change	0.1	-0.4	p<0.05
S.D.	0.9	1.3	(-1.0, -0.01)
n	48	42	

Table 3.7 continued

Test		No Surgery	Surgery	p value 95% C.I.
Red blood count ($x10^{12}$/L)				
	Mean change	-0.01	-0.17	N.S.
	S.D.	0.31	0.79	(-0.41, 0.09)
	n	45	40	
Mean cell volume (fl)				
	Mean change	-0.5	-1.1	N.S.
	S.D.	2.8	3.1	(-1.9, 0.6)
	n	48	42	
Platelet count ($x10^9$/L)				
	Mean change	33	20	N.S.
	S.D.	52	56	(-36, 10)
	n	48	42	
Ankle brachial pressure index	Mean change	-0.01	0.14	$p<0.001$
	S.D.	0.17	0.23	(0.07, 0.23)
	n	51	46	

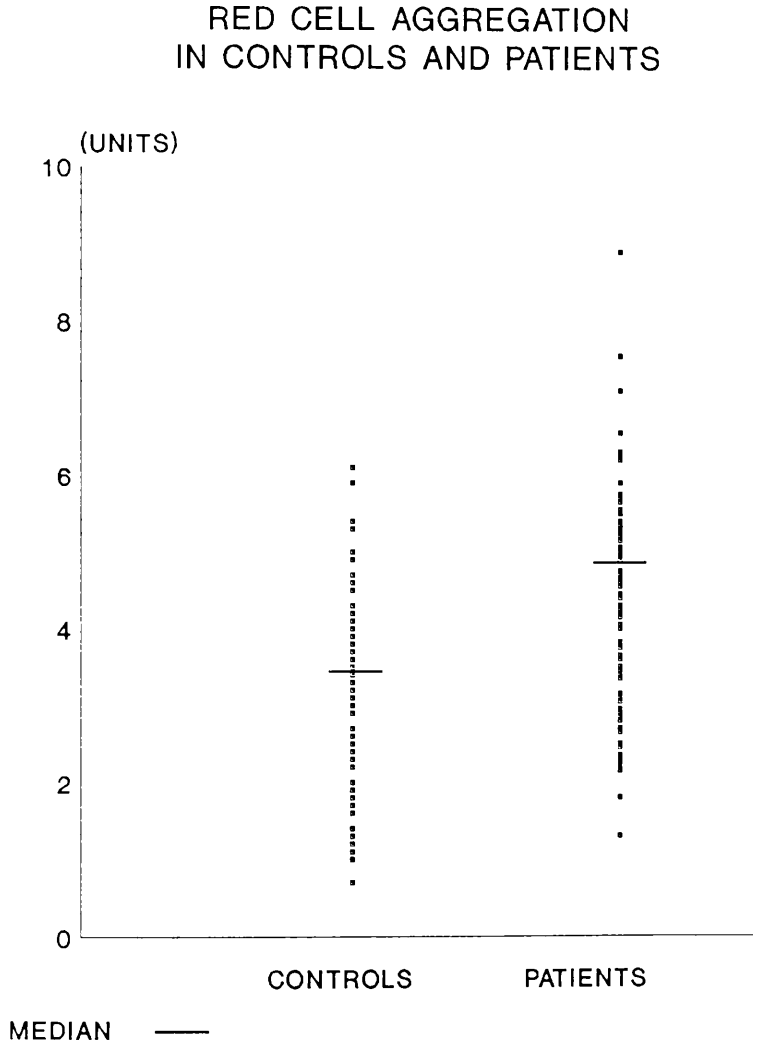

Figure 3.4

A comparison of red cell aggregation in controls and patients with peripheral arterial disease.

(median 5.30 units) compared to controls (median 3.30). One-way analysis of variance (Kruskal-Wallis) showed that red cell aggregation was significantly different between the groups ($p<0.01$). (Table 3.4 and Figure 3.5).

Red cell aggregation was higher in ex-smokers than current smokers in patients (Figure 3.6) and in controls (Table 3.5a, 3.5b). Multivariate analysis showed that the increased red cell aggregation in peripheral arterial disease was independent of this unexpected smoking related effect. (Table 3.6). Red cell aggregation did not correlate with age or sex.

Red cell aggregation did not alter in patients following reconstructive vascular surgery, nor in patients who did not have surgery. (Table 3.7)

Fibrinogen

Plasma fibrinogen level was significantly increased in patients with peripheral arterial disease compared to controls. The mean value for fibrinogen in patients was 3.47 g/l, compared to 2.97 g/l in the controls ($p<0.001$), (Figure 3.7).

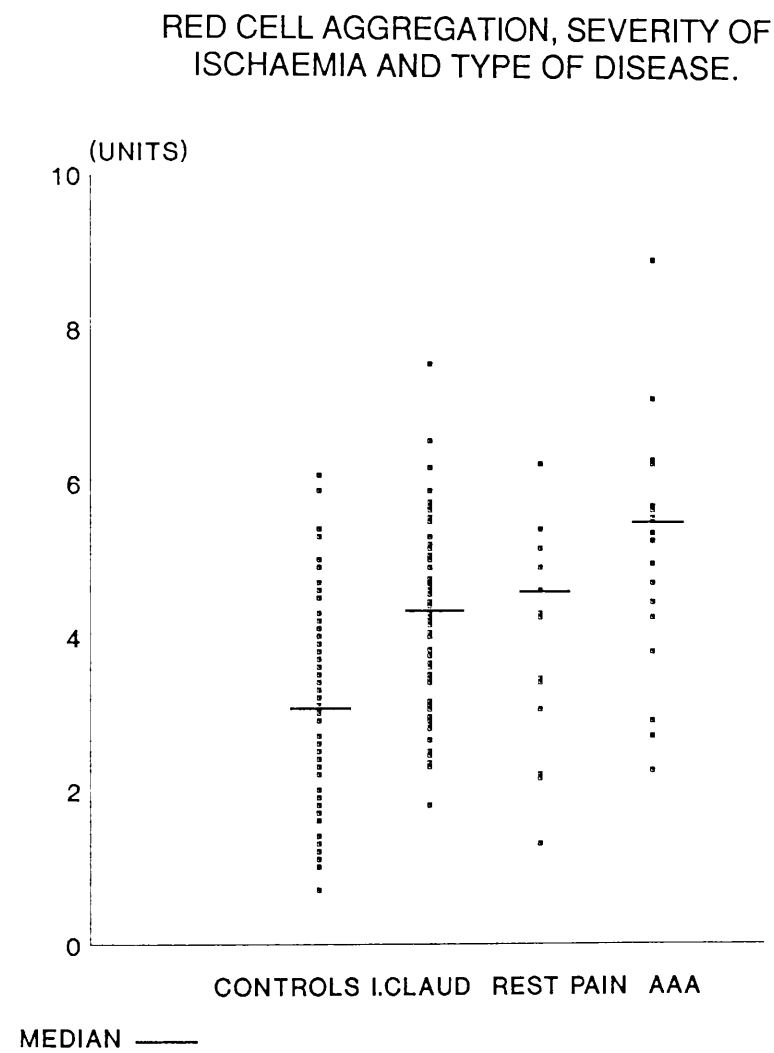

Figure 3.5

A comparison of red cell aggregation in controls and patients with intermittent claudication (I.CLAUD),ischaemic rest pain and abdominal aortic aneurysm (AAA).

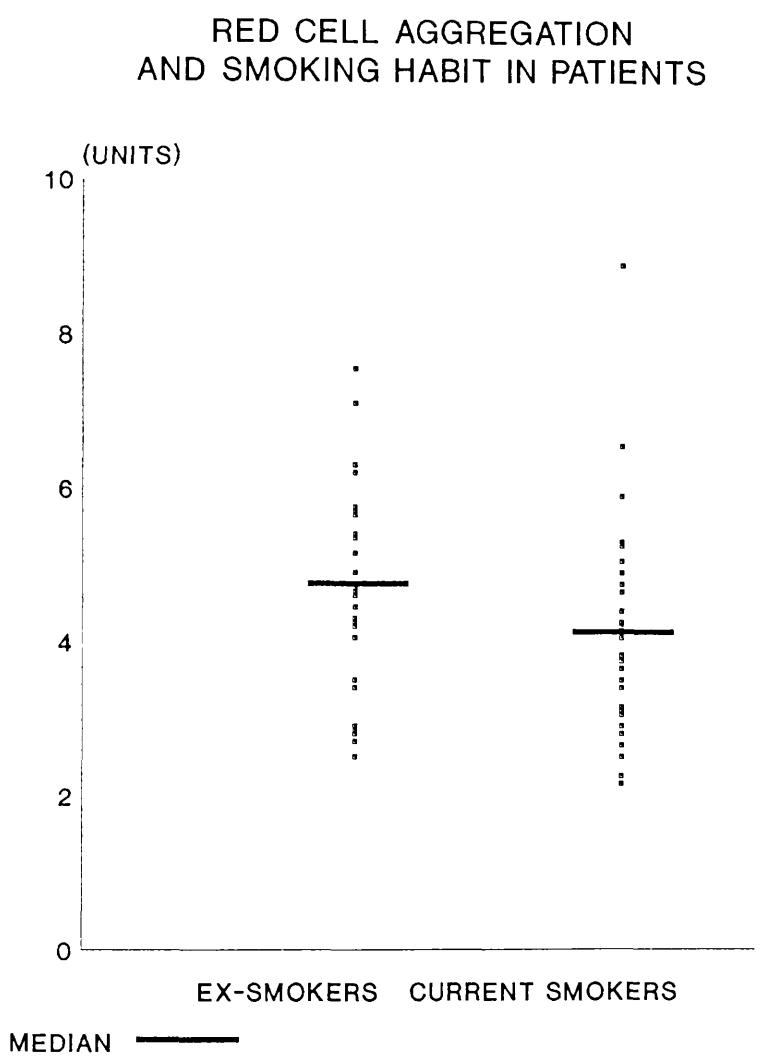

Figure 3.6

A comparison of red cell aggregation in ex-smokers and current smokers in patients with peripheral arterial disease.

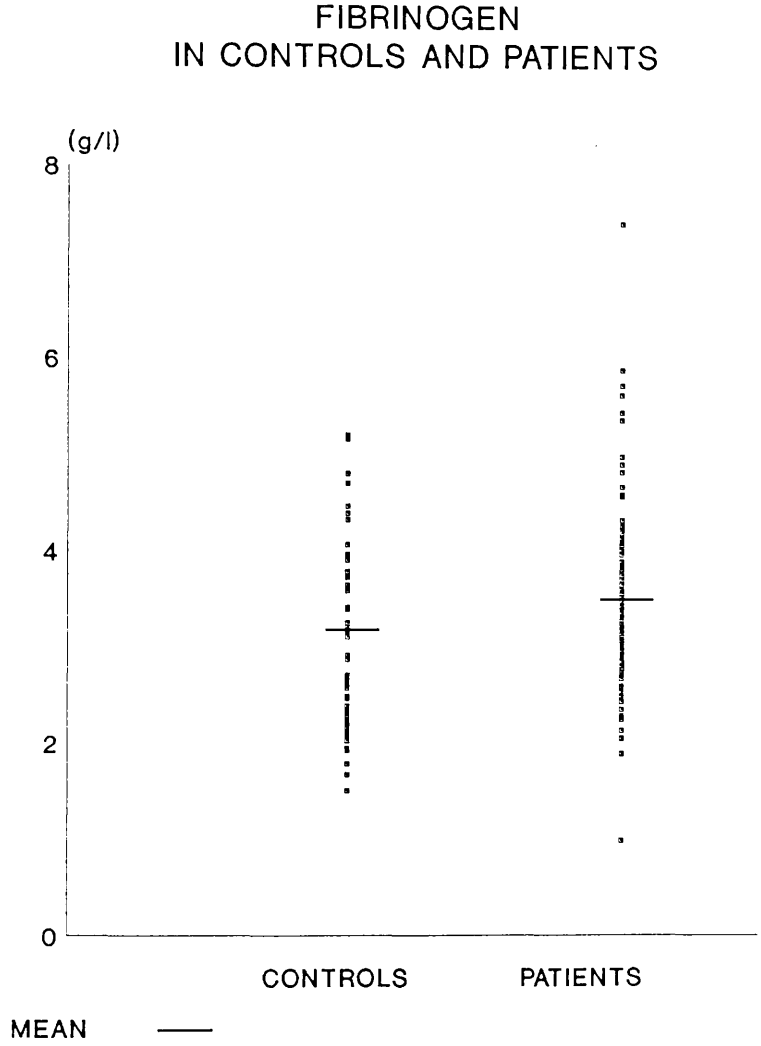

Figure 3.7

A comparison of levels of fibrinogen in controls and patients with peripheral arterial disease.

Fibrinogen correlated significantly in the patients with:

Plasma Viscosity	r=0.58	p<0.001
Platelet count	r=0.44	p<0.001
White Cell Count	r=0.29	p<0.01
Cross-linked Fibrin Degradation Products	r=0.26	p<0.01
but not with red cell aggregation	r=0.10	p=0.29

(See Table 3.3a).

Fibrinogen was significantly increased in patients with intermittent claudication (mean 3.34), ischaemic rest pain (mean 3.56) and in abdominal aortic aneurysm (mean 3.55) compared to controls (mean 2.97). However there was no difference in fibrinogen according to the severity of ischaemia or type of disease using analysis of variance. (Table 3.4, Figure 3.8)

Fibrinogen was significantly increased in current smokers compared to ex or non-smokers in both patients (Figure 3.9) and controls. (Table 3.5a, 3.5b).

Fibrinogen increased with age. Multivariate analysis showed that when cigarette smoking and age were taken into account there was no significant difference in fibrinogen level between patients with peripheral arterial disease and controls. Hence the effects of smoking and age accounted for the increased fibrinogen level in the patients with peripheral arterial disease (Table 3.6). There was no difference in fibrinogen level between males and females.

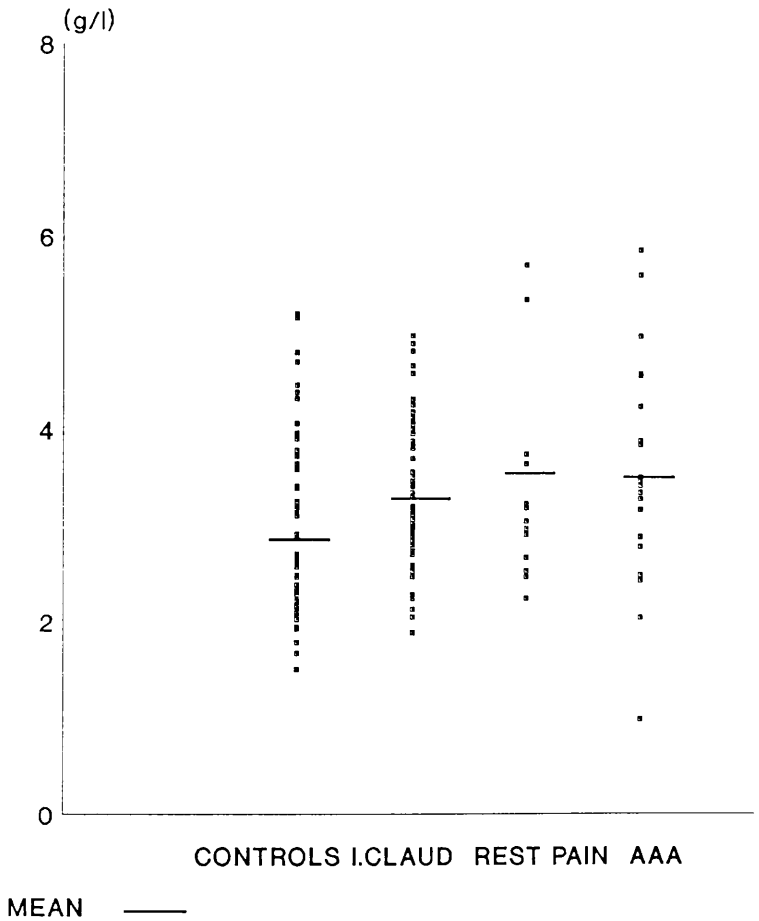

Figure 3.8

A comparison of levels of fibrinogen in controls and patients with intermittent claudication (I.CLAUD),ischaemic rest pain and abdominal aortic aneurysm (AAA).

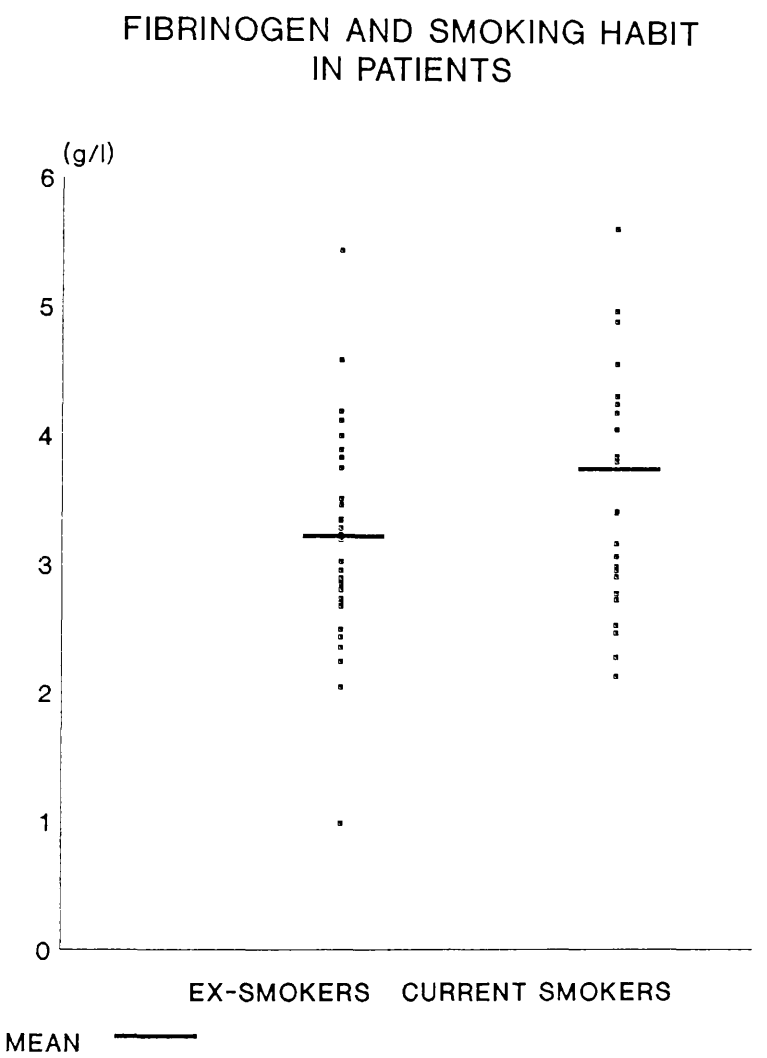

Figure 3.9

A comparison of levels of fibrinogen in ex-smokers and current smokers in patients with peripheral arterial disease.

Fibrinogen level decreased in patients at follow up after reconstructive vascular surgery, whereas it was not altered in those who did not have surgery. The difference is however not statistically significant (Table 3.7).

Plasma Viscosity

Plasma viscosity was significantly increased in patients with peripheral arterial disease compared to controls. The mean value of plasma viscosity was 1.329 mPa.s compared to 1.285 mPa.s in controls ($p<0.01$). (See Table 3.2 and Figure 3.10).

Plasma viscosity correlated significantly in the patients with:

Fibrinogen	r= 0.58	p<0.001
Platelet count	r= 0.43	p<0.001
Cross-linked FDP's	r= 0.27	p<0.01
White Cell Count	r= 0.25	p<0.01
Red blood count	r= 0.24	p<0.05
Age	r= 0.20	p<0.05
Ankle brachial pressure index	r=-0.20(inversely)	p<0.05

(See Table 3.3a).

Plasma viscosity was significantly increased in intermittent claudication (mean 1.313 mPa.s), ischaemic rest pain (mean 1.349 mPa.s), and abdominal aortic aneurysm (mean 1.344 mPa.s) compared to controls (mean 1.285 mPa.s). While

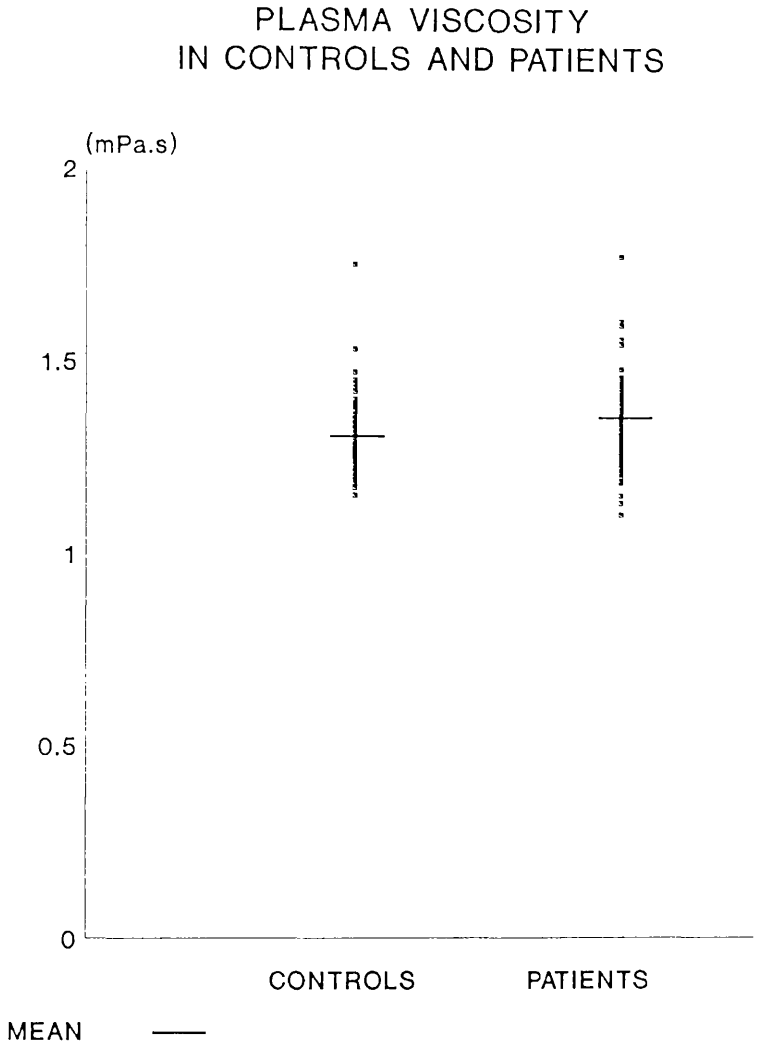

Figure 3.10

A comparison of plasma viscosity in controls and patients with peripheral arterial disease.

there was no significant difference in plasma viscosity according to the severity of ischaemia or type of disease using analysis of variance, plasma viscosity was higher in ischaemic rest pain than in intermittent claudication. Plasma viscosity was associated with ischaemia, correlating inversely with the ankle brachial pressure index ($r=-0.20$, $p<0.05$. Table 3.4 and Figure 3.11).

There was no significant difference between current smokers and ex- or non-smokers in patients and controls. (Tables 3.5a and 3.5b). Plasma viscosity increased with age. Multivariate analysis showed that when age was taken into account there was no significant difference in plasma viscosity between patients with peripheral arterial disease and controls. The effect of age accounted for the increased plasma viscosity level in the patients with peripheral arterial disease (Table 3.6). Plasma viscosity was not altered by sex: there was no difference in plasma viscosity between males and females.

Plasma viscosity did not alter significantly following reconstructive vascular surgery, nor did it alter in patients who did not have surgery (Table 3.7).

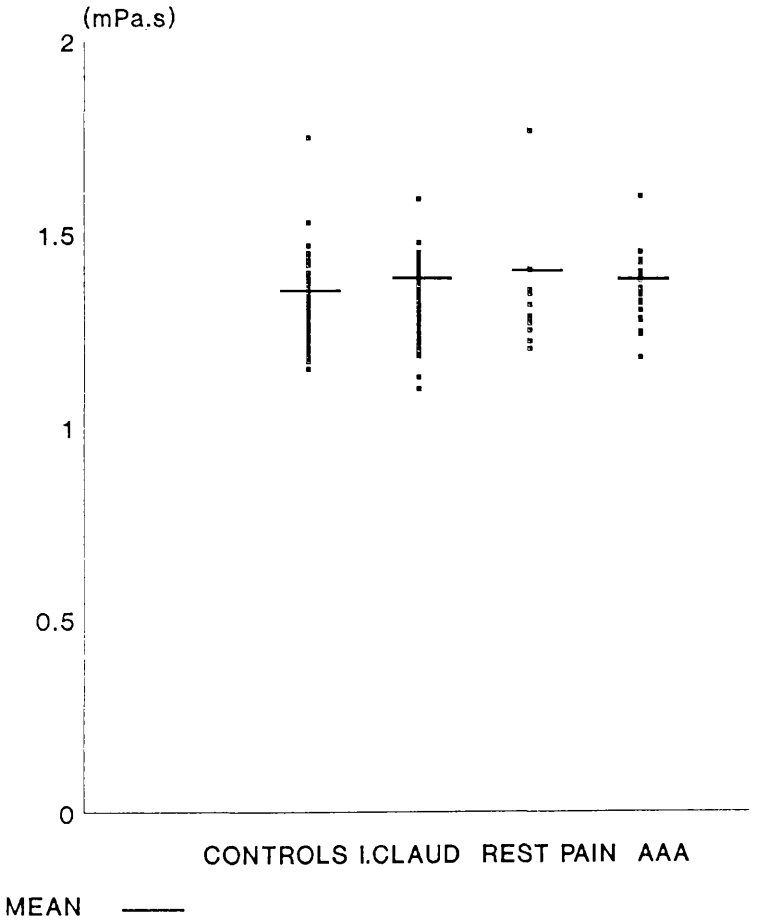

Figure 3.11

A comparison of plasma viscosity in controls and patients with intermittent claudication (I.CLAUD), ischaemic rest pain and abdominal aortic aneurysm (AAA).

White Cell Count

White cell count was significantly increased in patients with peripheral arterial disease compared to controls. The mean value for white cell count in patients was 8.1 X 10^9/l, compared to 6.5 X 10^9/l in controls. ($p<0.001$. Table 3.2 and Figure 3.12).

White cell count correlated significantly in the patients with:

Red blood count	r= 0.39	p<0.001
Haemoglobin	r= 0.37	p<0.001
Haematocrit	r= 0.37	p<0.001
Platelet count	r= 0.36	p<0.001
Fibrinogen	r= 0.29	p<0.01
Red Cell Aggregation	r=-0.27(inversely)	p<0.01
Plasma Viscosity	r= 0.25	p<0.01
ABPI	r=-0.22(inversely)	p<0.05
Carboxyhaemoglobin	r= 0.21	p<0.05

White cell count was significantly increased in intermittent claudication (mean 7.7 X 10^9/l) and ischaemic rest pain (mean 10.3 x 10^9/l) compared to controls (mean 6.5 X 10^9/l). Analysis of variance showed that there was a significant difference between the patients with ischaemic rest pain and those patients with intermittent claudication or abdominal aortic aneurysm ($p<0.001$. Table 3.4 and Figure 3.13). There was also a significant inverse

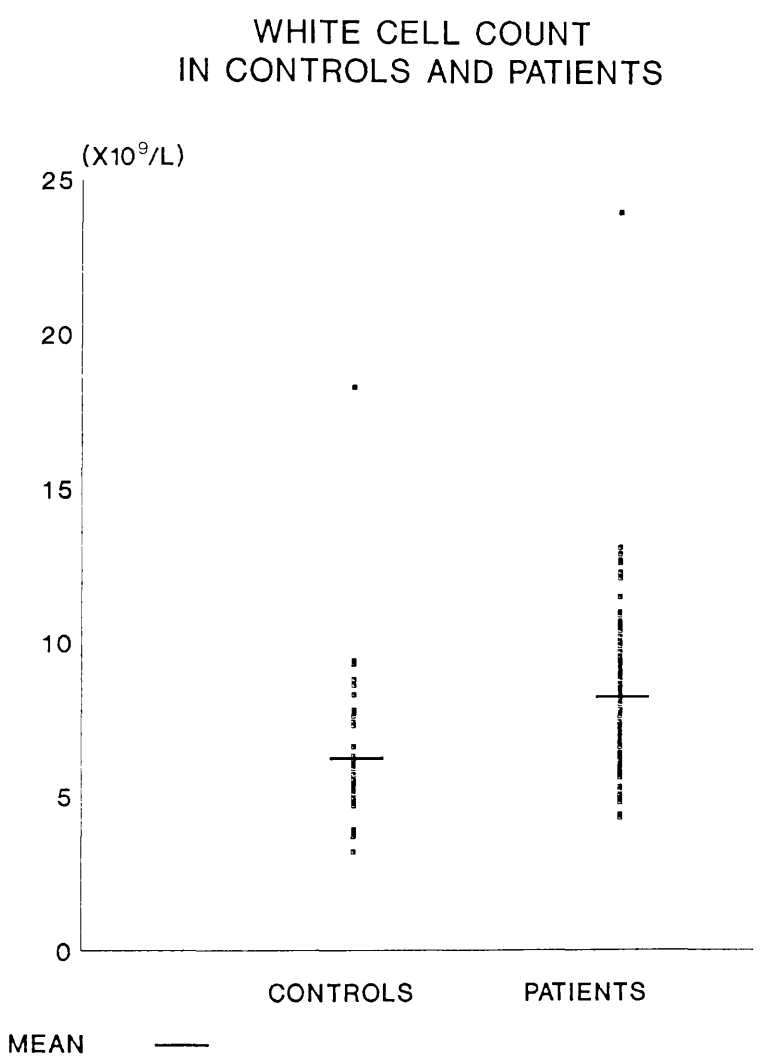

Figure 3.12

A comparison of white cell count in controls and patients with peripheral arterial disease.

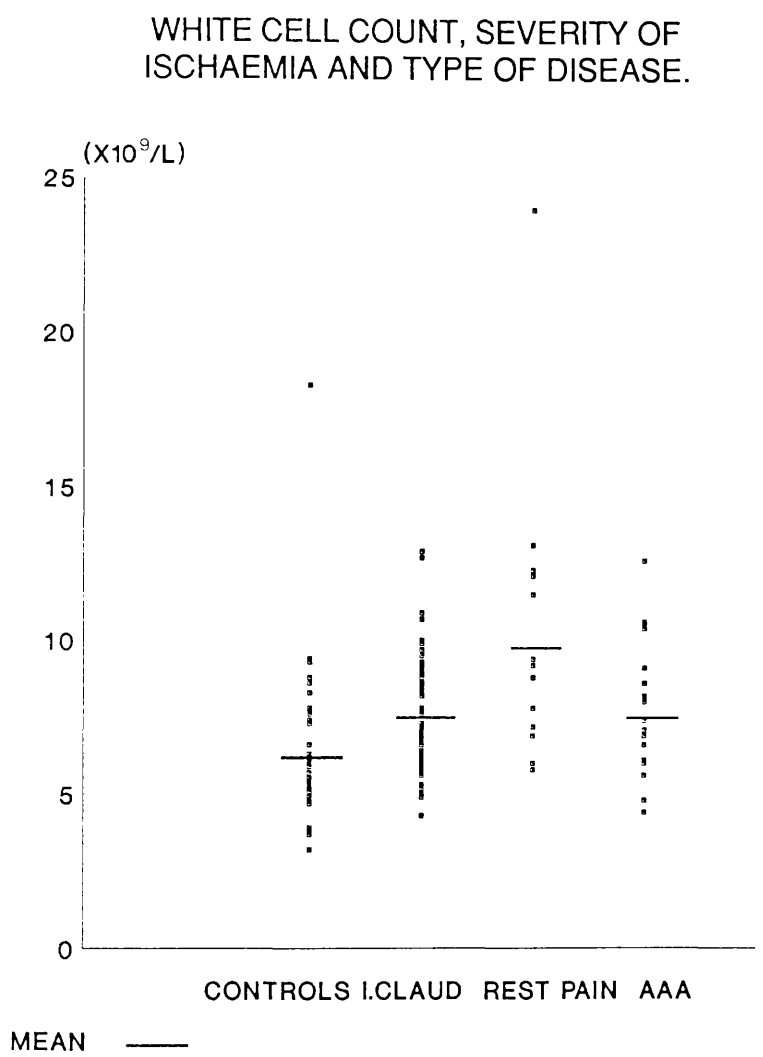

Figure 3.13

A comparison of white cell count in controls and patients with intermittent claudication (I.CLAUD),ischaemic rest pain and abdominal aortic aneurysm (AAA).

correlation with the ABPI ($r=-0.22$, $p<0.05$). However white cell count was not significantly different in patients with abdominal aortic aneurysm compared to controls.

White cell count was increased in current smokers compared with ex or non-smokers in both patients (Figure 3.14) and controls (Table 3.5a and 3.5b). White cell count correlated significantly with carboxyhaemoglobin level. Multivariate analysis showed that when the effect of smoking was taken into account there remained a significant difference between the patients with peripheral arterial disease and the controls (Table 3.6). The increase in white cell count in patients with peripheral arterial disease was independent of this smoking related effect. White cell count was not altered by age or sex.

White cell count did not alter significantly following reconstructive vascular surgery, nor in the patients who did not have surgery (Table 3.7).

Triglyceride

Triglyceride level was significantly increased in patients with peripheral arterial disease compared to controls. The mean value for triglyceride in patients was 1.89 mmol/l compared to 1.13 mmol/l in controls. ($p<0.001$. Table 3.2 and Figure 3.15).

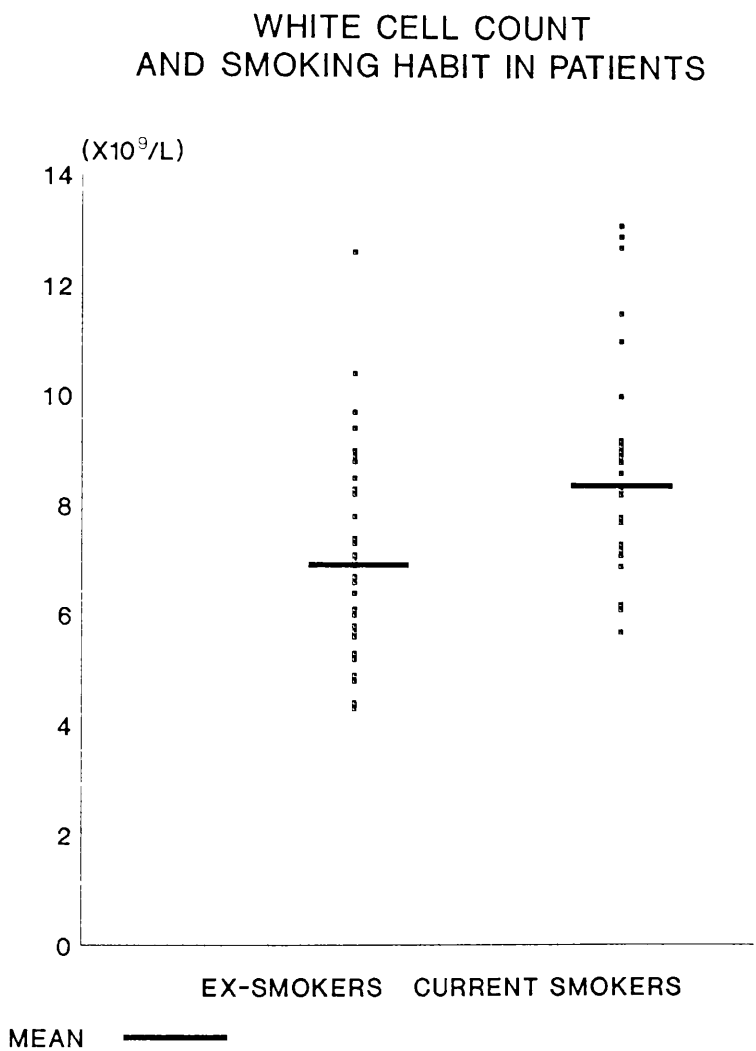

Figure 3.14

A comparison of white cell count in ex-smokers and current smokers in patients with peripheral arterial disease.

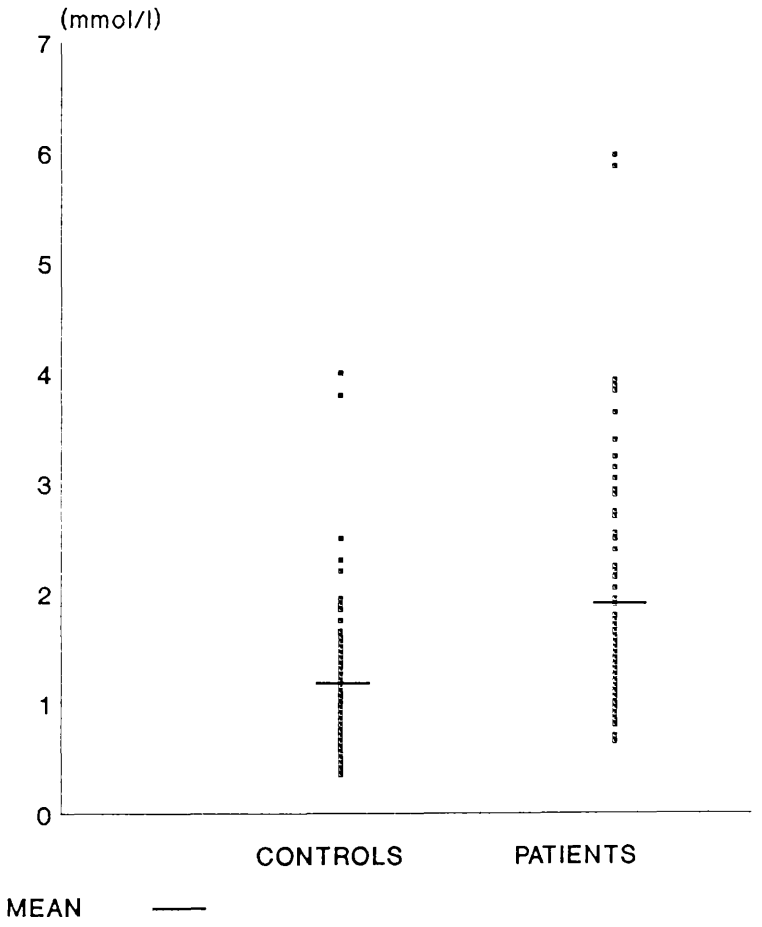

Figure 3.15

A comparison of levels of triglyceride in controls and patients with peripheral arterial disease.

Triglyceride correlated significantly in patients with:

Total cholesterol	r= 0.52	p<0.001
HDL cholesterol	r=-0.41(inversely)	p<0.001
Body mass index	r= 0.28	p<0.01
Haemoglobin	r= 0.27	p<0.01
Carboxyhaemoglobin	r= 0.26	p<0.05

(See Table 3.3a).

Triglyceride was significantly increased in intermittent claudication (mean 2.02 mmol/l), ischaemic rest pain (mean 1.69 mmol/l) and abdominal aortic aneurysm (mean 1.85 mmol/l) compared to controls (mean 1.13 mmol/l). However there was no significant difference according to the severity of ischaemia or type of disease, using analysis of variance. (Table 3.4).

There was no difference in triglyceride levels between smokers and ex-smokers in patients and controls (Tables 3.5a and 3.5b). Triglyceride was not altered by age or sex. Multivariate analysis showed that the increased triglyceride level in patients with peripheral arterial disease was independent of age, sex or smoking habit (Table 3.6).

HDL Cholesterol

HDL cholesterol was significantly decreased in patients with peripheral arterial disease compared to controls. The mean value for patients was 1.28 mmol/l compared to 1.66 mmol/l in the controls ($p<0.001$). (Table 3.2 and Figure 3.16).

HDL cholesterol inversely and significantly correlated with:

Triglyceride $r=-0.41$ (inversely) $p<0.001$

and Body mass index $r=-0.27$ (inversely) $p<0.05$

in the patients with peripheral arterial disease (Table 3.3a).

HDL cholesterol was significantly decreased in intermittent claudication (mean 1.31 mmol/l), ischaemic rest pain (mean 1.32 mmol/l) and abdominal aortic aneurysm (mean 1.14 mmol/l) compared to controls. There was no relation to the severity of ischaemia or type of disease, using analysis of variance (Table 3.4).

HDL cholesterol level was not different in current smokers compared to ex- or non-smokers in patients or in controls. (Table 3.5a and 3.5b). HDL cholesterol was not age dependent. HDL cholesterol level was altered by sex: HDL cholesterol level was lower in males than females ($p<0.05$). Multivariate analysis showed that the decreased HDL cholesterol level was independent of this sex related effect (Table 3.6).

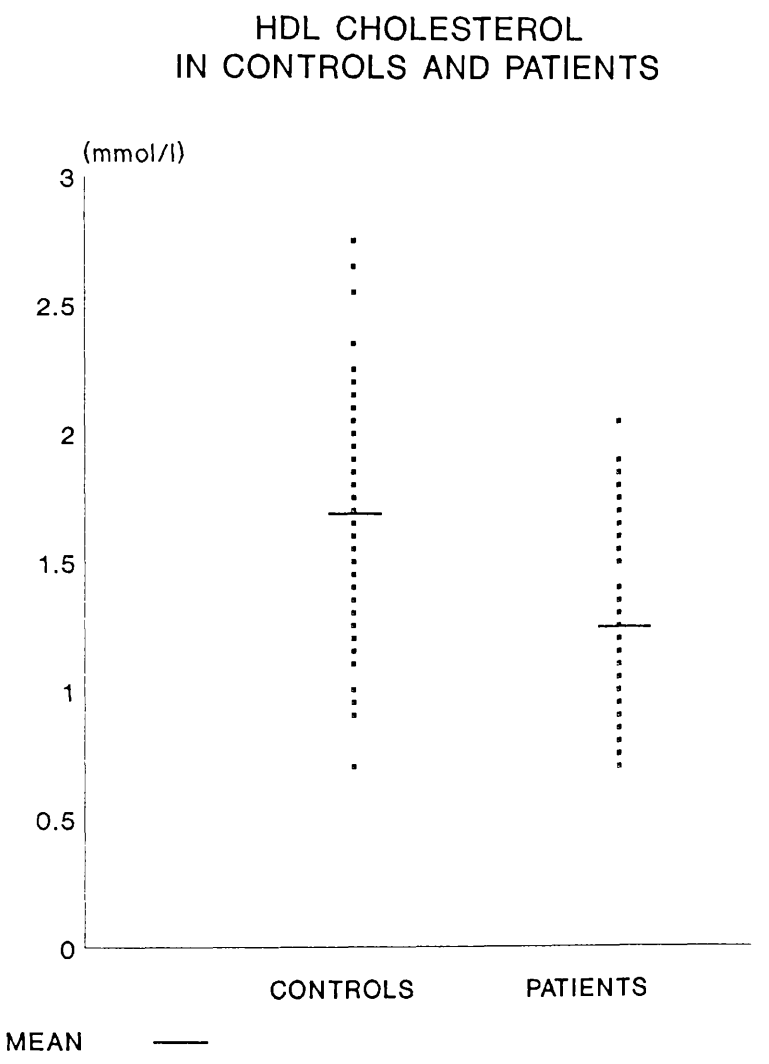

Figure 3.16

A comparison of levels of HDL cholesterol in controls and patients with peripheral arterial disease.

Total Cholesterol

Total cholesterol level was not significantly different in patients compared to controls (Table 3.2).

Total cholesterol correlated significantly in the patients with:

Triglyceride	r=0.52	p<0.001
Haematocrit	r=0.31	p<0.01
Body mass index	r=0.24	p<0.05
Haemoglobin	r=0.24	p<0.05
Red blood count	r=0.23	p<0.05

(See Table 3.3a).

There was no correlation with severity of ischaemia or type of disease (Table 3.4).

Total cholesterol was not related to smoking habit, age or sex (Tables 3.5a, 3.5b and 3.6).

Haematocrit

Haematocrit was not significantly increased in all patients with peripheral arterial disease compared to controls. The mean value for haematocrit in patients was 0.45 compared to 0.43 in controls (p=0.067. Table 3.2).

Haematocrit correlated significantly in the patients with:

Haemoglobin	r= 0.83	p<0.001
Red blood count	r= 0.79	p<0.001
Red Cell Aggregation	r=-0.38(inversely)	p<0.001
White Cell Count	r= 0.37	p<0.001
Cross-linked FDP's	r=-0.31(inversely)	p<0.001
Total Cholesterol	r= 0.31	p<0.01
Age	r=-0.31(inversely)	p<0.01

Haematocrit was significantly increased in patients with intermittent claudication (mean 0.45) and ischaemic rest pain (mean 0.48) compared to controls (mean 0.43). Patients with abdominal aortic aneurysm had a mean haematocrit of 0.43. There was not a significant difference between patients with abdominal aortic aneurysm and controls. Analysis of variance showed that patients with occlusive arterial disease (intermittent claudication and ischaemic rest pain) were significantly increased compared to patients with abdominal aortic aneurysm disease ($p<0.05$. See Table 3.4 and Figure 3.17).

Haematocrit was significantly increased in current smokers compared to ex-smokers in patients and in controls (Table 3.5a and 3.5b). Haematocrit was significantly higher in males compared to females and decreased with age. Multivariate analysis showed that when the effects of age

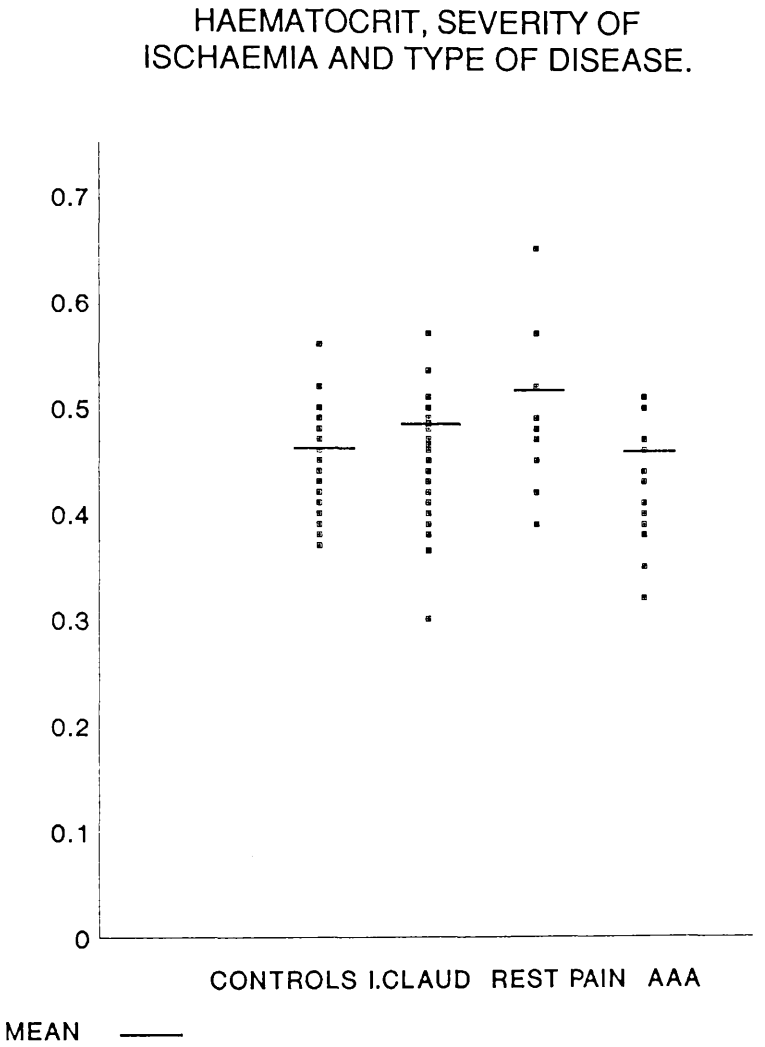

Figure 3.17

A comparison of haematocrit in controls and patients with intermittent claudication (I.CLAUD), ischaemic rest pain and abdominal aortic aneurysm (AAA).

and sex were taken into account there was no difference between all patients and controls (Table 3.6). Haematocrit did not alter significantly following reconstructive vascular surgery or in patients not having surgery (Table 3.7).

Full Blood Count

Haemoglobin

Haemoglobin was not significantly increased in patients with peripheral arterial disease compared to controls. The mean value for haemoglobin level in patients was 14.2 g/dl compared to 13.7 g/dl in controls (p=0.059. Table 3.2).

Haemoglobin correlated significantly in the patients with:

Haematocrit	r= 0.83	p<0.001
Red blood count	r= 0.83	p<0.001
White cell count	r= 0.37	p<0.001
Age	r=-0.35(inversely)	p<0.001
Carboxyhaemoglobin	r= 0.30	p<0.01
Triglyceride	r= 0.27	p<0.01
Cross-linked FDP's	r=-0.26(inversely)	p<0.01
Total cholesterol	r= 0.24	p<0.05
Body mass index	r= 0.22	p<0.05

(See Table 3.3a).

There was no correlation with severity of ischaemia or type of disease (Table 3.4).

Haemoglobin level was significantly increased in current smokers compared to ex-smokers in patients (p<0.05), but

not in the controls (Table 3.5a and 3.5b). Like haematocrit, haemoglobin level was significantly higher in males than females and decreased by age. Multivariate analysis showed that when the effects of sex and age were taken into account there was still no difference in haemoglobin level between patients and controls (Table 3.6).

At 3-12 months following reconstructive vascular surgery, haemoglobin was decreased by 0.4 g/dl. In the patients not receiving surgery there was a mean increase in haemoglobin by 0.1g/dl. This difference is statistically significant ($p<0.05$).

Red Blood Cell Count

Red blood count was not significantly increased in patients with peripheral arterial disease compared to controls (Table 3.2).

Red blood count correlated significantly with:

Haemoglobin	r= 0.83	p<0.001
Haematocrit	r= 0.79	p<0.001
White cell count	r= 0.39	p<0.001
Mean cell volume	r=-0.37(inversely)	p<0.001
Age	r=-0.29(inversely)	p<0.01
Plasma viscosity	r= 0.24	p<0.05
Total cholesterol	r= 0.23	p<0.05
Carboxyhaemoglobin	r= 0.23	p<0.05

in the patients (See Table 3.3a).

There was no correlation with severity of ischaemia or type of disease (Table 3.4).

Red blood count was not significantly different between current smokers, and ex or non-smokers in patients and controls (Table 3.5a and 3.5b).

Red blood count was significantly increased in males ($p<0.01$) and significantly decreased with age ($p<0.05$) in patients and controls. Red blood count was not altered following reconstructive vascular surgery or in patients not having surgery (Table 3.7).

Mean Cell Volume

Mean cell volume was not increased in peripheral arterial disease, and was not altered by severity of ischaemia, type of disease, sex, age, smoking habit or reconstructive vascular surgery.

Mean cell volume correlated significantly with red blood count $r=-0.37$ (inversely) $p<0.001$ in the patients.

Platelet Count

Platelet count was not significantly increased in patients compared to controls (Table 3.2). Platelet count correlated significantly with:

Fibrinogen	$r= 0.44$	$p<0.001$
Plasma Viscosity	$r= 0.43$	$p<0.001$

White Cell Count	r= 0.36	p<0.001
ABPI	r=-0.20(inversely)	p<0.05

(See Table 3.3a).

Platelet count was higher in patients with ischaemic rest pain (mean 328 X 10^9/l) than intermittent claudication (mean 282 X 10^9/l).

Platelet count was not altered by smoking, sex or age. Platelet count significantly increased in patients who did not undergo reconstructive vascular surgery after 3-12 months duration. A non significant increase was also present in patients who underwent reconstructive vascular surgery. There was no statistical significant difference between these two groups (Table 3.7).

Other Correlations

Age correlated significantly in the patients with:

	r value	p value
Cross-linked FDP`s	0.56	<0.001
Haemoglobin	-0.35 (inversely)	<0.001
Haematocrit	-0.31 (inversely)	<0.01
ABPI	-0.30 (inversely)	<0.01
Red blood count	-0.29 (inversely)	<0.01
Carboxyhaemoglobin	-0.21 (inversely)	<0.05
Plasma Viscosity	0.20	<0.05

ABPI significantly correlated in patients with:

	r value	p value
Cross-linked FDP's	-0.35 (inversely)	<0.001
Age	-0.30 (inversely)	<0.01
Body mass index	0.27	<0.01
White Cell Count	-0.22 (inversely)	<0.05
Platelet count	-0.20 (inversely)	<0.05
Plasma viscosity	-0.20 (inversely)	<0.05

Body mass index correlated significantly in patients with:

	r value	p value
Triglyceride	0.28	<0.01
ABPI	0.27	<0.01
HDL	-0.27 (inversely)	<0.05
Cholesterol	0.24	<0.05
Haemoglobin	0.22	<0.05
Red cell aggregation	0.20	<0.05

Carboxyhaemoglobin levels correlated significantly in the patients with:

	r value	p value
Haemoglobin	0.30	<0.01
Triglyceride	0.26	<0.05
Red blood count	0.23	<0.05
Age	-0.21 (inversely)	<0.05
White cell count	0.21	<0.05

This study has shown that fibrinogen, cross-linked fibrin degradation products, photometric red cell aggregation, plasma viscosity, white cell count and triglyceride were significantly increased in the patients with peripheral arterial disease compared to controls. HDL cholesterol was significantly decreased in patients compared to the controls.

Increased levels of fibrinogen, plasma viscosity, white cell count and triglyceride, and a decreased level of HDL cholesterol have previously been reported in peripheral arterial disease (Dormandy et al., 1973a; Alderman et al., 1981; Greenhalgh et al., 1971; Schrade et al., 1960; Fowkes, 1988).

Cross-linked Fibrin Degradation Products

The increased level of cross-linked fibrin degradation products indicates increased fibrin formation and lysis in patients with peripheral arterial disease. The level of cross-linked fibrin degradation products was significantly associated with the increased fibrinogen level found in these patients ($r=0.26$). This has not previously been reported. Whether an increased fibrinogen level causes increased fibrin formation and hence increased FDP's remains to be proven. An assessment of the fibrinolytic system will help to clarify this. In chapter 6 of this thesis plasminogen activator inhibitor was measured to determine if

fibrinolytic activity is impaired in peripheral arterial disease. Cross-linked fibrin degradation products increased with age, as did fibrinogen level. The correlation of cross-linked FDP's with plasma viscosity can probably be explained by mutual correlation with the increased fibrinogen level found in these patients.

The raised levels of cross-linked FDP's may be related to thrombus formation in these patients, e.g. deposition of fibrin mural thrombi on the artery wall, which has been frequently observed at necropsy (Martin, 1989). Cross-linked FDP's were highest in patients with abdominal aortic aneurysm, and were higher in patients with ischaemic rest pain than in patients with intermittent claudication. The high levels found in patients with aneurysm may be due to degradation of the laminated thrombus found in an aneurysm sac (See Plate 5). The raised levels in patients with ischaemic rest pain may be due to thrombus in the arteries distal to arterial occlusion; or possibly due to venous thrombosis (Whittaker et al., 1987) caused by stasis from limb dependency for relief of pain; or to microthrombi in the microcirculation in critical limb ischaemia (Lowe, 1990).

In contrast to fibrinogen levels, the level of cross-linked FDP's was not related to smoking. Statistical analysis showed that increasing age results in increased cross-linked fibrin degradation products, possibly due to increased

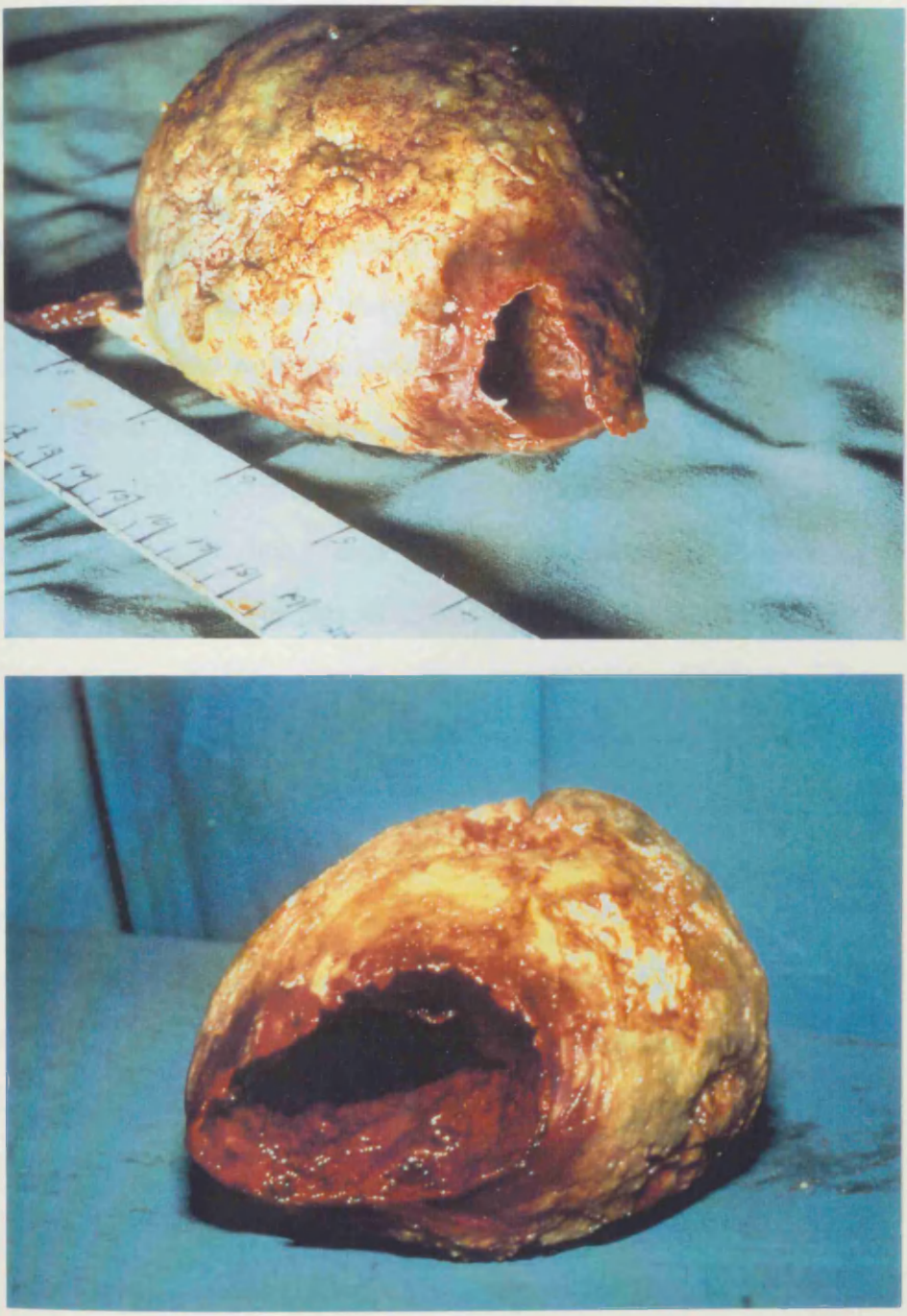

Plate 5

Laminated thrombus removed from the sac of an abdominal aortic aneurysm.

fibrin deposition with age. Multivariate analysis showed that even when this effect of age was taken into account cross-linked FDP's were still significantly higher in patients with peripheral arterial disease than in controls.

The increase in cross-linked FDP's in patients following reconstructive vascular surgery indicates increased fibrin formation. This was present at a mean duration of seven months from surgery, and may be caused by deposition of fibrin on the prosthetic graft. Deposition of fibrin on prosthetic grafts has been well documented (DeBakey et al., 1964; Anderson et al., 1986). It is possible that cross-linked FDP's could be prognostic markers for subsequent vascular graft occlusion. This merits further investigation.

Red Cell Aggregation

Patients with peripheral arterial disease had significantly increased red cell aggregation. This has not previously been reported. However the increased fibrinogen level found in these patients did not appear to account for this: fibrinogen did not correlate with red cell aggregation. This is surprising, since fibrinogen concentration is a major determinant of red cell aggregation (Rampling, 1988). While triglyceride did not correlate with red cell aggregation in the patients, there was a significant association between triglyceride level and red cell aggregation in controls. The cause of the raised red cell aggregation in peripheral

arterial disease is therefore unclear. Although the haematocrit level is said to have less effect on photometric red cell aggregation than the ESR (Myrenne aggregometer manual), a significant inverse association was noted between red cell aggregation and the haematocrit ($r=-0.38$, $p<0.001$), and this may reduce correlations between red cell aggregation and concentrations of aggregating proteins (e.g. fibrinogen and VLDL). Alternatively, some other plasma protein may be responsible for the increased red cell aggregation in peripheral arterial disease.

Red cell aggregation was highest in patients with abdominal aortic aneurysm and in ex-smokers, conditions where haematocrit was relatively low. The low haematocrit may explain these two associations. The effects of haematocrit on red cell aggregation in peripheral arterial disease are shown (Figure 3.18).

The increase in red cell aggregation in these patients could reflect an inflammatory process in atherosclerosis, since red cell aggregation is increased in other inflammatory conditions (Friederichs et al., 1984). This possibility is examined in Chapter 4 of this thesis.

One consequence of this increased tendency to red cell aggregation in peripheral arterial disease could be a reduction in blood flow. Low-shear conditions created by low perfusion pressure in ischaemic tissue favour red cell

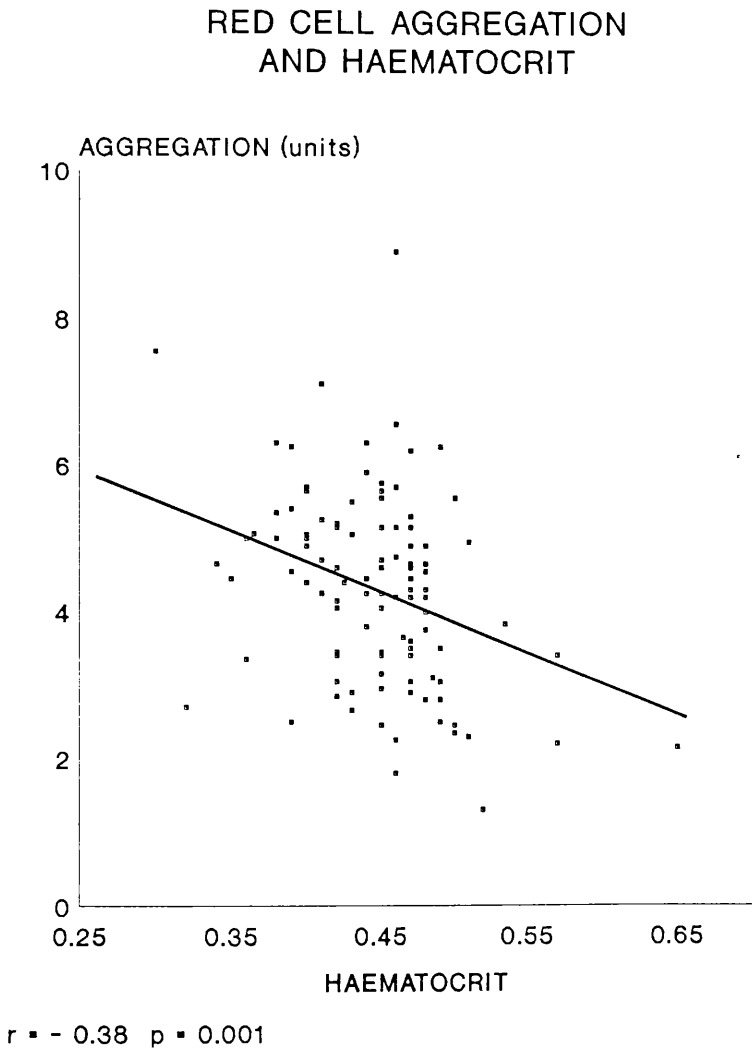

Figure 3.18

Red cell aggregation correlated with haematocrit.

aggregates, locally increasing blood viscosity, blocking arterioles and obstructing the microcirculation (Lowe et al., 1987a; Ditzel, 1959).

Fibrinogen

Fibrinogen was significantly increased in the patients with peripheral arterial disease compared to controls. This finding is consistent with previous studies (Hamer et al., 1973; Dormandy et al., 1973a; Hughson et al., 1978; Lowe et al., 1986). However multivariate analysis suggests that this difference can be explained by the effect of smoking. When the effects of smoking and age were taken into account, there was no significant difference in fibrinogen level between patients and controls. This explanation for the increased fibrinogen level in peripheral arterial disease has not been previously reported.

Age and smoking are both risk factors for developing peripheral arterial disease (Meade, 1987a; Hughson et al., 1978; Fowkes, 1988). Fibrinogen could act as a mediator for these two risk factors. There is evidence for a genetic component to both peripheral arterial disease and fibrinogen levels. A strong association has been shown between certain genotypes coding for the fibrinogen protein and plasma fibrinogen concentrations (Humphries et al., 1987).

The correlations of fibrinogen with white cell count and platelet count are probably due to a generalised inflammatory process in atherosclerosis (Reizenstein, 1979; Stuart et al., 1981). This possibility is further considered in chapter 4.

Plasma Viscosity

Plasma viscosity was significantly increased in patients with peripheral arterial disease compared to controls, consistent with previous studies (Dormandy et al., 1973a, Lowe et al., 1986).

Mulitvariate analysis suggests that this can be explained by the effect of age. Plasma viscosity increased with age ($r=0.20$, $p<0.05$). When this effect of age was taken into account there was no significant difference between patients and controls.

There was an expected strong correlation with fibrinogen, a major determinant of plasma viscosity (Harkness, 1971). Plasma viscosity also correlated with cross-linked FDP's, probably an association mediated through mutual correlations with fibrinogen. The correlations with platelet count and white cell count again suggest a general increase in factors related to inflammation in peripheral arterial disease including protein reactions.

White Cell Count

White cell count was significantly increased in peripheral arterial disease consistent with previous studies (Alderman et al., 1981; Ciuffetti et al., 1988).

White cell count was associated with ischaemia. It was significantly higher in ischaemic rest pain than in intermittent claudication or abdominal aortic aneurysm and correlated inversely and significantly with the ankle brachial pressure index.

White cell count was associated with cigarette smoking. The increase in white cell count in smokers has been previously reported and may persist after stopping smoking (Petitti & Kipp, 1986). Multivariate analysis showed that the increase in white cell count in patients with peripheral arterial disease was independent of the smoking related effect.

This increased white cell count could also promote ischaemia. White cells have a larger cell volume and are three orders of magnitude less deformable in capillaries than red cells (Chien, 1988). These rheological effects increase flow resistance and lead to pressure dependent plugging of capillaries in the low flow states that occur in ischaemia (Lowe, 1990). Ischaemia of the lower limbs causes

activation of white cells (Nash et al., 1988; Hickey et al., 1990). This may then increase adhesive forces between impacted leucocytes and capillary endothelium preventing full restoration of capillary flow (Lowe, 1987a, 1990; Ernst et al., 1987a).

Hence increased white cell count might contribute to ischaemia and activated white cells may contribute to tissue injury in ischaemic conditions.

Lipids

This study found increased triglyceride and decreased HDL cholesterol levels in patients with peripheral arterial disease compared to controls. Total cholesterol level was not significantly different between patients and controls.

Increased triglyceride and decreased HDL cholesterol are important risk factors for peripheral arterial disease (Hughson et al., 1978; Fowkes, 1988; Meerloo & Billimoria, 1979; Bihari-Varga et al., 1981). This is supported in this study. However it cannot be assumed that triglyceride alone is an important risk factor, since serum triglyceride concentrations are related inversely to HDL concentrations. The independent effect of triglyceride has not been adequately examined in peripheral arterial disease (Fowkes, 1989).

An increased HDL level is thought to afford some protection from the development of peripheral arterial disease (Thompson, 1987). The lower level of HDL cholesterol in males may partly explain the preponderance of males with peripheral arterial disease. There was no effect of smoking on HDL level in this study.

This study showed no increase in total cholesterol levels in patients with peripheral arterial disease compared to controls. The majority of case control studies conducted on hospital patients with peripheral arterial disease have found higher total cholesterol levels compared to controls (Juergens et al., 1960; Greenhalgh et al., 1971; Jacobsen et al., 1984; Bliss et al., 1972; Skrede & Kvarstein, 1975; Greenhalgh et al., 1981; Lipinska et al., 1979; Angquist et al., 1982; Trayner et al., 1980). However in other studies no major differences were found (Sirtori et al., 1974; Vyden et al., 1975; Schrade et al., 1960; Dormandy et al., 1973a). Hence the role of cholesterol in peripheral arterial disease is less certain (Fowkes, 1988).

Haematocrit

Haematocrit was not significantly increased in all patients with peripheral arterial disease compared to controls. This can be explained by the low haematocrit found in patients with abdominal aortic aneurysm. Patients with occlusive

disease (intermittent claudication and ischaemic rest pain) had a significantly increased haematocrit compared to controls, and compared to patients with abdominal aortic aneurysm.

In this study a high haematocrit was therefore associated with occlusive disease and not with aneurysmal disease.

The significantly higher haematocrit (and lower HDL cholesterol level) in males may partly explain the high incidence of peripheral arterial disease in males. The significant increase in haematocrit by cigarette smoking in both patients and controls confirms the previous studies (Dintenfass, 1975; Lowe et al., 1980).

Platelet Count

While platelet count was not significantly increased in patients compared to controls, there was an association with ischaemia. Platelet count inversely correlated with the ankle brachial pressure index.

Platelet count increased significantly in patients not operated upon, who were followed up for 3-12 months. This association with ischaemia and increase in platelet count after 3-12 months, suggests that platelets are associated with progression of disease. This is supported by Hess et al. who reported that aspirin and dipyridamole delayed progress of arterial disease when measured by serial arteriograms (Hess et al., 1985).

The correlations with fibrinogen, plasma viscosity and white cell count are probably related to a generalised reactive process.

Haemoglobin

While haemoglobin was not significantly increased in patients compared to controls, there was a significant effect following reconstructive vascular surgery. Haemoglobin decreased at 3-12 months following surgery, but increased in patients at 3-12 months who did not have surgery. The drop in haemoglobin level following reconstructive vascular surgery cannot be easily explained by blood loss at operation. This finding was present 3-12 months (mean 7 months) following surgery. This suggests that the ischaemic limb may stimulate an increase in haemoglobin concentration to carry oxygen to the tissues which does not occur with the improved circulation following revascularisation.

Other correlations

Age correlated inversely with the ankle brachial pressure index. Age is a risk factor for peripheral arterial disease and the correlation in these patients may simply be an indication of progressive arterial disease as the patients get older.

Ankle brachial pressure index correlated with body mass index. Associated atherosclerosis of the mesenteric vessels may interfere with food absorption (Watt, 1968, 1972).

Abdominal Aortic Aneurysm

During this present study ultrasound was performed to determine the incidence of abdominal aortic aneurysm in 100 patients with intermittent claudication. A 6% incidence of abdominal aortic aneurysm was found.

While it is generally accepted that atherosclerosis is the underlying pathology, abdominal aortic aneurysms differ from occlusive disease in some respects (Reid et al., 1990). In this study patients with abdominal aortic aneurysm had significantly higher cross-linked fibrin degradation products, red cell aggregation, decreased haematocrit and HDL cholesterol, compared to patients with occlusive peripheral arterial disease.

CHAPTER 4

The Role of Interleukin-6 in Peripheral Arterial Disease

INTRODUCTION

Plasma fibrinogen is raised in patients with peripheral vascular disease (see Chapter 3). The cytokine, interleukin-6 regulates liver synthesis of fibrinogen and other acute phase reactive proteins (Castell et al., 1988a). This chapter investigates the role of interleukin-6 (IL-6) in patients with peripheral vascular disease. This has not previously been reported.

Inflammation resulting from tissue injury causes an increase in the concentration of liver derived plasma proteins. This systemic response to injury, inflammation, and tissue damage is termed the "acute phase response" (Whicher & Dieppe, 1985). The plasma proteins produced by the liver have an important role in the inflammatory process. They are termed the acute phase reactive proteins. The acute phase response also consists of several systemic responses including fever, tachycardia, net catabolism and a leucocytosis. Fibrinogen and C-reactive protein (CRP) are two of the acute phase reactive proteins. These and others are shown in Table 4.1.

The hepatocyte is the major site of synthesis for the acute phase proteins, all of which can be synthesised by a single cell (Whicher & Dieppe, 1985).

Table 4.1
Acute Phase Reactive Proteins
(after Whicher & Dieppe, 1985)

Mediators

C-reactive protein

Complement components C1s, C2, C3, C4, C5, C9, Factor B

Kallikrein

Kinin

Factor VIII

Fibrinogen

Prothrombin

Plasminogen

Inhibitors

Antithrombin III

C1 1NH

Factor I

Factor H

Alpha-1-antitrypsin

Alpha-antichymotrypsin

Haptoglobin

Repair and Resolution

Orosomucoid

Alpha-1-antitrypsin

Alpha-1-antichymotrypsin

C1-1NH

Scavengers

Haptoglobin

Serum amyloid A protein

Caeruloplasmin

Immune Reaction

C-reactive protein

Orosomucoid

The regulation of the acute phase response is known to be mediated by interleukins. These peptides regulate many cellular functions including cell growth, cell growth inhibition, as well as cell differentiation and protein synthesis (Sporn & Roberts, 1988). Interleukin-6 is a multifunctional cytokine produced by lymphoid (T cells, B cells), and non-lymphoid cells (endothelial cells, fibroblasts and macrophages). It is also produced by monocytes, keratinocytes and by certain tumour cells (Hirano & Kishimoto, 1990).

IL-6 regulates the hepatic synthesis of acute phase reactive proteins, haemopoiesis, and the immune response. IL-6 enhances phagocytosis (Andus et al., 1987; Okano et al., 1989; Ishibashi et al., 1989a,b; Hirano et al., 1986; Lotz et al., 1988; Hirano & Kishimoto, 1990).

Hepatocyte Stimulation.

IL-6 has been shown to be identical to hepatocyte stimulating factor (HSF), (Andus et al., 1987). In vitro experiments have shown that it induces synthesis of fibrinogen and a variety of other acute phase reactive proteins in human primary hepatocytes and also in the human hepatoma cell line Hep G2 (Castell et al., 1988b).

Haemopoietic Progenitors

IL-6 increases the number of haemopoietic stem cells synergistically with IL-3 (Okano et al., 1989). Ishibashi et al. (1989a) demonstrated that IL-6 induces maturation of megakaryocytes in vitro. They showed that administration of IL-6 increases platelet numbers in mice (Ishibashi et al., 1989b). These studies indicate that IL-6 functions as a thrombopoietic factor. In accordance with these results Suematsu et al. (1989) found an increase in mature megakaryocytes in bone marrow of IL-6 transgenic mice.

The plasma half life of human IL-6 found free in the plasma is three minutes. However it has been shown to be bound to a carrier protein increasing its half life to about 55 minutes (Castell et al., 1988b). The carrier protein has been identified as alpha-2-macroglobulin (Matsuda et al., 1989), which does not inhibit IL-6 activity but protects it from degradation. IL-6 has been demonstrated to induce alpha-2-macroglobulin production in rats (Andus, 1987; Gauldie, 1987). Alpha-2-macroglobulin is therefore important for IL-6 function as it transports IL-6 from the site of its production to the liver, and to the haemopoietic progenitors. It therefore connects the site of injury with the process of the acute phase response. Alpha-2-macroglobulin also causes "clumped" red cell aggregation (Schmid-Schonbein et al., 1973, see Chapter 1).

In summary, Interleukin-6 has been shown to regulate the liver synthesis of fibrinogen in the acute phase response and also to regulate haemopoietic progenitors, inducing phagocytosis, maturing megakaryocytes and increasing the platelet count. The acute phase response is accompanied by a leucocytosis. Leucocytosis is also present in patients with peripheral arterial disease (Alderman et al., 1981). Serum IL-6 is raised in tissue injury, e.g. surgery (Cruickshank et al., 1990), and is known to be produced by endothelial cells, and by fibroblasts and macrophages (Hirano & Kishimoto, 1990). These cells are found histologically at the site of atherosclerotic plaques. This raises the possibility that if IL-6 was found to be elevated in patients with arterial disease, it may indicate an inflammatory response to the damaged vessel wall, which in turn might mediate the increased fibrinogen level and the increased white blood cell count in patients with peripheral arterial disease.

IL-6 induces production of alpha-2-macroglobulin which is its carrier protein (Andus et al., 1987; Gauldie, 1987). If IL-6 was found to be raised in peripheral arterial disease it could in part explain the increased red cell aggregation found in these patients (Chapter 3).

Aims

With this in mind, IL-6 serum concentrations were measured in patients and also controls with the following aims:

1 To determine if IL-6 is raised in patients with peripheral arterial disease.

2 To determine if IL-6 is associated with the increased fibrinogen level found in patients with peripheral arterial disease.

3 To determine if IL-6 is associated with the raised white cell count found in peripheral arterial disease or with red cell aggregation, cross-linked FDPs, platelets, plasma viscosity or lipids.

4 To determine if IL-6 is related to cigarette smoking (i.e. could cigarette smoking stimulate IL-6 production?).

5 To determine if IL-6 is associated with C-reactive protein levels (as an indicator of the acute phase response) in patients with peripheral arterial disease.

6 To determine if IL-6 is associated with red cell aggregation through an increased production of alpha-2-macroglobulin in patients with peripheral arterial disease.

Methods

Subjects

IL-6 serum concentration was measured in 28 patients with proven peripheral arterial disease and compared to 18 age matched controls without evidence of vascular disease. Of the 28 patients, 10 patients had intermittent claudication, nine had ischaemic rest pain, and nine had abdominal aortic aneurysms. No patient had an acute illness or sepsis and none of the patients with ischaemic rest pain had ulceration or gangrene. No patient had undergone surgery in the preceeding three months; no patient had a known inflammatory condition such as rheumatoid arthritis.

Protocol and Assays

Venous blood was taken. Serum IL-6 level was measured by the hybridoma growth stimulation assay using the mouse B-cell hybridoma 7TD1 line (Coulie et al., 1987). Cell numbers were evaluated colorimetrically using MTT, a tetrazolium salt cleaved by dehydrogenase enzymes present in living cells. Standardisation was performed using recombinant IL-6 which had been assigned a specific activity of 10^6 units/microgram, i.e. 1 unit approximate to 1 picogram. Serum samples were heat treated at 56 °C for 30 minutes before analysis to inactivate any inhibitors. The detection limit for this bioassay was approximately 12 units/ml and the imprecision (coefficient of variation) calculated from 20 consecutive assays was 19%. The specificity of the assay has been confirmed using a polyclonal neutralising IL-6 antibody.

C-reactive protein was measured by a fluorescence polarisation immunoassay (TDX, Abbott, Wokingham, Berkshire) which has a detection limit of 10 mg/l.

Alpha-2-macroglobulin was measured by an immunoturbidimetric assay using anti-human alpha-2-macroglobulin (Atlantic Antibody). The resulting turbidity was measured using an Encore analyzer (Baker).

In the patients, plasma levels of fibrinogen, cross-linked FDPs and plasma viscosity, red cell aggregation, carboxyhaemoglobin levels, haematocrit, total cholesterol, triglyceride and HDL cholesterol, white blood count, red blood count, haemoglobin, mean cell volume and platelet count were measured (for details of measurements see Chapter 2). The ankle brachial pressure index, height, weight and body mass were also measured.

Statistics

IL-6 and C-reactive protein were not normally distributed in these subjects. Comparison of these groups was therefore performed by Mann-Whitney U tests, and correlations were performed using Spearman's rank tests.

RESULTS

Serum IL-6 concentration was raised in patients with peripheral arterial disease compared to controls ($p<0.05$). (See Table 4.2 and Figure 4.1).

IL-6 correlated significantly with CRP. The correlation co-efficient for IL-6 versus CRP was 0.61 ($p<0.01$), (see Table 4.3). CRP was not increased in patients with peripheral arterial disease (the median value was under the detection limit for the assay).

IL-6 correlated inversely with the mean cell volume. The correlation co-efficient for IL-6 versus MCV was -0.43 ($p<0.05$).

While IL-6 did not correlate significantly with fibrinogen level or white cell count there were trends towards significant correlations. There were also trends towards significant correlations between IL-6 and plasma viscosity, and between IL-6 and triglyceride. Cross-linked FDP's, red cell aggregation, platelet count, haematocrit, full blood count, carboxyhaemoglobin, total cholesterol, HDL cholesterol, body mass index and the ankle brachial pressure index did not correlate significantly with IL-6. (See Table 4.3).

Table 4.2
Comparison of serum IL-6 concentrations (units/ml) in patients and controls.

Test		Controls	Patients with PVD	p value
Serum IL-6				
(u/ml)	Median	23.5	30	$p<0.05$
	IQR	28	11	
	n	18	28	

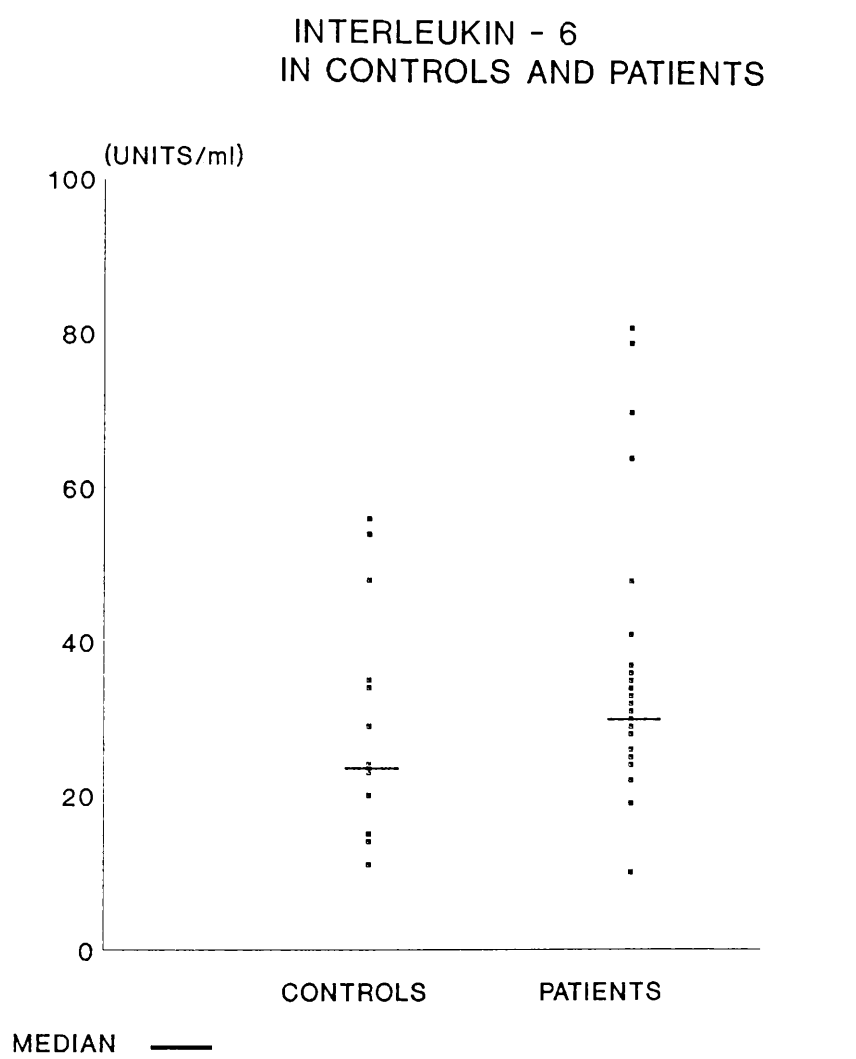

Figure 4.1

A comparison of levels of serum interleukin - 6 in controls and patients with peripheral arterial disease.

Table 4.3
IL-6 correlations (Spearman's rank) in patients with peripheral arterial disease.

Variable	r value	p value
C-reactive protein	0.61	p<0.01
Mean cell volume	-0.43 (inversely)	p<0.05
Fibrinogen	0.32	N.S. p=0.09
White blood cell count	0.26	N.S. p=0.17
Red cell aggregation	-0.01 (inversely)	N.S
Cross-linked Fibrin Degradation Products	-0.11 (inversely)	N.S.
Platelets	0.06	N.S.
Plasma Viscosity	0.29	N.S. p=0.12
Total Cholesterol	0.03	N.S
Triglyceride	0.28	N.S. p=0.18
HDL Cholesterol	-0.08 (inversely)	N.S.
Haematocrit	0.22	N.S.
Alpha-2-macroglobulin	0.06	N.S.
Red blood count	0.21	N.S.
Haemoglobin	0.06	N.S.
Body mass index	-0.05 (inversely)	N.S.
Age	0.16	N.S.
Carboxyhaemoglobin	0.05	N.S.
Ankle Brachial Pressure Index	0.10	N.S.

There was no significant difference between IL-6 levels in patients with intermittent claudication, ischaemic rest pain or abdominal aortic aneurysm: i.e. it did not correlate with the clinical severity of ischaemia or type of disease (See Figure 4.2).

There was no significant difference between the IL-6 levels in patients who were currently smoking and in patients who were definitely ex-smokers - confirmed by their carboxyhaemoglobin levels. (See Figure 4.3).

Alpha-2-macroglobulin level was decreased in patients with peripheral arterial disease compared to controls ($p<0.05$). Alpha-2-macroglobulin did not correlate significantly with IL-6 levels or red cell aggregation (See Tables 4.3 and 4.4).

DISCUSSION

The raised IL-6 levels found in patients with peripheral arterial disease indicates that there is an ongoing chronic inflammatory process. This raised level is not as high as levels found following major acute tissue injury (e.g. major surgery), suggesting that any inflammatory process occurring is a low-grade one (Cruickshank et al., 1990). However the raised level of IL-6 in the patients with peripheral arterial disease was similar to that in the patients undergoing minor surgery in the study by

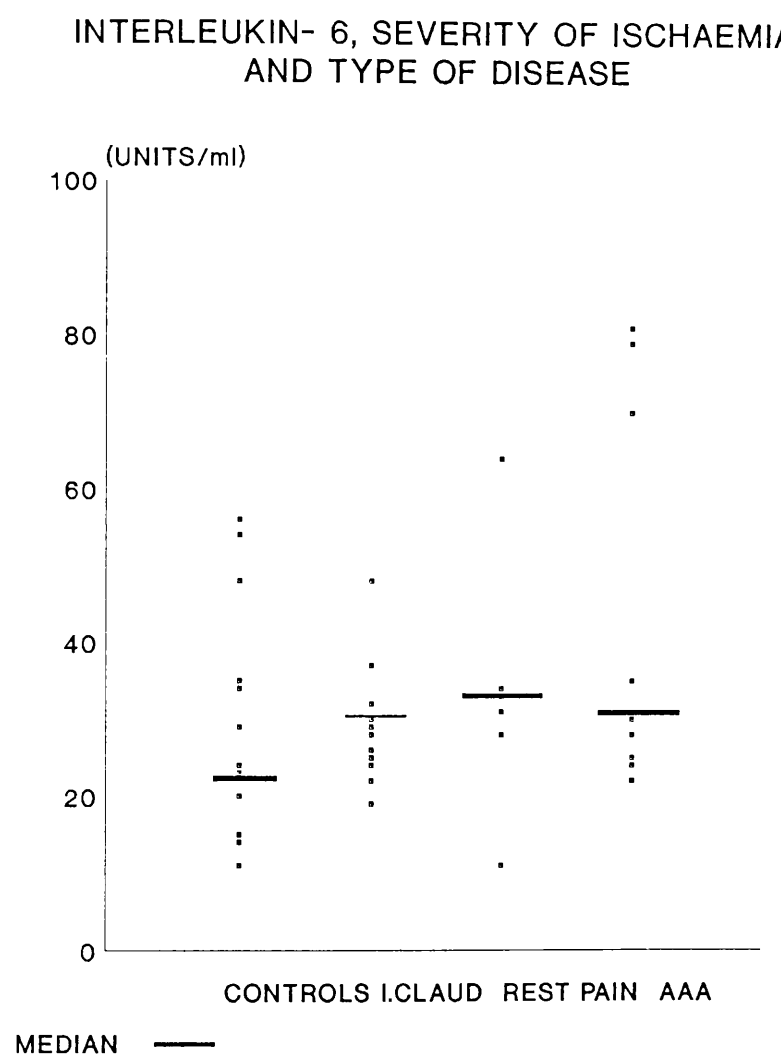

Figure 4.2

A comparison of levels of serum interleukin - 6 in controls and patients with intermittent claudication (I.CLAUD),ischaemic rest pain and abdominal aortic aneurysm (AAA).

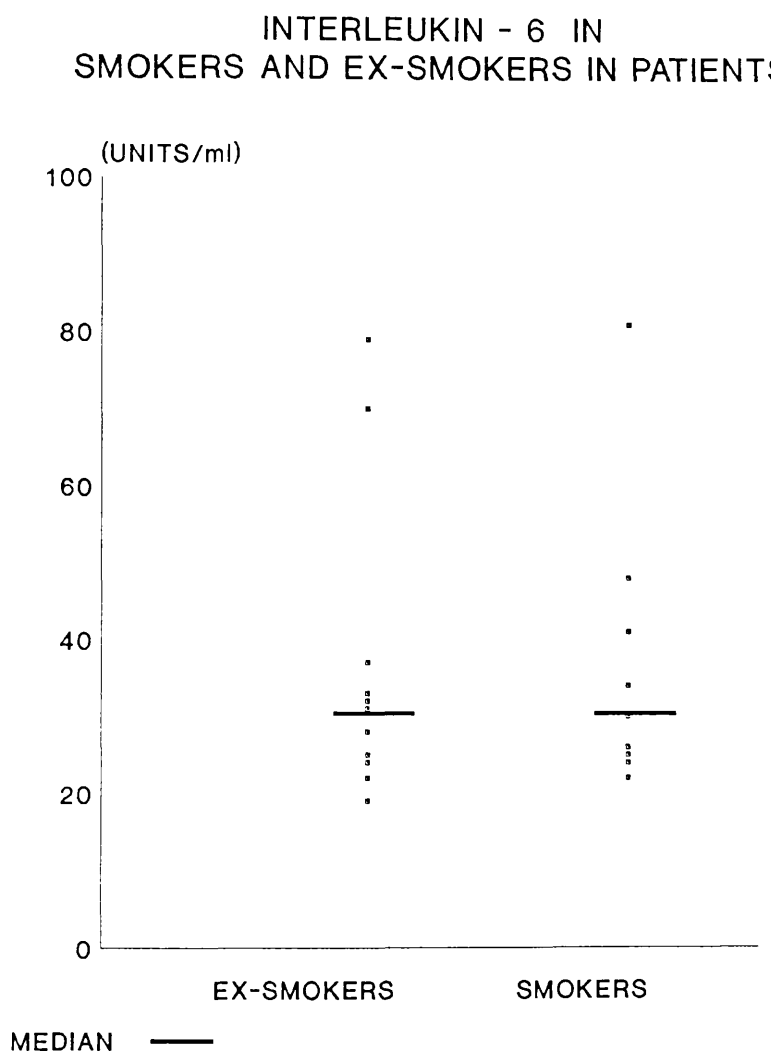

Figure 4.3

A comparison of levels of serum interleukin - 6 in ex-smokers and current smokers in patients with peripheral arterial disease.

Table 4.4
Comparison of serum alpha-2-macroglobulin in patients and controls

Test		Controls	Patients with PVD	p value
Serum alpha-2-macroglobulin				
(g/L)	Mean	2.95	2.07	p<0.01
	S.D.	0.95	0.65	
	n	18	28	

Cruickshank et al. (1990). Further evidence of the involvement of IL-6 in a low grade inflammatory process was the significant correlation of CRP with IL-6, although CRP was not increased in patients with peripheral arterial disease. The serum CRP concentration relates poorly to the extent of tissue damage, its response being an "all or nothing" phenomenon (Colley et al., 1983).

The raised levels of IL-6 may be due to tissue damage of the atheromatous vessel wall, i.e. the atheromatous process may be stimulating IL-6 production. The fact that IL-6 level did not correlate with carboxyhaemoglobin and was equal in smokers and ex-smokers suggests that cigarette smoking is not a direct stimulus for IL-6 production.

The increased IL-6 levels in peripheral arterial disease might explain some of the "non-smoking" related raised fibrinogen and raised white cell count levels in patients with peripheral arterial disease. The trend towards a significant correlation between IL-6 and fibrinogen suggests that IL-6 might be partly responsible for the increased fibrinogen level in peripheral arterial disease. There was also a trend towards a significant correlation between IL-6 and plasma viscosity.

It is possible that IL-6 may be partly responsible for the raised white cell count in peripheral arterial disease, since there was a trend towards a significant correlation between

IL-6 and white cell count. The trend gives some support to atherosclerosis being a chronic inflammatory process.

It is also interesting that there is a trend to a significant correlation between IL-6 and triglyceride. Triglyceride was increased significantly in patients with peripheral arterial disease in Chapter 3 and is recognised as a risk factor for peripheral arterial disease (Fowkes, 1988).

The reason for a significant correlation between IL-6 and mean cell volume is not clear.

The finding that IL-6 did not correlate significantly with cross-linked FDP's or red cell aggregation suggests that IL-6 is unlikely to be the major cause of their raised levels in peripheral arterial disease. Although Ishibashi et al. (1989b) showed that IL-6 increased platelet numbers in mice, there was no significant correlation between platelet count and IL-6 in this study.

Conclusions

IL-6 was moderately raised in patients with peripheral arterial disease and correlated with C-reactive protein, and inversely with mean cell volume.

IL-6 correlated poorly with fibrinogen and white cell count as well as with plasma viscosity and triglyceride levels, but

there were trends toward significant correlations. It must be concluded that this merits further investigation with larger numbers.

The lack of correlation with the other blood variables measured in this study suggests that IL-6 is not responsible for their raised levels in peripheral arterial disease. Cigarette smoking does not appear to be a direct stimulus for IL-6 production.

CHAPTER 5

Thrombosis and Artificial Surfaces

INTRODUCTION

Joseph Lister recorded that venous blood clotted more rapidly in a cup than in an India rubber tube. He concluded that blood coagulated when it came into contact with a foreign surface and in this regard the cup was more foreign than the India rubber (Lister, 1863).

Nowadays, many patients with symptomatic peripheral arterial disease can be successfully treated by reconstructive vascular surgery. This involves insertion of a prosthetic arterial graft. This material is a foreign surface which comes into direct contact with the blood flow.

When blood comes into contact with a foreign surface there is protein adsorption, platelet adhesion and activation of the intrinsic system of coagulation. Fibrinogen is preferentially adsorbed onto the artificial surface (Vroman et al., 1972; Vroman et al., 1980) and this enhances platelet accumulation on the surface. Fibrinogen is a predictor of subsequent graft occlusion (Hamer et al., 1973; Harris et al., 1978; Wiseman et al., 1989).

Patients with peripheral arterial disease have a significant risk of arterial thrombosis. Thrombus formation is often found on the atherosclerotic plaque and it is possible that atherosclerosis of the vessel wall might act as a foreign

surface and initiate the intrinsic system. The intrinsic system of blood coagulation is initiated by activation of factor XII on exposure of blood to a foreign surface.

Activation of factor XII leads to activation of the intrinsic system which results in the conversion of fibrinogen to fibrin.

In this chapter the role of activated factor XII is examined in its relation to fibrinogen, peripheral arterial disease and insertion of prosthetic arterial grafts.

Factor XII and the Intrinsic Pathway

Factor XII was first detected by its apparent absence in a railway brakeman called John Hageman (Ratnoff & Colopy, 1955). Factor XII is present as an inactive precursor in human plasma. It is synthesised in the liver.

The intrinsic system of blood coagulation is activated with blood: biomaterial contact; this is termed contact phase activation, and is initiated by the conversion of factor XII to factor XIIa, in the presence of prekallikrein and high molecular weight kininogen (Figure 5.1).

Adsorbed high molecular weight kininogen forms a complex with prekallikrein. This complex cleaves factor XII to factor XIIa. factor XIIa catalyses the conversion of the prekallikrein in this complex with high molecular weight

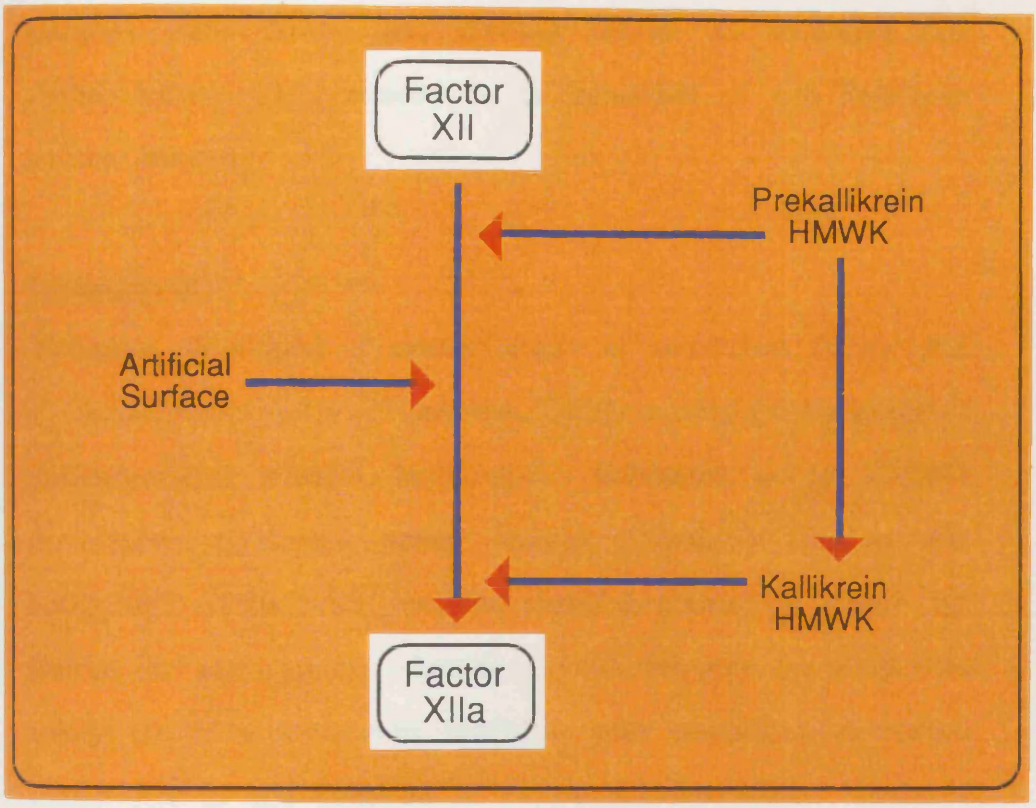

Figure 5.1

Feedback mechanism of the intrinsic system of coagulation in response to an artificial surface.

kininogen into kallikrein. Since kallikrein activates factor XII, a positive feedback mechanism is set up which ensures the autoactivation of factor XII on or close to the artificial surface. High molecular weight kininogen can also form a complex with factor XI, making factor XI available for factor XIIa. This results in progression of the intrinsic system cascade.

Measurements

Vinazzer developed a measurement of activated factor XII performed indirectly (Vinazzer, 1979) using a chromogenic substrate for plasma kallikrein. Gallimore et al. (1987) developed a direct assay using a soluble factor XII activator. This was modified into a microtitre assay by Walshe et al. (1987). Irvine (1989) further modified the assay using a kallikrein inhibitor and measured activated factor XII in the absence of factor XII activator. Irvine's modified assay has been used in this study since it is more suitable for the assessment of biomaterials and their effect on contact phase activation (Irvine, 1989).

Blood Alteration on Contact with a Foreign Surface

As well as activation of the intrinsic system of coagulation, blood contact with a foreign surface initiates many reactions, including fibrinogen and other plasma proteins, platelets, the complement system and blood cells.

Fibrinogen and other Plasma Proteins

Proteins adsorb rapidly and selectively to artificial surfaces. They form a layer termed the "conditioning layer" about 100 nm thick. The extent of adsorption is dependent on the nature of the surface (Vroman, 1983). This layer should not be regarded as passive (Bruck, 1980) since transient adsorption, denaturation or changes in conformation may occur (Ihlenfeld & Copper, 1979; Lee & Hairston, 1971; Forbes & Courtney, 1987).

Many different plasma proteins have been shown to adsorb onto artificial surfaces. These include fibrinogen (Vroman & Adams, 1969), albumin and globulins (Packham et al., 1969; Mason, 1972), with relatively high amounts of fibrinogen and albumin being adsorbed (van Damme & Feijen,1989).

Fibrinogen is preferentially adsorbed compared to other plasma proteins onto artificial surfaces (Vroman et al., 1972; Forbes & Courtney, 1987). Fibrinogen strongly attracts platelets: defibrinated or afibrinogenaemic plasma does not support platelet accumulation unless fibrinogen is added (Zucker & Vroman, 1969; Mason et al., 1971).

Adsorbed fibrinogen is replaced by high molecular weight kininogen (Vroman et al., 1980). It may also interact with leucocytes (Szycher, 1983). Hence fibrinogen has an important role in thrombosis on artificial surfaces.

Platelets

Circulating platelets adhere to the fibrinogen covered surface and change from discoid to spiny spheres. These form platelet aggregates with red and white blood cells, entrapped by fibrin (Salzman et al., 1979). This may progress to a mural thrombus (Muller-Mohnssen & Kratner, 1979).

Platelet adhesion is dependent on the nature of the "conditioning layer": surfaces coated with fibrinogen promote platelet adhesion (Whicher & Brash, 1978).

Following platelet adhesion, platelets undergo a release reaction discharging the contents of their granules into the circulation. These contents include ADP, serotonin and other compounds potentiating platelet aggregation. Simultaneously, release of membrane arachadonic acid leads to the production of thromboxane A2 (Holmsen et al., 1969; Szycher, 1983). Thromboxane A2 further increases platelet adhesion. As more platelets adhere, mounds of red and white cells are entrapped in fibrin (Salzman et al., 1979).

Leucocytes, Complement Activation and Erythrocytes

Leucocytes adhere to artificial surfaces (Kusserow et al., 1971). They appear to function in a similar manner to platelets. Their adhesion depends on prior adsorption of the protein monolayer (Szycher, 1983). The adherent white cells are polymorphonuclear leucocytes, and monocytes.

Complement activation by artificial surfaces is thought to occur via the alternative pathway (Forbes & Courtney, 1987). Leucocyte and platelet adhesion may be mediated by activation of complement components (Herzlinger & Cumming, 1980).

Erythrocytes are also attracted to the foreign surface in particular to regions where fibrin has formed (Feijen, 1977). Under low shear conditions red cells become entrapped by fibrin and form red thrombus (Bruck, 1980).

Summary

Fibrinogen has therefore an important role in thrombosis on an artificial surface. Atherosclerosis damages the vessel wall. It is possible that this vessel wall damage may present to the blood flow as an artificial surface. This would promote thrombosis through the intrinsic pathway of coagulation.

Aims

1 To determine if atherosclerosis on the arterial wall can act as a foreign surface initiating the intrinsic pathway of coagulation and thrombosis.

2 To determine if activated factor XII is associated with the increased levels of fibrinogen, cross-linked FDP`s, red cell aggregation, plasma viscosity, white cell count and triglyceride found in peripheral arterial disease.

3 To determine if sex, age or smoking habit alter activation of factor XII.

4 To determine if factor XIIa is increased by implanting a prosthetic arterial graft, and for how long does any increase last.

5 To determine the effects on inserting a prosthetic arterial graft on fibrinogen, cross-linked FDP`s, red cell aggregation ,white cell count, platelets, plasma viscosity and haematocrit.

METHODS

Sampling

Fresh venous blood was sampled and immediately transferred to a plastic tube containing 3.2 % citrate (1:9 anticoagulant:blood). It was then centrifuged at 2,500 g at

laboratory temperature (20-25°C) for 10 minutes. The same type of 20 ml plastic syringe and 19 gauge needle were used for all blood sampling to prevent contacting different foreign surfaces.

Assay

The assay (Irvine, 1989) utilises a chromogenic substrate (2ACOH - H - D - CHT - Gly - Arg - pNA, Channel Diag, Walmer, Kent) and a standard curve allowing direct correlation between absorbance values and factor XII activity).

Platelet poor plasma samples were pretreated with acetone (3:1), prior to dilution to prevent inhibitors of factor XIIa from interfering with the assay. Acetone treated samples were stored for a minimum of 30 minutes at 4°C, after which they were diluted 1:3 in Buffer A (0.05 M Tris - HCL, pH 7.9, 0.12 mmol/l methylamine. and 9.7 mmol/l Di Na EDTA).

A standard curve using acetone - treated standard plasma (Walshe et al., 1987) was prepared.

Kallikrein inhibitor (Channel Diagnositics) diluted 1:50 with Tris - HCl - Buffer (pH 7.9), and the substrate (1.5 mmol/l were prewarmed at 37°C. The pretreated samples

were assayed on a microtitre plate according to the following protocol:

1. 25 microlitres of sample and 25 microlitres of Buffer A were incubated for 10 minutes.

2 75 microlitres of Buffer containing kallikrein inhibitor (37°C) were added and incubated for one minute.

3 50 microlitres of chromogenic substrate (37°C) were added and incubated for 10 minutes.

4 The reaction was stopped with 50 microlitres of 30% acetic acid.

The absorbance values were read at 405 nm, using a microtitre plate reader (Dynatech Ltd) against a Buffer blank. Activity levels (units per ml) were calculated from the standard curve.

Subjects

Levels of activated factor XII were measured in 40 patients with peripheral arterial disease. 30 patients had intermittent claudication and 10 patients had abdominal aortic aneurysm. These patients also had measurements of fibrinogen, cross-linked FDP's, red cell aggregation, plasma viscosity, haematocrit, lipids and carboxyhaemoglobin. The 40 patients were compared to 10

controls who had measurement of activated factor XII.

Gelatin sealed knitted Dacron (Vascutek Ltd, UK) aortic grafts were implanted into 11 additional patients undergoing reconstructive vascular surgery. Seven patients had intermittent claudication and four patients had abdominal aortic aneurysms. Serial measurements of activated factor XII were performed in these 11 patients at induction of anaesthesia (baseline measurement), during operation at cross-clamping of the aorta before the implantation of the aortic graft, and one hour after blood flow was established through the graft. Measurement was also performed at one day and one week post-surgery. Measurement of fibrinogen, cross-linked FDP's, red cell aggregation, plasma viscosity, haematocrit, full blood count and platelet count was also performed.

All surgery was carried out with a preoperative prophylactic antibiotic (1 g of intravenous Cefotaxime), and 5,000 i.u. of systemic heparin was given two minutes prior to aortic clamping. Intravenous volume loading was carried out by the anaesthetist before declamping.

RESULTS

The level of activated factor XII in the patients with peripheral arterial disease was not significantly different from the controls (see Table 5.1, Figure 5.2). This was the case for patients with intermittent claudication and

Table 5.1
Comparison of levels of activated Factor XII in controls and patients

Test	Controls	Patients	p value
Activated Factor XII (units) Median	0.28	0.30	N.S.
IQR	0.05	0.10	
n	10	40	

Table 5.2
Comparison of levels of activated Factor XII in patients with intermittent claudication and with abdominal aortic aneurysm

Test	Intermittent claudication	Abdominal aortic aneurysm	p value
Activated Factor XII (units) Median	0.3	0.3	N.S.
IQR	0.1	0.1	
n	30	10	

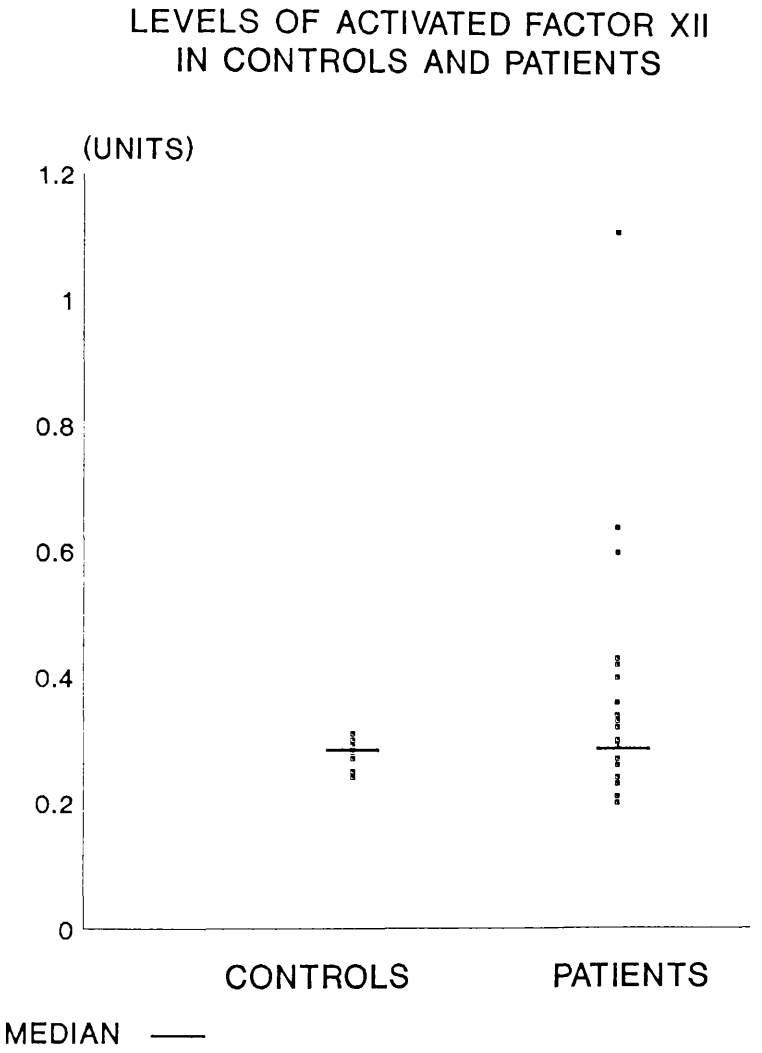

Figure 5.2

A comparison of levels of activated Factor XII in controls and patients with peripheral arterial disease.

abdominal aortic aneurysm. There was no difference in the level of activated factor XII between the 30 patients with intermittent claudication and the 10 patients with abdominal aortic aneurysm (Table 5.2).

Activated factor XII was not significantly associated with levels of fibrinogen, cross-linked FDP's, red cell aggregation, plasma viscosity, white cell count or platelet count in patients with peripheral arterial disease. It was not significantly associated with age, sex, carboxyhaemoglobin or the ankle brachial pressure index (Table 5.3).

There were 15 current smokers and seven ex-smokers confirmed by carboxyhaemoglobin levels. Activated factor XII was significantly higher in the ex-smokers compared to the current smokers ($p<0.05$), (Table 5.4).

Reconstructive Vascular Surgery

The levels of activated factor XII were significantly increased following surgery to expose and prepare the aorta for arterial grafting ($p<0.01$). This increase was also found following systemic heparinisation (Table 5.5 & Figure 5.3). There was an additional significant increase after one hour of blood: biomaterial surface contact ($p<0.01$).

The levels of activated factor XII returned to the baseline values when measured at one day and one week after surgery.

Table 5.3
Activated Factor XII correlations (Spearman's rank) in patients with peripheral arterial disease.

Variable	r value	p value
Fibrinogen	0.23	N.S.
XL - FDPs	-0.16	N.S.
Red cell aggregation	-0.01	N.S.
Plasma viscosity	0.26	N.S.
White cell count	-0.11	N.S.
Haematocrit	-0.05	N.S.
Haemoglobin	-0.19	N.S.
Red blood count	-0.24	N.S.
Mean cell volume	0.15	N.S.
Platelet count	0.17	N.S.
ABPI	-0.03	N.S.
COHB	-0.22	N.S.
Age	0.18	N.S.
Body mass index	0.40	$p<0.05$

Table 5.4
Comparison of activated Factor XII levels in ex-smokers and current smokers in patients with peripheral arterial disease

Test		Ex -smokers	Current smokers	p value
Activated Factor XII				
(units)	Median	0.34	0.29	$p<0.05$
	IQR	0.13	0.12	
	n	7	15	

Table 5.5
Measurements in 11 patients during and following reconstructive vascular surgery

Test	Baseline	Pre-graft	After blood-biomaterial contact		
			1 hour	1 day	1 week
Activated Factor XII (units)					
Median	0.34	0.4	0.46	0.35	0.34
IQR	0.06	0.13	0.14	0.11	0.10
p value	-	p<0.01	p<0.01	N.S.	N.S.
Fibrinogen (g/L)					
Mean	2.70	2.36	2.17	3.70	5.53
S.D.	0.80	0.37	0.72	0.06	1.43
p value	-	N.S.	N.S.	p<0.05	p<0.001
Cross linked FDPs (ng/ml)					
Median	153	153	201	721	1069
IQR	270	309	174	447	726
p value	-	N.S.	N.S.	p<0.05	p<0.05
Red cell aggregation (units)					
Median	3.7	1.4	5.0	6.3	7.2
IQR	2.2	2.3	1.5	2.9	2.5
p value	-	p<0.01	N.S.	N.S.	p<0.05
White cell count ($x10^9$/L)					
Mean	8.3	8.8	17.5	11.7	11.2
S.D.	2.4	2.5	5.8	1.8	2.2
p value	-	N.S.	p<0.001	p<0.01	p<0.05
Platelet count ($x10^9$/L)					
Mean	253	229	244	187	310
S.D.	59	55	74	72	76
p value	-	N.S.	N.S.	p<0.05	N.S.
Plasma viscosity (mPa.s)					
Mean	1.24	1.17	1.17	1.19	1.33
S.D.	0.10	0.07	0.05	0.11	0.13
p value	-	N.S.	p<0.05	N.S.	N.S.
Haematocrit					
Mean	0.40	0.38	0.36	0.34	0.34
S.D.	0.05	0.06	0.05	0.05	0.04
p value	-	N.S.	N.S.	p<0.05	p<0.05

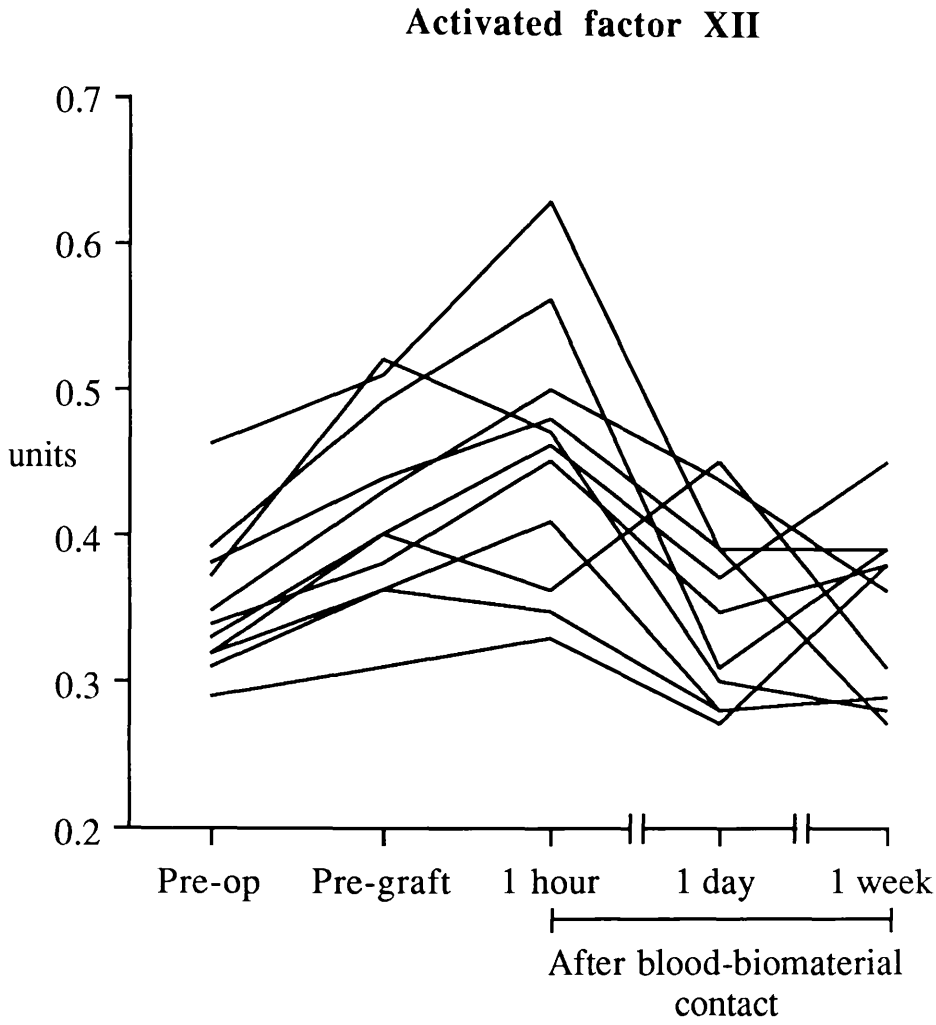

Figure 5.3

Serial measurements of levels of activated factor XII in eleven patients undergoing reconstructive vascular surgery (inserting a prosthetic aortic graft). Measurements were taken immediately pre-operatively, during operation at cross-clamping of the aorta before implantation of the graft and one hour, one day and one week after blood flow was established through the graft.

Fibrinogen

Fibrinogen level was significantly increased one day ($p<0.05$) and one week ($p<0.001$) following surgery. There was no change one hour after blood was flowing through the graft (see Table 5.5 & Figure 5.4).

Cross-linked FDP's

Cross-linked FDP's were similar to fibrinogen. The level of cross-linked FDP's was significantly increased one day ($p<0.05$) and one week ($p<0.05$) following surgery. Cross-linked FDP's were not increased one hour following blood: biomaterial contact (see Table 5.5 & Figure 5.5).

Red Cell Aggregation

The level of red cell aggregation was significantly reduced following surgery to prepare and expose the aorta for grafting and following systemic heparinisation ($p<0.01$, see Table 5.5 & Figure 5.6). The level of red cell aggregation was significantly increased one week following surgery compared to the baseline measurements ($p<0.05$). In two patients, red cell aggregation decreased following surgery. These two patients had high baseline red cell aggregation levels.

White Cell Count

White cell count was significantly increased, peaking one hour after blood: biomaterial surface contact ($p<0.001$). It

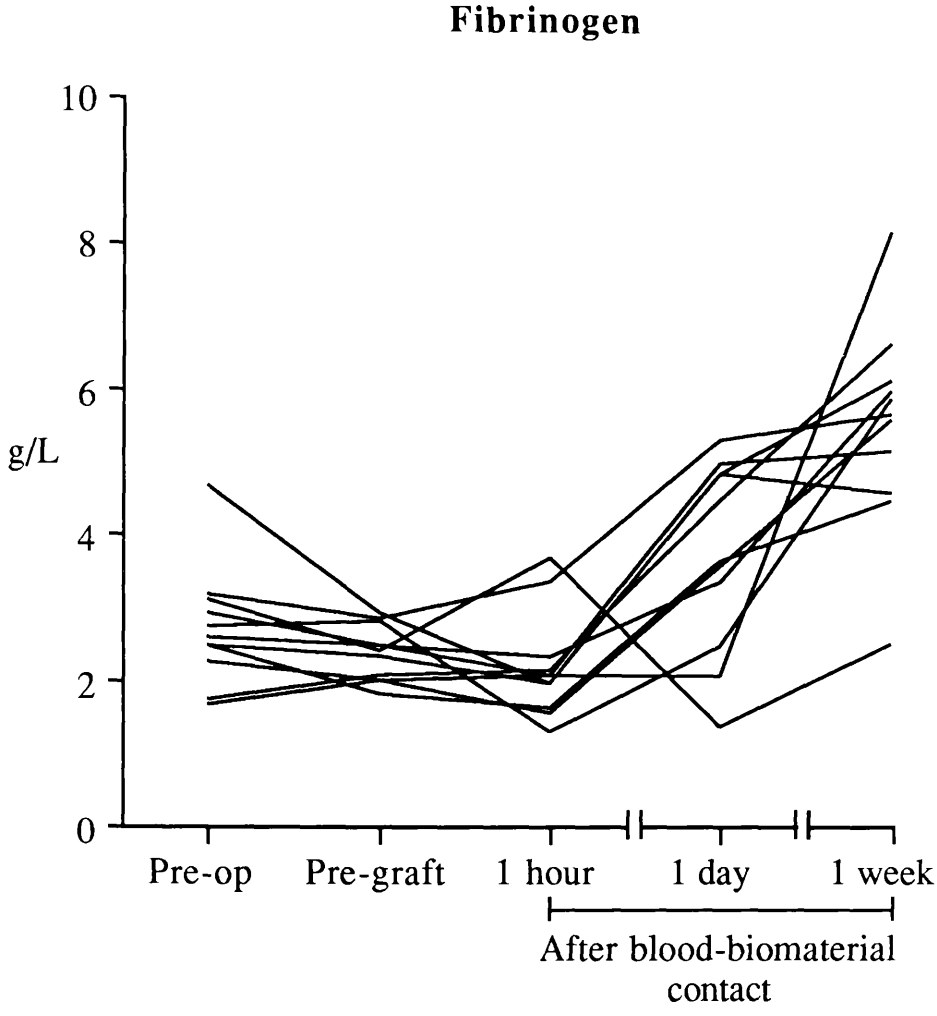

Figure 5.4

Serial measurements of levels of fibrinogen in eleven patients undergoing reconstructive vascular surgery (inserting a prosthetic aortic graft). Measurements were taken immediately pre-operatively, during operation at cross-clamping of the aorta before implantation of the graft and one hour, one day and one week after blood flow was established through the graft.

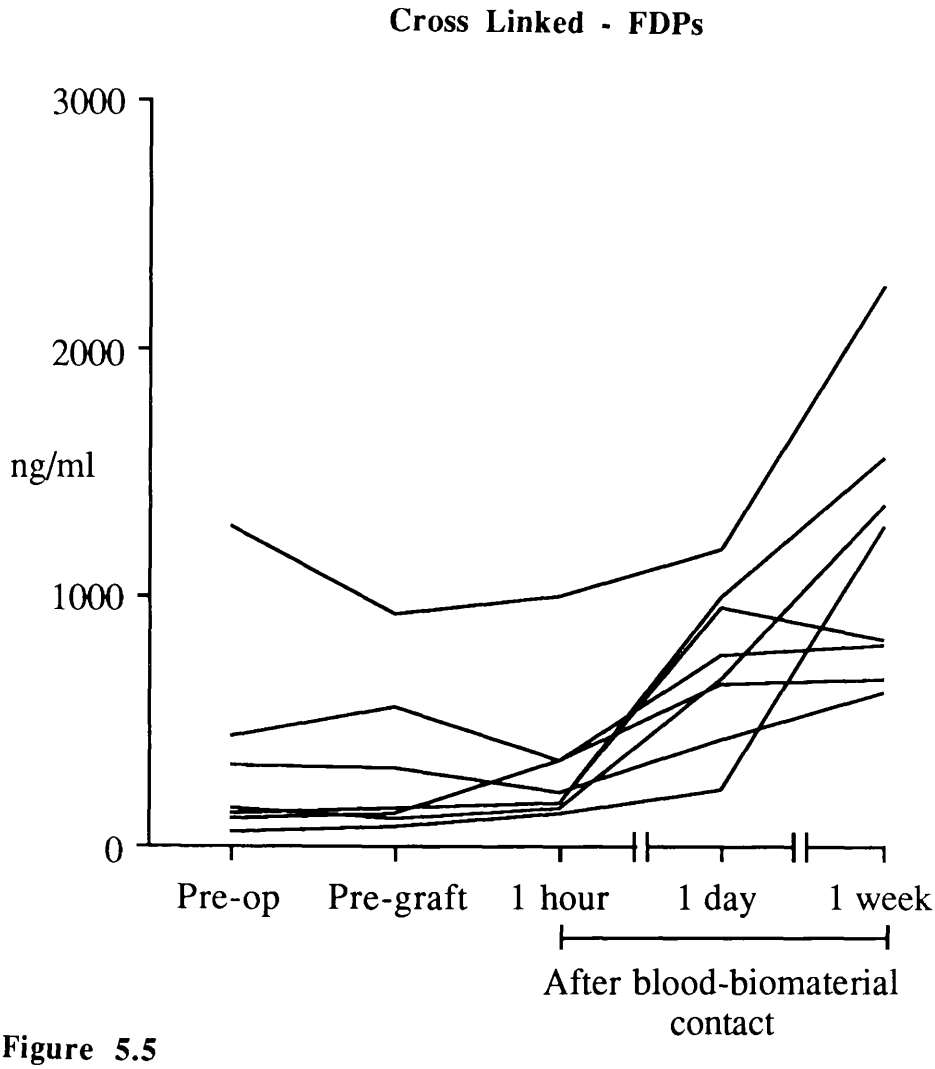

Figure 5.5

Serial measurements of levels of cross linked - FPDs in eleven patients undergoing reconstructive vascular surgery (inserting a prosthetic aortic graft). Measurements were taken immediately pre-operatively, during operation at cross-clamping of the aorta before implantation of the graft and one hour, one day and one week after blood flow was established through the graft.

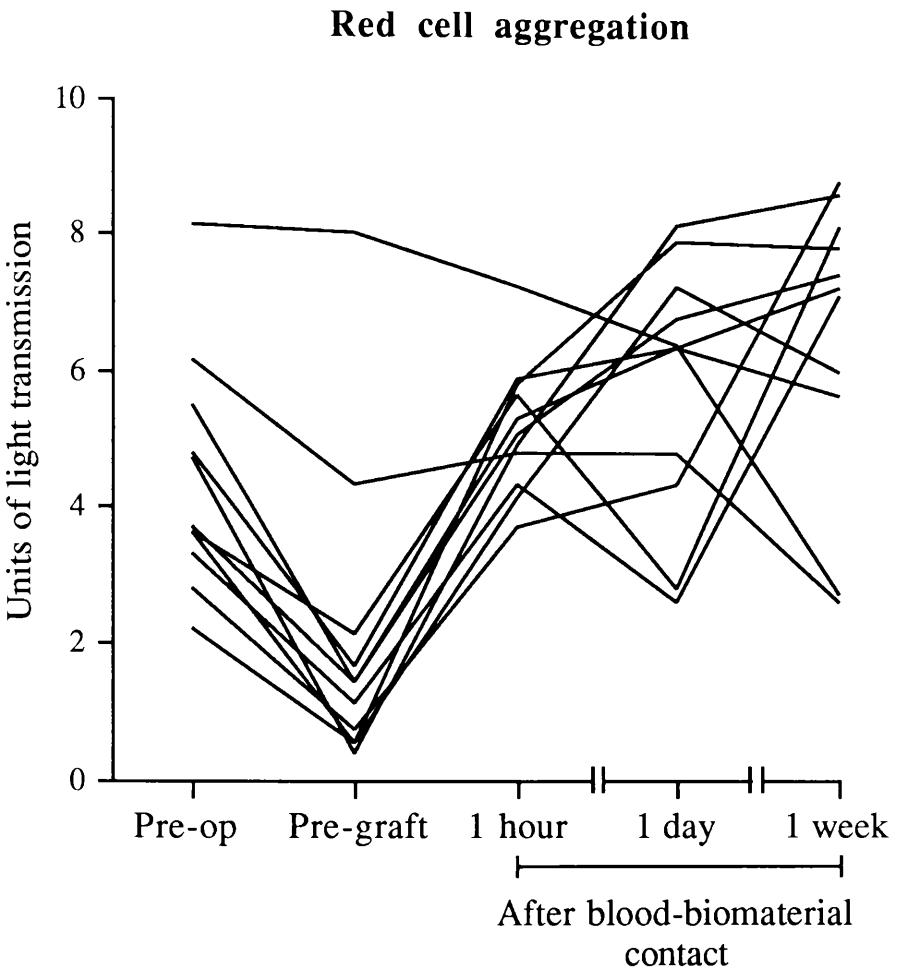

Figure 5.6

Serial measurements of red cell aggregation in eleven patients undergoing reconstructive vascular surgery (inserting a prosthetic aortic graft). Measurements were taken immediately pre-operatively, during operation at cross-clamping of the aorta before implantation of the graft and one hour, one day and one week after blood flow was established through the graft.

was also increased at one day ($p<0.01$) and one week ($p<0.05$) after surgery. This increase in white cell count was a neutrophilia (see Table 5.5 & Figure 5.7).

Platelet Count

Platelet count significantly decreased ($p<0.05$) at one day after surgery. There was a subsequent increase in platelet count (Table 5.5 & Figure 5.8).

Plasma Viscosity

Plasma viscosity was significantly decreased at one hour after surgery (Table 5.5 & Figure 5.9).

Haematocrit

Haematocrit significantly decreased after surgery at one day and at one week ($p<0.05$), (Table 5.5 & Figure 5.10).

DISCUSSION

The finding that levels of activated factor XII were not significantly different between patients with peripheral arterial disease and controls suggest that atherosclerotic plaques do not act as foreign surfaces activating the intrinsic system of coagulation. This was the case for patients with intermittent claudication as well as patients with abdominal aortic aneurysm.

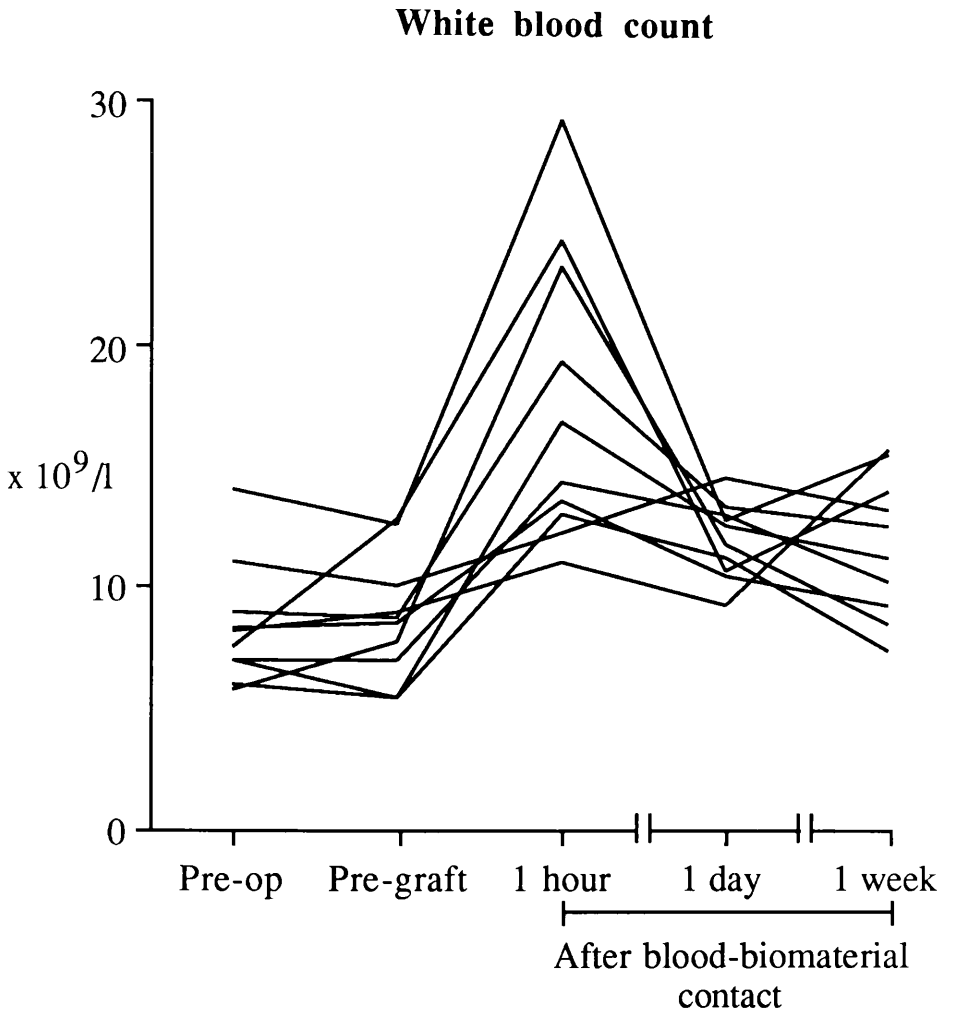

Figure 5.7

Serial measurements of white blood count in eleven patients undergoing reconstructive vascular surgery (inserting a prosthetic aortic graft). Measurements were taken immediately pre-operatively, during operation at cross-clamping of the aorta before implantation of the graft and one hour, one day and one week after blood flow was established through the graft.

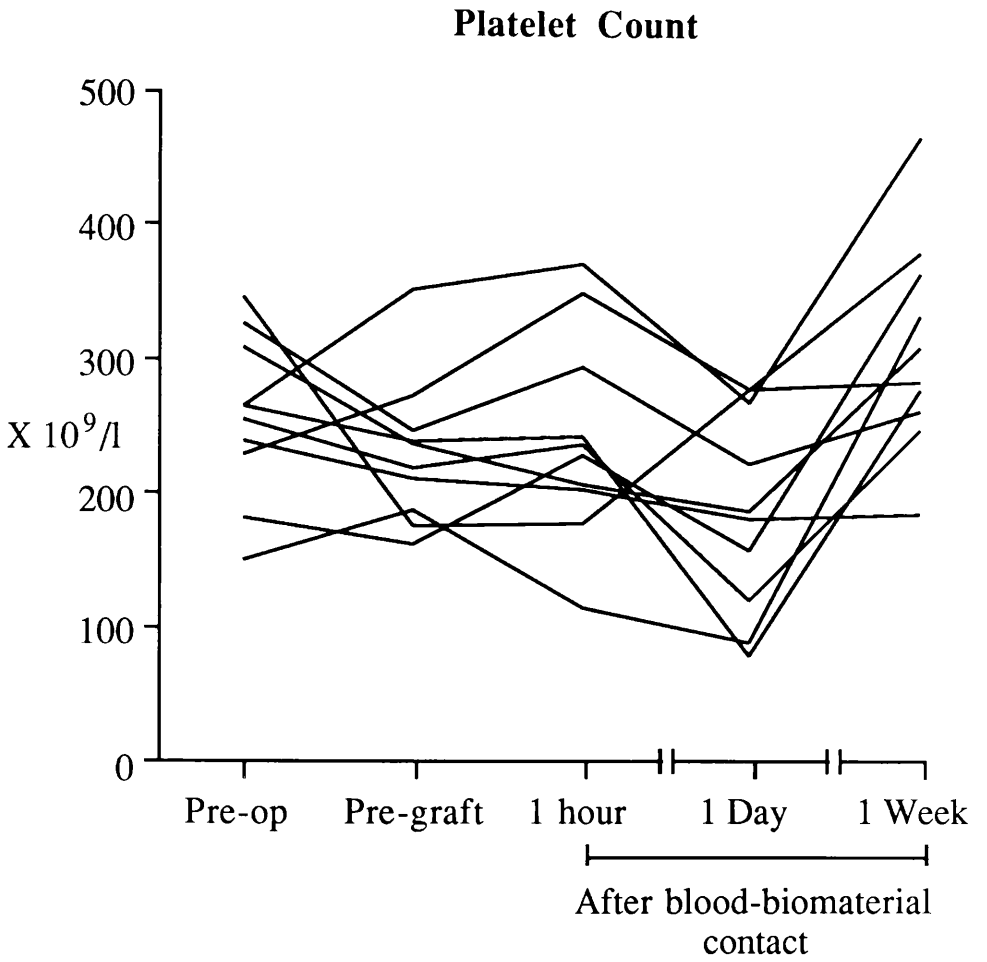

Figure 5.8

Serial measurements of platelet count in eleven patients undergoing reconstructive vascular surgery (inserting a prosthetic aortic graft). Measurements were taken immediately pre-operatively, during operation at cross-clamping of the aorta before implantation of the graft and one hour, one day and one week after blood flow was established through the graft.

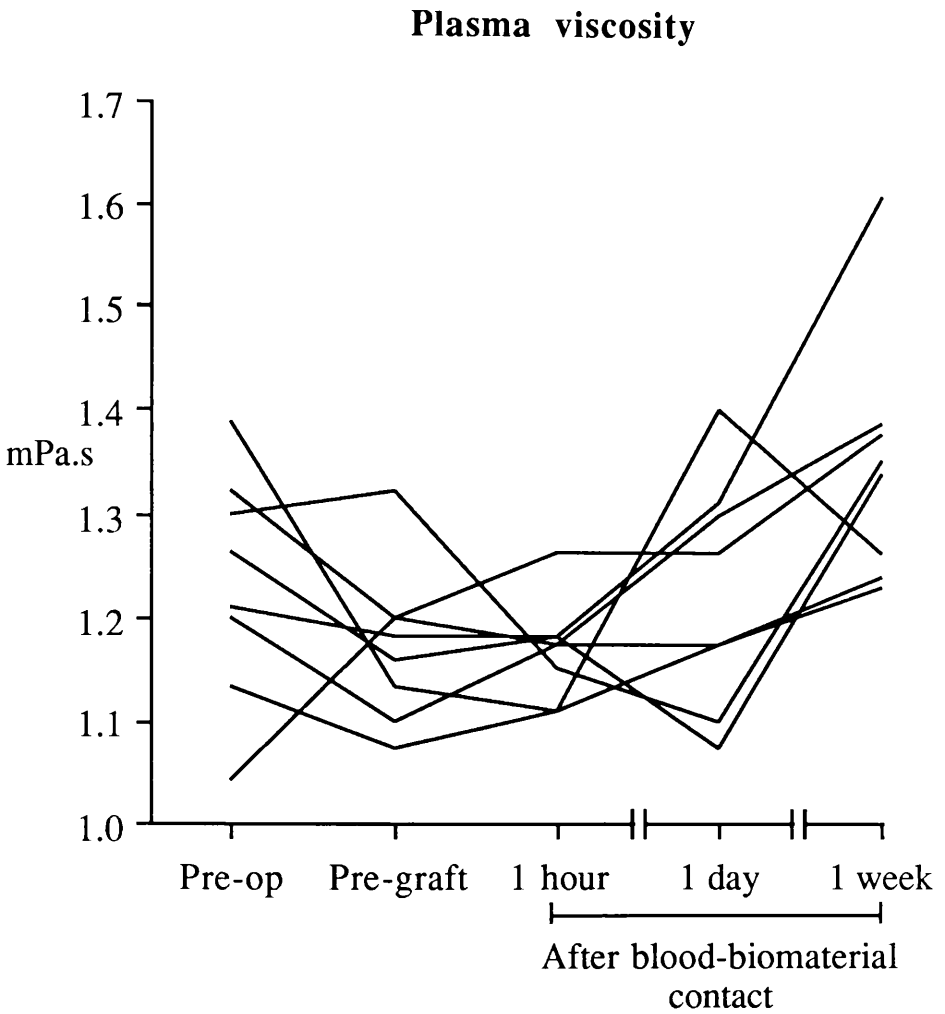

Figure 5.9

Serial measurements of plasma viscosity in eleven patients undergoing reconstructive vascular surgery (inserting a prosthetic aortic graft). Measurements were taken immediately pre-operatively, during operation at cross-clamping of the aorta before implantation of the graft and one hour, one day and one week after blood flow was established through the graft.

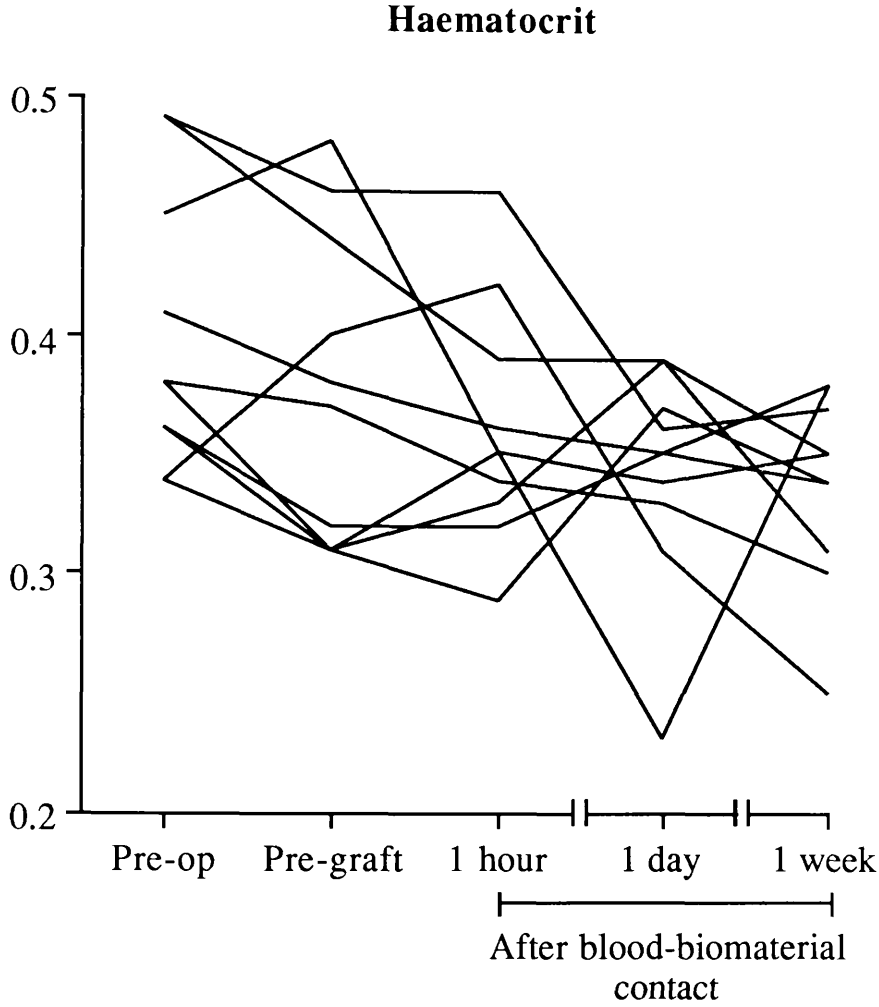

Figure 5.10

Serial measurements of haematocrit in eleven patients undergoing reconstructive vascular surgery (inserting a prosthetic aortic graft).
Measurements were taken immediately pre-operatively, during operation at cross-clamping of the aorta before implantation of the graft and one hour, one day and one week after blood flow was established through the graft.

Thrombus formation is common on atherosclerotic plaques and as laminated thrombus in abdominal aortic aneurysm. These results suggest that this does not result from activation of factor XII and the intrinsic pathway.

Activated factor XII did not correlate significantly with fibrinogen or cross-linked FDP's. This suggests that activation of the intrinsic system does not account for the increased levels of fibrinogen and cross-linked FDP's found in patients with peripheral arterial disease. Red cell aggregation, plasma viscosity and white cell count also do not seem to be associated with an increased intrinsic pathway of coagulation in patients with peripheral arterial disease.

While the level of activated factor XII was significantly higher in ex-smokers compared to current smokers there were only seven ex-smokers in this study, hence larger studies are required to confirm this finding. It is of interest that ex-smokers had higher plasma fibrinopeptide A levels than current smokers in another study (Lowe et al., 1991), which likewise suggests activation of coagulation.

Reconstructive Vascular Surgery

Heparin activates factor XII in the absence of a foreign surface possibly because of its anionic nature (Irvine, 1989; Fuhrer et al., 1987). This may explain the significant increase in activated factor XII before graft insertion

(following surgery to expose and prepare the aorta and following systemic heparinisation). Activation of factor XII by heparin is immediate (Irvine, 1989).

The gelatin sealed aortic graft significantly activated factor XII stimulating the intrinsic system of coagulation: activated factor XII was significantly increased after one hour of biomaterial surface contact. However the activation of factor XII by the graft was short lived and was not present at one day or one week after surgery. The absence of activation of factor XII at one day and one week may be due to coating of the foreign graft surface by a conditioning layer.

The increase of fibrinogen level at one day and one week after surgery may be part of the normal acute phase response to surgery. While fibrinogen level is a predictor of subsequent graft occlusion, (Wiseman et al., 1989), fibrinogen was not associated with activation of Factor XII and the intrinsic system of coagulation.

The increase in cross-linked FDP`s at one day and one week is similar to the increase in fibrinogen. This may be due to degradation of fibrin formed on the aortic graft. However it could also represent degradation of fibrin formed following surgical dissection, or of post-operative venous thrombosis.

The significant decrease in red cell aggregation before graft insertion is probably due to heparinisation. Heparin decreases red cell aggregation (Myrenne Aggregometer manual). Fluid volume loading cannot explain this decrease since there was no significant change in haematocrit during this interval. The decrease was reversed one hour after blood biomaterial contact, and the increase in red cell aggregation at one week may be related to the increase in fibrinogen level or other plasma proteins involved in an acute phase response.

The most significant change in white cell count was the increase one hour after blood biomaterial contact. This was one hour after the vascular clamp was removed from the aorta and blood flow was restored to the lower limbs. Cross-clamping of the aorta produces a period of severe lower limb ischaemia. Lower limb ischaemia is known to activate white cells (Nash et al., 1988; Paterson et al., 1989) and white cell count is significantly increased in patients with ischaemia (see Chapter 3). Hence this increase in white cell count is probably due to the period of lower limb ischaemia and reperfusion during graft insertion. Another possible reason for the leucocytosis is the introduction of a foreign surface to the blood stream. Neutrophil polymorphs are known to attract to foreign surfaces (Lederman et al., 1978).

The white cell count is very labile. Granulocytes which make up the majority of the circulating white cells are distributed in three pools: the marrow, the circulating pool and the tissue pool. Traffic among these pools may be acutely altered by physiological influences and pathological changes (Ernst et al., 1987a). The increase in white cell count at one day and one week is in keeping with an inflammatory response following surgery. Whatever the reason for the increase in white cell count it might tend to promote further ischaemia by pressure dependent plugging of microvessels reducing the microvascular blood flow (Ernst et al., 1987a).

The significant decrease in platelet count one day after surgery could represent consumption of platelets coating the graft surface and/or involved in haemostasis and wound healing.

The decrease in haematocrit after surgery can be accounted for by intravenous fluid volume loading and blood loss of operation. There was a similar decrease in red blood count and haemoglobin. The decrease in plasma viscosity at one hour after graft insertion was probably also related to intravenous fluid volume loading.

Conclusions

Levels of activated factor XII were not increased in peripheral arterial disease; and patients with intermittent claudication and abdominal aortic aneurysm did not differ in their levels of activated factor XII. This suggests that atherosclerosis does not act as a foreign surface that activates the intrinsic system of coagulation.

Introduction of a prosthetic graft during reconstructive vascular surgery activated factor XII, because the graft acts as a foreign surface. The activation of factor XII returned to baseline levels one day and one week after surgery. This was accompanied by an increase in fibrinogen, cross-linked FDP`s, red cell aggregation and white cell count. However the levels of activated factor XII did not significantly correlate with fibrinogen, FDP`s, red cell aggregation, white cell count, platelet count or any other measured variables.

The normal levels of activated factor XII at one day and one week suggests that the graft is coated with a conditioning layer and no longer acts as a foreign surface.

CHAPTER 6

Antiplatelets and anticoagulants in chronic peripheral arterial disease: the effect of minidose warfarin and low-dose aspirin on the hypercoagulable state and abnormal haemorheology in patients with intermittent claudication.

Summary

In the previous chapters, this thesis has reported that patients with peripheral arterial disease have a hypercoagulable state (increased plasma levels of fibrinogen and cross-linked fibrin degradation products) and abnormal haemorheology (increased fibrinogen, plasma viscosity, red cell aggregation and white cell count).

Patients with peripheral arterial disease have an increased risk of coronary, cerebral and limb arterial thrombosis (Reunanen et al., 1982). Currently there is no medical treatment available to produce regression of atherosclerosis. However, an improved management of peripheral arterial disease might be obtained by prevention of thrombosis and it is therefore reasonable to look at anticoagulant and antiplatelet therapy to try to prevent progress of peripheral arterial disease.

The aim of this chapter is to determine if low-dose aspirin or minidose warfarin significantly alter the hypercoagulable state and abnormal blood rheology found in patients with intermittent claudication. This is not only relevant to the management of claudication, but also tests the hypotheses that increased platelet or coagulant activity may be causally related to the increase in fibrinogen, cross-linked fibrin degradation products and viscosity levels in peripheral arterial disease.

Patients with chronic peripheral arterial disease (usually manifest as intermittent claudication) have a greatly increased risk of arterial thrombosis, not only in the limbs, but also in the cerebral and coronary arteries. Ischaemic heart disease and stroke are the major causes of death and disability in such persons (Reunanen et al., 1982). Epidemiological studies have reported that individuals with intermittent claudication have a two to three fold increase in cardiovascular mortality compared to age matched controls (Reunanen et al., 1982; Dormandy et al., 1989). 50% of all deaths are due to myocardial infarction, 15% occur in the cerebral region, and 10% in the abdomen (Dormandy et al., 1989).

Thrombosis is a feature in the progress of peripheral arterial disease. Chapters 3, 4, and 5 of this thesis have shown that patients with peripheral arterial disease have a hypercoagulable state (increased fibrinogen and cross-linked fibrin degradation products) and abnormal blood rheology (increased fibrinogen, red cell aggregation, plasma viscosity and white cell count).

Dormandy et al. (1973b) have shown that an increased fibrinogen level predicts subsequent deterioration of symptomatic peripheral arterial disease, and Wiseman et al. (1989) have shown that an increased fibrinogen level predicts vascular graft occlusion.

It is therefore reasonable to look at antithrombotic therapy to try to prevent thrombotic progression of peripheral arterial disease. Two simple medical treatments which might reduce the thrombotic component of arterial disease are minidose warfarin therapy and low-dose aspirin.

Warfarin

Karl Paul Link isolated dicoumarol in 1933 while investigating the cause of haemorrhagic sweet clover disease of cattle. Warfarin, an analogue of dicoumarol, was introduced in 1948 for rodent control and later became recommended for clinical anticoagulation (Link, 1948; Campbell & Link, 1940).

Warfarin inhibits synthesis of prothrombin and factors II, VII and X needed in prothrombin conversion to thrombin which is a potent platelet aggregating agent responsible for the conversion of fibrinogen to fibrin.

Warfarin has been studied in several prevention trials after myocardial infarction. Oral anticoagulation reduces mortality after myocardial infarction by about 20% (International Anticoagulant Review Group, 1970; Chalmers et al., 1977). A recent review of the effect of warfarin on mortality and reinfarction after myocardial infarction showed a reduction in mortality of 24% and a reduction of

34% in reinfarction. There was an increased risk of intracranial haemorrhage during oral anticoagulant therapy but this was outweighed by the significant reduction in "undifferentiated" and thrombo-embolic cerebrovascular events (Smith et al., 1990b).

Poller et al have shown that minidose warfarin (1mg per day) was effective in reducing the incidence of venous thrombosis after major surgery (Poller et al., 1987). Bern et al. (1990) in a randomised trial have shown that 1mg of warfarin daily prevents thrombosis in central venous catheters. Hence minidose warfarin significantly reduces the incidence of clinical thrombosis with minimal bleeding side effects and does not require blood monitoring.

Aspirin

Aspirin inhibits platelet aggregation, blocking the synthesis of thromboxane A2 in platelets by inhibiting the enzyme cyclooxygenase (Patrono et al., 1985). At high doses it also inhibits prostacyclin production. Prostacyclin is produced by the endothelial cells and may protect against platelet deposition and endothelial damage (Boobis & Bell, 1982). At low doses (75 mg/day) aspirin maximally inhibits thromboxane A2 production but has a minimal inhibition on prostacyclin (Hanley et al., 1981).

It is unlikely that aspirin will reverse atherosclerosis. Aspirin could however prevent progress of chronic peripheral arterial disease by an antithrombotic effect.

Hess et al. reported that aspirin and dipyridamole delayed the progress of peripheral arterial disease as measured by serial arteriograms, although the benefit from aspirin alone did not reach statistical significance (Hess et al., 1985). They used a scoring system based on the change in severity of stenosis and on the length of the lesion. However it is difficult to assess the clinical significance of such a scoring system. No data were provided on the results of clinically important outcome such as progression of symptoms or the need for surgical intervention (Clagett et al., 1989).

Aspirin has been shown to be of benefit in secondary prevention studies of myocardial infarction and stroke. A meta-analysis of 29,000 patients with cardiovascular and cerebrovascular disease compared antiplatelet therapy with placebo. Antiplatelet treatment significantly reduced overall vascular mortality by 15% and significantly reduced non-fatal myocardial infarction and non-fatal stroke by 30%. When different antiplatelet agents were examined, they were unable to find a treatment regimen better than aspirin alone (Antiplatelet Trialists' Collaboration, 1988). There is no good evidence that adding dipyridamole is likely to confer any additional benefit (Fitzgerald, 1987).

In the United Kingdom Transient Ischaemic Attack Aspirin Trial two different daily doses of aspirin (325mg and 1200mg), were compared to placebo in 2435 patients with transient ischaemic attack or minor stroke. In aspirin

treated patients there was a reduction in non-fatal myocardial infarction and non-fatal stroke compared to controls. There was no significant therapeutic difference between the two aspirin doses. However there was a definite dose response for upper gastrointestinal side effects and gastrointestinal haemorrhage. The lower dose of aspirin was significantly less gastrotoxic (UK-TIA Study Group, 1988).

In primary prevention, aspirin significantly reduced non-fatal myocardial infarction and significantly increased haemorrhagic stroke rate in the Physicians' Health Study in America. However total cardiovascular death was not significantly different with aspirin from placebo (The Steering Committee of the Physicians' Health Study Research Group, 1988).

In the United Kingdom, Peto et al. (1988) reported no significant primary prevention benefit for aspirin in ischaemic heart disease and stroke in 5,000 doctors.

Thus only for patients with vascular disease is there clear evidence that antiplatelet treatment reduces the overall incidence of fatal or disabling vascular disease. The subjects in the primary prevention studies were not patients at high risk of thrombotic events.

Aspirin has important side effects: causing gastric toxicity and gastrointestinal bleeding (UK-TIA Study Group, 1988).

Aspirin was also associated with an increased incidence of haemorrhagic stroke in the Physicians' Health Study (The Steering Committee of the Physicians' Health Study Research Group, 1988).

Low-dose aspirin (75mg daily) has been shown in pharmacological studies to inhibit thromboxane generation by platelets while preserving the ability of the arterial wall to produce prostacyclin (Patrono et al., 1985; Hanley et al., 1981; Pedersen & Fitzgerald, 1984).

Hence at this low-dose, aspirin might be a more effective antithrombotic agent than at higher doses and have reduced side effects. Aspirin could be of benefit in preventing progress of arterial thrombosis in patients with peripheral arterial disease as it has been shown to be of benefit in reducing the thrombotic component of cardiovascular and cerebrovascular disease. Low-dose aspirin remains pharmacologically active and has fewer side effects than higher doses.

Given the difficulty in regressing atherosclerosis by medication, improved management could be achieved by preventing thrombosis. At present there is no published experience of minidose warfarin or low-dose aspirin in patients with chronic peripheral arterial disease. It is important to evaluate medical treatment that might reduce hypercoagulability and hyperviscosity in patients with peripheral arterial disease as well as their tolerance and

feasibility. Such pilot studies are required prior to evaluation of their efficacy in reducing arterial events in large randomised trials.

Aims

1 To determine if a three month course of either warfarin (1mg daily) or aspirin (75mg daily) or placebo reduces the hypercoagulable state (plasma levels of fibrinogen, cross-linked fibrin degradation products) and/or the haemorheological disturbance (levels of blood viscosity, plasma viscosity, haematocrit, red cell aggregation, and white cell count) in patients with intermittent claudication.

2 To determine if these two treatment regimens are well tolerated as regards possible adverse effects including bleeding and gastrointestinal upset.

3 To determine significant correlations between fibrinogen and the other blood variables measured in 100 patients with intermittent claudication.

4 To determine if levels of three other haemostatic factors - plasminogen activator inhibitor, von Willebrand factor and factor VII - are elevated in peripheral arterial disease, and their response to warfarin or aspirin treatment. Before the methods are described, these three factors will be briefly reviewed.

Plasminogen Activator Inhibitor (PAI)

Thrombosis may be caused by stasis of the blood, hypercoagulability or deficient fibrinolysis (Winman et al., 1985). PAI is a rapid inhibitor of tissue plasminogen activator in human plasma (Verheijen et al., 1984; Kruithof, 1988). Recent studies have shown that the fibrinolytic system is impaired in patients suffering myocardial infarction due to high levels of PAI, and that elevated PAI levels predict recurrent myocardial infarction (Hamsten, 1987). High levels of PAI are also found in some patients with venous thrombosis (Nilsson et al., 1985), hypertension (Landin et al., 1990) and patients with systemic lupus erythematosis and a history of thrombosis (Violi et al., 1990).

Since patients with peripheral arterial disease also have an increased tendency to thrombosis, PAI was measured in this study. There is as yet no published report on PAI levels in peripheral arterial disease.

Von Willebrand Factor

Von Willebrand factor is a multimeric glycoprotein synthesised and secreted by endothelial cells (Jaffe et al., 1973, 1974). Platelet adhesion to the vessel wall and subsequent platelet aggregation at sites of vascular injury depend on von Willebrand factor binding to two platelet receptors: glycoprotein Ib.Ix and glycoprotein IIb.IIIa (Ruggeri et al., 1983). The glycoprotein IIb.IIIa is also the platelet receptor for fibrinogen (Kloczewick et al.,

1984). Von Willebrand factor forms a bridge between the platelet surface and areas of damaged vascular endothelium (Sadler, 1987). Von Willebrand factor binds with platelets and vessel wall components including collagen (Pareti et al., 1986, 1987) and crosslinked and noncrosslinked fibrin (Ribes & Francis, 1990). Fibrin stimulates rapid secretion of von Willebrand factor by endothelial cells (Ribes et al., 1987). It has been suggested that von Willebrand factor released from endothelial cells in the presence of fibrin will bind fibrin, facilitating platelet adhesion and aggregation (Ribes & Francis, 1990). Badimon et al. (1988) showed that the effect of von Willebrand factor occurs primarily at high shear conditions e.g. in the microcirculation (during haemostasis) or at advanced arterial stenotic lesions. This could initiate thrombus formation (O'Brien, 1990).

There are two mechanisms for platelet aggregation. One is the cyclooxygenase dependant pathway which is sensitive to aspirin. The other is a shear induced platelet aggregation (O'Brien, 1990). Several studies have shown that high shear activates a domain on glycoprotein IIb/IIIa platelet receptor and in the presence of von Willebrand factor as ligand, platelets can aggregate and also stick to collagen and pile up and form thrombi (Berliner et al., 1988; Coller et al., 1989; O'Brien, 1990).

Measurement of von Willebrand factor antigen was performed to determine if von Willebrand factor was elevated in peripheral arterial disease as suggested by

Christe et al. (1984) and to determine if aspirin or warfarin would reduce high levels.

Factor VII

High activities of the procoagulant clotting factor VII have been shown to be an important risk factor for ischaemic heart disease. The Northwick Park Heart Study demonstrated that high levels of factor VII coagulant activity were associated with the risk of subsequent ischaemic heart disease (Meade et al., 1980, 1986). This has been confirmed by others (Dalaker et al., 1985, 1987; de Sousa et al., 1988; Hoffman et al., 1988). Factor VII activity is rapidly and significantly reduced by warfarin even at low dosage (Meade, 1990). Poller et al. have recently reported that a minidose of warfarin significantly reduced factor VII coagulant activity in subjects with high levels (Poller et al., 1990).

Factor VII activity was measured to determine if high levels occur in patients with intermittent claudication (which has not been previously studied) and because minidose warfarin has been reported to reduce significantly factor VII in subjects with high levels (Poller et al., 1990).

Whole Blood Viscosity

In this chapter, the haemorheological measurement of whole blood viscosity was measured at high shear rate ($200s^{-1}$) at native haematocrit. Whole blood viscosity was corrected to a standard haematocrit of 0.45 using the formula of Matrai et al. (1987). (See Chapter 2)

METHODS

Study Design and Patient Selection

Patients were randomised in a controlled, double blind, parallel group study. This was approved by the Glasgow Royal Infirmary Ethical Committee.

102 patients attending the out-patient clinic, Unit for Peripheral Vascular Surgery, Glasgow Royal Infirmary, were entered into the study. All patients had stable intermittent claudication of at least three months duration. Every patient had a typical history of intermittent claudication and a Doppler ankle brachial pressure index of 0.9 or less in at least one leg.

Exclusions for entry into the study were failure to give informed consent, females of child bearing age, need for long-term aspirin or anticoagulation, peptic ulceration, recent cerebrovascular event, known diabetes mellitus, known allergy to aspirin or warfarin, severe hepatic or renal impairment, bacterial endocarditis, uncontrolled hypertension, other major illnesses, difficulty in attending clinic, or a known coagulopathy.

Investigations

Venous blood samples (25mls) were taken from an arm vein for the following measurements which are established in the Coagulation and Haemorheology Laboratory, Glasgow Royal Infirmary.

* A coagulation screen (activated partial thromboplastin time, prothrombin time, fibrinogen, factor VII activity) was measured in an automated coagulometer (Coag-a-mate X2) using standard reagents and standards (Organon-Teknika), except for the prothrombin time, and factor VII which were measured using the techniques described by Poller et al. (1987).
* Cross-linked fibrin degradation products.
* Plasminogen activator inhibitor.
* von Willebrand factor antigen.
* Whole blood and plasma viscosity at high shear rates, 37°C.
* Microhaematocrit.
* Red cell aggregation.
* Full blood count and platelet count.
* Carboxyhaemoglobin level.

The methodology for these measurements is described in detail in Chapter 2.

Method of Study

After explanation of the study and informed consent, and a baseline blood sample for the above tests, patients were given identical placebo, low-dose aspirin (75mg daily) or minidose warfarin (1mg daily). The allocation for this was randomised. The patient's general practitioner was informed by letter, and the patients were asked to contact myself in the event of any bleeding or other acute event occurring between scheduled visits to the clinic.

Details of intermittent claudication, smoking habit, past medical history, medication and alcohol intake were recorded. Height, weight and blood pressure were also recorded. Carboxyhaemoglobin level was used to confirm smoking habit.

The patients were reviewed after one week to check tolerance and prothrombin time (to exclude hypersensitivity to warfarin), then monthly for three months. A further review was made one month after discontinuing the trial medication. Review included history of intercurrent events (especially bleeding and gastrointestinal upset), general examination, measurement of haematocrit and prothrombin time, and tablet counts to assess compliance. Patients could be withdrawn from the study and their medication stopped at their request, in the event of bleeding or adverse events requiring withdrawal, or for non-compliance

with attendance or medication. All blood tests were repeated after three months of treatment, and again one month after stopping treatment or on premature discontinuing of trial medication.

The ankle brachial pressure index was repeated after three months of treatment.

Statistical Analysis

All data were entered onto computer in the Medical Statistics Unit, Department of Community Medicine, Edinburgh University. Changes in coagulation and haemorheological variables were compared in the three treatment groups before and at the end of three months treatment.

Differences in mean plasma levels of fibrinogen between cases of intermittent claudication and controls have been measured (Chapter 3). Several studies have shown that fibrinogen levels are elevated by 15-20% in claudicants (Dormandy et al., 1973a; Hamer et al., 1973; Lowe et al., 1986). The sample size of 100 patients was chosen because it was calculated that with 33 patients in each treatment group the study would have a power of 80% at the 5% significance level to detect a reduction of 9% in plasma fibrinogen level: such a reduction is likely to be of prognostic significance for cardiovascular events (Meade, 1987c).

Analysis of Blood Variables

The blood variables were analysed by analysis of covariance of the change from baseline to three months, fitting the baseline value for each patient as a covariate. The results are presented in terms of the least squares mean change from baseline to three months, standard error of the least squares mean, the number of patients for each treatment group, and the p-value from the overall F-test for any differences between the treatment groups. Least squares means are adjusted means which take into account any differences between the treatment groups at baseline. (It is important to look not only for statistical differences between treatment groups but also for clinically relevant changes).

There were nine blood variables where the change from baseline to three months did not have a reasonably normal distribution: factor VII, prothrombin time, von Willebrand factor, cross-linked FDP's, PAI, fibrinogen, red cell aggregation, platelets, and carboxyhaemoglobin. These were investigated using logarithmic transformation before using analysis of covariance. This was only successful for factor VII for which analysis of covariance on the change from baseline to three months in the logarithmically transformed values has been performed, with the untransformed baseline value for each patient as the covariate.

The results of the analysis of covariance of the logarithmically transformed values have been presented in terms of the least squares mean change from baseline logarithmic value to three months logarithmic value, standard error of the least squares mean, the number of patients in each treatment group and the p-value from the overall F-test for any differences between the treatments.

Because the change in logarithmically transformed values is on a scale which is difficult to interpret the exponential of the least squares mean has been used to obtain a value which estimates the mean of the ratio of the three month value to the baseline value, adjusting for any difference between the treatment groups at the baseline.

For the eight blood variables for which logarithmic transformation was unsuccessful in producing a normally distributed variable, non-parametric analysis was performed. The results of this analysis for the change from baseline to three months have been presented in terms of median change, interquartile range and numbers of patients for each treatment group, and the p-value from the Kruskal-Wallis test for any differences overall between the treatments.

Correlations

Spearman's rank correlations were performed. In view of the large numbers of correlation coefficients measured in

this chapter only r values which were significant at the 0.1% level provided strong evidence of any relationships between the blood variables.

RESULTS

Patients were aged between 28-82 (mean 64), 76 males, 26 females. Two patients were withdrawn from study: one patient withdrew at her own request after one week, the other patient was seriously injured in a road traffic accident and could not attend. This left 100 patients for study.

At entry to the study, each treatment group was similar for distribution of sex, age, body mass index, blood pressure, past medical history of cardiovascular disease, alcohol consumption, ankle brachial pressure index and smoking (See Table 6.1).

The baseline values of the blood variables are shown (Table 6.2).

To illustrate the effects of aspirin, warfarin and placebo on the blood variables between baseline and three months, the least squares mean changes and median changes are shown. The significance of differences between each treatment group are also shown (see Table 6.3 and Figures 6.1-6.10).

Table 6.1
Baseline values of age, sex, body mass index, systolic and diastolic blood pressure, ankle brachial pressure index, smoking habit, male and female alcohol consumption, and past medical history of cardiovascular disease in the three treatment groups

Test		Aspirin	Placebo	Warfarin
Age (years)				
	Mean	65.4	64.1	62.6
	S.D.	9.6	9.2	10.9
	n	34	33	34
Sex	Male	27	24	24
	Female	7	9	10
	Total	34	33	34
Body mass index (kg/m^2)				
	Mean	24.1	25.2	25.8
	S.D	3.0	3.4	4.0
	n	34	33	34
Systolic blood pressure (mmHg)	Mean	148	147	151
	S.D.	18	22	25
	n	34	33	30
Diastolic blood pressure (mmHg)	Mean	82	82	84
	S.D.	11	11	12
	n	34	33	30
Ankle brachial pressure index	Mean	0.78	0.75	0.80
	S.D.	0.19	0.20	0.19
	n	34	32	32
† **Smoking habit**				
	Current	19	19	25
	Ex-smoker	14	10	8
	Never	1	4	1
	Total	34	33	34

† Carboxyhaemoglobin proven

Table 6.1 continued

Test		Aspirin	Placebo	Warfarin
Male Alcohol consumption (units per week)	Median	4	2	4
	Min	0	0	0
	Max	20	50	80
	n	27	24	24
Female Alcohol consumption (units per week)	Median	0	0	0
	Min	0	0	0
	Max	15	6	20
	n	7	9	10
Past cardiovascular history	Yes	28	30	29
	No	6	3	5
	n	34	33	34

Table 6.2
Baseline values for variables in the three treatment groups and in all patients

Test	Aspirin	Placebo	Warfarin	All Patients
Fibrinogen (g/L)				
Median	3.10	3.07	2.81	3.02
IQR	0.84	1.18	0.85	0.87
n	34	32	34	100
Cross linked FDPs (ng/ml)				
Median	137	137	149	139
IQR	182	131	132	155
n	34	33	34	100
Red cell aggregation (units)				
Median	4.6	5.0	4.3	4.6
IQR	1.9	1.4	1.7	1.8
n	34	32	30	96
Plasminogen activator inhibitor (units/ml)				
Median	125	133	124	125
IQR	100	127	81	101
n	26	28	32	86
von Willebrand factor (%)				
Median	129	147	160	141
IQR	66	76	87	75
n	34	32	34	100
Factor VII (%)				
Median	110	110	113	110
IQR	58	34	56	46
n	34	31	34	99
Prothrombin time (sec)				
Median	13.8	13.8	13.4	13.6
IQR	0.9	1.2	1.1	1.0
n	34	32	34	100

Table 6.2 continued

	Aspirin	Placebo	Warfarin	All Patients
White cell count ($x10^9$/L)				
Mean	7.6	8.0	8.8	8.2
S.D	1.7	1.6	2.0	1.9
n	34	33	33	100
Plasma viscosity (mPa.s)				
Mean	1.37	1.38	1.35	1.36
S.D	0.10	0.10	0.09	0.10
n	32	32	33	97
Whole blood viscosity (mPa.s)				
Mean	3.40	3.56	3.58	3.51
S.D.	0.45	0.54	0.75	0.59
n	34	32	32	98
Corrected blood viscosity (mPa.s)				
Mean	3.35	3.41	3.37	3.37
S.D.	0.35	0.34	0.45	0.38
n	32	29	33	94
Haematocrit				
Mean	0.46	0.47	0.47	0.47
S.D	0.04	0.04	0.05	0.04
n	34	33	33	100
Activated Partial Thromboplasim time (sec)				
Mean	29.3	29.3	29.1	29.2
S.D.	2.8	3.4	3.0	3.1
n	33	31	28	93
Red blood count ($x10^{12}$/L)				
Mean	4.62	4.80	4.73	4.71
S.D	0.42	0.50	0.40	0.45
n	34	33	33	100
Haemoglobin (g/dl)				
Mean	14.4	14.8	14.9	14.7
S.D.	1.3	1.4	1.6	1.4
n	34	33	33	100

Table 6.2 continued

	Aspirin	Placebo	Warfarin	All Patients
Mean cell volume				
(fl) Mean	90	90	91	90
S.D.	7	6	6	6
n	34	33	33	100
Platelet count ($x10^9$/L)				
Median	304	289	287	296
IQR	83	89	90	86
n	34	33	33	100
Carboxy-haemoglobin (%)				
Median	2.8	3.3	3.3	3.3
IQR	3.5	4.3	5.0	4.1
n	31	31	33	95

Table 6.3
Least squares mean (LS mean) changes and median changes between baseline and three months in the three treatment groups

Variable	Aspirin	Placebo	Warfarin	p value
Fibrinogen (g/L)				
Median	-0.06	-0.08	0.15	N.S.
IQR	0.83	1.14	0.56	(Kruskal-Wallis)
n	34	29	31	
Cross linked FDPs (ng/ml)				
Median	0.5	-2	-5.5	N.S.
IQR	48	47	27	(Kruskal-Wallis)
n	33	28	30	
Red cell aggregation (units)				
Median	0.4	0.35	0.45	N.S.
IQR	1.7	1.7	2.0	(Kruskal-Wallis)
n	32	29	26	
Plasminogen Activator Inhibitor (units/ml)				
Median	3.5	5	11.5	N.S.
IQR	81	94	80	(Kruskal-Wallis)
n	22	24	21	
Von Willebrand Factor (%)				
Median	3	-9	0	N.S.
IQR	23	39	27	(Kruskal-Wallis)
n	34	29	30	
† **Factor VII** (%)				
LS Mean	0.206	0.140	0.003	N.S.
SE	0.073	0.079	0.076	(F-test)
n	33	28	30	
Prothrombin time (sec)				
Median	0.1	0.05	0.3	p<0.05)
IQR	0.9	0.8	0.7	(Kruskal-Wallis)
n	34	29	31	

† Logarithmic transformation

Table 6.3 continued

Variable	Aspirin	Placebo	Warfarin	p value
White cell count ($x10^9$/L)				
LS mean	0.5	-0.5	-0.3	p<0.05
SE	0.3	0.3	0.3	(F-test)
n	32	32	31	
Plasma viscosity (mPa.s)				
LSMean	-0.03	-0.04	-0.02	N.S.
S.E.	0.01	0.01	0.01	(F-test)
n	32	29	31	
Whole blood viscosity (mPa.s)				
LS mean	-0.17	-0.03	-0.08	N.S.
SE	0.08	0.09	0.08	(F-test)
n	34	27	29	
Corrected blood viscosity (mPa.s)				
LS mean	-0.10	0.09	-0.01	N.S.
SE	0.06	0.07	0.06	(F-test)
n	32	25	29	
Haematocrit				
LS mean	-0.9	-0.8	-1.0	N.S.
SE	0.5	0.5	0.5	(F-test)
n	34	29	31	
Activated partial thromboplastin time (sec)				
LS mean	0.3	-0.1	0.3	N.S.
SE	0.5	0.5	0.5	(F-test)
n	31	26	25	
Red blood count ($x10^{12}$/L)				
LS mean	-0.09	-0.10	-0.12	N.S.
SE	0.04	0.04	0.04	(F-test)
n	32	32	31	

Table 6.3 continued

Variable	Aspirin	Placebo	Warfarin	p value
Haemoglobin (g/dl)				
LS mean	-0.38	-0.22	-0.38	N.S.
SE	0.13	0.13	0.13	(F-test)
n	32	32	31	
Mean cell volume (fl)				
LS mean	1.1	0.0	0.8	N.S.
SE	0.4	0.4	0.4	(F-test)
n	32	32	31	
Platelet count ($x10^9$/L)				
Median	5	-4.5	2	N.S.
IQR	108	43	66	(Kruskal-Wallis)
n	31	32	31	
Carboxyhaemoglobin (%)				
Median	-0.2	0.5	0.0	N.S.
IQR	1.5	1.6	0.8	(Kruskal-Wallis)
n	29	27	28	

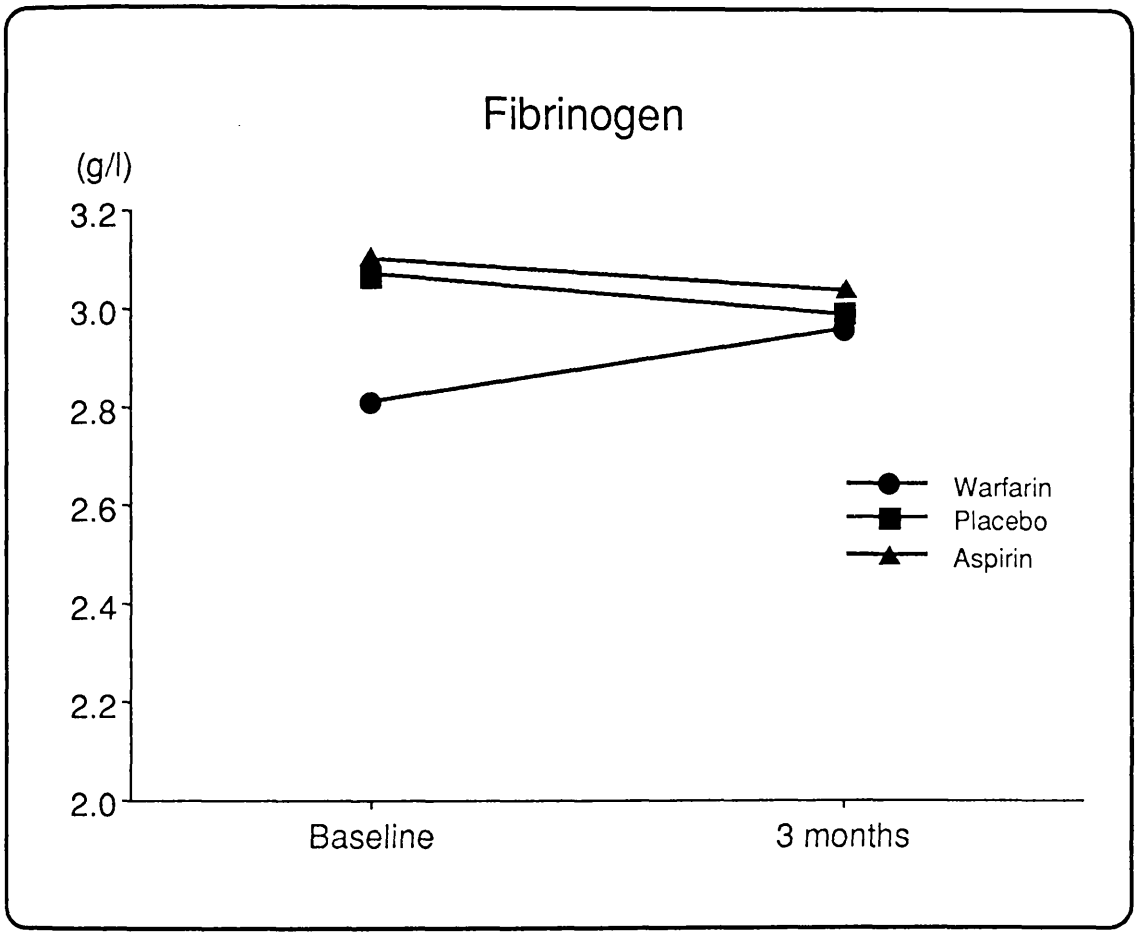

Figure 6.1

Median changes in plasma fibrinogen level between baseline and three months in the three treatment groups.

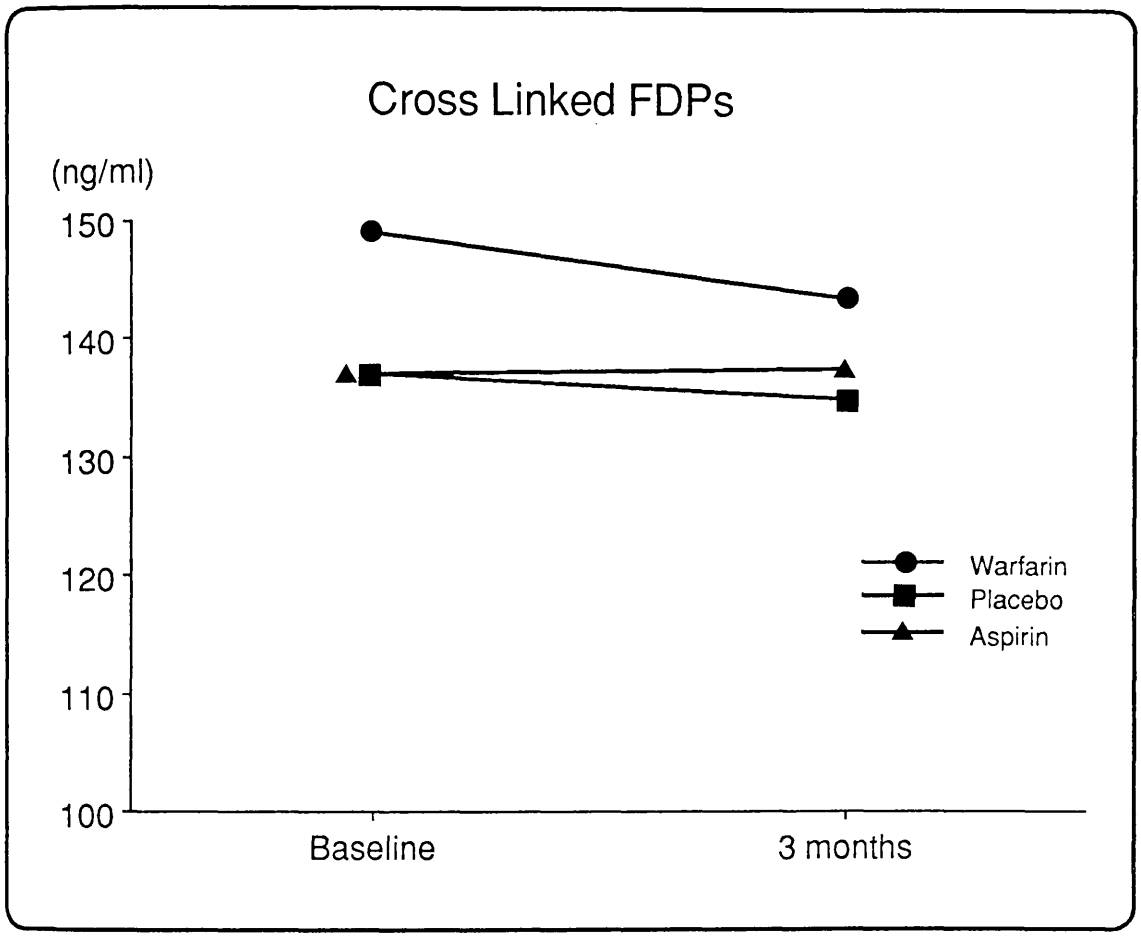

Figure 6.2

Median changes in plasma levels of cross linked fibrin degradation products between baseline and three months in the three treatment groups.

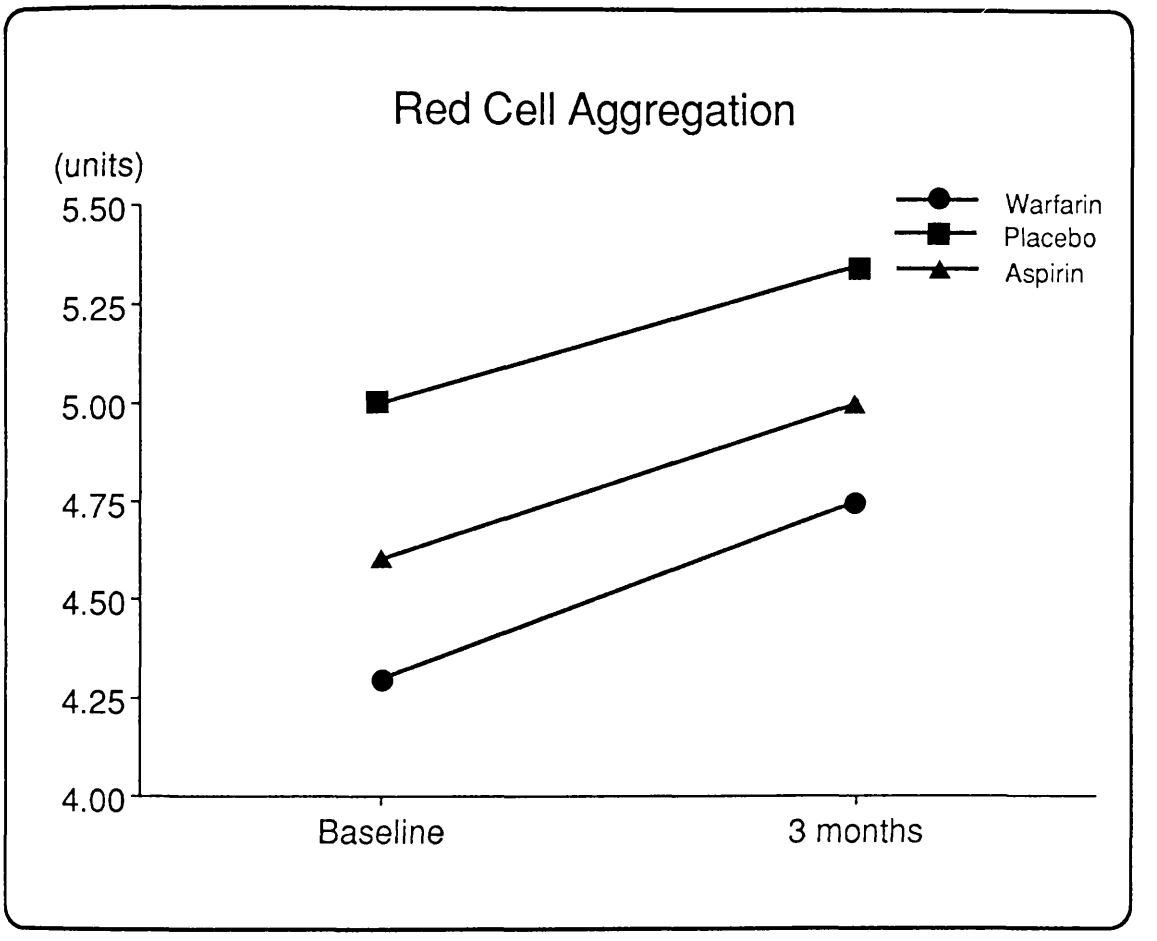

Figure 6.3

Median changes in red cell aggregation between baseline and three months in the three treatment groups.

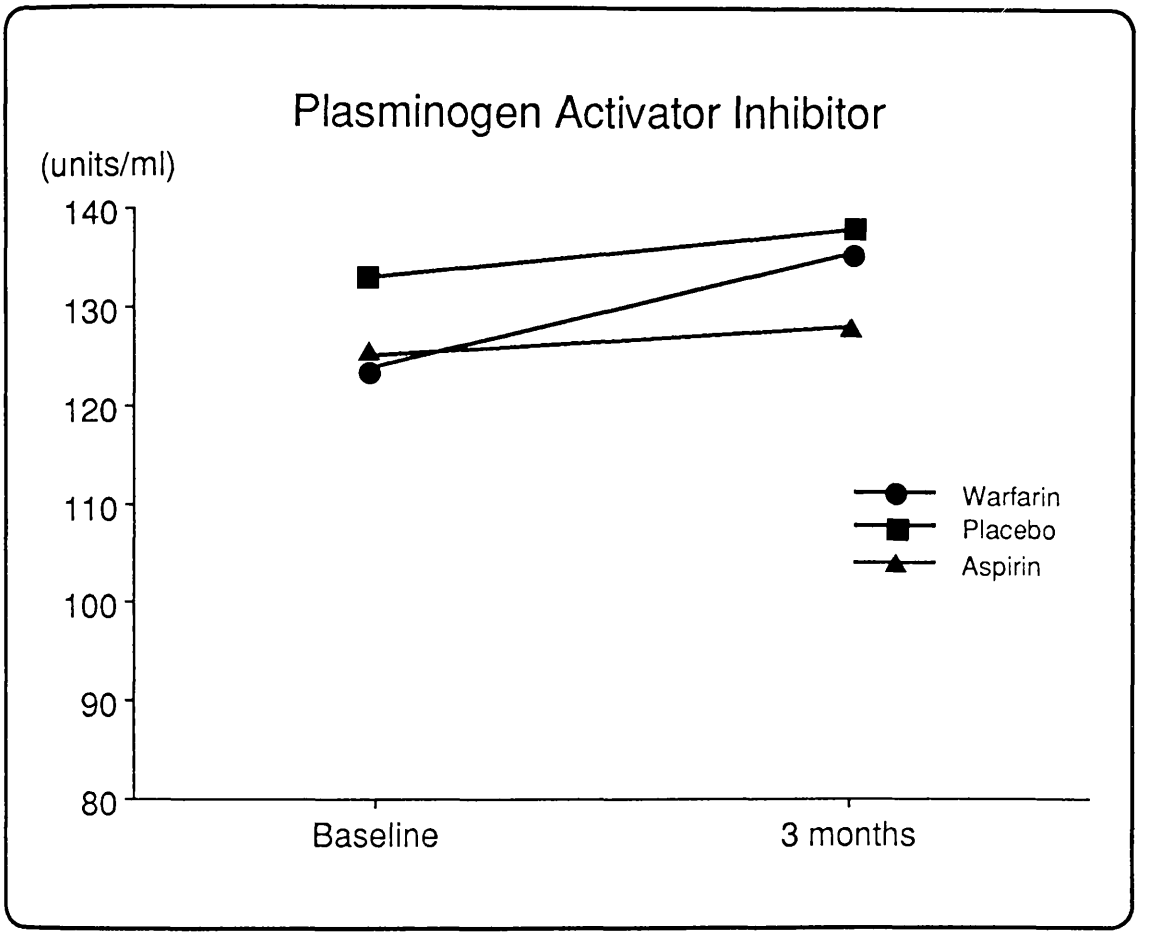

Figure 6.4

Median changes in levels of plasminogen activator inhibitor between baseline and three months in the three treatment groups.

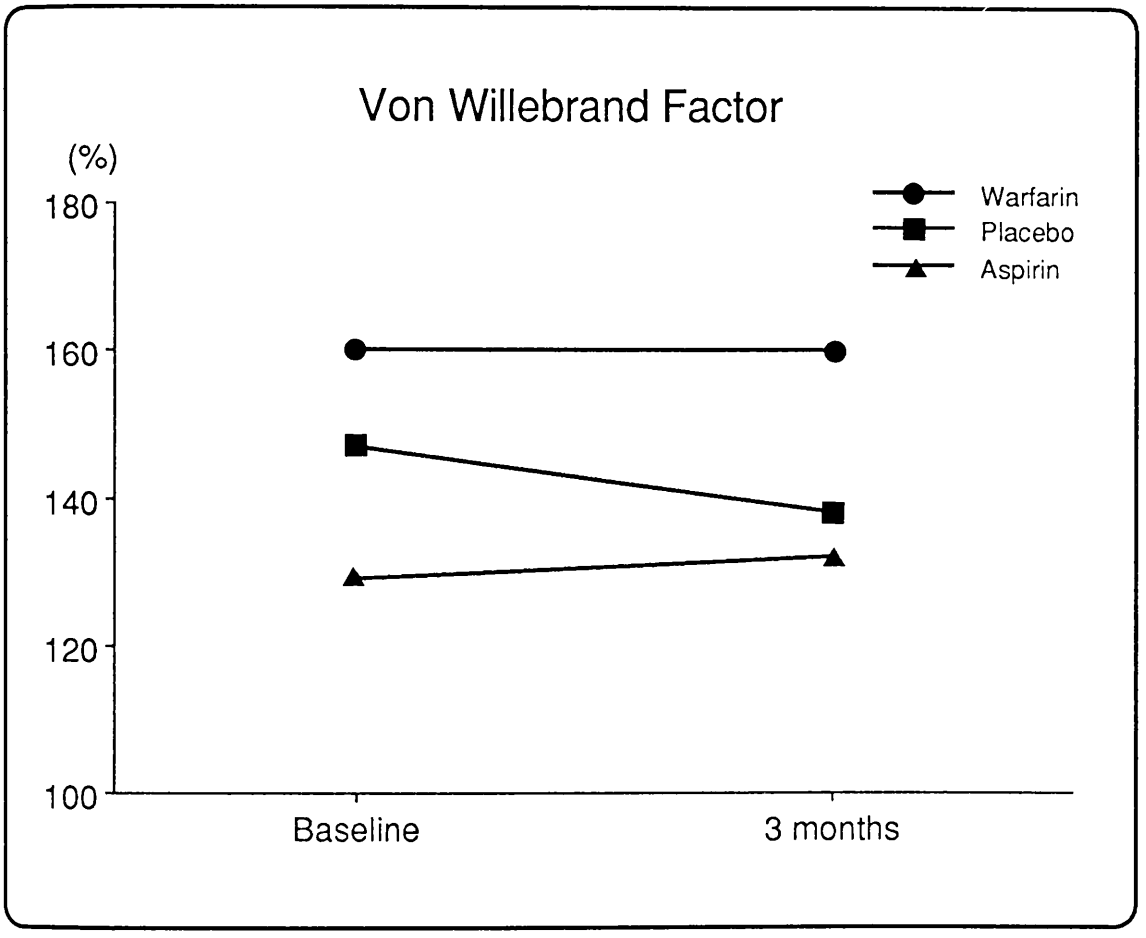

Figure 6.5

Median changes in levels of von Willebrand factor antigen between baseline and three months in the three treatment groups.

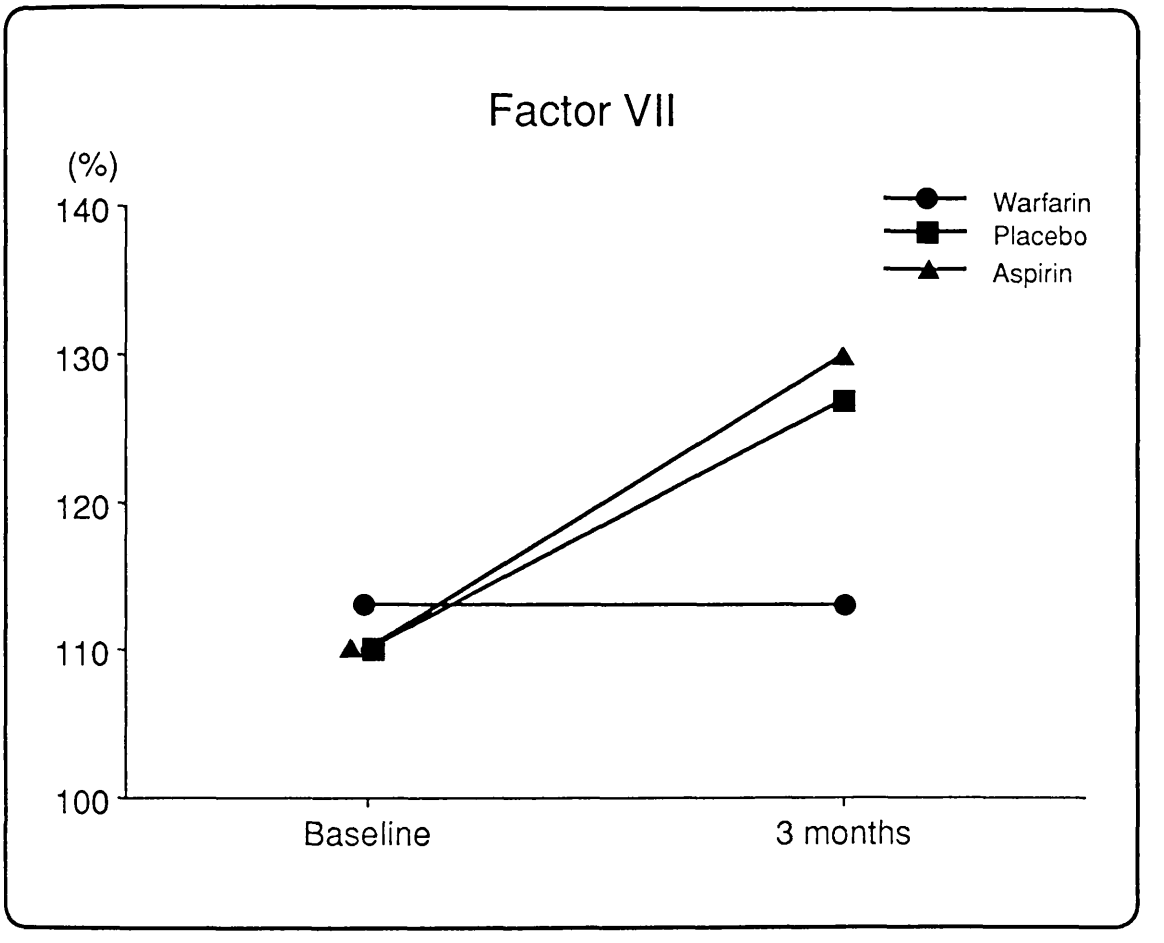

Figure 6.6

Least squares mean changes in Factor VII activity between baseline and three months in the three treatment groups.

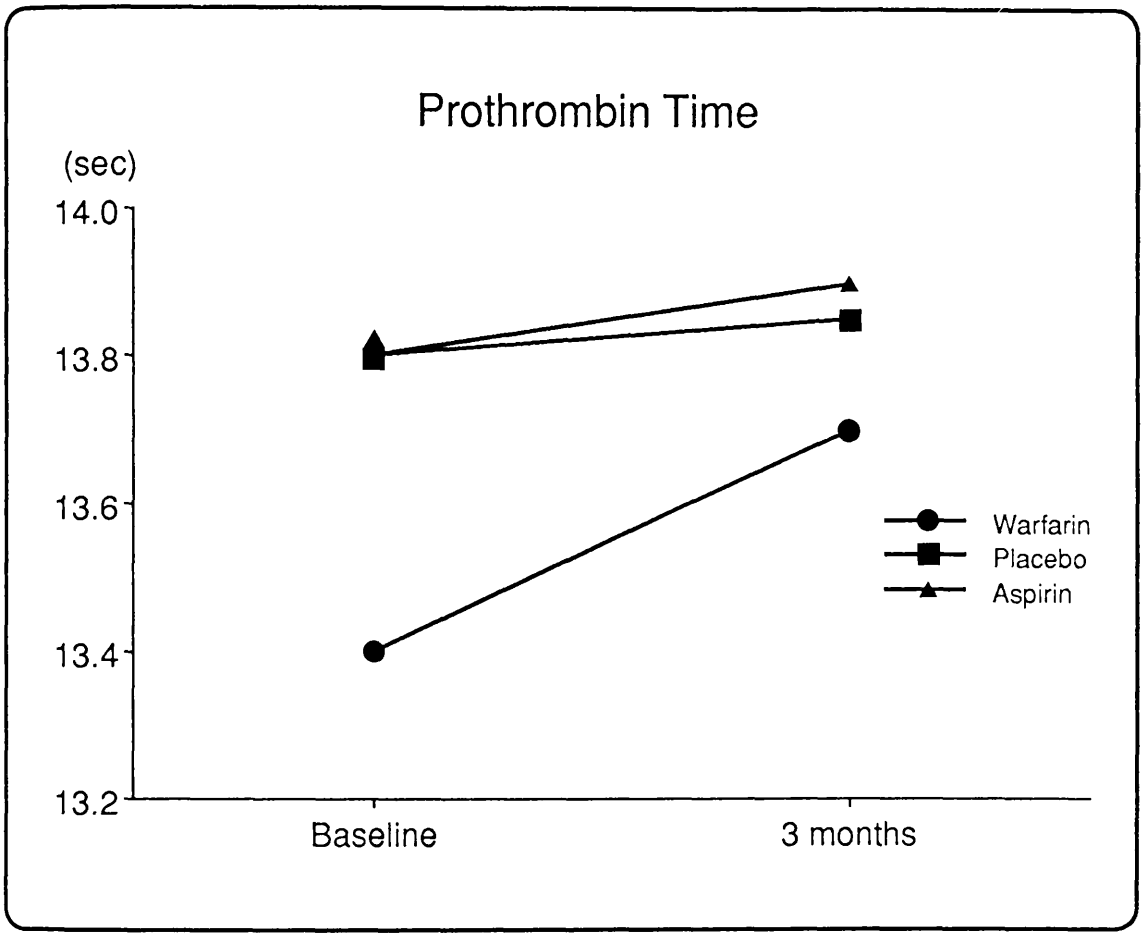

Figure 6.7

Median changes in prothrombin time between baseline and three months in the three treatment groups.

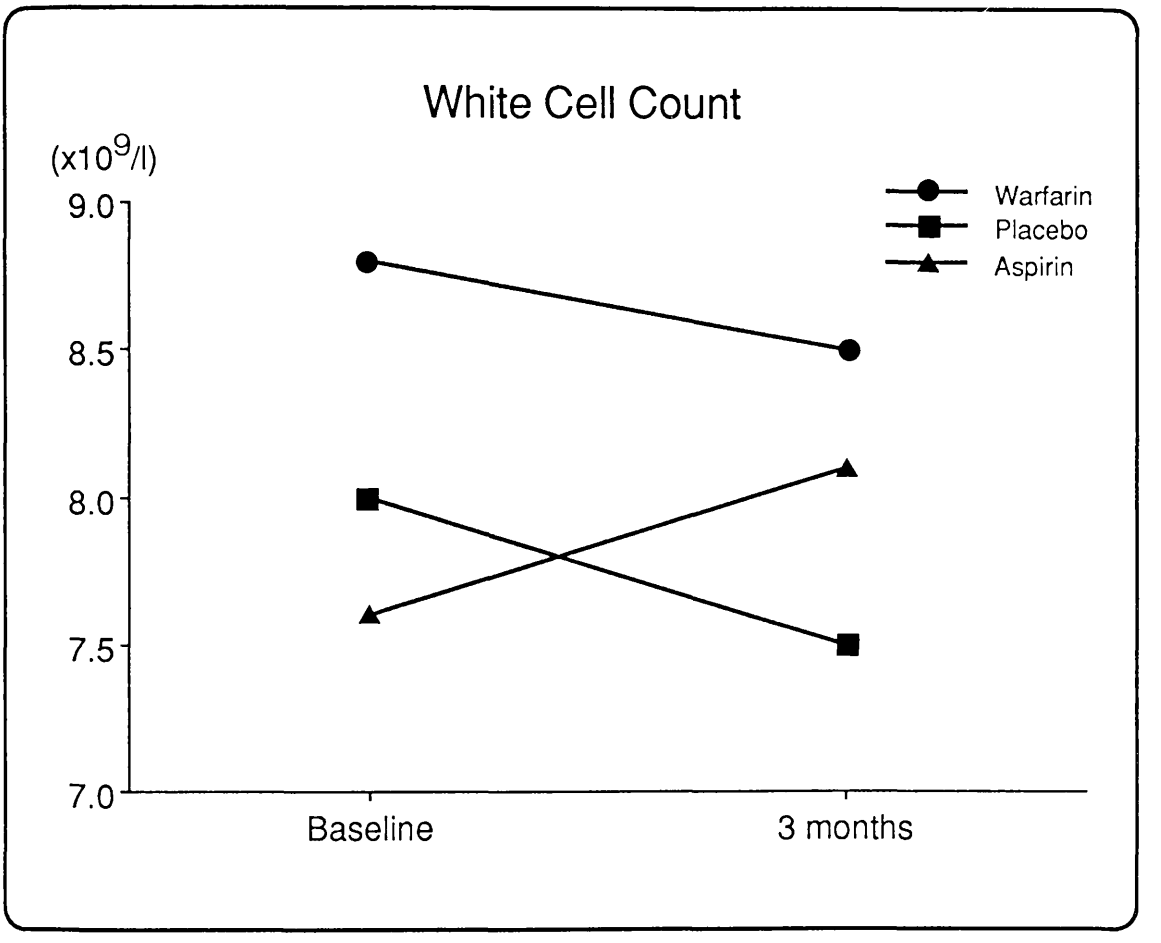

Figure 6.8

Least squares mean changes in white cell count between baseline and three months in the three treatment groups.

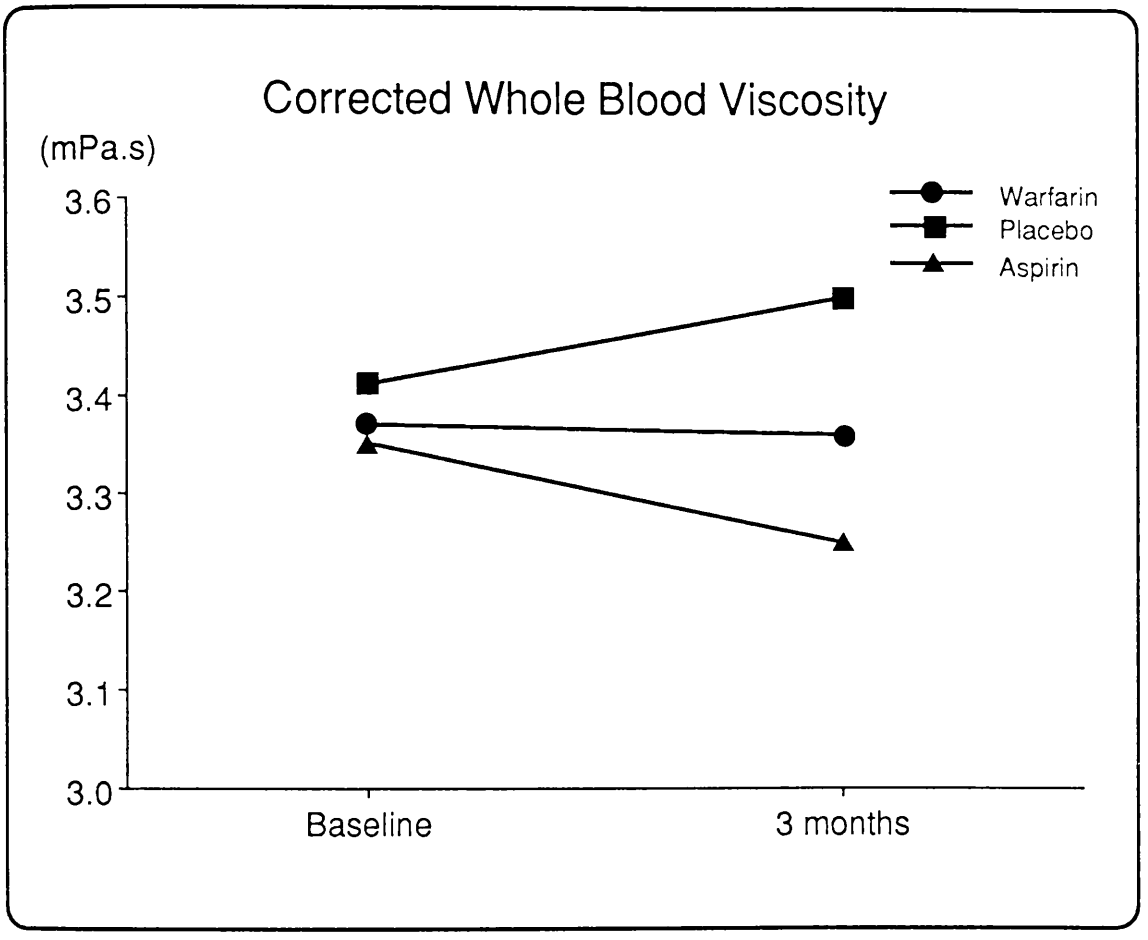

Figure 6.9

Least squares mean changes in corrected blood viscosity between baseline and three months in the three treatment groups.

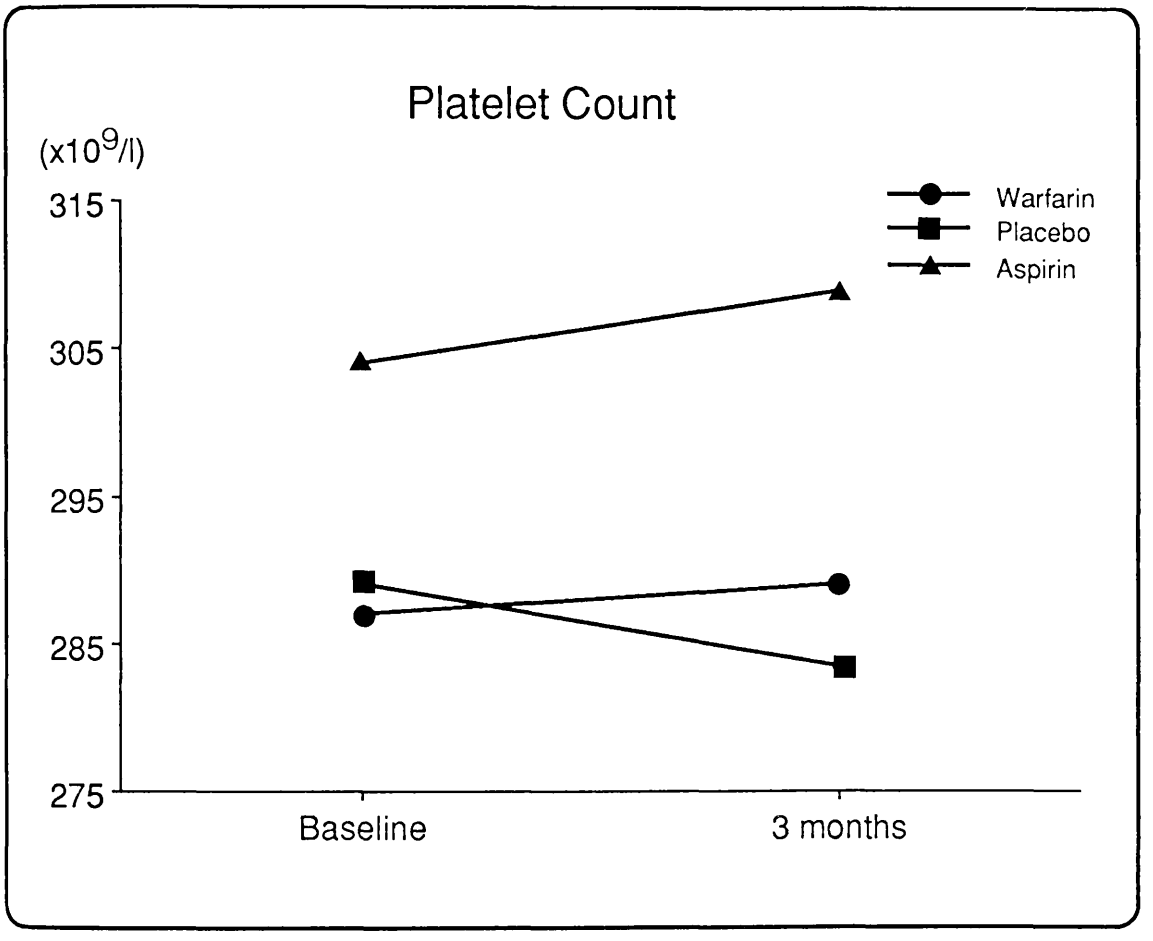

Figure 6.10

Median changes in platelet count between baseline and three months in the three treatment groups.

Only prothrombin time and white cell count were statistically significantly altered. Prothrombin time was significantly prolonged by 1 mg of warfarin daily, compared to the placebo and aspirin groups. White cell count was significantly increased by 75 mg of aspirin daily compared to the placebo and warfarin groups.

Levels of PAI were elevated in the patients (compared to the laboratory normal range: median 92 u/ml, interquartile range 45, n=780). However PAI was not altered by warfarin, aspirin or placebo. PAI correlated inversely with cross-linked FDP`s and inversely with age (See Table 6.4).

The patients had high levels of von Willebrand factor antigen (compared to laboratory normal range: median 94%, interquartile range 48, n=263). Warfarin, aspirin or placebo did not significantly alter von Willebrand Factor antigen level. Von Willebrand factor antigen did not correlate with any other variable at the 0.1% significance level.

High levels of factor VII activity were found in the patients, (compared to the laboratory normal range: median 98%, interquartile range 66, n=21). The level of factor VII activity was not significantly altered by 1 mg of warfarin in this study, nor by low-dose aspirin or placebo. Factor VII activity did not correlate with any other blood variable at the 0.1% significance level.

Table 6.4

Spearman's Rank Correlation Co-efficients

• p<0.05 •• p<0.01 ••• p<0.001

	White cell count	Red blood count	Haemoglobin	Mean cell volume	Platelets	Prothrombin time	Active partial thromboplastin time	Fibrinogen	von Willebrand factor	Factor VII	Red cell aggregation	Plasma viscosity	Whole blood viscosity	Haematocrit	Corrected blood viscosity	Cross linked FDPs	Carboxy-haemoglobin	Plasminogen activator inhibitor
White cell count																		
Red blood count	-																	
Haemoglobin	-	••• 0.73																
Mean cell volume	-	•• -0.32	•• 0.31															
Platelets	• 0.21	••• -0.34	••• -0.40	-														
Prothrombin time	-	-	-	• -0.20	-													
Active partial thromboplastin time	-	-	-	-	-	•• 0.27												
Fibrinogen	-	-	-	-	-	-	-											
von Willebrand factor	-	-	-	-	-	-	•• -0.32	-										
Factor VII	-	-	-	-	-	• -0.26	-	-	-									
Red cell aggregation	-	-	-	-	-	-	-	••• 0.38	-	-								
Plasma viscosity	-	-	-	-	-	-	-	••• 0.40	-	• -0.21	• 0.25							
Whole blood viscosity	-	••• 0.56	••• 0.65	-	•• -0.24	-	-	-	-	-	-	••• 0.37						
Haematocrit	-	••• 0.62	••• 0.77	• 0.22	•• -0.29	-	-	• -0.20	• -0.22	-	-	-	••• 0.71					
Corrected blood viscosity	-	•• 0.30	•• 0.33	-	-	-	-	-	-	-	-	••• 0.43	••• 0.86	•• 0.29				
Cross linked FDPs	-	• -0.20	••• -0.32	-	-	-	-	-	-	-	-	• 0.22	-	•• -0.26	-			
Carboxy-haemoglobin	-	-	••• 0.36	•• 0.33	-	•• -0.20	-	-	-	-	-	-	-	• 0.22	-	• -0.23		
Plasminogen activator inhibitor	-	-	-	• 0.21	-	-	-	-	-	-	-	-	-	-	-	••• -0.42	•• 0.33	
Age	• -0.21	-	•• -0.26	••• -0.34	-	•• 0.28	-	• 0.20	• 0.22	-	-	-	-	• -0.20	-	••• 0.33	••• -0.40	••• -0.36
Body mass index	-	-	• 0.20	-	-	-	-	-	-	-	-	-	-	-	-	• -0.24	-	•• 0.34
Systolic BP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Diastolic BP	-	-	-	-	-	• -0.26	• -0.21	-	-	-	-	-	-	-	-	-	-	-
Ankle brach. pressure index	-	-	-	-	-	•• -0.28	-	• -0.21	-	-	-	-	-	-	-	• -0.24	-	-

Whole blood viscosity was also elevated in the patients with intermittent claudication but was not altered in any of the treatment groups. Whole blood viscosity significantly correlated with plasma viscosity, haematocrit, haemoglobin and red blood cell count.

Correlations

Table 6.4 shows the significant correlations found between the blood variables at the baseline measurements in the 100 patients.

Side Effects

Seven patients complained of side effects. Five of these were in the aspirin treatment group, one patient was in the warfarin treatment group, and one patient in the placebo treatment group. No patients with side effects stopped medication.

Aspirin

One patient had an episode of haemoptysis. One patient had an episode of haematuria. Two patients complained of upper abdominal pain. One patient complained of a rash.

Warfarin

One patient complained of an episode of haemoptysis.

Placebo

One patient complained of upper abdominal pain.

Adverse Events

Five adverse events occurred. All were in patients in the warfarin treatment group. Two patients suffered strokes. One patient died suddenly. One patient occluded a prosthetic femoropopliteal bypass graft. One patient suffered a myocardial infarction.

DISCUSSION

The baseline measurements of clinical presentation together with the baseline blood variables are equivalent in each treatment group. This is in keeping with the double blind randomisation method employed. Baseline results showed similar abnormalities to those previously observed in intermittent claudication in Chapter 3.

Neither low-dose aspirin or minidose warfarin appeared to alter significantly the hypercoagulable state or abnormal blood rheology in patients with intermittent claudication. Only prothrombin time and white cell count were statistically significantly altered.

As expected, prothrombin time was prolonged (by a mean of 0.3 seconds) after 3 months of warfarin treatment. The increase in prothrombin time was similar to that found by Poller et al. (1990) and Bern et al. (1990). This is unlikely to be of clinical significance for bleeding since most patients remained in the normal range. As expected, there was individual variation, four patients showing sensitivity

to warfarin having significantly prolonged prothrombin times beyond the normal range. This however did not require a change of treatment.

Neither warfarin nor aspirin altered factor VII activity. The high levels of factor VII activity in the patients suggest a tendency to thrombotic events. The median level of 110% factor VII activity found in these patients is similar to the level considered high risk for development of ischaemic heart disease in the Thrombosis Prevention Trial (Meade, 1990). While Poller et al. (1990) showed that 1 mg of warfarin significantly reduced high factor VII levels, Bern et al. (1990) found minimal change in factor VII levels, as did this present study.

It is possible that the dose of warfarin was too low to alter significantly the hypercoagulable state and abnormal blood rheology. In the Thrombosis Prevention Trial an average of 4.6mg of warfarin daily was required to reduce factor VII coagulant activity from a high risk level of 120% to a low risk level of 70% (Meade, 1990). The aim of that trial is to reduce the incidence of ischaemic heart disease in men at high risk by 30%. However Poller et al. (1987) demonstrated that minidose warfarin (1mg daily) given for a mean of 20 days pre-operatively reduced deep venous thrombosis following major surgery, despite a prothrombin time within the normal range immediately before surgery. Minidose warfarin was continued throughout the patients stay in hospital. Poller et al. also reported that 1mg of

warfarin statistically significantly reduced factor VII coagulant activity in subjects with high levels (Poller et al., 1990). This was not confirmed by the present study in claudicants.

White cell count was significantly increased in the aspirin treatment group by a mean of 0.5 X 10^9/l. However this may not be an effect due to aspirin. The white cell count in the placebo treatment group was reduced by the same amount after three months. White cell count is very labile and can be influenced by many factors including smoking habit (Petitti & Kipp, 1986). An increase in white cell count of 0.5 X 10^9/l is unlikely to be of clinical relevance in patients with intermittent claudication.

Warfarin or aspirin did not alter PAI levels. Baseline high levels of PAI suggest increased inhibition of the fibrinolytic system, which may enhance the tendency to thrombosis in patients with peripheral arterial disease. Since increased PAI levels should inhibit lysis of fibrin, an inverse correlation with cross-linked FDP's might be expected, and indeed was observed. This has not been previously reported. PAI correlated inversely with age, in this study. While PAI levels decrease with age, cross-linked FDP's increase with age.

Warfarin or aspirin did not alter the levels of von Willebrand factor antigen. The high baseline levels of von Willebrand factor antigen confirm the study by Christe et

al. (1984), and may indicate endothelial damage due to atherosclerosis. It is possible that areas of high shear at the sites of arterial stenoses in patients with peripheral arterial disease increase von Willebrand factor levels. Fibrin is known to stimulate von Willebrand factor secretion from endothelial cells and also to bind to von Willebrand factor (Ribes & Francis, 1990; Ribes et al., 1987). Fibrin present in atherosclerotic plaques may stimulate secretion of von Willebrand factor, which may result in platelet aggregation and adhesion at the sites of atherosclerotic plaques, thus promoting thrombosis.

Levels of von Willebrand factor antigen might be increased because of high shear conditions occurring at stenoses in patients with peripheral arterial disease. Inhibition of shear induced platelet activation merits further investigation. Monoclonal antibodies to von Willebrand factor and the platelet receptor glycoprotien IIb/IIIa have been shown to inhibit thrombus formation in animal studies more effectively than aspirin (Coller et al., 1986; Hanson et al., 1988; Yasuda et al., 1988; Bellinger et al., 1987).

In a recent study of the effects of warfarin on mortality and reinfarction after myocardial infarction, Smith et al.

(1990b) reported an increased risk of intracranial haemorrhage during anticoagulation. It is therefore important to achieve a balance of benefit and risk. The objective of this study was to try to reduce the hypercoagulable state and abnormal haemorheology with a warfarin dose with minimal side effects, which did not require monitoring. Because four subjects showed increases in prothrombin time into the "therapeutic range", clearly doses higher than 1 mg per day would require monitoring and dose adjustment.

It is possible that the aspirin dose was too low in this study. However, at a low-dose (75mg daily) aspirin's effect maximises the inhibition of thromboxane while minimising the inhibitory effect on prostacyclin (Meade, 1990). The degree of inhibition of cyclooxygenase dependent platelet aggregation is similar over a range of aspirin from 50mg-1500mg daily (Patrono et al., 1985). In the UK TIA Trial there was no therapeutic difference between 325mg and 1200mg daily, and the lower dose had less side effects (UK TIA Study Group, 1988).

Correlations

Fibrinogen correlated significantly with red cell aggregation in this study (compared to chapter 3). This suggests that the increased fibrinogen level is in part responsible for the

increased red cell aggregation found in these patients. Increases in fibrinogen concentration greatly increase red cell aggregation in vitro (Merrill et al., 1969). As expected, fibrinogen also correlated significantly with plasma viscosity.

There was an inverse correlation of cross-linked FDP's with plasminogen activator inhibitor (PAI). Where there is increasing levels of PAI there is inhibition of the fibrinolytic system, and hence possibly a consequent decrease in fibrin degradation. Since high levels of PAI are present in patients with intermittent claudication, this may tend to allow a deposition of fibrin since there is inhibition of fibrinolysis. High levels of cross-linked FDP's and PAI in patients with intermittent claudication indicate excessive fibrin formation.

The positive correlation of cross-linked FDP's with age confirms this finding in chapter 3. The inverse correlation of cross-linked FDP's with haemoglobin may simply reflect the correlation of FDP's and haemoglobin with age. There was a reduction of haemoglobin and an increase in FDP's with increasing age.

PAI correlated inversely with age. As patients get older PAI decreases with a resultant decrease in inhibition of fibrinolysis.

The correlation of whole blood viscosity with plasma

viscosity, haematocrit, red blood count, haemoglobin, and corrected viscosity is expected since haematocrit and plasma viscosity are the major determinants of whole blood viscosity (Lowe, 1987b).

Von Willebrand factor antigen and factor VII activity had no significant correlations at the 0.1 % level.

The reason for inverse correlations between platelet count and red blood cell count, and haemoglobin is unclear.

Side Effects

Seven patients complained of side effects during the trial. Five patients were in the aspirin treatment group, one patient was in the warfarin treatment group and one was on placebo.

Aspirin

One patient with chronic obstructive airways disease had one episode of haemoptysis which resolved. One patient with prostatitis had an episode of haematuria one day after starting aspirin medication. Two patients complained of upper abdominal pain and one patient complained of a rash which resolved.

Warfarin

One patient complained of haemoptysis at one month. Her chest x-ray was normal and her prothrombin time was significantly prolonged. The haemoptysis did not recur

during the course of the trial.

Placebo

One patient complained of upper abdominal pain after three months.

The side effects were minimal in all treatment groups. Treatment was not stopped because of any side effects.

Adverse Events

All five adverse events occurred in the warfarin treatment group: probably a chance finding. This number of events would be expected from a study of 100 patients with peripheral arterial disease followed up for four months. Two patients suffered strokes. Both patients had CT scans which excluded haemorrhagic stroke. The first patient had a past history of a stroke and also suffered from angina. The second had a past history of a myocardial infarction and an aortic bifurcation graft. One patient died suddenly one week after taking warfarin. He had been complaining of chest pain and had an ischaemic ECG recorded on the day he died. His prothrombin time had increased by 0.6 seconds (12.8 to 13.4 sec). This was within the normal range. One patient occluded a prosthetic femoropopliteal bypass graft inserted one year previously. One patient suffered a myocardial infarction three weeks after completing warfarin treatment for three months.

Further larger studies are required to determine whether either long term aspirin or warfarin reduces the thrombotic risk in peripheral arterial disease. In a prospective randomised trial of warfarin compared to placebo, Kretschmer et al. (1987) showed that warfarin significantly improved the long term patency of femoropopliteal vein bypass grafts in patients with ischaemic rest pain and gangrene, but not in patients with intermittent claudication. This may reflect the fact that patients with critical limb ischaemia have a greater tendency to thrombosis than claudicants. Kretschmer et al. later showed that warfarin significantly reduced mortality in these patients compared to placebo (Kretschmer et al., 1988). Aspirin and warfarin might give clinical benefit in more severe ischaemia where there is reduced blood flow and where stagnation thrombosis can occur.

Summary

Patients with intermittent claudication have increased fibrinogen, a hypercoagulable state and abnormal blood rheology. Neither low-dose aspirin nor minidose warfarin altered these changes in the blood. The elevated levels of factor VII activity, von Willebrand factor antigen and PAI provide additional evidence of a hypercoagulable state and evidence of deficient fibrinolysis in peripheral arterial disease.

CHAPTER 7

General Discussion and Suggestions for Further Work

Introduction

Fibrinogen may have a role in peripheral arterial disease through its effects on atherogenesis, thrombosis and haemorheology.

Rokitansky (1852) proposed that fibrin deposition in the arterial wall initiated the atheromatous process. Duguid (1948) demonstrated the presence of fibrin in plaques and recently Smith et al. (1990a) have demonstrated fibrinogen and a range of fragments mainly derived from cross-linked fibrin in atherosclerotic plaques, and suggested that the degradation products of fibrinogen and fibrin are atherogenic.

This thesis has investigated the role of fibrinogen and its relationship to thrombosis and blood rheology in patients with peripheral arterial disease. The conversion of fibrinogen to fibrin is the final pathway of coagulation, and thrombosis is a feature in the progression of peripheral arterial disease. Fibrinogen has a rheological effect, increasing blood viscosity through its effects on plasma viscosity and on red cell aggregation. Two new techniques were used in this study: measurement of cross-linked FDP's (an index of fibrin formation from fibrinogen) and measurement of red cell aggregation - by a new photometric method. Both these techniques can be rapidly assayed and

could be potentially useful in clinical practice. The main aim of this thesis was to evaluate their relationships as well as that of fibrinogen, to several aspects of peripheral arterial disease.

Fibrinogen

This thesis has shown in a case - control study (Chapter 3) that fibrinogen is increased in patients with peripheral arterial disease, confirming previous studies (Dormandy et al., 1973a; Hamer et al., 1973). The important message of the current study was that multivariate analysis (including age) showed that the increased fibrinogen level was largely attributable to the effect of smoking. Multivariate analysis including smoking has not been performed in previous studies of fibrinogen in peripheral arterial disease. Fibrinogen was significantly increased in current smokers compared to ex or non-smokers, in both patients and controls. Fibrinogen is a possible mechanism through which the effect of smoking could cause peripheral arterial disease to progress. While further epidemiological studies of the relationship between peripheral arterial disease, fibrinogen and smoking are required, the results of the present study suggest that the increased smoking habit in peripheral arterial disease may largely explain the increased fibrinogen level.

Other possible influences on fibrinogen in peripheral arterial disease include genetic factors. Certain families show a predisposition to peripheral vascular disease and increased fibrinogen levels could occur in such families. Humphries et al. (1987) have shown a strong association between certain genotypes coding for the fibrinogen protein and plasma fibrinogen concentrations. They found a genotype with high fibrinogen levels and it would be worthwhile to study genotype distributions in peripheral arterial disease patients and in controls.

In the acute phase response, the cytokine interleukin-6 is known to regulate hepatic synthesis of fibrinogen. This thesis showed that serum interleukin-6 level was significantly increased in patients with peripheral arterial disease compared to controls. This has not previously been reported and is in keeping with an inflammatory process in peripheral arterial disease as suggested by Stuart et al. (1981). This is consistent with the increased white cell count found in peripheral arterial disease, and the significant correlation of interleukin-6 level with C-reactive protein ($r=0.56$ $p<0.01$) in chapter 4.

While there was not a "significant" correlation between interleukin-6 and fibrinogen level, the correlation co-efficient of 0.29 indicates a trend towards significance ($p=0.09$). The raised interleukin-6, possibly released by activated monocytes and other cells in atherosclerotic plaques may induce the raised fibrinogen level in peripheral

arterial disease. It would be of interest to examine the effects of interleukin-6 antagonists on fibrinogen levels in peripheral arterial disease (Hirata et al., 1989).

The fibrinogen level is significantly increased one day and one week following reconstructive vascular surgery. However this was probably an acute phase response because this increase was not maintained when measured at 3-12 months following reconstructive vascular surgery.

The increased fibrinogen level found in chronic peripheral arterial disease in theory could result in an increased tendency to thrombosis, and increased blood viscosity. Fibrinogen level correlated significantly with cross-linked FDP`s (suggesting an increased tendency to form fibrin), and also with plasma viscosity (Chapter 3), and red cell aggregation (Chapter 6). The clinical importance of the increased fibrinogen level is that it is known to predict for subsequent deterioration of symptomatic peripheral arterial disease (Dormandy et al., 1973b; Hamer et al., 1973) and is known to predict vascular graft occlusion (Hamer et al., 1973; Harris et al., 1978; Wiseman et al., 1989). Fibrinogen level is also a risk factor for ischaemic heart disease (Meade, 1987a). The results of the present study suggest that fibrin formation and red cell aggregation are two mechanisms by which increased fibrinogen levels may promote progress of peripheral arterial disease and ischaemic heart disease.

Cross-linked FDP's

A new method of measurement of cross-linked fibrin degradation products was used in this thesis using a specific monoclonal antibody against the D-dimer antigen of cross-linked FDP's. This is an indicator of in-vivo fibrin formation and lysis, and is a sensitive and specific assay that is easy to perform.

Cross-linked FDP's were increased in patients with peripheral arterial disease compared to controls, i.e. an increase in fibrin formation and lysis was present in these patients.

There are several possible reasons to account for this increased level. Cross-linked FDP's correlated significantly with fibrinogen level. One explanation is that the increased fibrinogen level in peripheral arterial disease promotes fibrin formation. Alternatively, FDPs formed might increase hepatic synthesis of fibrinogen, creating a vicious circle.

Cross-linked FDP's correlated with severity of ischaemia. They were higher in patients with ischaemic rest pain than intermittent claudication, and also were inversely correlated with the ankle brachial pressure index. The correlation with severity of ischaemia may be due to increased fibrin formation and lysis on atherosclerotic plaques, or increased intravascular thrombosis. Cross-linked FDP's were highest in abdominal aortic aneurysm, probably due to degradation of the aortic thrombus.

Cross-linked FDP's correlated strongly with age but were not related to smoking habit. Increased FDP levels could mean that there is an increased tendency to thrombosis in peripheral arterial disease. There is continuous formation of cross-linked fibrin and continuous fibrinolysis within the intima - both processes generating fragments that may be atherogenic (Smith et al., 1990a).

The plasma level of cross-linked FDP's significantly increased at one day and one week following reconstructive vascular surgery. This increase was also found when measured at 3-12 months after surgery and was not present in patients with peripheral arterial disease who did not have surgery. This observation indicates ongoing fibrin formation in patients following reconstructive vascular surgery and merits further investigation. It may be caused by deposition of fibrin on the graft wall. Levels of cross-linked FDP's could therefore be a prognostic marker for subsequent vascular graft occlusion. However Wiseman et al. (1989) stated that cross-linked FDPs showed no predictive value for femoropopliteal vein graft occlusion at one year. Further studies are required.

The importance of finding elevated levels of cross-linked FDP's in peripheral arterial disease is that they are a measure of the severity of ischaemia, they indicate an increased tendency to thrombosis, they may be atherogenic, and they could be possible markers of subsequent graft occlusion.

Red Cell Aggregation

Red cell aggregation was measured by a new method, the Myrenne photometric aggregometer. This is a rapid method which is reproducible and very easy to use.

Red cell aggregation was significantly increased in patients with peripheral arterial disease compared to controls. This has not previously been reported using this method. It is consistent with previous reports of increased low shear blood viscosity in peripheral arterial disease (Dormandy et al. 1973a).

Fibrinogen is known to promote red cell aggregation. While no clear correlation was found between fibrinogen and red cell aggregation in Chapter 3, a strong correlation was present in Chapter 6. It is likely that the increased fibrinogen level in peripheral arterial disease is partly responsible for the increased red cell aggregation. Red cell aggregation correlated inversely with haematocrit (as is known).

Red cell aggregation was higher in ischaemic rest pain than intermittent claudication and was highest in abdominal aortic aneurysm. The reason for this did not appear to be the fibrinogen level, which was not highest in aneurysm patients.

Following reconstructive vascular surgery red cell aggregation was significantly increased at one week but this increase was not found at 3-12 months. The temporary increase in red cell aggregation was probably due to an acute phase response in aggregating proteins following surgery.

An unexpected finding was the increased red cell aggregation in ex or non-smokers compared to current smokers in both patients and controls. The reason for this is not clear.

Increased red cell aggregation could be detrimental to patients with peripheral arterial disease. In ischaemia, reduced perfusion pressure creates low shear conditions which are favourable for red cell aggregation to occur. Red cell aggregates locally increase blood viscosity and may obstruct the microcirculation, further reducing blood flow and worsening the ischaemic conditions (Lowe, 1987a; Ditzel, 1959).

An important practical finding in this study was the significant inverse correlation between red cell aggregation and haematocrit. It is therefore suggested that in future clinical studies, blood samples be adjusted to a standard haematocrit prior to measurement.

Smoking

Cigarette smoking is a recognised and important risk factor for peripheral arterial disease. The association of smoking and peripheral arterial disease was confirmed in this study. Fibrinogen and white cell count were significantly increased in current smokers compared to ex or non-smokers in both patients and controls. White cell count correlated significantly with carboxyhaemoglobin levels. Fibrinogen and white cell count could each mediate some of the effects of smoking in peripheral arterial disease by their influence on thrombosis and haemorheology. While there was no significant correlation between fibrinogen and carboxyhaemoglobin, this might be explained by the fact that fibrinogen levels take several years to decrease to non-smoker levels after stopping smoking whereas carboxyhaemoglobin takes only days (Lee et al., 1990).

Red cell aggregation was higher in ex or non-smokers compared to current smokers in both patients and controls. The reason for this unexpected effect of smoking is unclear and was not related to a change in haematocrit.

Cross-linked fibrin degradation products were not altered by smoking.

Severity of Ischaemia and Abdominal Aortic Aneurysm

Patients were grouped according to severity of ischaemia and type of disease, i.e. intermittent claudication, ischaemic rest pain or abdominal aortic aneurysm. Cross-linked fibrin degradation products and red cell aggregation were highest in patients with abdominal aortic aneurysm compared to ischaemic rest pain or intermittent claudication using analysis of variance. Cross-linked FDP's correlated inversely with the ankle brachial pressure index, being higher in ischaemic rest pain than intermittent claudication. This has already been discussed.

White cell count was highest in ischaemic rest pain compared to intermittent claudication or abdominal aortic aneurysm using analysis of variance. (Patients with gangrene, tissue necrosis or infection were excluded from study). The white cell count was related to the severity of ischaemia, and it correlated inversely with the ankle brachial pressure index. A raised white cell count could tend to promote ischaemia through its rheological effects on the microcirculation. In lower limb ischaemia white cells become activated; in intermittent claudication white cells become activated with exercise but not at rest (Hickey et al., 1990). Activated white cells may promote tissue and endothelial damage.

The ankle brachial pressure index correlated inversely with cross-linked FDP's, white cell count, age and platelet count.

Patients with abdominal aortic aneurysm had increased red cell aggregation and cross-linked FDPs, and decreased haematocrit and HDL cholesterol, compared to patients with occlusive disease. This is in keeping with the view that abdominal aortic aneurysm is a different entity from occlusive disease and may have a different aetiology (Reid et al., 1990).

Reconstructive Vascular Surgery

Fibrinogen, cross-linked FDP's, and white cell count were significantly increased one day and one week following reconstructive vascular surgery. Red cell aggregation was significantly increased one week following reconstructive vascular surgery. The increase in fibrinogen, white cell count and red cell aggregation are probably due to an acute phase response since they were not increased at 3-12 months.

An interesting finding was that the white cell count also significantly increased one hour after release of the aortic clamp. Aortic cross-clamping produces a period of severe lower limb ischaemia. Lower limb ischaemia is known to activate white cells. Also, white cell count was highest in patients with ischaemic rest pain in this study (Chapter 3).

Only cross-linked FDP's were still significantly increased when measured in patients at 3-12 months following reconstructive vascular surgery. This indicates ongoing fibrin formation following reconstructive vascular surgery.

The importance of this is that it may be caused by deposition of fibrin on the graft surface and that levels of cross-linked FDP's could be prognostic markers for vascular graft occlusion.

Fibrinogen is rapidly and preferentially adsorbed onto an artificial surface. When blood comes into contact with a foreign surface, activation of factor XII initiates the intrinsic system of coagulation . Activated factor XII has been measured in this thesis by a new assay (Chapter 5). Levels of activated factor XII were not increased in patients with peripheral arterial disease compared to controls. This suggests that atherosclerosis does not act as a foreign surface activating the intrinsic system of blood coagulation. This however may not apply where ulceration occurs on the atherosclerotic plaque. Activated factor XII did not correlate with fibrinogen level.

Reconstructive vascular surgery introduces an artificial surface which resulted in significant activation of factor XII in this study. However these levels of activated factor XII were only temporarily increased, returning to baseline levels when measured at one day and one week after surgery. It is probable that the graft surface is rapidly coated with a "conditioning layer" and no longer acts as a foreign surface.

Antithrombotic Therapy

Having established that patients with peripheral arterial disease have a hypercoagulable state and abnormal blood rheology the next aim of this thesis was to determine if either minidose warfarin (1 mg daily) or low-dose aspirin (75 mg daily) significantly altered this in a placebo controlled, randomised trial.

Minidose warfarin and low-dose aspirin did not significantly alter the hypercoagulable state or abnormal haemorheology in patients with intermittent claudication.

Warfarin prolonged the prothrombin time compared to aspirin or placebo. However the prothrombin time remained within the normal range.

The white cell count was increased in the aspirin treatment group. However this effect may be chance variation since the white cell count in the placebo treatment group was reduced by the same amount. Also the white cell count is known to be labile.

This study confirmed that minidose warfarin and low-dose aspirin had minimal side effects.

Five adverse events occurred during the trial. These could be expected from a study of 100 claudicants followed up for a four month period. All adverse events observed were in the warfarin treatment group and were thrombotic events.

Low-dose aspirin or minidose warfarin did not significantly alter fibrinogen, cross-linked FDP's, red cell aggregation or the other blood variables of coagulation or blood rheology studied. It therefore appears that neither activation of blood platelets nor coagulation activity play a role in causing the abnormalities in haemorheology and coagulation in patients with peripheral arterial disease.

Aspirin and warfarin might still have benefit in patients with more severe ischaemia than intermittent claudication where there is reduced blood flow and where stagnation thrombosis can occur. Aspirin given over a more prolonged period or a higher dose of warfarin could have clinical benefit in preventing thrombotic events in patients with severe peripheral arterial disease (ischaemic rest pain and gangrene). This trial does not exclude patient benefit from long-term prevention of thrombotic events.

The failure of aspirin and warfarin to alter many of the blood abnormalities studied in this thesis suggests that other therapies which may reduce these abnormalities should be considered.

This thesis confirmed the raised plasma and whole blood viscosity, white cell count, triglyceride, and also the decreased HDL cholesterol levels previously reported in peripheral arterial disease. It confirmed the raised haematocrit previously reported in patients with intermittent

claudication and ischaemic rest pain. Total cholesterol was not significantly increased in peripheral arterial disease compared to controls in this study.

Other Blood Factors

The elevated levels of PAI, von Willebrand factor and factor VII (shown in Chapter 6) introduce three new possible thrombotic risk factors in peripheral arterial disease. The elevated level of PAI may inhibit fibrinolysis in vivo, creating an imbalance of haemostasis. PAI correlated inversely with cross-linked FDP's. It is therefore possible that despite elevated FDP levels, patients with peripheral arterial disease have greater fibrin deposition in vivo due to high PAI levels. PAI also correlated inversely with age, suggesting that the highest PAI levels occurred in patients with premature peripheral arterial disease.

The elevated level of von Willebrand Factor may be associated with endothelial cell damage in atherosclerosis, because von Willebrand Factor is released from endothelial cells. It is also possible that areas of high shear at sites of arterial stenoses account for the elevated level of von Willebrand factor. Von Willebrand factor is essential for platelet adhesion and aggregation at sites of endothelial cell damage.

The elevated level of factor VII may be another part of the hypercoagulable state found in patients with peripheral arterial disease.

Prospective studies of all these factors are required to assess their predictive value in peripheral arterial disease.

A better medical management for patients with peripheral arterial disease could be achieved by reducing plasma fibrinogen level. Stopping cigarette smoking has been the most effective clinical method of reducing fibrinogen; unfortunately most patients with peripheral arterial disease do not readily stop smoking. Recently a new fish oil concentrate ESKIMO-3 has been reported to decrease plasma fibrinogen levels in subjects by 23% over a six month period (Haglund et al., 1990). ESKIMO-3 also lowered triglyceride level by 64% and total cholesterol by 8%. There was also an increase in HDL cholesterol. Treatment with ESKIMO-3 was dependent of both dose and duration, and had no adverse effects (Haglund et al., 1990). This is a promising study and may point to future studies.

The probable effects of fibrinogen on thrombogenesis through fibrin formation, viscosity and atherosclerosis point to the multipotential nature of fibrinogen, and the need to lower raised levels in peripheral arterial disease.

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